# Genetic and Pharmacologic analysis of the Mechanisms of Selenium toxicity in *Caenorhabditis elegans*

by

Kathleen L. Morgan

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This dissertation was presented

by

Kathleen L. Morgan

It was defended on

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and approved by

**Robert Bowser, PhD**: Associate Professor, Department of Pathology

**Stephen Grant, PhD**: Associate Professor,

Department of Environmental and Occupational Health

**Lewis Jacobson, PhD**: Professor, Department of Biological Sciences

**A. Paula Monaghan-Nichols, PhD**: Assistant Professor, Department of Neurobiology

**Dissertation Advisor: Miguel Estevez, MD, PhD**: Assistant Professor,

Department of Neurology

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Selenium is an essential nutrient that is beneficial at daily intakes of 50-200µg/day and is detrimental at intake rates beyond 800µg/day. Selenium toxicity is an increasing environmental problem due to being a waste product of metal, coal, and oil refining. High selenium exposure causes developmental defects in wildlife, motor neuron degeneration in livestock and has been epidemiologically associated with the human motor neuron disease amyotrophic lateral sclerosis (ALS). In order to begin to define the cellular damage pathways activated by selenium, we have developed a genetic model of selenium toxicity using *Caenorhabditis elegans.* In this dissertation, we have begun to identify both environmental and genetic factors that affect selenium toxicity (like temperature, bacterial metabolism from the food source, calcium in the media), as well as a potential source of selenium's toxic effects, an increase in reactive oxygen species. We have also begun to determine which potential mechanism(s) of cell death are activated using strains with reduction-of-function mutations in cell death genes and pharmacologic treatments. In the second part of thesis, we characterize the neuronal damage caused by selenium because of its potential disease relevance. We demonstrate that selenium toxicity causes a decrease in cholinergic signaling that results in increased cytosolic protein catabolism in muscle which is predictably suppressed by nicotinic agonists and the acetylcholinesterase inhibitors. Combined, these data demonstrate that selenium toxicity causes muscle denervation, mimicking the pathology observed in ALS. We also demonstrate that

selenium causes similar denervation of the muscles mediating egg-laying in *C. elegans*. Finally, we have investigated oxidative stress pathways regulated by DAF-2, a major stress response pathway in *C. elegans*, and identified a gene target of DAF-2 regulation, an iron-manganese superoxide dismutase (*sod*-2), that is a "protective factor" affecting sensitivity to selenium. This work demonstrates that selenium-related oxidative stress causes a progressive movement impairment due to motor neuron injury.

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# 1.0 INTRODUCTION

## 1.1 SELENIUM

Selenium is an essential nutrient that has a narrow exposure window between beneficial and detrimental effects. This can be appreciated by simply looking at a periodic table. The element selenium falls in the same period as oxygen and sulfur, suggesting that it has properties essential for life. In its vertical group, selenium falls between arsenic and bromine, suggesting that it could also have toxic properties. While much research has been done on understanding the essential properties of selenium, little formal research has been done to investigate its potential toxic properties.

Selenium is found in a variety of forms, including elemental, inorganic (sodium selenite and sodium selenate), and organic (selenomethionine and selenocysteine) (Nuttall 2006). In whatever form it is found, it is eventually converted to an organic form and then incorporated into proteins. Selenomethionine is incorporated randomly in place of methionine, while selenocysteine incorporation is tightly regulated (Berry et al. 1991; Muller et al. 1997). Proteins that require selenocysteine for their function are referred to as selenoproteins; these include glutathione peroxidases, iodothyronine deiodinases, thioredoxin reductases, among others (Behne and Kyriakopoulos 2001).

Use of selenocysteine in proteins is conserved throughout the animal kingdom (Lee et al. 1990). Knockout of selenocysteine tRNA in mice have been shown to be embryonic lethal and conditional knockouts of selenocysteine tRNA in the liver caused mice to die within 1 to 3 months (Bosl et al. 1997; Carlson et al. 2004). In heterozygous selenocysteine tRNA knockout animals, selenoprotein expression was decreased 50-80%, with the exception of glutathione peroxidase, suggesting a critical role in an organism's survival. Knockout mice of glutathione peroxidase 1 are variable and normal in body weight, but are susceptible to myocardial ischemia and diquot-induced oxidative stress (Cheng et al. 1997; Yoshida et al. 1997; Fu et al. 1999). However, mice contain four glutathione peroxidases and a knockout mouse of all four has yet to be developed.

Selenocysteine is often referred to as the 21$^{st}$ amino acid due to the specific incorporation. Specific incorporation of selenocysteine is nearly universal from prokaryotes to eukaryotes, utilizing the UGA stop codon along with a 3' UTR (Berry et al. 1991; Berry et al. 1993; Fischer et al. 2007). *Caenorhabditis elegans* differ from mammals by using AUGA as the selenocysteine insertion sequence, directing selenium insertion into thioredoxin reductases, the only known selenoproteins in *C. elegans* identified to date (Buettner et al. 1999; Gladyshev et al. 1999).

The requirement of proteins to incorporate and utilize selenocysteine instead of cysteine is due to the difference in chemical properties of selenium compared to sulfur (Young et al. 1982; Tinggi 2003). Cysteine contains a thiol side group that has a pKa of around 8.5. With selenocysteine, a selenium atom replaces the sulfur and the pKa of selenide side group is 5.2. At physiological pH, the thiol side group is partially ionized, while the selenium containing side

chain is fully ionized (Young et al. 1982). The ionization state of selenium is likely key to its essential role in selenoproteins.

The random incorporation of selenomethionine is believed to provide storage of selenium (Behne and Kyriakopoulos 2001). However, the properties of selenium raise an interesting question about the random substitution of selenomethionine in place of methionine, since every protein contains at least one methionine amino acid (the ATG start codon for methionine). Can this substitution affect protein function or stability, in general? While this is not a widely researched topic, there are a couple of studies that investigated selenomethionine substitution affects the final protein product. One study compared selenomethionyl thymidylate synthase between wild type and a methionine auxotrophic strain of *E. coli*. In this study, the authors found that while the binding kinetics of the enzyme was not altered between the two strains, the selenium containing enzyme was eight-times less stable than when exposed to heat and twice as sensitive to oxygen (Boles et al. 1991). Another study investigating phosphomannose isomerase, using a similar experimental design with a methionine auxotrophic strain of *E. coli*, reported that substitution with selenomethionine resulted in a four-fold increase in its pKa (Bernard et al. 1995). These studies demonstrate that selenomethionine incorporation can affect the final protein product, but the effects vary greatly, likely depending on where selenomethionine is located in the tertiary and/or quandary protein structure.

## 1.2 PROTECTIVE AND ESSENTIAL ROLES FOR SELENIUM

An essential biological role for selenium was not proposed until the 1930's, when selenium was detected in a variety of plants (Trelease and Trelease 1938). The essential nature of selenium for animals was not understood until the 1950's, when it was discovered that liver necrosis induced by a vitamin E deficient diet could be prevented by selenium supplementation (Schwarz and Foltz 1999). Since then, a group of proteins has been identified, collectively known as selenoproteins, which require selenium to function by incorporation of selenocysteine. Of the 30 mammalian selenoproteins identified to date, including glutathione peroxidases, iodothyronine deiodinases, and thioredoxin reductases, only half have known functions (Behne and Kyriakopoulos 2001).

Glutathione peroxidase (GPx) was the first identified selenoprotein; it was discovered after finding that dietary selenium could protect erythrocytes from oxidative damage (Rotruck et al. 1972; Rotruck et al. 1973). GPx is a selenoprotein that is conserved in mammals, plants, and viruses (Rotruck et al. 1973; Zhang et al. 1999; Fu et al. 2002). The function of glutathione peroxidase is to convert hydrogen peroxide into water, protecting the cell from oxidative damage (Behne and Kyriakopoulos 2001). As mentioned in the previous section, there are multiple glutathione peroxidases in mammalian systems and an individual knockout of GPx1 has no affect on viability but do affect susceptibility to oxidative stress (Fu et al. 1999).

The two other main classes of selenoproteins in mammals with known functions are iodothyronine deiodinases and thioredoxin reductases. There are three iodothyronine deiodinases responsible for activating and inactivating thyroid hormones that are involved in development and controlling a variety of metabolic processes (Behne and Kyriakopoulos 2001). Iodothyronine deiodinase 1 and 2 knockout mice are viable with no gross abnormalities (Schneider et al. 2001;

Schneider et al. 2006). Iodothyronine deiodinase 3 knockout mice were found to have decreased fertility and reduced viability (Hernandez et al. 2006; Hernandez et al. 2007). Thioredoxin reductases are responsible for reducing oxidized thioredoxin in a NADPH–dependent manner, serving a key function in regulating the cellular redox status (Holmgren 1989). Single and double knockout mice for thioredoxin reductases are viable but have decreased fertility (Gauthier et al. 1999; Gauthier et al. 2001; Plateroti et al. 2001). While no knockout on an individual selenoprotein were embryonic lethal, they did affect an aspect of overall health with respect to oxidative stress and fertility.

Inadequate dietary selenium has been linked to Keshan disease, a cardiomyopathy, and Kaschin-Beck disease, an osteoarthropathy (Behne and Kyriakopoulos 2001). In Keshan disease, a coxsackie virus remains dormant in the presence of selenium but becomes virulent in its absence due to alterations in the viral genome (Beck et al. 2003). The mechanism involving selenium deficiency in Kashin-Beck disease remains elusive (Sudre and Mathieu 2001).

Deficient dietary intake of selenium alters immune system responses in mice. Selenium deficiency exacerbates flu symptoms and alters cytokine responses in mice fed a selenium-deficient diet compared to those fed an adequate selenium diet (Beck et al. 2003). Dietary selenium supplementation has been shown to attenuate lipopolysaccharide-induced expression of the pro-inflammatory agents, cyclooxygenase-2 and tumor necrosis factor-alpha in macrophages (Vunta et al. 2008). Selenium deficiency also causes hyperoxidant production in T cells, which results in suppression of T cell proliferation (Shrimali et al. 2008). These recent findings demonstrate an emerging and important role for selenium in immune system function.

Selenium also has a variety of anti-cancer properties (Zafar et al. 2003; Goel et al. 2006). Selenocysteine induces apoptosis in human cancer cells (and not in normal cells) by increasing

reactive oxygen species (Goel et al. 2006; Chen and Wong 2008). Selenium compounds also induce cell cycle arrest in human prostate cancer cells (Zhao and Brooks 2007). These mechanisms appear to be mediated through p53, altering sensitivity to chemotherapy and radiation (Fischer et al. 2006; Zhao et al. 2006; Zhao et al. 2006; Zu et al. 2006; Fischer et al. 2007). Selenium's sensitizing effect on cancer cells has resulted in ongoing clinical trials using selenium treatments in conjugation with cancer treatments, with mixed results (Clark et al. 1996; Block et al. 2008).

Selenium is also reported to have a variety of neuroprotective effects. Sodium selenite protects peripheral neurons from methyl-mercury exposure (Chang 1983). Motor neuron degeneration resulting from trauma is retarded by selenium (Hall and Braughler 1986; Hall 1987). Selenium treatments are neuroprotective in Parkinson's models of neurodegeneration (Imam et al. 2001; Zafar et al. 2003; Dodig and Cepelak 2004). In an Alzheimer's disease model, sodium selenite was recently found to inhibit gamma-secretase activity, which could potentially slow β-amyloid production (Tung et al. 2008). Despite the variety of selenium's essential roles, it can be toxic at levels only four times the daily recommended dose in humans (Lemly 1997).

## 1.3 SELENIUM TOXICITY

Initial observations describing selenium toxicity can be traced back to Marco Polo's travels through China during the 13$^{th}$ century (Young et al. 1982). While traveling through high selenium areas in China, Marco Polo's group was warned of "poisonous" plants that we now know to be part of the selenium accumulating plant genus *Astragalus*, that if eaten by "beasts of burden" (horses) would cause their hooves to fall off (Spallholz 1994). In humans, the

recommended daily level of selenium consumption ranges between 50-200µg per day but consumption of as little as 850-900µg per day leads to symptoms of selenium toxicity (Lemly 1997). Signs of toxicity in humans include nausea, hair loss, nail changes, and with high enough exposure, can occasionally progress to death (Young et al. 1982).

### 1.3.1 The complexity of selenium toxicity

It is interesting that for an element which has long been understood to be toxic, little is understood about its toxic properties. One source estimates that selenium is 5 times more toxic than arsenic, yet arsenic is more widely recognized as being poisonous (Harris 1991). There are many reasons for this confusion. Foremost, selenium is an essential nutrient utilized by various selenoproteins, as previously described in Section 1.1 (Behne and Kyriakopoulos 2001). Second, there is a large and continuously growing body of literature citing the numerous health benefits from selenium ranging from helping immune function to having anti-cancer properties as described in Section 1.2. Arsenic is not essential in mammals, known to be poisonous, and has previously been used as a pesticide (Oremland and Stolz 2003).

Adding to the confusion is the complexity of selenium toxicity. While all forms of selenium are toxic, the concentrations to achieve similar toxic phenotypes vary drastically. Given orally to rats, elemental selenium was found to have an $LD_{50}$ (lethal dose, 50% or the dose required to kill 50% of a population) of 6700mg Se/kg of body weight (Nuttall 2006). At the other end of the spectrum, sodium selenite was found to have an $LD_{50}$ of 7mg Se/kg of body weight (Nuttall 2006). Also, inorganic selenium was found to be more toxic at lower doses in adult animals while a more significant decrease in weight was observed in weaning animals

whose mothers were fed organic selenium when compared to offspring of mothers fed inorganic selenium (Kim and Mahan 2001).

In addition to the form of selenium causing toxicity, other factors also need to be considered, such as an individual's diet. The essential role of selenium was found when rats fed a vitamin E-deficient diet could be rescued with selenium (Schwarz and Foltz 1999). Conversely, selenium toxicity in rats was alleviated by dietary supplementation with methionine and vitamin E (Levander and Morris 1970). Selenium levels could be equal in the diets of 2 individuals. One individual could be eating a well balanced diet containing excess vitamin E and be physically normal, while the other individual could be eating a diet with less vitamin E and show signs of selenium toxicity. With the variety of factors affecting signs of selenium toxicity, like type of selenium (elemental, organic, or inorganic), as well as dietary components (like vitamin E), it is understandable that identifying damage caused by excess selenium is difficult.

### 1.3.2 Selenium toxicity is an increasing environmental problem

Selenium toxicity has become an increasing environmental concern because of bioaccumulation attributable to increased industrial refining of metals and petroleum and irrigation of seleniferous soils (Harris 1991; Lemly 1997; Lemly 2004). Bioaccumulation occurs as selenium waste is incorporated into a bioavailable form, as either selenocysteine or selenomethionine, by bacteria and plants (Lemly 1997; Lemly 2004). Plant uptake of selenium can be affected by selenium concentration, moisture, pH, salinity, sulfate concentrations, reoxidation conditions, and species of plants (Wu 2004). As bacteria and plants are ingested by higher organisms, selenium is concentrated and enters the food supply (Lemly 1997).

Selenium's toxic effects on fish and aquatic birds have been studied in Belews Lake, North Carolina and at the Kesterson Reservoir Wildlife Refuge in California (Harris 1991; Lemly 1997). In both locations, bioaccumulation resulted in selenium concentrations 100-3000 times greater than neighboring areas (Lemly 1997). Fish and birds laid fewer eggs and fewer eggs hatched of those laid (Ohlendorf et al. 1986; Lemly 1997). Those that did hatch were more likely to have congenital defects, including deformed spines, heads, limbs, and eyes (Ohlendorf et al. 1986; Lemly 1997).

Kesterson Reservoir provided a lesson in bioaccumulation and insight into the complexity of selenium toxicity (Ohlendorf et al. 1986; Harris 1991; Lemly 2004). Selenium toxicity in Kesterson resulted from an attempt to revitalize marshlands that had been drained for irrigation purposes. The plan was to revitalize the marshes by directing irrigation drainage from upstream of Kesterson. Initial reports testing water samples suggested that the plan to revitalize the marshlands with irrigation drainage would work lacking side-effects (Harris 1991). However, scientists who had visited the location were hesitant because of things they had not observed, like birds or insects (Harris 1991). They also observed crystal clear water in the marshland (Harris 1991). These observations led to sampling of soil, plants, and where they could be found, fish, in addition to the previously mentioned water samples. While selenium levels in the water were low, even undetectable at times, levels in plants and the few animals tested were startlingly high (Harris 1991). This example demonstrates how selenium is actively taken up by organisms and that simply measuring selenium concentration in water provided an inadequate representation as to selenium content in the overall environment.

### 1.3.3 Comparative selenium toxicity

As mentioned in Section 1.3.1, selenium is found in a variety of forms, from inorganic to organic to elemental, each with varying degrees of toxicity. A few experimental studies addressing selenium toxicity have investigated whether the form of selenium (inorganic or organic) affected overall toxicity. One such study that examined equivalent amounts of sodium selenite and seleno-DL-methionine in mallards and found that seleno-DL-methionine was more teratogenic (causing developmental abnormalities) while sodium selenite was embryotoxic (Heinz and Hoffman 1996). An experimental study of post-implantation rat embryos found all selenium sources to cause teratogenic deformities in the nervous system in all forms but at different concentrations (Usami and Ohno 1996). Forms of inorganic selenium (sodium selenite followed by sodium selenate) were found to have more toxic effects at lower concentrations when compared to organic selenium (Usami and Ohno 1996). Seleno-DL-cysteine and seleno-DL-methionine were also found to be toxic, but at concentrations 50-times greater than sodium selenite (Usami and Ohno 1996).

Another experimental study, this one in pigs, compared seleno-DL-methionine, sodium selenate, and *Astragalus bisulcatus* (Panter et al. 1996). Pigs were divided into groups and fed equivalent selenium amounts in their feed for up to a six week period (Panter et al. 1996). Phenotypes observed in severely affected animals included signs of chronic selenosis (hair and hoof lesions) as well as ataxia and paralysis that correlated to the development of lesions similar to poliomyelomalacia ("polio" refers to paralysis; "myelomacia" is softening of the spinal cord) and polioencephalomalacia (cerebrialcortical necrosis) (Panter et al. 1996). All animals given feed containing selenium from *Astragalus bisulcatus* developed paralysis at a faster rate than other forms (Panter et al. 1996). Animals fed sodium selenate showed the next most severe

symptoms from selenium-supplemented feed, with four animals developing CNS problems (posterior ataxia to paralysis) and the other animal showing signs of chronic selenosis (Panter et al. 1996). Also of interest was an analysis of the selenium content of various organs. Feeding with seleno-DL-methionine resulted in the greatest selenium content in the tissues of all selenium sources, but showed the least toxicity (2 animals showing CNS signs and 3 showing signs of chronic selenosis) (Panter et al. 1996). *Astragalus bisulcatus* and sodium selenate fed animals had lower selenium concentrations in their tissues than seleno-DL-methionine animals, but selenium concentrations in all were still greater than in the non-selenium treated controls. This study demonstrated that the severity of neurological damage caused by selenium toxicity does not correlate with selenium tissue content.

Another experimental study looked at selenium effects on the nervous system of mice fed organic (seleno-L-methionine) and inorganic (sodium selenite) selenium in drinking water (Tsunoda et al. 2000). Sub-lethal doses of inorganic selenium increased some dopamine metabolites in the striatum compared to controls or seleno-L-methionine-treated animals (Tsunoda et al. 2000). As dopamine is a neurotransmitter, this may suggest a mechanism of neurotoxicity by altering neurotransmission, it does not account for seleno-L-methionine toxicity in animals.

Yet another experimental study addressed various concentrations of inorganic and organic selenium and the emergence of selenosis in growing-finishing pigs (Kim and Mahan 2001). Again, the authors found that both forms of selenium caused toxicity. However, inorganic selenium caused more severe symptoms and the symptoms presented faster than in adult pigs fed organic selenium (Kim and Mahan 2001). Long term exposure to selenium in these pigs confirmed findings observed in birds and fish that led to increased reproductive problems (few

live pigs being born) (Kim and Mahan 2001). Further, organic selenium appears to lead to greater problems in the offspring due to higher selenium content in the tissue.

Here, the complexity of selenium toxicity is apparent and provides insight as to why selenium toxicity is poorly researched. In adults, inorganic selenium is more toxic that organic forms (Panter et al. 1996; Kim and Mahan 2001). In animals where the mother is fed selenium, organic forms of selenium are more toxic that inorganic when the progeny were examined (Heinz and Hoffman 1996). Adding to this, adult animals fed selenium from the same source yielded phenotypes that varied from hair and nail lesions to neurological signs (Panter et al. 1996).

### 1.3.4 Selenium and oxidative stress

The mechanism(s) of selenium toxicity is not known but one possibility is that it results from increased oxidative stress. This may seem counterintuitive since selenium is widely considered to be an antioxidant, since selenium is utilized in the active site of glutathione peroxidases, which catalyze the conversion of hydrogen peroxide to water (Behne and Kyriakopoulos 2001). An equation proposed by Seko *et al*. in 1997 suggested that selenite reacts with glutathione and hydrogen selenide to produce superoxide anion. This was later confirmed in vitro using chemiluminescence (Spallholz 1994; Seko and Imura 1997).

A variety of literature supports the idea that increased oxidative stress can result from both organic and inorganic selenium exposure. A superoxide dismutase mimetic alleviated sodium selenite toxicity in rat hepatocytes (Kitahara et al. 1993). In human hepatoma cells, sodium selenite was found to induce oxidative stress and subsequent apoptosis (Shen et al.

1999). Selenomethionine caused detection of oxidized protein in gastric cancer cells (Verma et al. 2004). Injections of reduced glutathione alleviated sodium selenite toxicity in buffalo calves (Deore et al. 2005). In *Saccharomyces cerevisiae*, both selenomethionine and sodium selenite resulted in decreased growth of yeast strains involved in DNA repair and oxidative stress, suggesting both selenium sources cause reduced growth due to oxidative damage (Seitomer et al. 2008). From the variety of literature cited, various selenium sources were found to cause increased oxidative stress or detect oxidative damage in a variety of cell types, supporting the role of oxidative stress in selenium toxicity.

## 1.4 DISEASE ASSOCIATION TO HIGH ENVIRONMENTAL SELENIUM

There is an increasing awareness of and focus on linking diseases to environmental factors. Previous research reveals an association between high environmental selenium and the motor neuron disease amyotrophic lateral sclerosis (ALS), more commonly referred to as Lou Gehrig's disease. Further evidence supporting toxicity in response to selenium exposure comes from accidental selenium poisoning in livestock, which will be discussed later.

### 1.4.1 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) was first described by the French neurobiologist Jean-Martin Charcot in 1869 and was originally known as Charcot's sclerosis. ALS is a neurodegenerative disorder affecting the motor neurons in the ventral horns of the spinal cord, motor cortex, and brainstem (Cleveland and Rothstein 2001). Interestingly, it spares sensory

neurons, neurons involved in cognitive processes, and a small specific group of motor neurons controlling sphincter and urethral function (Kunst 2004). ALS is a rapidly progressive and fatal disease. Death occurs as a result of denervation of the respiratory tract, causing suffocation (Kuncl et al. 1988).

The average age of onset for the disease occurs between 50-60 years, with death occurring within 2-5 years of onset (Williams and Windebank 1991). Typically, patients present with progressive weakness in the arms and legs (Williams and Windebank 1991). There is another form of the disease, differentiated because it presents with swallowing problems and difficulty talking, called bulbar ALS (Hillel et al. 1999). At any time, 1-2 individuals per 100,000 have ALS (Cleveland and Rothstein 2001). As a result, the lifetime incidence for ALS is approximately 1 in 800 (Yoshida et al. 1986). There is currently only one drug treatment for ALS patients, the anti-glutamatergic drug riluzole, which extends the patients life by an average of 3 months (Bensimon et al. 1994).

Until the early 1990's, there was no known cause of ALS. At that time, researchers studying families with the inherited form of the disease identified mutations in the superoxide dismutase 1 (SOD1) gene as a cause of familial ALS (Rosen et al. 1993). However, familial ALS accounts for only 10% of all ALS cases (Cleveland and Rothstein 2001). Also, it is now known that mutations in SOD1 have been identified in approximately 20% of familial ALS cases, thereby accounting for approximately 2% of all ALS cases (Cleveland and Rothstein 2001). There are more that 90 mutations in SOD-1 linked to familial ALS, causing confusion as to the toxic properties of mutant SOD-1 (Gaudette et al. 2000; Andersen 2001). Yet, research on SOD1 mutations remains the focus the majority of ALS studies.

Since identification of the SOD-1 mutation, other genetic mutations linked to familial ALS have been identified. These genes have been identified as ALS1-ALS8 (Kunst 2004). Of these, only ALS2 (Alsin) and ALS4 (Sentaxin) have known protein products (Ikemoto et al. 2002; Tudor et al. 2005). Alsin is a guanine nucleotide exchange factor that is predicted in interact with kinases that are involved in cytoskeleton rearrangement which plays a role growth cones development in neurons (Tudor et al. 2005). Sentaxin/syntaxin is a synaptic vesicle associated protein that is differentially expressed in ALS patients (Ikemoto et al. 2002). However, the function of both protein products in disease is poorly understood.

Recently, there has been increasing focus on genetic susceptibility factors in ALS. This is a result of research describing decreased expression of EAAT2, a glial glutamate transporter, in more than half of the sporadic ALS patients tested (Rothstein et al. 1995). Other genetic factors associated with ALS by affecting disease progression include vascular endothelial growth factor (VEGF), neurofilamant, and one allele of Apolipoprotein E (ε4 allele), among others (Rouleau et al. 1996; Drory et al. 2001; Lambrechts et al. 2003). Although, it is not clear if these factors are necessary and/or sufficient to cause the disease (Kunst 2004). Recently, multiple genome-wide scans were performed of sporadic ALS patients, attempting to identify single nucleotide polymorphisms (SNPs) found in ALS patients (Dunckley et al. 2007; Kasperaviciute et al. 2007; Schymick et al. 2007; Blauw et al. 2008). These studies provided a list of loci implicating genes with know and unknown functions (Dunckley et al. 2007; Kasperaviciute et al. 2007; Schymick et al. 2007). Many of the known genes are involved in cytoskeleton regulation (Dunckley et al. 2007; Kasperaviciute et al. 2007; Schymick et al. 2007). Given that susceptibility factors are present in non-disease individuals suggests that a combination of factors and/or an environmental trigger may be involved in developing sporadic amyotrophic lateral sclerosis.

The growing body of evidence suggests that studying SOD1 mutations to model ALS is inadequate to understand non-SOD1 ALS cases. Recent research that focused on identifying ubiquitin-positive inclusion in frontotemporal lobar degeneration led to a new class of disorders referred to as TAR-DNA binding protein 43 (TDP-43) proteinopathies (Neumann et al. 2007). TDP-43 positive inclusions were identified in sporadic ALS and some familial ALS, but not familial SOD-1 mutant cases (Neumann et al. 2007). Another study reported that TDP-43 was found in SOD-1 transgenic mice, but it was not associated with ubiquinated inclusions like it is with sporadic ALS cases (Robertson et al. 2007). Together, these reports suggest that while clinical progression of familial and sporadic ALS is nearly identical with a few exceptions of adolescence onset, there is a divergence in the molecular mechanisms of the diseases. A variety of drugs have been developed to treat SOD1 mutant mice, only to fail spectacularly when the drugs reach clinical trials (Benatar 2007; Scott et al. 2008). This combination of evidence reinforces the need for a model of sporadic ALS.

### 1.4.2 Epidemiologic studies linking high environmental selenium and ALS

The initial association between high environmental selenium and motor neuron disease can be traced back to the 1930's in South Dakota where animals that grazed in high selenium areas were found to develop alkali disease and "blind staggers" (Tinggi 2003). Later in the 1970's, the original modern connection between high environmental selenium and motor neuron disease was made when an ALS cluster was noted in a town whose livestock suffered from endemic selenium toxicosis (Kilness and Hichberg 1977).

More recent evidence linking environmental selenium exposure to ALS comes from an epidemiologic study examining the effects of long term exposure to increased environmental

selenium through drinking water in an Italian town and found an increased incidence of ALS (Vinceti et al. 1996). Investigators identified a cohort of residents that had continuously drunk from the town's water supply containing inorganic selenium during a 5 year period. Of 5,182 individuals, 4 developed sporadic ALS (Vinceti et al. 1996). All 4 individuals diagnosed with sporadic ALS fell into a sub-cohort of 2,065 individuals that was identified for continuously having drunk from the municipal tap water for 12 consecutive years (Vinceti et al. 1996). This is a significant increase of about 1 in 500-1000 ALS incidence compared to normal (1-2 per 100,000) which demonstrates a correlative association between high environmental selenium and an increased risk for developing ALS.

### 1.4.3 Selenium levels in ALS patients

Since the observations linking environmental selenium to ALS, investigations into an association between selenium levels within the body and ALS patients have been performed. A follow up study in the Italian town correlation incidence of ALS with high selenium in the drinking water could not link tissue selenium levels to ALS status when looking at biomarkers in toe nail clipping (Bergomi et al. 2002). However, as the authors of the report note, there is a limited potential for using toenail clippings as biomarkers for metal exposure (Bergomi et al. 2002). Another report found no increase in selenium levels in the urine of ALS patients compared to controls where as another report investigated selenium levels in hair, plasma, and blood and found that selenium concentrations in plasma decrease as ALS progressed (Moriwaka et al. 1993; Norris et al. 1993).

There are multiple problems with the findings described above. First, these reports examine selenium concentrations after disease onset and do not account for exposure prior to

disease diagnosis (for example, selenium has a half-life in human tissues between 65-115 days) (Mestek 1997). Second, selenium concentrations alone do not determine toxicity; the form of selenium is equally important (Panter et al. 1996; Usami and Ohno 1996). In addition, it is possible that a relatively brief window of exposure to high selenium could cause cellular damage, in a way that might take years to fully manifest.

The tissue tested for selenium is also critical as selenium is better retained in the nervous system. Rats fed selenium deficient diets for multiple generations resulted in a minimal selenium decrease in the nervous system when compared to other tissues (Savaskan et al. 2003). When the spinal cords of patients with motor neuron disease were examined, selenium levels were found to be increased compared to controls (Ince et al. 1994; Markesbery et al. 1995). While these reports do not demonstrate a causative relationship between selenium and motor neuron disease, it does suggest a potential relationship. Specifically, that selenium could be involved in a fraction of overall sporadic ALS cases, similar to SOD1 mutations in the familial ALS cases.

### 1.4.4 Selenium and Gulf War Veterans

There is a growing body of evidence that demonstrates a relationship between military service and increased risk of developing sporadic ALS. A review of the literature by the National Academies Institute of Medicine concurred that those in military service are at an increased risk of developing sporadic ALS (Weisskopf et al. 2005; Institute of Medicine (U.S.). Committee on the Review of the Scientific Literature on Amyotrophic Lateral Sclerosis in Veterans. 2006). Also of note is the increased incidence of ALS in Gulf War veterans. Two epidemiologic studies, using different methodology, identified increased incidence of sporadic ALS in troops deployed during the Gulf War, suggesting an environmental or war time trigger (Haley 2003; Horner et al.

2003). One report tracked the expected number of sporadic ALS compared to diagnosed cases and found that by 7 years post-Gulf War (the last year tracked in the study) that the number of observed sporadic ALS cases was more than twice than expected (Haley 2003). The increased incidence of developing sporadic ALS from Gulf deployment provides a unique opportunity at identifying an environmental or war-time trigger.

A possible culprit was identified in a report by Richardson *et al.*, in which the authors reported near toxic levels of selenium in the Mesopotamian marshes of Iraq (Richardson et al. 2005). In addition to the marsh, selenium is a major waste product of oil refining and selenium content in the oil in the region is some of the highest in the world, containing 1.10ug Se/g of oil (compared to 0.369ug/g in Venezuela oil and 0.0094ug/g in Alberta oil) (Yen 1975). This converts to an estimated 140-170g of selenium per barrel of crude oil. During the first Gulf War, over 700 hundred oil wells were set on fire and required nearly 9 months to contain (Osman 1997). Burning from 2-6 million barrels of crude oil each day and released tons of selenium into the atmosphere (Osman 1997). These data provide another correlative relationship between selenium exposure and ALS.

### 1.4.5 Supporting studies from selenosis in livestock

Further evidence suggesting selenium acts as a motor neuron toxin come from reports of accidental selenium poisoning reported in livestock due to excess selenium supplementation of their feed. Veterinary analysis was sought after animals (pigs) developed paralysis (Stowe et al. 1992). After excluding microbial and viral pathogens, investigations ultimately discovered that the selenium concentrations in the feed exceeded toxic levels (Stowe et al. 1992). This study reported neuronal loss and vacuolation of the neurons, glial cell proliferation in the ventral horn,

skeletal muscle swelling, and myocardium vacuolation (Stowe et al. 1992). Accidental selenium poisoning has been reported multiple times in the literature, with the most common observation being neuronal loss in the spinal cord (Harrison et al. 1983; Goehring et al. 1984; Casteel et al. 1985; Frank and Bergeland 1988; Stowe et al. 1992; Penrith and Robinson 1996). Another series of studies experimentally induced motor neuron degeneration with selenium to provide a biological basis for a causative role in the paralytic outbreaks (Harrison et al. 1983; Wilson et al. 1983; Wilson et al. 1988; Baker et al. 1989; Panter et al. 1996).

These reports of selenium toxicity in animals are significant in that they mirror ALS symptoms. Initial symptoms of affected animals included limb weakness and an inability to stand upright (Harrison et al. 1983; Stowe et al. 1992; Casteignau et al. 2006). The majority of ALS patients' initial symptoms include weakness in the hands, arms, or legs (Williams and Windebank 1991). Severely affected selenium-treated or exposed animals became quadriplegic and had to be euthanaized (Harrison et al. 1983; Casteel et al. 1985; Stowe et al. 1992). Similarly, ALS patients progress from muscle weakness to paralysis and eventual death (Williams and Windebank 1991). In addition to neuronal loss, glial proliferation in the spinal cord was observed of paralyzed selenium-treated animals, a pathology which has also been reported in ALS tissue (Harrison et al. 1983; Casteel et al. 1985; Stowe et al. 1992; Anneser et al. 2004; Casteignau et al. 2006). Further, selenium-treated animals also have neuronal vacuolation, which is also observed in ALS (Levine et al. 1999). The damage caused by selenium exposure mimics that observed in ALS patients.

## 1.5 STUDYING SELENIUM TOXICITY IN THE GENETICALLY TRACTABLE ORGANISM *CAENORHABDITIS ELEGANS*

Genetic model systems, including bacteria, yeast, *Drosophila*, *C. elegans*, and mice have proven useful in modeling processes that occur in higher organisms. While all systems could be used to investigate selenium toxicity, *C. elegans* provides a number of advantages. First, *C. elegans* has the shortest generation time of the multicellular systems; approximately 3 days from when an egg is laid until sexually maturity (Brenner 1974). The adult hermaphrodite has 959 somatic cells while the male has 1031 cells, including 302 neurons. Transparency of the animal makes it possible to visualize selenium-induced cellular changes throughout the animals' life (Wood 1988). Animals are easily maintained on agar with *E. coli* as a food source. *C. elegans* have 2 sexes, self-fertilizing hermaphrodites, which account for 99.9% of naturally occurring population, and males, which account for 0.1% of the population (Wood 1988). The presence of self-fertilizing hermaphrodites allows for maintenance of a mutant clonal population starting from a single animal, while the presence of males allow for genetic variability (Brenner 1974).

Due to the conservation of biological processes and the many useful experimental features of *C. elegans,* the model has proved to be a powerful tool for modeling basic processes like apoptosis, and for developing new research tools like RNAi that have been applied across multiple fields (Ellis and Horvitz 1986; Fire et al. 1998). The *C. elegans* nervous system is highly conserved in function when compared to mammalian systems, using the same neurotransmitters and second messengers as well as pre-synaptic and post-synaptic receptor orthologs (Bargmann 1998; Bargmann and Kaplan 1998). For this Reason, *C. elegans* have also been useful in modeling other complex neurodegenerative disorders such as Huntington's,

Parkinson's, Alzheimer's disease, and necrotic neuronal death, suggesting it could prove useful in modeling selenium toxicity (Driscoll and Gerstbrein 2003).

Before delving into biochemical and genetic analysis of selenium toxicity, we have established a model using *Caenorhabditis elegans* for studying selenium toxicity. Previous studies in our laboratory, we observed decreased fertility and increased occurrence of developmental defects observed in *C. elegans* (Lemly 1997).

## 1.6 RESEARCH PLAN

Literature searches of selenium recover little in the way of understanding why selenium is toxic. Further, there is a well-documented link between high selenium exposure and anatomical motor neuron loss, with a more debatable link to the specific motor neuron disease, ALS. Previously in our laboratory, we have observed this high selenium exposure causes decreased fertility and an increased number of developmental defects. This is similar to what has been observed in other organisms, demonstrating the usefulness of using *C. elegans* as a system for studying selenium toxicity (Lemly 1997). Chapter 2 investigates various factors that can affect toxicity in *C. elegans*, such as temperature, bacterial metabolism, and minimal/adequate calcium levels, as well as a potential source of toxicity, increased oxidative stress. Chapter 3 examines neuronal damage caused by selenium toxicity, specifically decreased cholinergic signaling resulting in muscle denervation and damage to the *C. elegans* egg-laying circuit. Chapter 4 then examines the insulin-like pathway in response to selenium exposure. In Chapter 5, we discuss findings from Chapters 2-4 and how they are relevant to motor neuron loss and future directions for this study.

# 2.0 BASIC SELENIUM TOXICITY IN *CAENORHABDITIS ELEGANS*

## 2.1 ABSTRACT

Despite the fact that selenium is well-known to be an antioxidant, at high concentrations it is a powerful cytotoxin. Work in the existing literature has shown both organic and inorganic selenium to be toxic *in vivo* and *in vitro*. Similarly, earlier work in our lab demonstrated that exposure to high selenium levels in the genetically tractable organism *Caenorhabditis elegans* causes reproductive and developmental defects similar to those reported other species. Here we show that both forms are toxic. Selenium exposure to adult animals causes a progressive serious of dysfunctional behaviors leading to death. Selenium exposure in adult animals causes a progressive impairment of motor function culminating in death. Several key environmental factors exacerbate or alleviate selenium toxicity, including calcium levels, temperature, and bacterial metabolism. In addition, we determined that the two canonical cell death mechanisms, apoptosis and necrosis, were not responsible for selenium-induced cell death. Finally, we determined that selenium exposure leads to evidence of increased reactive oxygen species (ROS) that could be related to cellular damage and motor impairment that could be suppressed by antioxidants.

## 2.2 INTRODUCTION

Selenium was discovered to be essential in the 1950's when scientists found that selenium supplementation in rats fed a vitamin E-deficient diet could prevent liver necrosis (Schwarz and Foltz 1999). It is now understood that the organic forms of selenium, selenomethionine and selenocysteine, are incorporated into proteins. Selenomethionine can incorporate randomly into proteins, while selenocysteine has been found to incorporate specifically using a UGA codon that normally codes for stop along with a 200 nucleotide sequence in the 3' UTR (Berry et al. 1991; Muller et al. 1997). The essential function of selenium is limited to selenocysteine incorporation while selenomethionine incorporation functions as "storage".

Despite its protective functions, selenium is also toxic. In fact, the earliest written accounts of selenium's toxicity date back to Marco Polo's travels through China in the 1200's (Young et al. 1982; Spallholz 1994). A majority of the literature on "selenium toxicity" is composed of descriptive reports that discuss selenium's effects on wildlife and accidental selenosis in livestock along with a few reports of selenium poisoning in humans (Carter 1966; Casteel et al. 1985; Koppel et al. 1986; Ohlendorf et al. 1986; Stowe et al. 1992; Lemly 1997; Lemly 2004; Casteignau et al. 2006; Nuttall 2006; Sutter et al. 2008). Only studies of its anti-cancer properties suggest specific mechanisms of toxicity, involving activation of apoptosis (Lu et al. 1994; Jiang et al. 2001; Zhu et al. 2002; Shilo et al. 2003; Goel et al. 2006; Xiao et al. 2006; Zhao et al. 2006; Zhao et al. 2006; Chen and Wong 2008). There is little research into what causes selenium to be toxic and what results from the toxicity. Literature studying selenium usually investigates physiological levels and beneficial effects.

The few studies investigating selenium toxicity have found organic and inorganic forms of selenium to be toxic, but to varying degrees. For example, equivalent amounts of

selenomethionine and sodium selenite given to mallard ducks demonstrated that organic selenomethionine caused developmental defects whereas inorganic sodium selenite was lethal to embryos (Heinz and Hoffman 1996). Another study in post-implantation rat embryos found inorganic selenium to be fifty-times more toxic than organic selenium (Usami and Ohno 1996). Comparative toxicity in adult vertebrate animals found selenium from the selenium accumulating plant species *Astragalus bisulcatus* to be more toxic than inorganic selenium (sodium selenite) which was more toxic than organic selenium (selenomethionine) (Panter et al. 1996).

Additional reports of selenium toxicity come from accidental selenium poisoning in livestock, where selenium was found to be the cause of paralytic outbreaks. In one study, post-mortem analysis of affected animals identified skeletal muscle swelling and disintegration and vacuolation of the myocardium (Stowe et al. 1992). Damage to the gastrointestinal and hematopoietic systems has also been reported as a result of selenium toxicity in livestock (Raisbeck 2000). One pathological finding that the reports shared was that selenium toxicity caused neuronal loss, vacuolation, and gliosis in the spinal cord of affected animals, demonstrating that excess selenium acts as a neuronal toxin (Harrison et al. 1983; Casteel et al. 1985; Frank and Bergeland 1988; Stowe et al. 1992; Casteignau et al. 2006).

Selenium toxicity is becoming an increasing environmental problem, as it is a major waste product from fossil fuels and soil irrigation. Fossil fuels contain high concentrations of selenium. Crude oil can contain over 2g Se/L and refined oils contains up to 250µg Se/L that enters the atmosphere when burned (Lemly 2004). Excess environmental selenium resulted in reproductive failure and developmental defects in a variety of fish (including *Lepomis cyanellus*, *Pomoxis annularis*, *Micropterus salmoides*, *Gambusia affinis*, *Notropis latrensis*, and *Ictalurus punctatus*) at Belews, NC where waste from a coal burning plant was released into a nearby

water basin (Ohlendorf et al. 1986; Harris 1991; Lemly 1997). Selenium is an increasing concern in landfills, as selenium is being used more frequently in electronics due to its photosensitive and semi-conductive properties (Sharma 1983; Lemly 2004). In addition, areas of the western United States have high selenium in the soil due to shale deposits (Lemly 1997). As these areas are arid and require irrigation for crop production, irrigation results in selenium leaching from the shale into the ground water (Lemly 1997). Such practices resulted in poisoning of wildlife in Kesterson, CA (Ohlendorf et al. 1986; Harris 1991; Lemly 1997). With increasing amounts of selenium being released into the environment each year, more research is needed to understand the potential impact on the human population as selenium is known to be toxic and has been documented to act as a motor neuron toxin in other mammals (Casteel et al. 1985; Panter et al. 1996; Casteignau et al. 2006).

The genetic model *C. elegans* is a useful tool for dissecting the mechanisms of selenium toxicity. The worm provides a simple model to address potential genetic and environmental factors that may influence selenium toxicity and when culturing animals, there are environmental conditions that can be altered that can differentiate the phenotypes of mutant animals. Maintenance of *C. elegans* was first described by Sydney Brenner (Brenner 1974). For example, culturing some mutants at 25°C, a non-permissive temperature, instead of the permissive 20°C, can cause the mutants to enter an alternate developmental stage called dauer larva and wild-type animals have a decreased lifespan at 25°C (Klass 1977; Riddle 1977). One consequence of growing wild-type animals at increased temperatures is an increase in metabolic rate, subsequently increasing oxidative stress (Van Voorhies and Ward 1999).

Another factor to be considered when working with *C. elegans* is a potential role for bacterial metabolism from their *E. coli* food source, strain OP50. Bacterial metabolism has been

a limiting factor in drug screens since the bacteria metabolize the drug compound before it can reach the animal and affect lifespan or another observable phenotype (Kaletta and Hengartner 2006; Saiki et al. 2008). To address possible contributions of bacterial metabolism to selenium toxicity, chemically defined media can be used. The idea of culturing animals on/in chemically defined media has been around since Brenner's initial paper (Brenner 1974). However, until recent understanding of *C. elegans* carbohydrate requirements, culturing animals on chemically defined media would also stress the animals (Lu 1993; Szewczyk et al. 2003).

Calcium is another factor in *C. elegans* media that could potentially contribute to toxicity. Calcium is a second messenger signaling molecule and is an important molecule in regulating neurotransmission (Rasmussen et al. 1976; Reid et al. 2003). Increases in intracellular calcium have been implicated in models of stroke, and heart disease, among others (Braun 1983; Gelmers 1985). In *C. elegans*, calcium is normally added to the media in addition to other ions. Media without additional calcium has no observable phenotype on wild-type animals. However, in animals with mutated calcium channels, minimal calcium (where no additional calcium is added to the media) increases the observed mutant phenotypes (unpublished observations from our laboratory with *unc-2*). It is possible that the difference between no additional calcium and physiological calcium concentrations established by Brenner could increase selenium toxicity and subsequent cell death.

Until 1972, cell death was widely thought to be a passive process where cells would die when they were too damaged to function. The idea of "programmed cell death" grew out of developmental observations that specific cells would die during the course of development (Kerr et al. 1972). Currently, cell death is conceptually divided into two categories; necrosis and

programmed cell death. Both types of cell death are active areas of research in the *C. elegans* community and numerous genetic tools for studying these processes are readily available.

Necrosis is a cell death process characterized by cellular swelling leading to membrane rupture resulting in inflammation of surrounding tissues. Necrosis was once thought to be a passive process, but has now been shown to have specific molecular signaling components. Necrosis begins by cells receiving an insult like an influx of ions via the degenerin channel or calcium channel (Driscoll and Chalfie 1991; Bianchi et al. 2004). Subsequently, intracellular calcium levels increase, leading to calpain activation (Samara and Tavernarakis 2003; Bianchi et al. 2004). Activated calpain then proceeds to rupture the lysosome (Artal-Sanz et al. 2006). The interior of the lysosome is acidic and the pH is maintained by a V-ATPase (Syntichaki et al. 2005). Lysosomal rupture results in cellular acidification, releasing cathepsins that proceed to degrade various cellular components (Kourtis and Tavernarakis 2007).

In contrast, programmed cell death can be divided into 2 sub-categories; programmed cell death I (apoptosis) and programmed cell death II (autophagic cell death). Apoptosis is characterized by cellular shrinking, DNA degradation, and engulfment of cellular corpses without cytoplasmic contents "leaking" out and causing inflammation in neighboring cells (Savill and Fadok 2000; Nathan 2002). In *C. elegans*, apoptotic cells increase in refractility that is easily observed under Nomarski differential interference contract optics (Ellis and Horvitz 1986). The initial work in this field identified *ced-3* (CEll Death abnormal) as being involved in developmental programmed cell death (Ellis and Horvitz 1986). CED-3 was later found to be similar to mammalian interleukin1β-converting protein that acts as a cysteine protease, or caspase (Yuan et al. 1993). Subsequent work demonstrated that *ced-4* (*C. elegans* ortholog of mammalian APAF) could activate CED-3. Since then, work in programmed cell death has

exploded to identify other genes involved in the killing phase (ex: *egl-1* and *ced-9*) as well as genes involved in DNA degradation (*nuc-1* and *crn-6*) and corpse engulfment (*ced-1*, *ced-10*, etc.) (Conradt and Xue 2005).

Here, we demonstrate that both organic and inorganic selenium sources cause toxicity in *C. elegans*. Various environmental factors were found to affect toxicity, such as temperature and calcium levels. Bacterial metabolism was not required for selenium toxicity, but may be protective. Additionally, we have made use of genetic information relating to cell death mechanisms in *C. elegans* to attempt to define how selenium toxicity leads to death.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Strain maintenance and growth conditions

The following strains were obtained from the *Caenorhabditis* Genetics Center located in Minneapolis, MN; wild-type N2, MT1522: *ced-3(n717)IV*, MT2547: *ced-4(n1162)III*, MT4470: *ced-9(n1950)III*, TJ1: *cep-1(gk138)I*, KJ216: *crt-1(jh101)V*. Strains PJ1: *cad-1(j1)II* and PJ13: *cad-1(j013)II* were kind gifts from Lew Jacobson. With the exception of the experiment testing chemically defined media (2.4.4), animals were grown on standard nematode growth media (NGM) plates.

NGM plates were made as follows: 3g NaCl, 2.5g Bactopeptone, 17g agar to 975ml of de-ionized water. The media was autoclaved for 60 minutes. After autoclaving, the following reagents were added (in order), swirling the flasks after each addition: 1ml of nystatin (0.446g of nystatin in 28ml EtOH and 4ml $H_2O$), 1ml cholesterol (5mg/ml in EtOH), 1ml 1M $MgSO_4$, 25ml of 1M potassium phosphate buffer (pH 6.0). For the experiments addressing altered sensitivity to selenium with calcium, 1ml of 1M $CaCl_2$ was added to media after the cholesterol and before the magnesium, for a final concentration of 1mM (Brenner 1974).

After the plates were poured (10ml for small plates and 30ml for large plates) and solidified, the uracil auxotrophic *E. coli* strain OP50 was added as a food source. Animals were maintained and experiments performed at 20°C unless otherwise stated.

### 2.3.2 Developmental synchronization

Developmental synchronization of *C. elegans* is required to remove potential variability of age from affecting relative selenium sensitivity. Developmental synchronization was obtained in one of 2 ways.

1) Hypochlorite/NaOH (Bleach Method): Plates containing hundreds of gravid adult hermaphrodites were washed with de-ionized water into 15ml conicals. The wash, with worms, was brought to 8.5ml. 1ml of bleach and 500ul of 5N NaOH was added to the tube and capped. The tube was shaken vigorously for at least 30 seconds then gently shaken for a minimal total time of 5 minutes. The shaking times were extended to account for a larger number of worms or microbial contamination to a maximum time of 8 minutes. Prolonged exposure to NaOH/bleach solution was avoided because it compromises egg viability. At that time, tube(s) were placed into a swinging bucket centrifuge for 2 minutes at 2000rpm. The supernatant was aspirated to waste leaving the eggs and adult debris as a pellet at the bottom of the tube. The NaOH/bleach solution was washed away through 3-4 washes of autoclaved de-ionized water (washes were of an equal volume as the NaOH/bleach solution). After the final wash, 3-4ml of M9 buffer (6g $Na_2HPO_4$, 3g $KH_2PO_4$, 5g NaCl, 1ml of 1M $MgSO_4$ per 1 L) was added. The tube(s) were then placed overnight on a shaker with gently agitation and with the caps slightly agar (1/4 of a turn) to allow for ventilation. The hatched eggs developmentally arrest in the first larval stage (L1). The next day, the hatched animals were plated (Stiernagle 2006).

2) Alternatively, developmentally synchronized worms were identified and transferred in the final larval stage (L4) to a new plate. L4 stage animals are easily recognizable with a white crescent in the posterior third of the body that contains a dark spot in the center of crescent

(Wood 1988). This method requires manual transfer of each animal to a new plate. As a result, it is used when fewer animals are required.

### 2.3.3 Selenium treatment

Initially, seleno-L-methionine (SeMet) was purchased from both Spectrum Chemical (Gardena, CA, USA) and later from Sigma-Aldrich (St. Louis, MO, USA). Seleno-L-methionine plates were made by dissolving SeMet in de-ionized water and adding to plates to achieve the desired concentration for each experiment. Sodium selenite, purchased from Spectrum Chemical (Gardena, CA, USA), was made into a 100mM stock solution in de-ionized water. An appropriate volume was added to plates immediately before use to obtain the desired final concentration. The sodium selenite solution was stored at 4°C for no more than 30 days.

### 2.3.4 Movement assay for selenium damage

A behavioral assay was developed in order to measure/monitor selenium damage. In normal, untreated *C. elegans*, an animal move forward with sinusoidal body turns when touched on the tail with a platinum wire (considered a "harsh" touch compared to a "soft" touch with an eyelash). When an animal is moving forward and tapped on the head, it will reverse direction. Exposure to selenium causes several movement deficits. In our initial studies (inorganic versus organic selenium and SeMet not working), we scored movement as "normal", paralyzed (i.e. failure to complete one sinusoidal turn of their body with in five seconds of tapping the tail), or dead (i.e. lack pharyngeal pumping).

Subsequent observation of animals noted an additional more subtle movement deficit resulting from selenium exposure, a failure to reverse direction. An animal will move forward in a sinusoidal motion when touched on the tail. But when the head is tapped to induce it to reverse direction, the animal pauses and continues forward. When scoring for the backing deficit, an animal had to fail this test twice to be scored as having impaired backing.

From Figure 3 on, the three movement deficits, movement, paralyzed, and death are grouped together as being damaged. The Y-axis for this assay contains the percent of the population of animals with normal movement behavior. Animals were considered "percent (%) motile" if they were able to complete one sinusoidal wave backward or forward within 5 seconds after tactile stimulation to the head or tail, respectively.

If any animals crawled onto the side of the plate, they were not scored or included in the final count because we could not tell if the animals died due to selenium exposure or desiccation. When calculating the percent of the population that was motile, the animals on the plate were scored as described. The population number was then adjusted to reflect the number of animals on the plate. Calculation for percent of population normal:

(# of normal animals/# of animals on plate) *100= percent motile

### 2.3.5 Treatment with weak bases to protect from necrotic death

Treatment with weak bases, like acridine orange and ammonium chloride, partially protect against necrotic cell death (Artal-Sanz et al. 2006). Water soluble acridine orange was obtained from Molecular Probes-Invitrogen (Carlsbad, CA, cat. # A3568). Ammonium chloride was purchased from Sigma-Aldrich (St. Louis, MO, cat # 9434). Concentrations of ammonium chloride and acridine orange were obtained from a previous publication (Artal-Sanz et al. 2006).

Ammonium chloride ($NH_4Cl$) was added to selenium treated and untreated NGM plates to a final concentration of 5mM. Acridine orange was added to selenium treated and untreated plated for final concentrations of 50μM, 100μM, and 150uM. Animals were scored for movement deficits as described in 2.3.4.

### 2.3.6 Reactive Oxygen Species (ROS) detection

Developmentally synchronous N2 animals were obtained as described in Section 2.3.2 and grown to young adults without being allowed to starve. To detect changes in ROS levels, a non-fluorescent dye was used that fluoresces when oxidized. The oxidation sensitive dye, Carboxy-H2DCFDA [5-(and-6)-carboxy-2', 7' dichlorodihydrofluorescein diacetate], was purchased from Molecular Probes-Invitrogen (Carlsbad CA, cat # C-400). Animals were plated on untreated or 5mM sodium selenite treated plates in the presences and absence of 50μM carboxy-H2DCFDA dissolved in 100% ethanol (Smith and Luo 2003).

*For imaging:* At one hour intervals after plating, 10-15 animals were removed to fresh plates for each condition (with no carboxy-H2DCFDA or sodium selenite) and allowed to clear their intestine, since the carboxy-H2DCFDA also causes the bacteria to fluoresce. Individual animals were analyzed and documented by visualization on a Leica DM IRB microscope equipped with fluorescence optics and a DC300F imaging system (Leica Microsystems Inc., Bannockburn, IL). The images were processed for publication using the Adobe Creative Suite 2 premium software package (Adobe Systems Inc., San Jose, CA).

*For quantification:* Animals were treated for 6 hours with 5mM sodium selenite and 50μM carboxy-H2DCFDA or with 50μM carboxy-H2DCFDA. Animals were washed off the plates in M9 buffer into 15ml conicals. Animals were washed three times with M9 buffer,

allowing the animals to sediment and removing the supernatant between each wash. Animals were analyzed using the COPAS Biosorter (Union Biometrica, Holliston MA) using a fluorescein filter. Theresholds were set to eliminate non-adult animals (by size) and background fluorescence. The Biosorter then measured the percentage of animals in the populations that were positive for green fluorescence.

### 2.3.7 Antioxidant treatments

Several antioxidants were tested to determine if they protected selenium induced movement impairment. Reduced glutathione (G4251, Sigma-Aldrich, St Louis, MO) was re-suspended in de-ionized water and used at 200µg/ml. Quercetin dihydrate (cat # 32782, Riedel-de Haën, Seelze, Germany) was re-suspended in 1N NaOH and used at 800ug/ml. α-tocopherol or Vitamin E (T3376, Sigma-Aldrich, St Louis, MO) was dissolved in 100% ethanol and tested at 200µg/ml. Plates without antioxidants (contained 1N NaOH for quercetin or ethanol equivalent for α-tocopherol) were tested simultaneously as controls. Animals were scored for movement deficits as described in Section 2.3.4.

### 2.3.8 Statistical analysis

Statistical analysis was performed using Microsoft Excel software (Microsoft Corporation, Redmond, WA). The means and standard deviations (SD) reported were determined by averaging data obtained from all the plates of each strain or population type (eg. treated or untreated) counted. P values were determined by applying a one-tailed Student's t-test with unequal variance. A one-tailed test was chosen since normal motility was being calculated

and each experiment started with a fixed population number, subsequently, a fixed number for normal motility. Therefore, deviations from normal motility could only go in one direction. Unequal variance was used instead of equal variance because it was more stringent for calculating P values. Comparisons were defined as significant if they were determined to have a p value less than 0.05. Graphs were initially drawn using Excel and were prepared for the dissertation using Adobe Illustrator CS2 (Adobe Systems Inc., San Jose, Ca.).

## 2.4 RESULTS

### 2.4.1 Organic and inorganic selenium cause a dose dependent effect in wild-type *C. elegans*

Selenium is found in a variety of forms in the environment. But ingested selenium is eventually metabolically incorporated into an organic form as either selenomethionine or selenocysteine (Behne and Kyriakopoulos 2001). Many literature reports have found all forms of selenium to be toxic but at varying concentrations (Panter et al. 1996; Kim and Mahan 2001; Nuttall 2006). However, it has also been repeatedly reported that inorganic forms of selenium (sodium selenite) are more toxic to adult animals than organic forms of selenium (Panter et al. 1996; Usami and Ohno 1996; Kim and Mahan 2001). Selenium has also been implicated in as a causative a causative agent in paralytic outbreaks in swine (Panter et al. 1996; Casteignau et al. 2006). We hypothesized that selenium toxicity in *C. elegans* could be monitored by observing the emergence of paralysis and subsequent death after exposure to selenium. To determine whether both organic and inorganic forms of selenium cause toxicity in *C. elegans*, developmentally synchronized adult N2 hermaphrodites were placed on NGM plates treated with increasing concentrations of organic selenium (seleno-L-methionine or SeMet), and an inorganic form of selenium (sodium selenite or NaSe). Animals were scored at 24-hour intervals for survival compared to paralysis and death (Figure 1).

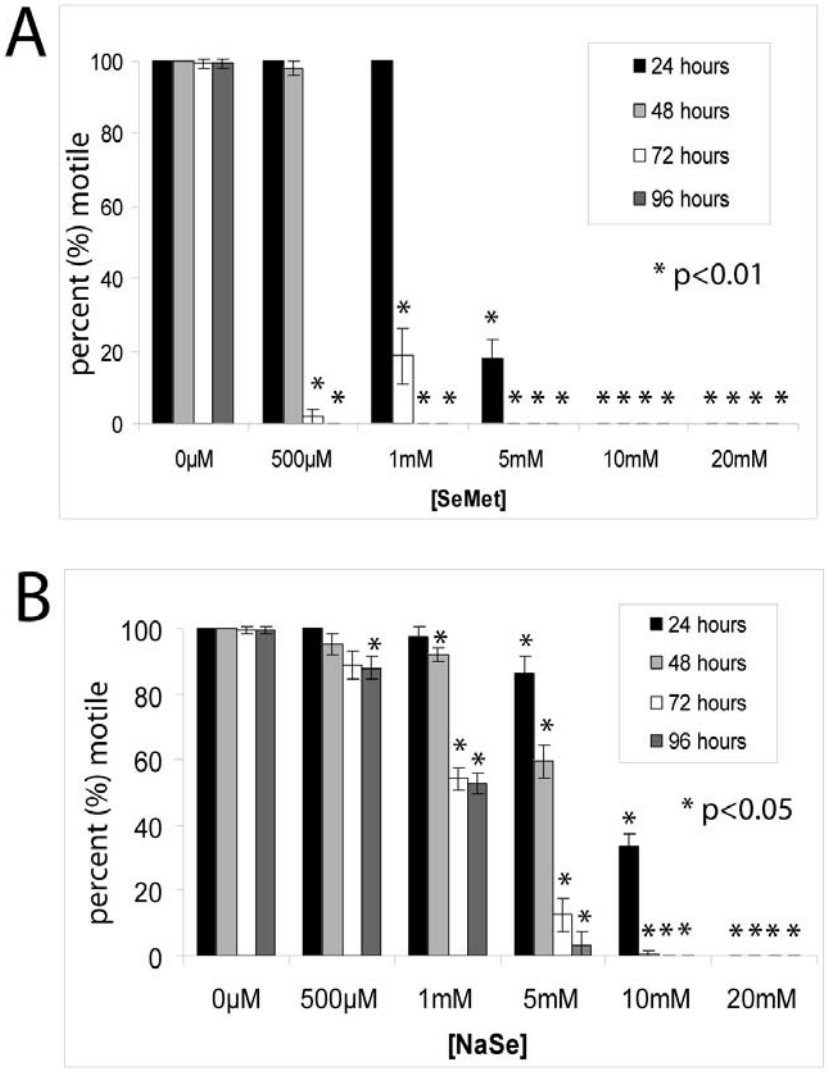



**Figure 1 Comparative selenium toxicity between seleno-L-methionine and sodium selenite**

Both organic selenium (**A**-seleno-L-methionine or SeMet) and inorganic selenium (**B**-sodium selenite or NaSe) caused a dose dependent increase in paralysis and mortality over time. Fifty developmentally synchronized adult N2 hermaphrodites were placed on standard NGM plates treated with increasing concentrations of an organic selenium source, SeMet (A), or an inorganic selenium source, NaSe (B). Animals were scored for normal movement versus paralysis and death at 24 hour intervals. Statistically analysis was performed using a one-tailed student's t-test with unequal variance as described in Materials and Methods Section 2.3.9 comparing each concentration (SeMet or NaSe) to untreated (0μM) animals. The graph above is the average of 3 populations (n=150). The experiment was repeated 3 times with similar results.

Both organic and inorganic forms of selenium were found to have a dose-dependent toxic effect over time on the paralysis and mortality of wild-type N2 animals (Figure 1A and 1B). Exposure to 500μM seleno-L-methionine maintained a greater than 90% normal motility over the first 48 hours of SeMet exposure with a significant decrease in normal motility at 72 hours when compared to untreated animals (Figure 1A, $p<0.01$). At 500μM of sodium selenite, greater than 80% of normal motility was observed after 96 hours of exposure; this is a significant decrease when compared to non-selenium treated animals (Figure 1B, $p<0.05$). This demonstrates that both organic and inorganic forms of selenium are toxic in *C. elegans* as in vertebrates. Comparing equivalent concentrations of organic and inorganic selenium, organic selenium would appear to be more toxic in this system (Figure 1A and 1B→10mM $p<0.005$, not indicated in Figure 1). This is different from previously published reports of comparative selenium toxicity which have generally found that selenomethionine to be less toxic than sodium selenite (Panter et al. 1996; Usami and Ohno 1996; Kim and Mahan 2001). It is possible that this difference in toxicity reflects differences in absorption of the two forms in *C. elegans*. For example, SeMet is better absorbed in mammalian systems and may more efficiently reach physiological toxic levels in the nematode (Panter et al. 1996).

A problem with these experiments was the variably severity of the toxicity even when using the same batch of SeMet. For example, there would be only 2% survival after 72 hours of 500μM seleno-l-methionine exposure (Figure 1A). But a repeat experiment would show a 60-80% survival on 500μM seleno-l-methionine after the same exposure (Figure 2-Spectrum). Variability was observed both from lot to lot and from day to day, although it is unknown if the stability of SeMet was an issue. This led us to test seleno-L-methionine from multiple suppliers to find a more stable source. As shown in Figure 2, animals still had a greater than 50% normal

motility rate after 72 hours of selenium exposure from two different sources despite earlier experiments that demonstrated nearly 100% mortality at this time point. While all sources of SeMet induced statistically significant mortality and paralysis compared to untreated animals ($p<0.05$), no source of SeMet was found that gave reproducible toxicity.

Methodological testing of various potential factors including temperature, age of plates, age of food source, and remaking/replacing each plate component, also failed to identify the source of this variability (data not shown). Subsequent experiments use 5mM sodium selenite as the selenium source due to the reproducible steady decline in movement over 96 hours at that concentration (Figure 1B).

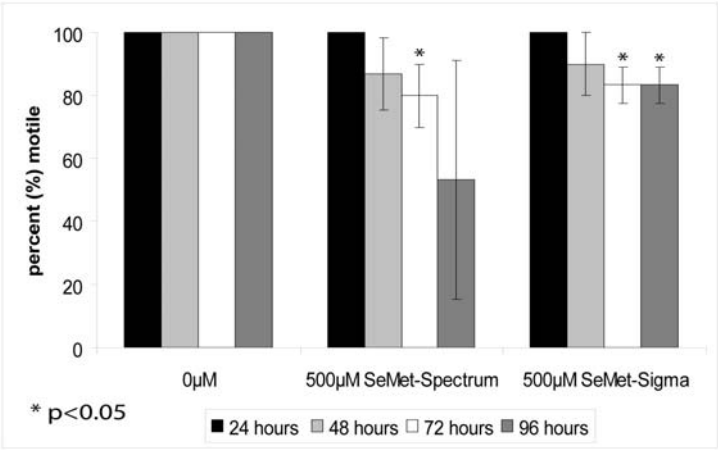



**Figure 2 Seleno-L-methionine variability**

Organic selenium from two sources (Spectrum Chemical and Sigma-Aldrich) was variable as to the severity of toxicity. Fifty developmentally synchronized N2 hermaphrodites were placed on standard NGM plates treated with 500µM seleno-l-methionine from either Spectrum Chemical or Sigma-Aldrich. Animals were scored at 24 hour intervals normal movement compared to paralysis and death. Statistically analysis was performed using a one-tailed student's t-test with unequal variance as described in Materials and Methods Section 2.3.9 by comparing selenium treated animals to untreated animals at the corresponding time points. The graph above is a compilation of 3 populations (n=150).

### 2.4.2 Selenium causes progressive movement problems

Observation of selenium-treated animals resulted in the identification of an additional movement deficit that was not seen in the controls. In addition to high selenium leading to paralysis and death, animals displayed a backing problem. Animals would move forward normally when touched on the tail but would fail to reverse direction when tapped on the head. There also appeared to be a progressive order of these behaviors. The number of individual animals that were observed to have a backing problem at one time point was similar the number of individual animals that were paralyzed at the next time point. Similarly, the number of individual animals that were observed to be paralyzed one day would be dead the next day. Because of this correlation, we hypothesized that there was a progressive order to these behaviors. Initially after selenium exposure, animals behaved as untreated animals, moving forward and reversing directions upon tapping the tail and head, respectively. After some time, animals would fail to reverse direction when touched on the head after they had been stimulated to move forward by a touch to the tail. Eventually, animals would slow or fail to respond to the touch (paralyzed) but were still alive as determined by the pharyngeal bulb pumping. Lack of pharyngeal bulb pumping was used to determine that the animal had died.

To determine whether the observed behaviors were progressive in response to exposure to selenium, single age-synchronous adult animals were scored for movement deficits at 6 hour intervals after being placed on NGM plates treated with 5mM sodium selenite. Table 1 demonstrates that the observed movement deficits, abnormal backing, paralysis, and death, occurred in a stereotypic order, suggesting they represent progressive nervous system injury. For example, in Table 1, Animal 3 behaved normally for 24 hours. Six hours later, the animal displayed backing problems and 6 additional hours after that the animal became paralyzed, and

six hours after that, the animal was dead. Animal 10 displayed a much different temporal development of movement deficits on selenium. The animal behaved normally for only the first 6 hours. Afterwards, the animal displayed a backing problem for the remainder of the assay. However, not all animals developed backing deficits within the time points observed. For example, Animal 11 never displayed the backing problem (or it progressed too rapidly to be observed at the set time points for this assay) and progressed to paralysis and death during the observed 6 hour intervals. The variability in the timetable in which animals developed scoreable movement deficits led us to represent selenium induced injury as the number of animals remaining fully motile. These studies demonstrate that even within a developmentally synchronized, clonal population of animals, individual animals progress to more severe deficits at different rates when exposed to high levels of selenium.

**Table 1 Progressive movement deficits observed after selenium exposure**

Selenium exposure causes a progressive sequence of movement deficits. Twenty wild-type N2 hermaphrodites were placed on individual NGM plates and scored as normal (**N**) forward and backward movement in response to touch; failure to back (**B**) in response to touch; paralysis (**P**) the failure to complete one sinusoidal turn with in 5 seconds of being touched, and death (**D**) as failure to respond to touch and lack of pharyngeal pumping at 6 hour intervals. Untreated wild type animals do not display this progression of behavioral deficits (data not shown).

| Animal # | 6hr | 12hr | 18hr | 24hr | 30hr | 36hr | 42hr | 48hr |
|---|---|---|---|---|---|---|---|---|
| 1 | N | N | N | N | N | B | B | P |
| 2 | N | N | N | N | B | B | B | B |
| 3 | N | N | N | N | B | P | D | D |
| 4 | N | N | B | B | B | B | B | B |
| 5 | N | N | B | B | B | B | B | B |
| 6 | N | N | B | B | B | B | P | P |
| 7 | N | N | B | B | B | P | P | P |
| 8 | N | N | B | B | B | P | D | D |
| 9 | N | N | B | B | B | D | D | D |
| 10 | N | B | B | B | B | B | B | B |
| 11 | N | P | D | D | D | D | D | D |
| 12 | B | B | B | P | P | P | P | P |
| 13 | B | P | D | D | D | D | D | D |
| 14 | B | P | D | D | D | D | D | D |
| 15 | P | D | D | D | D | D | D | D |
| 16 | P | D | D | D | D | D | D | D |
| 17 | P | D | D | D | D | D | D | D |
| 18 | P | D | D | D | D | D | D | D |
| 19 | P | D | D | D | D | D | D | D |
| 20 | D | D | D | D | D | D | D | D |

The observed movement deficits also suggest that selenium is either damaging the nervous system or causing muscle damage sufficient to impair movement, or both. Part of our movement assay mirrors a previously described assay used to map the mechanosensory circuit in *C. elegans* (Kaplan and Horvitz 1993). In their assay, a "soft" touch using an eyelash to touch on the head to reverse was used as a behavioral assay for investigating which ciliated neurons were involved in avoidance. Genetic mutants and laser ablation of individual neurons identified as ASH, FLP, and OLQ as being necessary for "soft" touch avoidance (Kaplan and Horvitz 1993).

Similar assays have been used to determine the specific neurons required for backing. Forward and backing movement in response to touch involves sensory neurons, interneurons, and motor neurons (Wicks and Rankin 1995). Using a platinum wire to deliver a "harsh" touch, laser ablation studies have shown PVD neurons to be involved in backing (Way and Chalfie 1989; Ernstrom and Chalfie 2002). Therefore, the current understanding of the regulation of backward movement suggests that backing deficits induced by selenium exposure are due to neurons being damaged.

The slow progression of movement dysfunction (i.e., in worm life-span terms given the fact that they go from egg to adult in 3.5 days and live 2 weeks in well-fed conditions) from backing to the observed paralysis and eventual death suggests that cellular damage accumulates over time (Brenner 1974). This notion of progressive, cumulative neuronal damage leading to movement deficits mirrors the disease course of the motor neuron disease ALS. Intriguingly, an epidemiologic study has shown that long-term exposure to selenium in the drinking water is associated with an increased risk of sporadic ALS, suggesting that selenium could be one environmental risk factor for developing this disease (Vinceti et al. 1996; Cleveland and Rothstein 2001).

### 2.4.3 Temperature alters *C. elegans* sensitivity to selenium

*C. elegans* can be grown at different temperatures, with the most common temperatures being 20°C and 25°C. Twenty-degrees Celsius is considered to be a permissive temperature, at which a majority of experiments reported in the literature are performed, while twenty-five degrees Celsius is considered as a non-permissive and is used to induce metabolic stress. Wild-type animals grow faster, have a shorter lifespan, and have a smaller brood size at the non-permissive

temperature (Klass 1977). Certain mutant phenotypes are enhanced or only expressed at the higher temperature. For example, animals with the reduction-of-function mutation in the gene encoding an insulin-like receptor, *daf-2*, enter an alternate development larval stage when grown at a non-permissive temperature (Riddle 1977). Although the mechanisms by which higher temperature cause these phenotypic changes are likely complex, increased metabolism resulting from increased oxidative stress may be one of its most important factors.

Selenium is also known to cause oxidative stress (Seko and Imura 1997; Shen et al. 1999; Verma et al. 2004). We have found that increased temperature enhanced selenium toxicity in *C. elegans* roughly three-fold (Figure 3, $p<0.001$). Given the pro-oxidative affects of excess selenium and the increased temperature in *C. elegans*, our results suggest that selenium induced movement deficits are caused by oxidative damage. If true, the decreased survival at higher temperature may be due to an increase in damage from oxidative stress. Consistent with this hypothesis, mutations in DAF-2 have been shown to control a large number of genes, including antioxidant and antimicrobial proteins (McElwee et al. 2003; Murphy et al. 2003; Oh et al. 2006). *C. elegans* lifespan in wild-type animals can be extended by treatment with superoxide dismutase and catalase mimetics (Sampayo et al. 2003). Similarly, co-enzyme Q10 has been shown to extend lifespan by lowering oxidative stress and quercetin, a component of *Gingko biloba* leaf extract, has also been shown to extend lifespan in *C. elegans* by reducing oxidative stress (Ishii et al. 2004; Kampkotter et al. 2008).

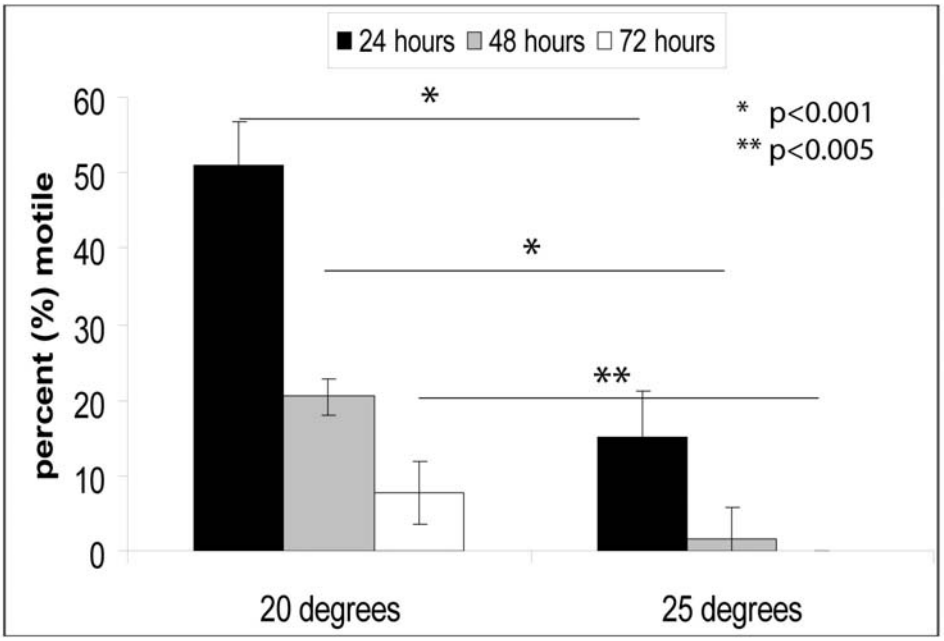



**Figure 3 Increased temperature accelerates movement deficits caused by selenium toxicity**

Increasing the temperature to non-permissive conditions accelerates the movement deficits caused by selenium toxicity. N2 animals were grown at 20°C and 25°C on NGM plates. Twenty developmentally synchronized N2 hermaphrodites were placed on NGM plates treated with 5mM sodium selenite as adults and returned to either 20°C or 25°C. Animals were scored at 24 hour intervals for movement deficits as described in Section 2.3.4. Statistical analysis was performed as described in Section 2.3.9 comparing selenium treated animals at 20°C to 25°C. Animals grown and treated with selenium at 25°C were significantly more sensitive to selenium at all three time points ($p<0.001$ at 24 and 48 hours, $p<0.005$ at 72 hours). The graph above represents 6 plates with 20 animals per plate (n=120). Untreated animals at 20°C and 25°C had a greater than 90% normal motility at 72 hours (data not shown).

### 2.4.4 Selenium toxicity does not require bacterial metabolism (in collaboration with Nate Szewczyk)

In their natural habitat, the soil, *C. elegans* ingest a variety of bacteria. However, under laboratory conditions, the *C. elegans* food source is an auxotrophic strain of *E. coli*, OP50 (Brenner 1974). While this contributes to the ease of culturing animals, it has also caused problems. Bacterial metabolism has been a limiting factor in using *C. elegans* for drug screens due to the bacteria metabolizing the drug before it can reach the animals (Kaletta and Hengartner 2006). A recently developed chemically defined liquid medium known as *C. elegans* Maintenance Medium (CeMM) provided us with a method of addressing the role, if any, of bacterial metabolism on selenium toxicity (Szewczyk et al. 2003).

Essentially, if bacterial metabolism contributes to selenium toxicity by metabolizing sodium selenite into by-products that are the real toxic factor, selenium should be non-toxic to *C. elegans* in CeMM. We worked with a collaborator using CeMM to address the role of bacterial metabolism in selenium toxicity (Szewczyk et al. 2003). Four populations of one hundred animals were grown in either normal CeMM or CeMM supplemented with 5mM sodium selenite. Looking at survival only, sodium selenite treated animals had a 29.75%±11.81 survival after 24 hours where the untreated controls had a 100% survival ($p<0.001$) demonstrating that bacterial metabolism is not required for selenium toxicity in *C. elegans*.

### 2.4.5 Calcium increases selenium toxicity

Calcium has been shown to be a central cellular signal involved in cell death induction in vertebrate models. Multiple lines of evidence have demonstrated that induction of cell death is closely linked to the regulation of intracellular calcium ($Ca^{2+}$) levels in the vertebrate nervous system. For example, in the presence of apoptotic stimuli, calcium influx through the IP3 and ryanodine receptors can activate of calpain, calcineurin, and subsequent activation of apoptosis (Hajnoczky et al. 2000).

To determine whether calcium levels might play a role in the toxicity of selenium in *C. elegans*, developmentally synchronized N2 populations were grown on standard NGM (with a final $Ca^{2+}$ concentration of 1mM) plates and minimal $Ca^{2+}$ plates (without an additional $Ca^{2+}$). Animals were transferred as adults to plates containing the equivalent calcium concentration that they were grown on, with or without 5mM NaSe (i.e., the concentration of NaSe that leads to approximately 50% paralysis over 48 hours). Growth and movement of non-selenium treated animals on NGM plated with and without calcium supplementation did not lead to any significant movement deficits over the 4 day interval of the experiment. However, selenium treated animals that were grown on NGM plates containing 1mM calcium showed a 2-fold increase in the emergence of movement deficits (Figure 4). By 48 hours of selenium treatment, significantly more animals had movement deficits on calcium plates (Figure 4, $p<0.05$), a trend that became more prominent by the 72 hour time point (Figure 4, $p<0.005$). Also, NGM plates contain 25mM phosphate, which chelates free $Ca^{2+}$, making the actual concentration of $Ca^{2+}$ well below 1mM. Even with such low levels, there was a detectable difference in an animal's sensitivity to selenium when they were grown on the different $Ca^{2+}$ concentrations. Given the relationship of selenium and motor neuron disease and the increased calcium observed in motor

neurons of ALS patients, it is possible that evolutionarily conserved cell toxicity mechanisms are related to the movement deficits induced by selenium exposure in *C. elegans* (Siklos et al. 1996).

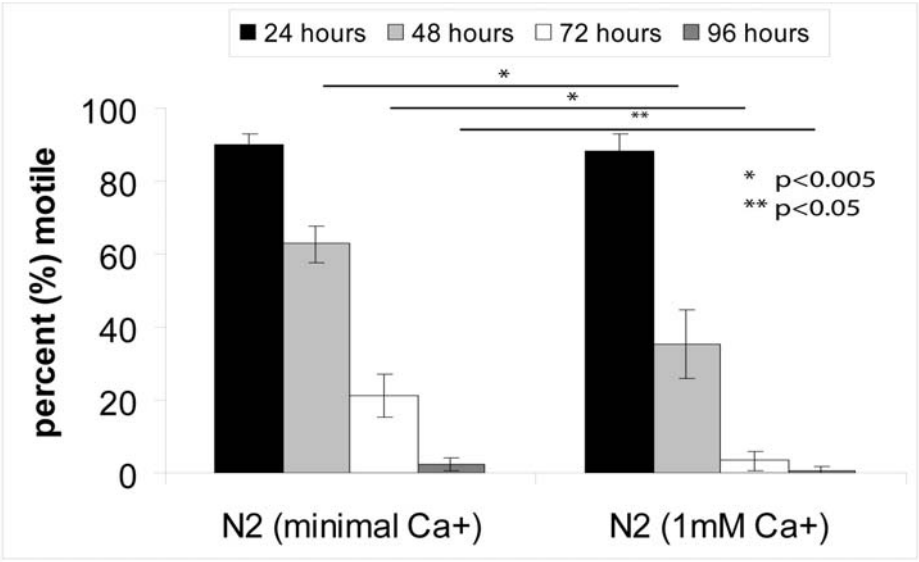



**Figure 4 Calcium enhances selenium toxicity**

Increased calcium levels increase the rate at which movement deficits were observed after treatment with selenium. No difference in survival was observed at 24 hours. Significant differences in survival were observed at the remaining time points. (48hours: $p<0.005$, 72hours: $p<0.005$, 96hours $p<0.05$). Developmentally synchronous wild-type N2 hermaphrodites were placed on NGM plates containing minimal calcium or 1mM calcium (1mM Ca+) in addition to 5mM sodium selenite. Animals were scored at 24 hour intervals for movement deficits as described in Section 2.3.4. Statistical analysis was performed using a one-tailed student's t-test with unequal variance as described in Section 2.3.9, comparing minimal calcium to 1mM calcium at the corresponding time points. Significance is indicated on the graph as * ($p<0.005$) or ** ($p<0.05$). This graph represents six populations of 20 animals per population (n=120). Non-selenium treated animals had a greater that 90% normal movement at 96 hours (data not shown).

### 2.4.6 Cell death mutants are not resistant to selenium exposure

Programmed cell death I, or apoptosis, was first identified in *C. elegans* by identifying mutants that prevented death of cells that normally die during development (Ellis and Horvitz 1986). The *C. elegans* ortholog of mammalian caspase, *ced-3,* and the ortholog of mammalian APAF (Apoptotic Protease Activating Factor), *ced-4,* are responsible for the killing phase of apoptosis, and loss-of-function in either of these genes results in survival of cells that would normally undergo developmental apoptosis (Ellis and Horvitz 1986; Yuan and Horvitz 1992). *ced-9,* the *C. elegans* ortholog of mammalian BCL-2, inhibits cell death and the loss-of-function of *ced-9* causes embryonic lethality (Hengartner et al. 1992). These three genes have been shown to act in a simple pathway, where *ced-9* is upstream of *ced-4*, inhibiting cell death (Hengartner et al. 1992; Conradt and Xue 2005). *ced-3* is downstream of *ced-4*, both promoting developmental cell death (Yuan and Horvitz 1992; Conradt and Xue 2005). Yet another player in cell death regulation is *cep-1*, the *C. elegans* ortholog of p53, which is a tumor suppressor that has been shown to be involved in DNA repair and apoptosis. Loss-of-function of the gene results in animals that are sensitive to hypoxia and have decreased longevity as a result of starvation-induced stress (Derry et al. 2001).

To test this hypothesis that programmed cell death is responsible for the movement deficits and organismal death caused by selenium exposure, three strains containing mutations in genes involved in the final stage of apoptotic cell death *ced-3(n717)*, *ced-4(n1142)*, *ced-9(n1950)* were tested for altered resistance/sensitivity to 5mM sodium selenite. The *ced-3* and *ced-4* mutants tested are loss-of-function mutants, and the *ced-9(n1950)* contains a point mutation that results in a gain-of-function phenotype (i.e. the mutation results in increased survival of cells that would normally undergo apoptosis) (Ellis and Horvitz 1986; Yuan and Horvitz 1992; Hengartner

and Horvitz 1994). Interestingly, animals containing mutations for apoptotic genes were found to have significantly increased sensitivity to high selenium relative to N2 (*ced-3*, *ced-4*, and *ced-9*, in Figure 5, $p<0.001$ by a student's t-test comparison of the percentage of the population without movement deficits in N2 versus cell death mutant strain). Similarly, *cep-1(gk138)* was also found to be more sensitive (Figure 5, $p<0.01$ at 24 hours). This suggests that apoptosis that was defined by developmental cell death is not involved in cell death caused by selenium.

Since developmental apoptosis does not appear to be involved in selenium toxicity, we then hypothesized that necrosis could be involved. Expression of the *C. elegans* ortholog of calreticulin, *crt-1*, is induced by stress and CRT-1 has been shown to bind to calcium and suppress heat induced protein aggregation. Reduction-of-function mutants of calreticulin in *C. elegans* have been shown to block necrosis (Smith 1992; Park et al. 2001; Xu et al. 2001). Wild-type animals treated with NaSe develop movement deficits that progressively proceed to paralysis and eventual death. Since these movement deficits suggest loss of neurons, we hypothesize that necrosis is induced by selenium exposure.

One of the final steps in necrosis involves release of cathepsin D from the lysosome, which contributes to cellular degradation leading to cell death (Kourtis and Tavernarakis 2007). If a cell was dying by necrosis, decreased cathepsin D expression should result in increased survival. However, animals that contained mutations that caused reduced cathepsin D expression also had significantly increased sensitivity to selenium (Figure 5, *cad-1(j1)* and *cad-1(j13)*, $p<0.001$ at 24 hours). Null mutations of calreticulin have been shown to block necrosis induced by the degeneration mutation (Xu et al. 2001). The reduction-of-function mutant for calreticulin (*crt-1*) was also found to be sensitive to selenium (Figure 5, $p<0.001$ at 24 hours). These data

suggests that the canonical versions of cell death are not involved in death from selenium exposure.

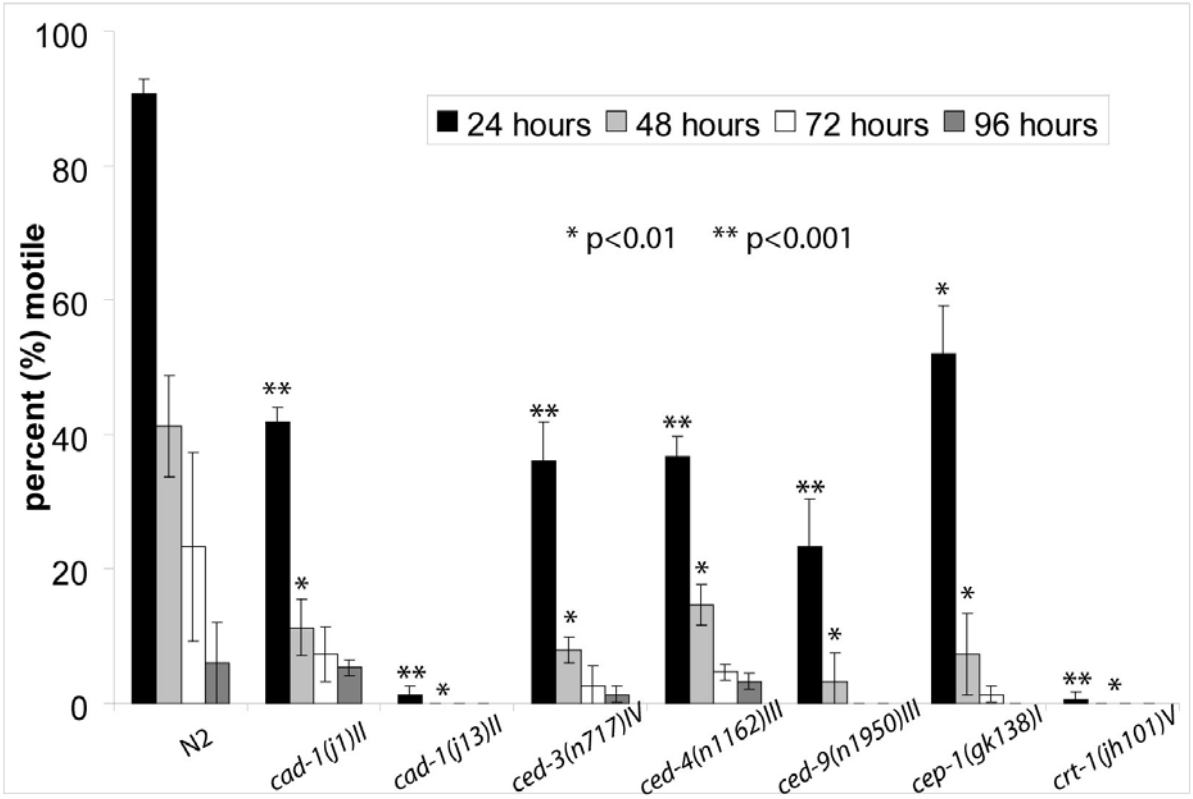



**Figure 5 Animals containing mutations for apoptotic and necrotic cascades have increased sensitivity to selenium.**

Animals containing mutations in genes involved in cell death cascades are not resistant to selenium. Mutants tested included; *cad-1(j1)* which has 90% reduced Cathepsin D expression, *cad-1(j13)* which had 30% reduced cathepsin D expression, *ced-3(n717)* an ortholog of mammalian caspase, *ced-4(n1162)* an ortholog of mammalian APAF, *ced-9(n1950)* an ortholog of mammalian BCL-2, *cep-1(gk138)* an ortholog of p53, and *crt-1(jh101)* an ortholog of calreticulin. All mutants were found to have a statistically increased sensitivity to selenium at the 24 hour and 48 hour time points. Twenty developmentally synchronized adult hermaphrodites were placed on NGM plates treated with 5mM sodium selenite and scored at 24 hour intervals for movement deficits described in Section 2.3.4. Statistical analysis was performed using a one-tailed students T-test with unequal variance as described in Section 2.3.9 comparing mutants strains treated

with selenium to wild-type populations (N2). Significance was indicated on the graph as * ($p<0.01$) or ** ($p<0.001$). The graph above is the average of three populations of 20 animals per population (n=60). The experiment was repeated three times with similar results. Non-selenium treated animals had greater than 90% normal movement after 96 hours (data not shown).

### 2.4.7 Weak bases do not protect wild-type animals from selenium damage

Necrosis, a type of cell death, is characterized by cellular swelling and subsequent membrane rupture and secondary inflammation. Cells undergoing necrosis cell develop decreased cytoplasmic pH, demonstrating that the pH shift is integral to necrotic death. Consistent with this, reduction-of-function mutations in a vacuolar $H^+$-ATPase that regulates acidification of the intracellular compartment protects against necrosis induced by the degeneration cascade (Syntichaki et al. 2005). Similarly, increasing intracellular pH by utilizing lysotropic bases such as ammonium chloride and acridine orange can alleviate necrotic cell death in the same neurodegeneration model (Artal-Sanz et al. 2006).

To determine whether necrosis is involved in wild-type N2 animals dying from selenium exposure, animals were also exposed to weak bases to prevent cellular acidification. Animals were simultaneously exposed to 5mM sodium selenite and 5mM ammonium chloride and 50μM, 100μM, and 150μM acridine orange, concentrations that were found to suppress MEC-4(d) degeneration in *C. elegans* (Artal-Sanz et al. 2006). Animals scored at 24 hour intervals for the movement deficits described in Section 2.3.4 demonstrated that $NH_4Cl$ and acridine orange conferred no protection against the selenium-induced movement deficits and mortality (Figure 6, $p>0.05$ as determined by a student's t-test comparing the percent of the population that did not exhibit movement deficits with and without weak bases).

The motility of animals exposed to 5mM ammonium chloride, a concentration that was found to suppress MEC-4(d) degeneration in *C. elegans,* in addition to selenium were found to be statistically the same as animals treated only with selenium at all time points (Figure 6, $p>0.05$) (Artal-Sanz et al. 2006). Similarly, the motility of animals treated with selenium and 50μM acridine orange were also not different statistically when compared to selenium treated animals (Figure 6, $p>0.05$). Animals treated with selenium and 100μM and 150μM acridine orange were found to have significantly decreased survival by the 24 hour time point compared animals treated with selenium alone. These data suggest that lysosomal degradation is protective against selenium induced movement deficits and death and shows that necrosis is not the etiology of these deficits.

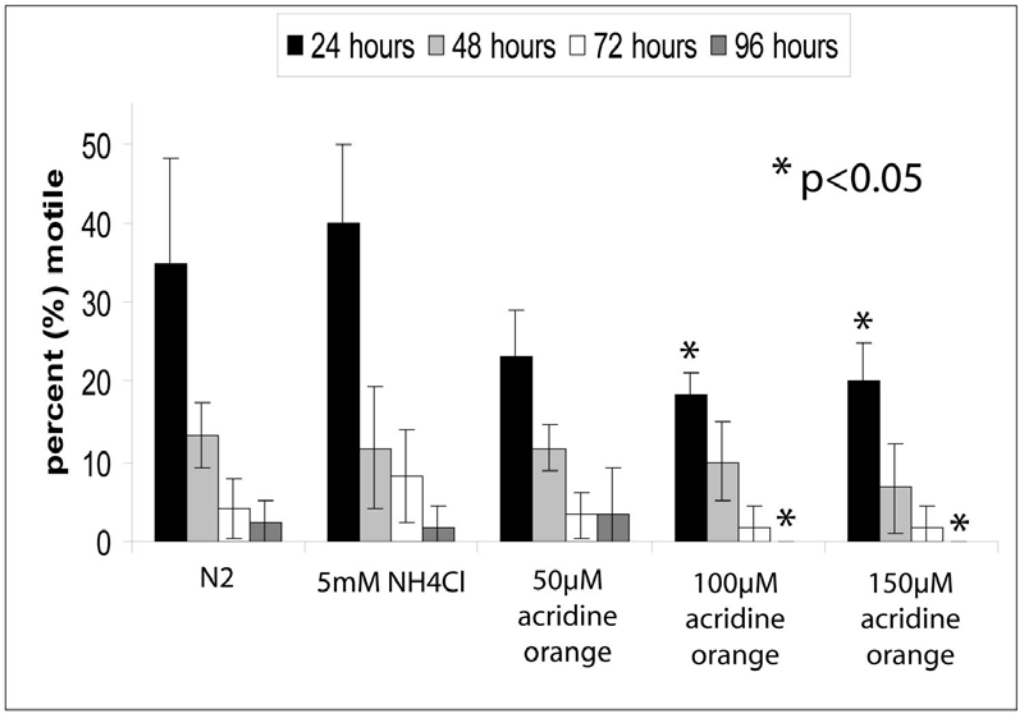



**Figure 6 Weak bases do not protect from selenium toxicity**

Treatment of wild-type animals exposed to lysotropic bases (acridine orange and ammonium chloride) does not protect animals from selenium damage. Significant increases in movement deficits was observed at the 24 hour and 96 hour time points of 100μM and 150μM acridine orange ($p<0.05$). Twenty developmentally synchronous wild-type animals were placed on NGM plates treated with 5mM sodium selenite and either 5mM $NH_4Cl$, 50μM acridine orange, 100μM acridine orange, or 150μM acridine orange and scored at 24 hour intervals for movement deficits as described in Section 2.3.4. Statistical analysis was performed as described in Section 2.3.9 using a 1-tailed student's t-test comparing each populations treated with NaSe + base to NaSe-treated populations with significance indicated as * on the graph. The graph above is the average of 3 populations of 20 animals per population (n=60). The experiment was repeated two times with similar results. Non-selenium treated animals exposed to 5mM NH4Cl, 50μM acridine orange, 100μM acridine orange, and 150μM acridine orange had greater than 90% normal movement at 96 hours (data not shown).

### 2.4.8 ROS detection

Existing literature has shown that when some forms of selenium are combined with reduced glutathione, superoxide anions can be detected (Seko and Imura 1997; Spallholz 1997). Since selenium is known to produce superoxide anions, we predicted that selenium exposure might cause a detectable increase in levels of ROS in our model. Using a peroxide sensitive dye, $H_2$DCFDA, we assayed levels of ROS in populations of N2 and selenium exposed populations of N2. Essentially, age-synchronous adult animal populations were placed on NGM plates with $H_2$DCFDA or NGM plates with $H_2$DCFDA and 5mM sodium selenite. These plates were incubated at 20°C for 6 hours and then the animals were washed off the plates and the oxidation of $H_2$DCFDA was visualized by observing green fluorescence (Figure 7A or 7B) or quantified (Figure 7C). The six hour time point was chosen to allow enough exposure to selenium to cause

selenium-induced damage but not enough time where a large fraction of the animals were dead (Table 1). Selenium-treated animals have a visually observed increase in green fluorescence (Figure 7B) when compared to non-selenium treated control animals (Figure 7A-images were captured using equivalent exposure times) after 6 hours of selenium exposure. When quantified, 0.2% of non-selenium-treated animals were positive for green fluorescence while 4.4% of the selenium-treated population was positive, a twenty-fold increase, demonstrating that an increase in ROS is a contributing factor of selenium toxicity (Figure 7C).

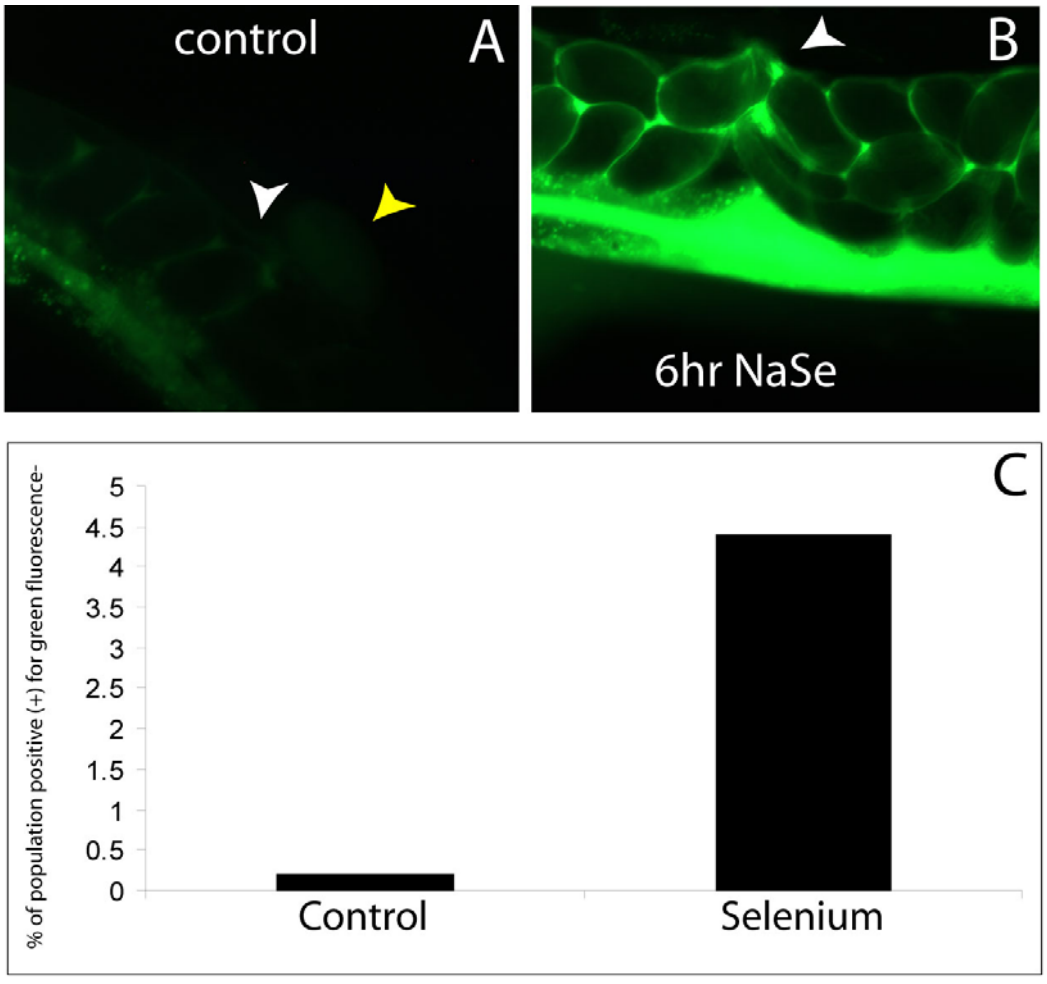



**Figure 7 Selenium toxicity causes an increase in ROS**

Wild-type animals treated with 5mM sodium selenite have increased levels of ROS when compared to untreated animals. Developmentally synchronized adult wild-type animals were transferred to NGM plates containing 50uM carboxy-$H_2$DCFDA (Invitrogen, Carlsbad, CA) with and without 5mM sodium selenite. (**A**) An untreated animal examined around the vulva. White arrowhead is pointing to the vulva. Yellow arrow is pointing to an egg that has been expulsed by the animal. (**B**) N2 animal after 6 hours of selenium treatment. White arrowhead is pointing to the vulva. Note: despite the extra pressure from the cover slip and extra eggs being retained, no eggs have been extruded (will be addressed in Chapter 3). (**C**) Quantification of green fluorescent positive animals in the population. Selenium treatment results in a twenty fold increase in green fluorescent animals in the population. (control: n=893, selenium treated: n=481).

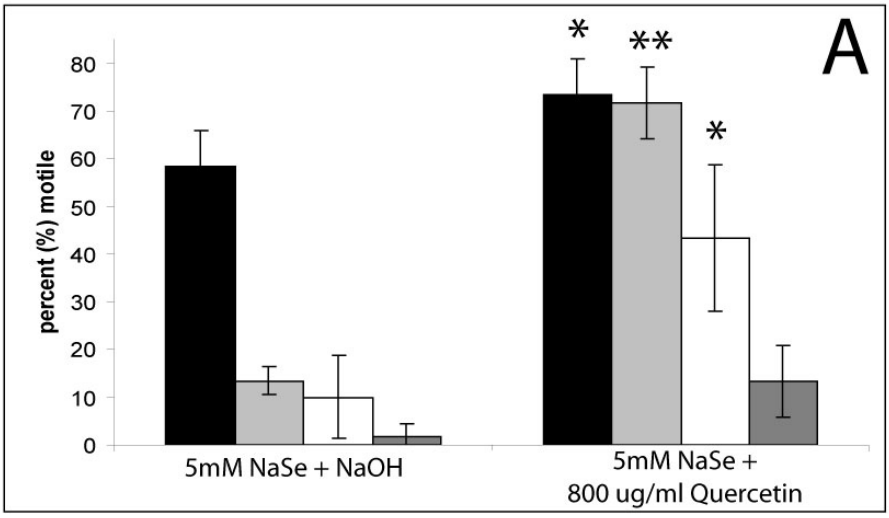
### 2.4.9 Movements deficits caused by selenium toxicity can be alleviated by antioxidants

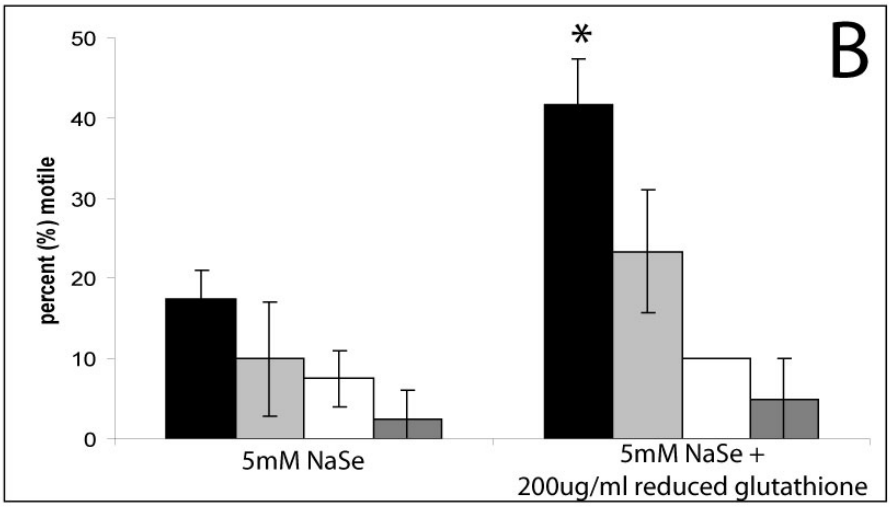
Selenium is often considered to be an antioxidant since selenocysteine occupies the active site of glutathione peroxidase, an enzyme that catalyzes the breakdown of hydrogen peroxide into water and oxygen (Behne and Kyriakopoulos 2001). However, as noted before, multiple sources of evidence suggest that selenium causes oxidative damage (Kitahara et al. 1993; Seko and Imura 1997; Spallholz 1997; Shen et al. 1999; Verma et al. 2004; Deore et al. 2005; Seitomer et al. 2008). Given these studies, we hypothesized that co-treatment with antioxidants might alleviate the movement deficits caused by exposure to high selenium.

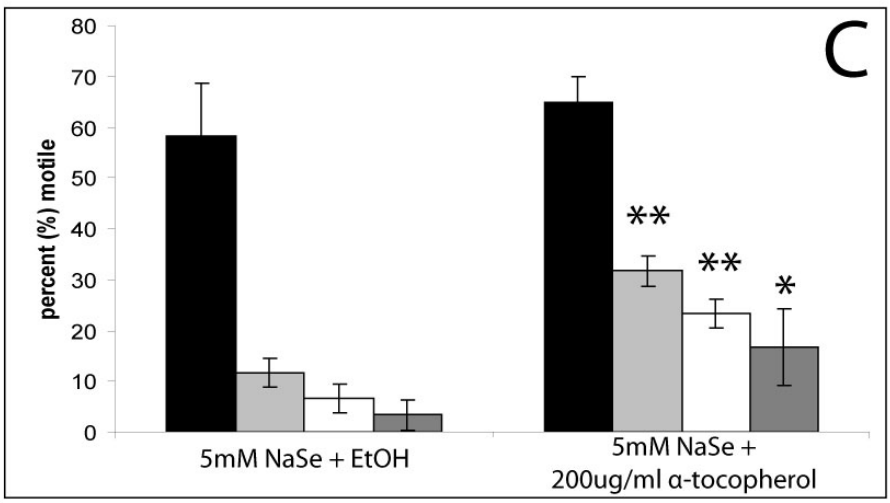
Vitamin E (α-tocopherol), reduced glutathione, and quercetin were tested for their ability to suppress movement deficits and death caused by exposure to selenium. Vitamin E (or α-tocopherol) has a well documented history with selenium, since the essential nature of selenium was understood only when researchers found that it could suppress liver damage that resulted

from a vitamin E deficient diet (Schwarz and Foltz 1999). Reduced glutathione was tested because it is a cofactor for glutathione peroxidase and has been shown to alleviate selenium toxicity in a vertebrate model (Deore et al. 2005). Quercetin is a flavinoid which has been the focus of growing interest in medical research, as it has been shown to be a free radical scavenger, prevents lipid peroxidation, suppress ROS production, as well as having numerous neuroprotective effects (Smith and Luo 2003).

To determine whether the above mentioned antioxidants could suppress the behavioral deficits observed from selenium exposure, developmentally synchronized adults were placed on NGM plates with increasing concentrations of the antioxidants with and without 5mM sodium selenite. The concentrations that provided the maximum protection are graphed in Figure 8. As would be predicted by the increased ROS detected by $H_2$DCFDA in Figure 7, these studies show that quercetin, reduced glutathione, and vitamin E all conferred significant protection against selenium as observed by increased observation of normal motility (Figure 8A $p<0.05$ at 24hours, 8B $p<0.05$ at 24 hours, and 8C $p<0.005$ at 48 hours). Given these findings, it is possible that the movement deficits and mortality that we observe are due to increased oxidative cellular damage. The damage may occur predominantly or exclusively in neurons or muscle, an issue that we will address in a subsequent chapter. These experiments were performed separately, which makes it difficult to approximate each antioxidants relative protection from selenium to another. α-tocopherol was dissolved in ethanol and was observed to precipitate into oil-like drops on the surface of the plates, resulting in variable exposure of the antioxidant to individual *C. elegans*. However, under the conditions tested, quercetin provided the best protection from selenium, with significant increases in normal motility when compared to selenium-treated animals observed at 24 hours, 48 hours, and 72 hours.




24 hours 48 hours 72 hours 96 hours

* p< 0.05 ** p< 0.005

**Figure 8 Antioxidants partially suppress selenium induced movement deficits**

Co-treatment with one of three antioxidants, quercetin, reduced glutathione, and α-tocopherol (vitamin E) was found to partially, but significantly suppress the movement deficits caused by selenium exposure. **(A)** 800 μg/ml of quercetin was found to significantly suppress the movement deficits at the first three time points (24 hours: $p<0.05$, 48hours: $p<0.005$, 72hours: $p<0.05$) **(B)** 200μg/ml of reduced glutathione was found to significantly suppress the movement deficits at the 24 hours time point ($p<0.05$) **(C)** 200 μg/ml of α-tocopherol was found to significantly suppress the movement deficits at three time points (48hours: $p<0.005$, 72hours: $p<0.005$, 96hours: $p<0.05$). Twenty developmentally synchronized wild-type hermaphrodites (N2) were placed on NGM plates treated with the specified antioxidant and 5mM sodium selenite and scored at 24 hour intervals for movement deficits described in Section 2.3.4. Statistical analysis was performed as described in Section 2.3.9 using a one-tailed student's t-test with unequal variance, comparing selenium co-treatment with an antioxidant to selenium treatment alone. Significance is indicated on the graph as either * ($p<0.05$) or ** ($p<0.005$). Non-selenium treated controls (including the corresponding vehicle as NaOH control for quercetin and EtOH control for α-tocopherol) had greater than 90% normal movement after 96 hours (data not shown).

## 2.5 DISCUSSION

Here, we show that inorganic and organic forms of selenium are toxic in *C. elegans*, as has also been reported in vertebrate models (Panter et al. 1996; Usami and Ohno 1996). Further, we identified a series of movement deficits that occur progressively in response to selenium exposure that suggests that neurons are being damaged. These movement deficits mimic deficits observed in a vertebrate model of selenium toxicity and some symptoms of human ALS patients (Kuncl et al. 1988; Panter et al. 1996). We further demonstrate that exposure to selenium causes an increase in ROS, contributing to toxicity. Other factors that we investigated are more complicated in their overall implications, specifically the role of calcium in selenium exposure and mechanisms of cell death.

### 2.5.1 Bacterial metabolism and selenium toxicity

Bacterial metabolism was found not to be a factor contributing to selenium toxicity. However, bacterial metabolism may be protecting animals from selenium. In CeMM, the average survival in a population after 24 hours was 30% whereas 24 hours on NGM plates result in greater that 50% survival in the population (Figures 1B and 3). Additionally, bacterial metabolism has been shown to alter lifespan in *C. elegans* (Saiki et al. 2008). While not formally tested, evidence for a potential protective affect from bacterial metabolism was observed when sodium selenite was added to seeded (with OP50 as a food source) and unseeded (no OP50)

plates. Unseeded plates did not change color while seeded plated turned orange over time. This is significant because elemental selenium is know to be orange in color and significantly less toxic (Nuttall 2006). It is likely in this case that bacterial metabolism is providing a protective function by metabolizing sodium selenite into elemental selenium or by altering the pH of the plate, causing selenium to precipitate into its elemental form. This could also explain the large variability of toxicity observed between experiments, and between individual plates throughout this dissertation. While it was determined that bacterial metabolism was not necessary to observe the toxic effects of selenium, it was not determined whether bacterial metabolism could have provided protection. This could be examining wild-type animals for movement deficits on UV irradiated seeded plates (plates containing OP50 as a food source) and comparing them to non-irradiated plates treated plates, both containing selenium. Another possible test would involve altering the pH of the media and test for changes in animal's movement deficits when exposed to selenium.

### 2.5.2 Potential role of calcium in selenium toxicity

In these studies we have shown that exposure to high levels of selenium gives rise to a progressive paralysis in the genetic model *C. elegans* as in a previous vertebrate experimental model (Panter et al. 1996). This paralysis was dose-dependent, suggesting a direct association between selenium exposure and the subsequent neuronal or muscle damage leading to paralysis. Our results also suggest that pre-existing stores of intracellular calcium exacerbate selenium toxicity and that elevated calcium levels may act as a co-toxin. If this model is

correct, increased levels of calcium during selenium exposure may represent an environmental factor influencing the severity of toxicity.

Ongoing and future work from this observation has involved testing animals containing mutations in genes involved in calcium regulation for altered sensitivity to selenium. Mutants being tested for altered sensitivity include L-type calcium channel (*egl-19 gf* and *rf*), IP3 receptor (*itr-1(rf)*), ryanodine receptor (*unc-68(rf)*), and calcineurin (*tax-6(rf)*). Differential sensitivity/resistance to a gain- or loss-of-function in *egl-19* will help us elucidate the role of calcium in response to selenium toxicity. Given our results with calcium enhancing selenium toxicity (Figure 4), we hypothesize that gain-of-function mutations which increase the flow of calcium and subsequently, calcium signaling, will enhance the emergence of the movement deficits caused by selenium exposure, while reduction-of-function mutations which decrease calcium signaling, will suppress the emergence of these movement deficits. Intriguingly, increased calcium has been implicated in the pathology of sporadic ALS and a SNP of the IP3 receptor has been implicated in sporadic ALS (Siklos et al. 1999; van Es et al. 2007). This hypothesized role for calcium in a motor neuron disease is not surprising, given that calcium is involved in a variety of cell death mechanisms (Hajnoczky et al. 2000; Arundine and Tymianski 2003). The potential implication here is that calcium signaling increases the emergence of the observed movement deficits in response to selenium exposure, and similar alterations in calcium signaling have been implicated in sporadic ALS, adding to the possibility that selenium toxicity could be used to model sporadic ALS.

### 2.5.3 Cell death caused by selenium toxicity

Although mutations preventing apoptosis are available in *C. elegans* (e.g., *ced-3(rf), ced-9(gf)*, and *ced-4(rf)*), these mutations actually paradoxically enhanced the emergence of selenium induced movement deficits resulting in mortality. There are several potential problems with the interpretation of our data that have been addressed by the experiments in this chapter. The data suggests at least superficially that neither classical apoptotic nor necrotic mechanisms are the cause to the movement deficits and mortality seen in selenium exposed animals. In fact, the data paradoxically suggest that apoptosis and necrosis are protective.

An alternative explanation for these results is that in the absence of apoptotic pathways, there is a super-physiologic enhancement to necrotic and autophagic (programmed cell death II) cell death mechanism. Conversely, in the absence of key proteins required for necrotic-mediated cell death, apoptotic pathway proteins maybe super-physiologically active. Hence, it may not be as simple as the notion that both apoptotic and necrotic signaling pathways somehow confer protection. One way to address this conundrum would be to make a *cad-1(rf);ced-3(rf)* double mutant and test its sensitivity to selenium. However, again an alternative cell death mechanism (autophagy) may be "hyper-activated" in the absence of normal apoptotic and necrotic signals leading to increased selenium sensitivity. The potential for developmental effects on viability in general in the absence of these two pathways can also not be discounted. Future work will attempt to address these issues further using inducible RNAi inactivation of apoptotic and necrotic pathway genes in adults. Despite these problems, our data clearly show that selenium-induced oxidative stress gives rise to the movement deficits and mortality in our model. In addition, given our results with necrotic and apoptotic pathway genes, it is likely that individuals

with mutations in these genes may have altered susceptibility to selenium exposure in the environment.

Another mechanism of cell death, autophagic cell death or programmed cell death II, was not investigated here. However, autophagy is not only a cell death mechanism but is involved in cellular degradation and organelle turnover (Gozuacik and Kimchi 2004). One hypothesis for this process would be that increasing autophagy would be protective by degrading oxidized proteins/organelles damaged by selenium exposure. Indeed, ongoing studies in the lab investigating genes encoding proteins required for formations of the autophagosome complex suggests autophagy protects against selenium-induced movement deficits and death, as has been shown for the *C. elegans* model of Huntington's disease (Jia et al. 2007). In addition, these data are consistent with my findings that decreased cathepsin D function, an autophagy-related protease, increases the rate at which movement deficits emerge when exposed to selenium (Figure 5).

### 2.5.4 Conclusion

This chapter provided insight into mechanisms of selenium toxicity by identifying environmental factors that provide insight into potential signaling mechanisms (like calcium). Here, we also demonstrated that canonical cell death mechanisms are not involved in the observed movement deficits and mortality. Finally, we have observed an etiology behind selenium toxicity, an increase in reactive oxygen species. This was further confirmed by partial suppression of movement deficits and mortality by treatment with multiple antioxidants.

# 3.0 SELENIUM TOXICITY AND NEURONAL DAMAGE

## 3.1 ABSTRACT

Selenium is an essential nutrient that has been demonstrated to cause motor neuron damage in vertebrates and has been epidemiologically linked to the motor neuron disease, ALS. Here, treatment with high selenium is observed to cause neuronal swelling in the genetic model *C. elegans*, while gross morphology of muscle fibers is unchanged. In addition, selenium exposure was found to cause an increase in cytosolic muscle protein catabolism that could be suppressed by acetylcholinesterase inhibitors and nicotinic agonists, demonstrating cholinergic denervation. Further, selenium-damaged animals were found to have a decrease in cholinergic signaling, demonstrating motor neuron damage. Selenium was also shown to cause neuronal damage to a specific neuron-muscle circuit responsible for egg-laying, which is significantly rescued by serotonin.

## 3.2 INTRODUCTION

Selenium toxicity has been repeatedly shown to cause decreased fertility and developmental defects in wildlife (Ohlendorf et al. 1986; Lemly 1997; Lemly 2004). Excess selenium exposure has also been found to cause neuronal damage in developing embryos and adult livestock (Panter et al. 1996; Usami and Ohno 1996). Post-implantation rat embryos treated with various forms of selenium were all found to develop abnormalities in the nervous system, ranging from swollen rhombencephalon, small/swollen optic vesicles, deformed otic vesicles, hypoplastic telencephalon, and a zigzag neural tube (Usami and Ohno 1996).

Comparisons of selenium toxicity in livestock revealed that all forms of selenium led to behavioral indications of nervous system damage, ranging from posterior ataxia to complete paralysis (Panter et al. 1996). Post-mortem analysis of the spinal cord and brain of these animals found a positive correlation between the behavioral abnormalities indicating nervous system damage and the severity of lesions found in the spinal cord (Panter et al. 1996). Interestingly, the animals that did not exhibit behavioral abnormalities did have indicators of chronic selenosis (hair abnormalities and hoof lesions) and animals that exhibited signs of nervous system damage did not have any signs of selenosis, suggesting that distinct damage pathways can be activated by this metalloid (Panter et al. 1996).

Other evidence of neuronal damage from high selenium exposure have been reported from numerous cases of accidental selenium poisoning in livestock. Several cases of outbreaks of swine paralysis have been linked to excess selenium supplementation in feed (Harrison et al. 1983; Casteel et al. 1985; Stowe et al. 1992; Casteignau et al. 2006). Post-mortem analysis of

these animals revealed loss of motor neurons in the spinal cord that correlated with paralysis. Other studies have experimentally reproduced incidents of accidental selenosis in pigs and confirmed that excess selenium causes loss of motor neurons in the spinal cord (Harrison et al. 1983; Wilson et al. 1988). In addition to neuronal loss, other pathological observations included gliosis and vacuolation (Harrison et al. 1983; Stowe et al. 1992; Casteignau et al. 2006).

In addition to the selenium livestock studies, epidemiologic studies have been published linking high environmental selenium to the motor neuron disease, ALS. The first study linked a cluster of 4 unrelated patients with ALS living in an area with high ground selenium that was also known to have animals suffering from blind staggers (Kilness and Hichberg 1977). In another formal epidemiologic study, a town was identified that had high inorganic selenium in the municipal water supply and increased risk of developing sporadic ALS (Vinceti et al. 1996). In a population of 5,182 that was found to have used the water supply continuously for 5 years, 4 individuals developed sporadic ALS (Vinceti et al. 1996). Analysis of this population was further refined to identify a group within the cohort that drank continuously from the municipal water supply for 10 years and all 4 ALS cases fell within this group (Vinceti et al. 1996). This is significantly higher than the baseline incidence of ALS (1-2 individuals in 100,000) (Cleveland and Rothstein 2001).

In Chapter 2, the movement assay we developed monitored a progressive series of movement deficits that mimics an assay developed in a vertebrate model to measure severity of nervous system damage caused by selenium toxicity (Panter et al. 1996). This assay detected progressive weakness and eventual paralysis and death as is observed in ALS patients (Williams and Windebank 1991). However, we did not definitively address whether the observed movement deficits were due to muscle or neuronal damage in the model system *C. elegans*.

While paralysis could be a result of muscle damage or neuronal damage, the initial backing deficit observed suggests that the neurons are damaged first, as it is a neuronally controlled behavior (Way and Chalfie 1989; Wicks and Rankin 1995; Ernstrom and Chalfie 2002). Subsequent paralysis could be due to either muscle or neuronal damage.

Here, we investigate potential neuronal damage resulting from selenium toxicity as suggested by the backing deficit that has been used to observe selenium damage in Chapter 2. We examine neuronal morphology to determine if selenium exposure causes any alterations in structure when compared to untreated animals and found neuronal swelling with a corresponding loss in chromatin staining, axonal blebbing along the ventral cord, and egg retention that were not observed in untreated controls.

Similarly, we then examined muscle structure to determine is selenium exposure altered the structure and discovered no gross differences in muscle morphology. However, this does not reveal the cytosolic state of the muscle cells. Altered cytosolic protein catabolism has been demonstrated in a variety of neuromuscular diseases, including ALS (McKeran et al. 1977; Kar and Pearson 1978; Corbett et al. 1982). Examination of cytosolic muscle protein revealed an increase in protein catabolism that could be suppressed by a nicotinic agonist or by an acetylcholinesterase inhibitor, suggesting decreased cholinergic signaling. Further, selenium-treated animals displaying movement deficits had increased resistance to the cholinesterase inhibitor aldicarb, also suggesting a decrease in cholinergic signaling in response to selenium exposure. This is significant as cholinergic receptors are decreased in ALS patients (Manaker et al. 1988; Berger et al. 1992). In addition, animals paralyzed by selenium exposure hyper-contracted when exposed to the nicotinic agonist, levamisole, demonstrating that the muscles were still functional.

Finally, we examined the egg-laying circuit in response to selenium exposure, since decreased egg-laying was observed as a result of selenium exposure. Egg-laying provides another measure of determining muscle or neuronal damage, since egg-laying is controlled by a well-defined circuit which includes uterine muscles and two types of neurons. We observed a significant increase in egg-laying that was partially, but significantly, rescued by serotonin, demonstrating that the muscles could still function in the egg-laying process. The incomplete rescue was then determined to be due to increased apoptosis occurring in the germ line of selenium treated animals. The combined data suggest that the phenotypes observed resulting from selenium exposure are due to neuronal damage.

## 3.3 METHODS AND MATERALS

### 3.3.1 Strains and strain maintenance

Animals were maintained as described in Section 2.3.1 on nematode growth media (NGM) without additional calcium. The following strains were obtained from the *Caenorhabditis* Genetics Center; wild-type N2, RB756: *gar-2(ok520)III*, and OH441: *otIs45(unc-119::GFP)*. Animals expressing *myo-3::GFP* were a kind gift from Lew Jacobson.

### 3.3.2 Nuclear staining of *C. elegans*

Developmentally synchronized animals from the strain expressing *unc-119::GFP* were transferred to NGM plates that were untreated or contained 5mM sodium selenite. After 24 hours, animals were transferred to a microcentrifuge containing 10μg/ml of ethidium bromide (EtBr) for 1 hour. Animals were then transferred onto new NGM plates and allowed to recover for 1 hour before being analyzed by fluorescence microscopy.

### 3.3.3 Fluorescence Microscopy

Individual animals were analyzed and documented by visualization on a Leica DM IRB microscope equipped with fluorescence optics and a DC300F imaging system (Leica Microsystems Inc., Bannockburn, IL). The EtBr staining was visualized using a rhodamine filter, while a GFP-specific filter was used for observing both the muscle- and neuron-specific

GFP fluorescence. The images were processed for publication using the Adobe Creative Suite 2 premium software package (Adobe Systems Inc., San Jose, CA).

### 3.3.4 Staining for cytosolic Lac-Z in the muscle (in collaboration with Nate Szewczyk)

Age-synchronized adult animals with *unc-54::lac z* fusion were transferred to untreated NGM plates or NGM plates supplemented with 5mM sodium selenite for 24 hours. Subsets of these control and selenium-treated populations were also co-treated with the nicotinic agonist levamisole (1mM) or the cholinesterase inhibitor aldicarb (40μM, 80μM, and 400μM). X-Gal staining was performed as previously described (Szewczyk et al. 2000).

### 3.3.5 Aldicarb assay to determine pre-synaptic damage

Developmentally synchronized adult N2 hermaphrodites were plated on 5mM sodium selenite or untreated plates as adults and incubated for 24 hours at 20°C. Selenium-treated animals tested for aldicarb resistance/sensitivity were animals that failed the backing test. This was to test for animals that had minimal damage from selenium since selenium damage was determined to be progressive (Table 1). Animals that still moved normally (moved and reversed direction like untreated controls) were not included, as they would have skewed the population towards aldicarb sensitivity. Also, paralyzed and dead animals were also not included in this assay, since it would be difficult to determine if animals were paralyzed/dead due to selenium or aldicarb exposure. Untreated controls and selenium damaged animals that failed the backing test of the touch assay were plated on 0.5mM aldicarb and were scored for paralysis at 30 minute intervals (Mathews et al. 2003).

### 3.3.6 Levamisole assay for determining post-synaptic damage

N2 animals were developmentally synchronized and treated with 5mM sodium selenite for 24 hours at 20°C as adults. Animals picked for this assay demonstrated paralysis (failure to complete one sinusoidal turn within 5 seconds of touching the tail with a platinum wire). Animals with little (backing deficit) to no damage were not included as they were likely to be sensitive to levamisole and skew the results toward levamisole sensitivity, resulting in a false positive. Animals were plated on 100mM levamisole and examined after 30 minutes for muscle hypercontraction.

### 3.3.7 Egg-laying assay

Developmentally synchronized adult wild type N2 or *gar-2(ok520)III* hermaphrodites were plated on 5mM sodium selenite treated and untreated plates and incubated at 25°C. At one hour intervals following plating, 10 animals were moved to a new, untreated plate and returned to the 25°C incubator. After 24 hours on the untreated plates following the selenium treatment, the adult animals were removed and the eggs/progeny were counted. As the eggs/progeny were counted, they were removed by aspiration. Total egg numbers for each plate were calculated. Then the number of eggs was divided by the number of animals on the plate to determine the number of eggs per animal. If adult hermaphrodites died, they were removed from the number of adult animals on the plate, since they likely died as a result of the transfer.

### 3.3.8 Serotonin treatments for egg-laying

To determine if the decreased egg laying was due to neuronal or muscle damage, animals were treated with the neurotransmitter, serotonin. N2 animals were plated on untreated and 5mM selenium treated plates for 6 hours and transferred to a plate containing 3mg/ml of 5-hydroxytryptamine hydrochloride (5-HT, H2195 Spectrum Chemical, Gardena, CA, USA), for 1 hour. Afterward, the adult hermaphrodites were removed and the eggs were counted to determine the rate of egg-laying per animal per hour in response to serotonin.

### 3.3.9 Acridine orange treatment for apoptotic bodies in germ line

N2 animals were developmentally synchronized and grown to gravid adults. Animals were either left untreated or transferred to 5mM sodium selenite containing plates. After 24 hours of selenium exposure, animals were transferred to NGM plates containing 0.02mg/ml of acridine orange (cat # A3568, Invitrogen, Carlsbad, CA) for one hour (Lettre et al. 2004). Then animals were transferred to a new plate for one hour to reduce acridine orange staining in the gut lumen and analyzed by fluorescence microscopy for fluorescent puncta.

## 3.4 RESULTS

### 3.4.1 Selenium exposure causes gross neuronal damage

As mentioned in Chapter 2, the behavioral assay that was developed to measure relative resistance to high selenium scored normal movement, backing deficits, paralysis, and death. The backing deficits suggest that neuronal damage was an early event resulting from selenium exposure, since backing is neuronally controlled (Way and Chalfie 1989; Wicks and Rankin 1995; Ernstrom and Chalfie 2002).

In order to determine whether selenium caused gross neuronal damage, animals expressing green fluorescent protein (GFP) under control of the pan-neuronal promoter for *unc-119* were utilized. Developmentally synchronized animals were treated with 5mM sodium selenite as adults. Animals were examined after 24 hours of selenium exposure since approximately half of selenium exposed animals have some type of movement deficit at that time point (Figure 1B). Neuronal swelling was observed in selenium treated animals that was not observed in untreated animals (Figure 9A and 9B). Selenium-treated animals lost a majority of chromatin staining in the swollen neurons (Figure 9D) when compared to untreated controls (Figure 9C). Further analysis of selenium-treated animals revealed axonal blebbing (Figure 9F) that was not present in the control (Figure 9E). In addition, selenium treated animals had more eggs including late stage embryos in hermaphrodites (Figure 9H) that were not observed in the untreated control (Figure 9G). These observations suggest that selenium toxicity is damaging

neurons in adult hermaphrodites, as has been reported with selenium toxicity in vertebrates (Casteel et al. 1985; Stowe et al. 1992; Panter et al. 1996).

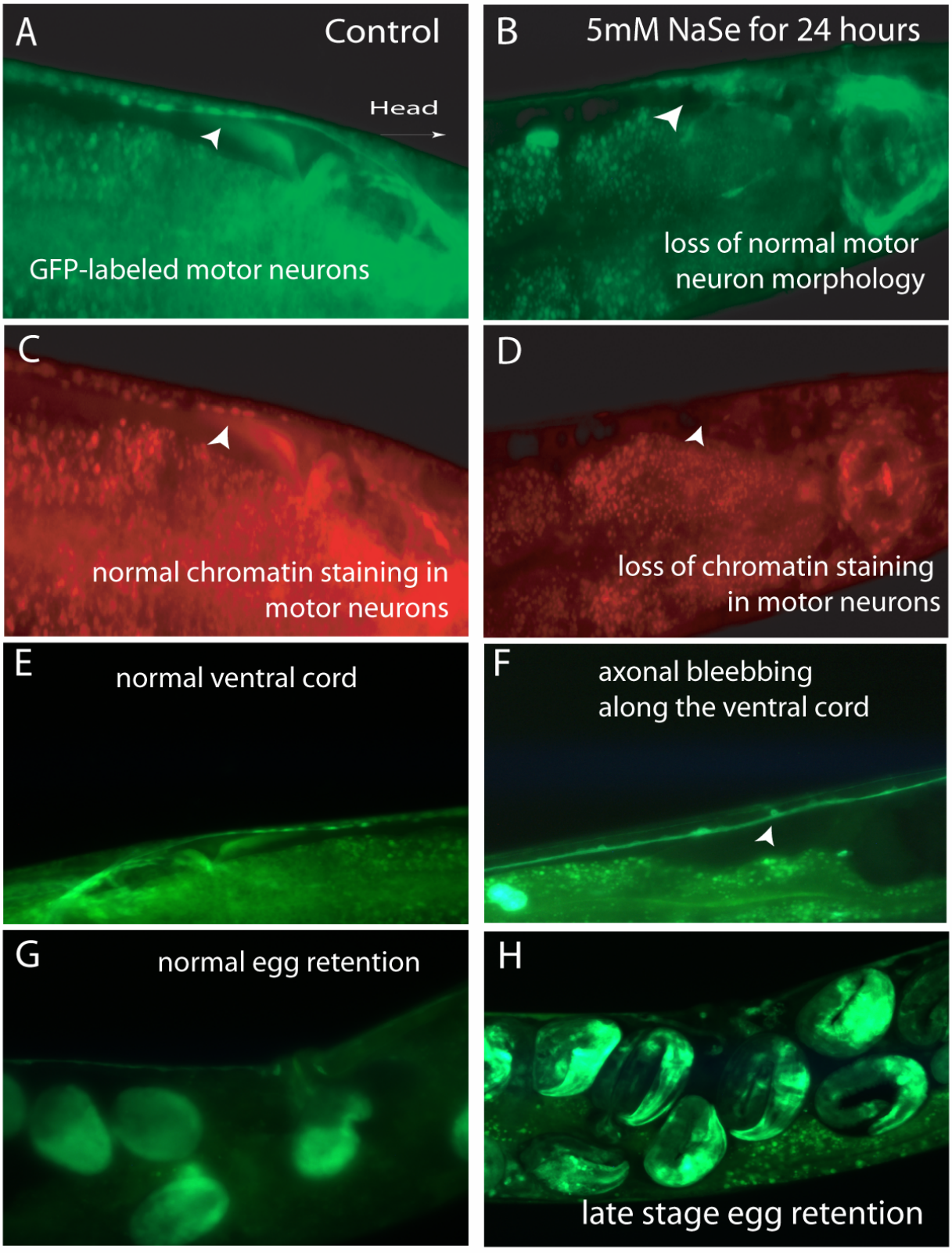

A
Control
Head
GFP-labeled motor neurons
B
5mM NaSe for 24 hours
loss of normal motor neuron morphology
C
normal chromatin staining in motor neurons
D
loss of chromatin staining in motor neurons
E
normal ventral cord
F
axonal bleebbing along the ventral cord
G
normal egg retention
H
late stage egg retention

**Figure 9 Neuronal morphological damage observed at 24hours of 5mM sodium selenite (NaSe) treatment**

Analysis of animals expressing the pan-neuronal marker *unc-119::GFP*. Several morphological changes were observed in NaSe-treated animals, including neuronal swelling, loss of chromatic staining, axonal blebbing along the ventral cord, and egg retention. Panels **A**, **C**, **G**, and **E** represent untreated controls while panels **B**, **D**, **F**, and **H** are images of neuronal damage from 24 hours of 5mM NaSe treated worms. **B** shows neuronal swelling in NaSe-treated worms as compared to untreated control in **A**. In **D**, loss of chromatin staining was visualized by EtBr but colloquial staining was observed in the neurons observed in **B**. **E** normal ventral cord. **F** Axonal blebbing in a NaSe-treated animal along the ventral cord. **G** shows normal egg retention, while 24 hour exposure to NaSe causes increased egg retention. **H** Animals expressing *unc-119::GFP* were developmentally synchronized and placed on untreated NGM plates or NGM plates treated with 5mM NaSe and examined after 24 hours by fluorescence microscopy.

### 3.4.2 Gross morphology of muscle fibers appear normal

In chapter 2, selenium exposure was shown to cause progressive movement deficits (Table 1) that begin with backing problems, and proceed to paralysis and eventual death. As discussed earlier, the observed backing problem suggests early nervous system damage that was grossly observed in Figure 9. The next abnormal behavior observed was paralysis, or failure to complete one sinusoidal body turn within 5 seconds of a harsh touch to the tail with a platinum wire. Selenium-induced paralysis could also be due to either muscle or neuronal damage. Previously, it has been shown that aging causes breakdown of the body wall muscle sarcomeres between 4 days and 18 days, by examining an animal that expresses *myo-3::GFP* (Herndon et al. 2002).

To determine whether muscle sarcomeres are breaking down as a result of selenium toxicity, we used animals expressing *myo-3::GFP* to label the body wall muscle sarcomeres.

Developmentally synchronized animals were examined after 48 hours of selenium treatment. Analysis of selenium-treated animals (Figure 10b) and untreated controls (Figure 10A) after 48 hours led to no observable difference between the two groups. This suggests that the muscle sarcomeres are not being damaged (when sarcomeres are damaged, myosin fibers appear as short segments rather than a continuous fiber). While the structure appears undamaged, it does not demonstrate whether cytosolic differences that may be occurring as a result selenium exposure, which were next examined.

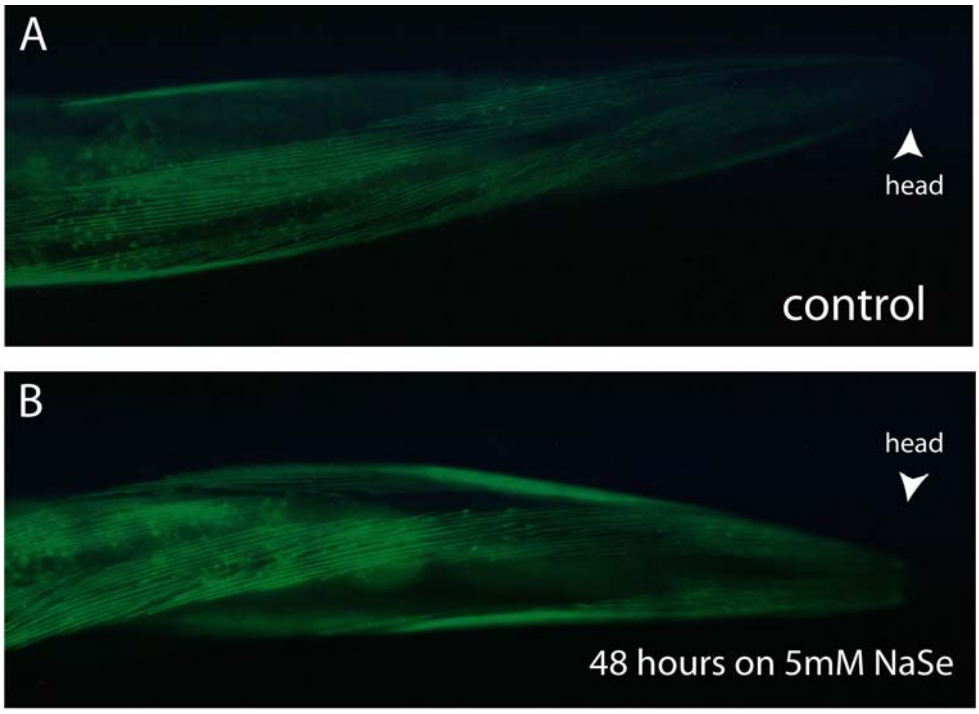



**Figure 10 Gross muscle morphology appears normal after 48 hours of selenium exposure**

No gross differences in muscle morphology were observed in selenium-treated animals. Developmentally synchronized animals expressing *myo-3::GFP* were placed as adults on untreated NGM plates or NGM plates containing 5mM sodium selenite (NaSe). Animals were examined by fluorescent microscopy after 48 hours for changes in muscle morphology. No obvious differences were noted between untreated animals (**A**) and selenium-treated animals (**B**).

### 3.4.3 Evidence of cytosolic muscle protein turnover in response to selenium exposure (in collaboration with Nate Szewczyk)

Another possibility is that selenium exposure alters the intracellular status of cytosolic muscle proteins. Decreased cholinergic signaling has been found to affect cytosolic protein turnover by turning acetylcholine synthesis off using a temperature sensitive reduction-of-function mutation in the choline acetyltransferase gene, *cha-1* (Szewczyk et al. 2000). As a result, the stability of soluble muscle proteins can be used as an indirect assay for the synaptic function of cholinergic motor neurons.

To test the hypothesis that selenium toxicity involves decreased cholinergic signaling to the muscle, synchronized wild-type adult populations expressing a β-galactosidase (β-gal) transgene in muscle were placed on NGM plates with or without selenium and tested for maintenance of β-gal activity, with and without addition of the addition of the direct nicotinic agonist (levamisole) and acetylcholisterase inhibitor (aldicarb) (Figure 11). The selenium-treated animals showed significant loss of β-gal enzymatic activity (Figure 11A, 24 and 48 hours time points). The direct nicotinic agonist levamisole prevented selenium-induced breakdown of soluble muscle proteins, consistent with the requirement for nicotinic acetylcholine receptor(s) activation for muscle protein stability (Figure 11B). This loss of β-gal activity could also be rescued using an acetylcholinesterase inhibitor, aldicarb, across a range of concentrations (Figure 11C). This rescue of β-gal activity suggests that decreased cholinergic signaling was at least partially responsible for the observed protein catabolism. These studies provide evidence that selenium toxicity causes cholinergic denervation, a classical finding in the pathology of human ALS (Gould et al. 2006).

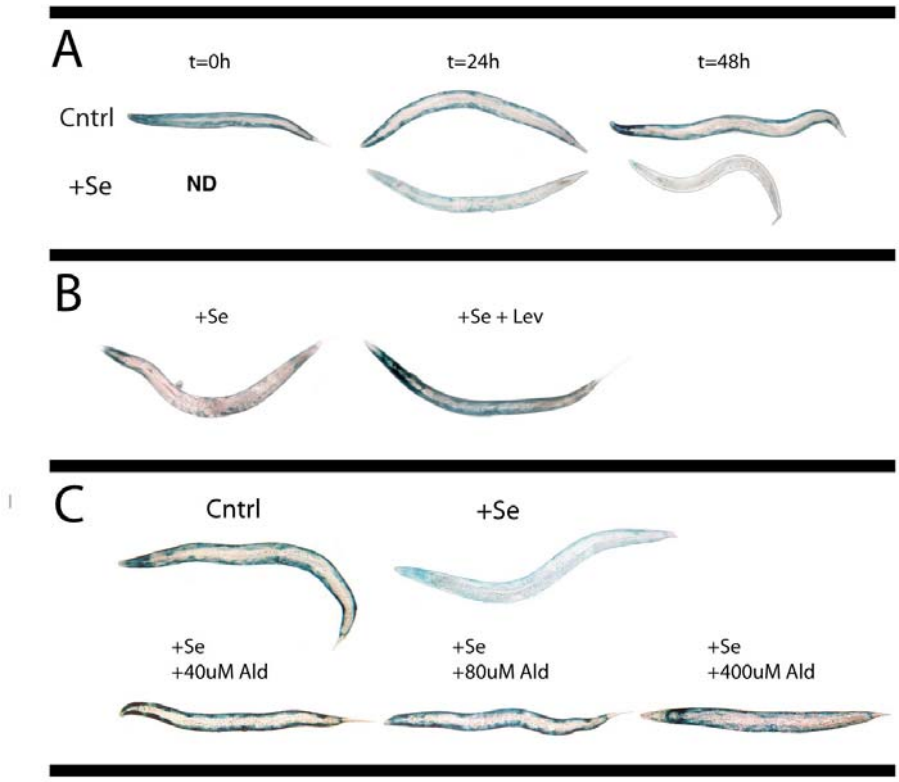



**Figure 11 Selenium exposure causes increased cytosolic muscle protein catabolism as observed by loss of cytosolic β-gal (figure by Nate Szewczyk)**

Selenium exposure causes increased cytosolic protein catabolism that can be suppressed by the nicotinic agonist, levamisole, and the acetylcholinesterase inhibitor, aldicarb. (**A**) Animals expressing cytosolic β-galactosidase in the muscle were exposed to untreated NGM plates or NGM plates treated with 5mM sodium selenite (represented in figure as Se). Untreated animals are in the top. Selenium-treated animals for the 24 and 48 hours time points are in the bottom row. (**B**) Selenium-treated animals were also treated with the nicotinic agonist levamisole showing that levamisole can suppress the cytosolic protein catabolism (as observed by β-gal staining) caused by selenium treatment. (**C**) Selenium-treated animals co-treated with the cholinesterase inhibitor, aldicarb (40μM, 80μM, and 400μM) which was also found to suppress the cytosolic muscle protein catabolism caused by selenium treatment.

### 3.4.4 Determining whether selenium damage is pre-synaptic and/or post-synaptic

As previously mentioned, the movement deficits and eventual paralysis resulting from exposure to sodium selenite could be due to either post-synaptic damage, to muscles, or pre-synaptic damage, to the neurons, or both, although analysis of cellular morphology suggests that the neurons are being damaged resulting in increased muscle protein catabolism. Pre-synaptic/post-synaptic damage can be differentiated by observing the behavior of selenium damaged animals treated with an acetylcholinesterase inhibitor, aldicarb, and a nicotinic agonist, levamisole.

Aldicarb prevents the breakdown of acetylcholine in the synaptic clefts (Riddle 1997). As a result, aldicarb treatment of animals with normal cholinergic signal causes super-physiologic acetylcholine accumulation causing paralysis and death (Riddle 1997). Animals containing gene mutations have been identified that are resistant to aldicarb, a majority of which have been shown to be involved in cholinergic signaling (Nguyen et al. 1995). The principle behind the assay is that if mutations are involved in cholinergic signaling, paralysis and death will be delayed or prevented when animals are exposed to aldicarb, as has been observed in mutants for UNC-2, a voltage-gated calcium channel that controls cholinergic and GABAergic signaling in motor neurons (Mathews et al. 2003).

To determine whether cholinergic signaling was decreased as a result of selenium exposure, selenium-damaged animals were tested for resistance to aldicarb-induced paralysis. For this assay, we specifically tested the selenium-treated animals that failed the backing portion of the movement assay were tested for aldicarb resistance. This subset of animals was selected to avoid false negatives (animals that respond normally to the touch assay) and false positives (animals that were already paralyzed). After 30 minutes of aldicarb treatment, there was no difference in paralysis between selenium-treated animals and untreated controls (Figure 12, as

determined by a one-tailed student t-test with unequal variance). No significant difference was observed after 60 minutes of aldicarb treatment, although the two populations began to trend apart (Figure 12). After 90 minutes of aldicarb treatment, the selenium-treated population was statistically more resistant to aldicarb-induced paralysis ($p<0.01$). This resistance was also observed at 120 minutes of aldicarb treatment ($p<0.01$). These results demonstrate an apparent decrease in cholinergic signaling, although not as prominent as mutants resistant to aldicarb, like UNC-2 (Mathews et al. 2003). However, potential post-synaptic damage must also be addressed.

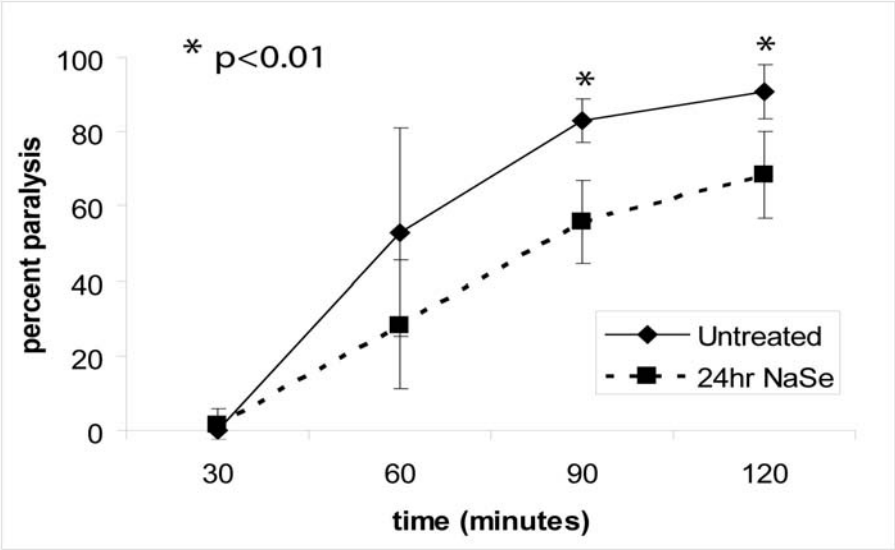



**Figure 12 N2 animals damaged by selenium have partial resistance to the AChE inhibitor, aldicarb**

Animals that displayed backing deficits resulting from selenium treatment had increased resistance to the acetylcholinesterase inhibitor, aldicarb. Untreated and selenium-treated animals were measured at 30 minute intervals for paralysis in response to 0.5mM aldicarb treatment. At 30 minutes, there was no difference between untreated animals and selenium damaged animals. After 60 minutes, untreated animals trend towards greater paralysis, but were determined to be statistically not different ($p>0.05$). By 90 minutes of aldicarb treatment, selenium-treated animals were statistically more resistant to aldicarb induced paralysis ($p<0.01$). Increased resistance was also observed at 120 minutes of aldicarb treatment ($p<0.01$). Statistical significance was determined as described in Section 2.3.9 using a one-tailed student's t-test with unequal variance comparing untreated to selenium-treated populations.

After determining that selenium damaged animals have an increased resistance to aldicarb, we next needed to determine if there was postsynaptic damage. By determining whether selenium damaged animals would respond to the nicotinic agonist, levamisole. In wild-type animals, levamisole acts post-synaptically on muscles to cause the body wall muscles to hyper-contract resulting in paralysis (Culetto et al. 2004). For this assay, selenium-treated animals that failed to complete one sinusoidal turn within 5 seconds of being tapped on the tail were tested for altered sensitivity/resistance to the nicotinic agonist, levamisole, since they are the most severely affected animals that are still alive. Less severely affected animals will likely be sensitive to levamisole since they are less likely to have developed muscle damage and potentially skew the data with potentially false positives.

In Figure 13A, 10 untreated wild-type animals were plated within a 1cm circle on a plate. After 5 minutes (Figure 13B), all but one animal had moved outside of the 1cm circle, confirming their motility. In Figure 13C, fifteen animals that failed to complete one sinusoidal turn within 5 seconds of a harsh touch after selenium treatment were plated within a 1cm circle. After 5 minutes (Figure 13D), the animals had moved, but all fifteen remained inside the 1cm circle. This demonstrates the motility of the untreated animals and the relative paralysis of the selenium-treated animals. Animals from Figure 13A,B (untreated) and Figure 13C,D (animals paralyzed by selenium) were then treated with levamisole. Figure 13E shows a N2 control animal while Figure 13F shows the same animal post-levamisole treatment. The animal appears shorter and eggs are extruded after levamisole treatment, both phenotypes due to muscle hypercontraction. Similar results are observed with levamisole treatment of selenium paralyzed animals (Figure 13G and 13H). This data suggests that the muscles can still function.

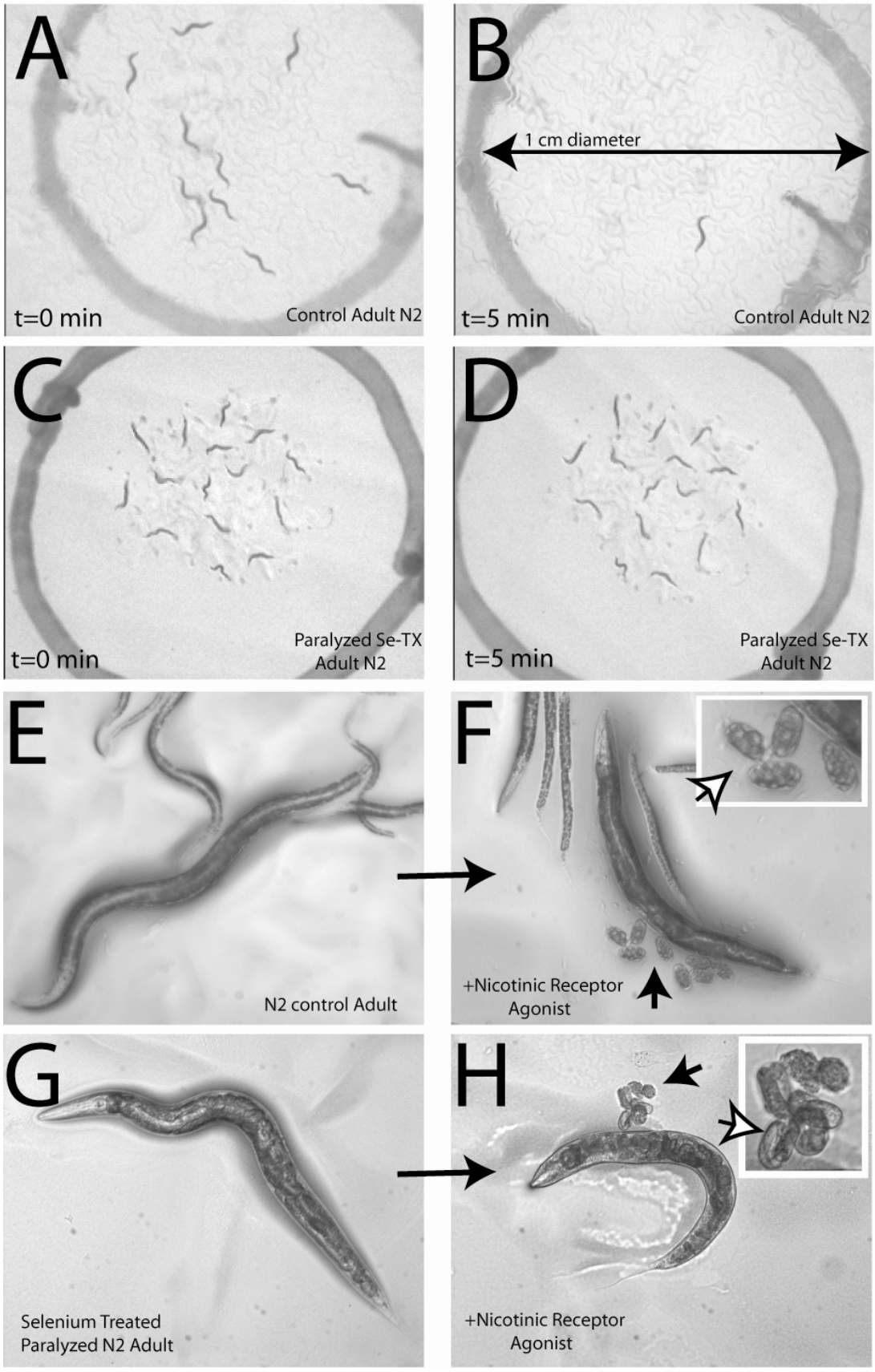

A
t=0 min
Control Adult N2
B
1 cm diameter
t=5 min
Control Adult N2
C
t=0 min
Paralyzed Se-TX
Adult N2
D
t=5 min
Paralyzed Se-TX
Adult N2
E
N2 control Adult
F
+Nicotinic Receptor
Agonist
G
Selenium Treated
Paralyzed N2 Adult
H
+Nicotinic Receptor
Agonist

**Figure 13 Levamisole causes hypercontraction in sodium selenite-paralyzed animals**

The muscles of animals paralyzed by selenium hyper-contract when treated with levamisole, demonstrating that they are still functional. **(A)** Untreated animals placed in a 1cm circle at t=0. **(B)** Untreated animals plated in a 1cm circle at t=5minutes. **(C)** Animals paralyzed by selenium treatment placed inside a 1cm circle at t=0. **(D)** Animals paralyzed by selenium treatment were placed inside a 1cm circle at t=5 minutes. **(E)** Untreated animal, prior to levamisole treatment. **(F)** Untreated animal after levamisole treatment. Black points to post-levamisole treatment. Animal is hypercontracted as observed by decreased length. White arrows point to early staged eggs released due to levamisole-induced hypercontraction. **(G)** Animal paralyzed by selenium before levamisole treatment. **(H)** Animal paralyzed by selenium after levamisole treatment. Black arrow points to eggs released by levamisole-induced muscle hypercontraction.

### 3.4.5 Egg-laying is decreased by selenium exposure

One of the initial observations in selenium-treated animals was that they retained eggs, as indicated by the presence of late stage embryos (Figure 9H). This phenotype provides another method of differentiating whether selenium causes neuronal or muscle damage, since egg-laying in *C. elegans* is a well-defined circuit involving two hermaphrodite specific neurons (HSNs), six ventral cord neurons (VC), and 16 vulval muscles (White 1986; Desai et al. 1988; Bany et al. 2003). Mutants that have abnormal egg-laying phenotypes are termed egg-laying defective or *egl*. This category is divided into two subcategories; egg-laying defective (Egl-d) and egg-laying constitutive (Egl-c).

Early work in *C. elegans* demonstrated that exogenous treatment of wild-type animals with the neurotransmitter serotonin causes them to lay eggs (Horvitz et al. 1982). Subsequently, HSNs were shown to contain serotonin and acetylcholine, suggesting a stimulatory role of

serotonin in the HSNs (Desai et al. 1988; Riddle 1997). Mutants that lack HSNs were found to be Egl-d (Desai et al. 1988). Laser ablation of the HSNs also caused defective egg laying, that could be rescued by treatment with exogenous serotonin (Waggoner et al. 1998). Combined, these studies demonstrate a stimulatory role of serotonin from the HSNs in the egg-laying phenotype. Animals in which the vulval muscles were laser ablated failed to respond to serotonin, demonstrating that serotonin acts on the uterine muscles to stimulate egg-laying (Trent et al. 1983).

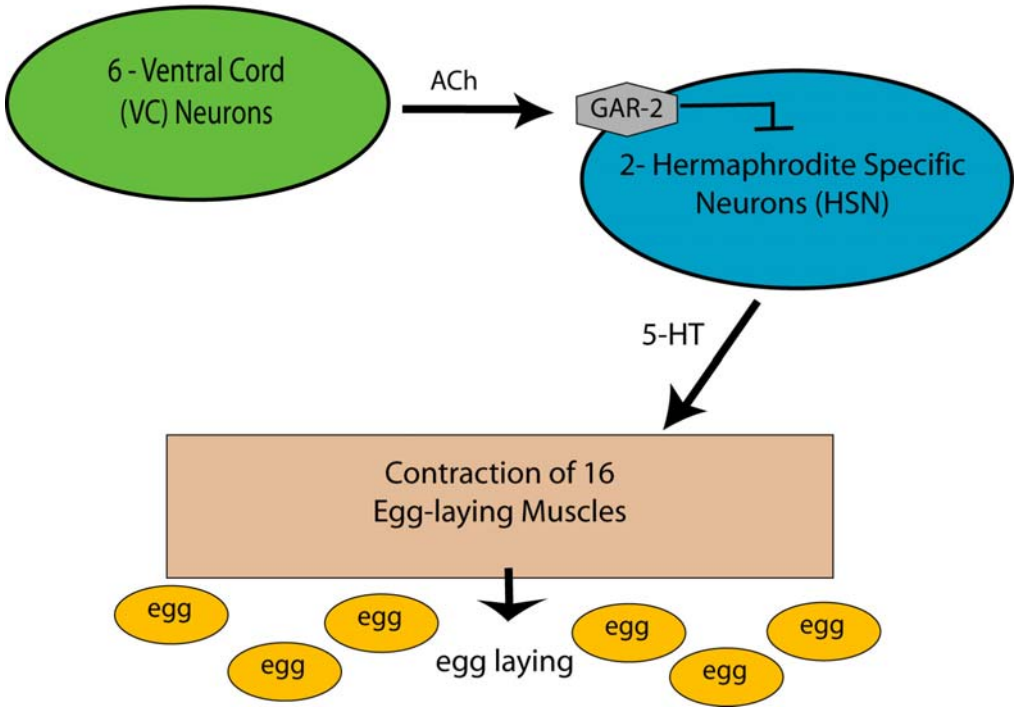



**Figure 14 Egg laying circuit in *C. elegans***

Modified from (Bany et al. 2003). Ventral cord neurons (VCs) synapse to the hermaphrodite-specific neurons (HSNs). Serotonin (5-HT) is released from the HSNs to trigger muscle contraction in the vulval muscles.

If selenium exposure caused neuronal damage, treatment with exogenous serotonin would rescue the egg-laying phenotype. Conversely, if selenium exposure caused muscle damage, the muscles would fail to respond to serotonin and the egg-laying phenotype would not be altered. To test this hypothesis, we first needed to determine how long after selenium exposure we could measure a significant decrease in the number of eggs laid when compared to untreated controls. Developmentally synchronized adult N2 animals were placed on untreated NGM plates or NGM plates containing 5mM sodium selenite. Animals were transferred to new plates at one hour intervals and incubated for 24 hours to determine the rate of eggs laid per animal per hour. After 6 hours, animals were found to have a statistically significant decrease in egg laying (Figure 15A). A significant decrease in egg lay was also observed after 5 hours of selenium treatment. However, the large deviation in the untreated populations made this time point less desirable than the 6 hour time point. This large deviation is likely due to a switch between active and inactive periods of egg laying, which has previously been reported and can be observed in Figure 15A-untreated (Hardaker et al. 2001). Untreated N2 animals had an average egg lay of 5.7+/-1.1eggs laid per animal per hour, while selenium treated animals had an average egg lay of 1.4+/-0.1 eggs laid per animal per hour ($p<0.01$).

Next, we determined whether the decreased egg-laying phenotype observed in selenium-treated animals was due to muscle damage by treating animals that had been exposed for 6 hours to 5mM sodium selenite with exogenous serotonin (Figure 15). First, untreated animals and selenium-treated animals were compared to confirm that selenium-treated animals had a statistically significant decrease in egg-laying, demonstrating that the selenium treatment damaged the animals as observed in Figure 15A (Figure 15B, $p<0.01$). Non-selenium-treated animals that were treated with serotonin were found to increase their egg-laying from 3.9+/-0.5

eggs/animal per hour to 15.5+/-1.1 eggs per animals per hour (Figure 15B, $p<0.001$). Selenium-treated animals also significantly increased the egg-lay in response to serotonin, from 1.8+/-0.3 eggs/animals/hour to 11.1+/-0.9 eggs/animal/hour (Figure 15B, $p<0.001$). It was also noted that there was a significant difference between untreated and selenium-treated animals when exposed to serotonin, demonstrating that the egg-laying was not completely rescued (Figure 15B, $p<0.01$).

There are several possible reasons for the incomplete rescue of egg-laying. This experiment only addressed the role of serotonin and the HSNs, and not the role of acetylcholine in the VCs. Cholinergic signaling is also involved in egg-laying, and acetylcholine is found in both HSNs and VCs (Bany et al. 2003). Agonists of nicotinic receptors have been found to stimulate egg-laying (Kim et al. 2001). However, the ventral cord expresses a muscarinic acetylcholine receptor that has an inhibitory role in egg-laying (Bany et al. 2003).

Selenium has also been shown to trigger apoptosis in a variety of cell types (Shen et al. 1999; Zhou et al. 2003; Goel et al. 2006; Xiao et al. 2006; Zhao et al. 2006; Zhao et al. 2006; Chen and Wong 2008). However, animals containing mutations for apoptotic and necrotic genes were found to be sensitive to selenium exposure (Figure 5). However, apoptosis in the worm was identified in a developmental setting as opposed to death induced by an environmental factor (Ellis and Horvitz 1986). While developmental apoptosis may not be responsible for death in the adult hermaphrodite, it may be responsible for cell death in the germ line. Both of these possibilities are subsequently addressed.

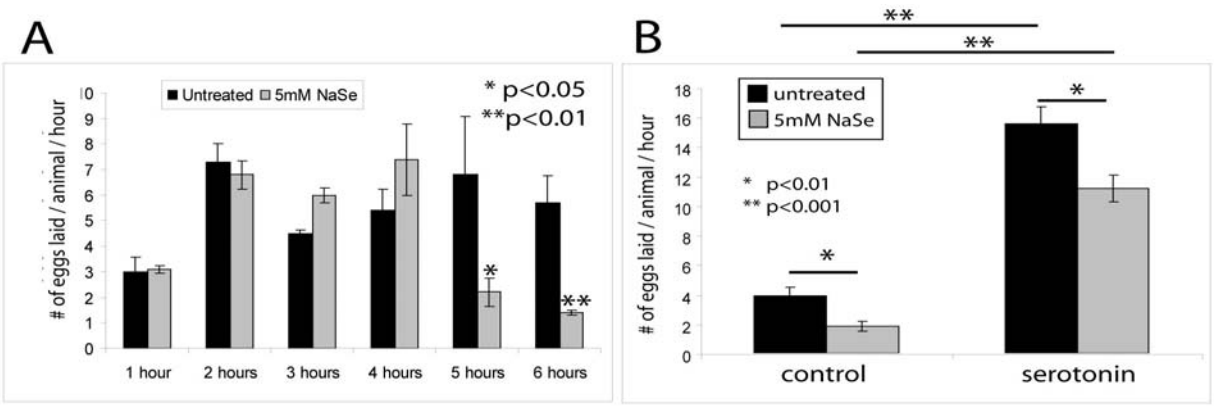



**Figure 15 Decreased egg-laying in N2 hermaphrodites observed after 6 hour selenium exposure**

Treatment of type hermaphrodites with 5mM sodium selenite causes decreased egg-laying that is partially rescued by the serotonin. **(A)** A significant decrease in egg-laying was observed after 6 hours of selenium exposure. Developmentally synchronized adult N2 hermaphrodites were placed on untreated NGM plates or NGM plates containing 5mM NaSe. At one hour intervals of NaSe, 10 animals were transferred to fresh untreated NGM plates for 24 hours. Adult hermaphrodites were then removed, and the eggs/progeny were counted and the average number of eggs laid per animal per hour was calculated (average of 3 plates). A student t-test (two-tailed, unequal variance) determined this difference to be significant between untreated and NaSe-treated populations at corresponding time points ($p<0.05$ or 0.01 as indicated on the graph). The animals were grown and the experiment performed at 25°C. **(B)** Treatment with the serotonin partially but significantly restored the egg-laying deficit. All conditions were found to yield statistically different numbers of eggs laid. Untreated hermaphrodites were found to lay significantly more eggs than selenium-treated animals ($p<0.01$). Serotonin treatment of non-selenium-treated animals caused a significant increase in egg-laying compared to non-selenium-treated animals ($p<0.001$). Serotonin treatment of selenium-treated animals caused a significant increase in egg-laying compared to selenium-treated animals ($p<0.001$). However, serotonin-treatment of selenium treated animals did not completely rescue egg-laying behavior when compared to serotonin treatment of non-selenium-treated animals ($p<0.01$). Developmentally synchronized N2 hermaphrodites were placed on untreated or 5mM NaSe supplemented NGM plates for 6 hours. Three hermaphrodites were then transferred to plates containing 3mg/ml of serotonin. After one hour, the adult animals were removed from the plates, the eggs were counted, and the number of egg laid per animal was calculated.

### 3.4.6 Reduction-of-function mutation of a G-protein coupled muscarinic acetylcholine receptor confers resistance to selenium

One possible explanation for the incomplete rescue of egg-laying in selenium-treated animals treated with serotonin could be due to the role of acetylcholine. While HSNs are both cholinergic and serotonergic, the ventral cord neurons are primarily cholinergic (Wood 1988). Egg-laying can be stimulated acutely by nicotinic agonists acting on the vulva muscle and adapt over time, resulting in egg-laying returning to pre-nicotinic agonist rates (Waggoner et al. 2000). When analyzed, a majority of Egl-c mutants were found to have defects in ventral cord structure. Laser ablation of the VCs led to hyperactive egg laying, suggesting an inhibitory role from the VCs in the egg-laying phenotype (Bany et al. 2003). Further, mutants that have decreased acetylcholine synthesis also exhibit hyperactive egg-laying (Schafer et al. 1996). This evidence suggests that the VCs, which contain acetylcholine, have an inhibitory role in egg-laying.

When originally designing the experiment to determine whether serotonin could rescue the decreased egg-laying phenotype in selenium-treated animals, we sought to use a mutant for the G-protein coupled acetylcholine receptor, GAR-2. GAR-2 is a muscarinic type acetylcholine receptor but also has distinct unrelated properties that is expressed mid-embryogenesis through adulthood in the VCs, HSNs, as well as in ciliated head neurons. GAR-2 has been shown to have an inhibitory effect on egg-laying by inhibiting serotonin release from the HSNs (Bany et al. 2003). If this inhibitory mechanism on egg-laying could be removed, exposure to exogenous serotonin and nicotine might fully rescue egg-laying.

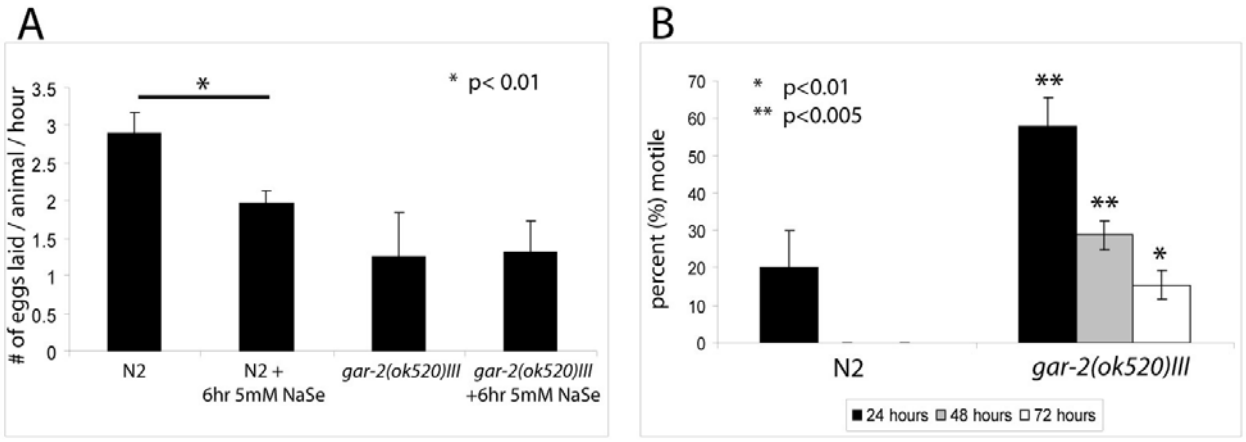



**Figure 16 Reduction-of-function mutation in a AChR, GAR-2, have increased resistance to 5mM sodium selenite**

A reduction-of-function mutation in the muscarinic acetylcholine receptor, *gar-2*, confers resistance to the effects of selenium exposure on egg-laying and motility. (**A**) The egg-laying assay was performed as described in Section 3.3.7. N2 animals were found to have a statistically significant decrease in egg-laying when exposed to selenium ($p<0.01$). *gar-2(ok520)* was found to have no affect on egg-laying after exposure to selenium ($p=0.5$). Developmentally synchronized adult hermaphrodites were plated on 5mM NaSe supplemented plates and untreated plates for 6 hours at 25°C. Four hermaphrodites were then transferred to fresh, unsupplemented plates for 24 hours. The adult hermaphrodites were then removed and the eggs/progeny were counted to calculate rate of egg lay. Statistical analysis was performed as described in Section 2.3.9, with the exception of using a two-tailed test. (**B**) Adult *gar-2(ok520)* hermaphrodites were found to have an increased resistance to selenium at all time points, as observed by retaining normal motility (24 hours: $p<0.005$, 48 hours: $p<0.005$, and 72 hours: $p<0.01$). Developmentally synchronized adult *gar-2(ok520)* hermaphrodites were grown at 25°C and then placed on NGM plates treated with 5mM sodium selenite and scored at 24 hour intervals for movement deficits. Statistical analysis was performed using a one-tailed student t-test with unequal variance as described in Section 2.3.9 comparing mutant *gar-2* to N2 at corresponding time points.

As seen in Figure 16A, a significant decrease in egg-laying was observed in the N2 animals ($p<0.01$), demonstrating that 6 hours of selenium exposure was sufficient to decrease egg-laying in wild-type animals. However, when the rate of egg-laying was calculated for animals with a reduction-of-function mutation in the G-protein coupled acetylcholine receptor, *gar-2(ok520)*, no decrease was observed between untreated and selenium treated animals. However, this measure for lack of neuronal damage is limited due to a statistical concept called floor or basement effect. Untreated *gar-2(ok520)* animals have an average egg lay of 1-1.5 eggs per animal per hour, which could make it difficult to obtain a measurable decrease with selenium exposure. Eggs could accumulate in the adult hermaphrodites as a result of selenium exposure, resulting in some eggs randomly being extruded due to lack of physical space and a vulval opening.

To confirm that the *gar-2(ok520)* mutations conferred resistance to selenium, adult animals were examined using the motility assay. As shown in Figure 16B, mutant *gar-2(ok520)* animals have a statistically significantly increased resistance to selenium treatment at all time points tested in this assay (Figure 16B, 24hours: $p<0.005$, 48hours: $p<0.005$, 72hours $p<0.01$). Hence, *gar-2(ok520)* provides resistance to selenium toxicity. It is intriguing that wild-type animals exposed to selenium have a decrease in cholinergic signaling as determined by aldicarb resistance and a mutation in an acetylcholine receptor provides protection to selenium exposure. While these combined data could support the hypothesis that neuronal dysfunction causes impaired movement and decreased egg-laying, it must also be remembered that when a gene is mutated, other genes can alter expression to compensate. For example, compensatory up-regulation of other receptors may alter these two phenotypes.

In order to develop firm hypothesizes and conclusions, additional information would need to be obtained about the *gar-2(ok520)* mutant. For example, *gar-2(ok520)* is resistant to the aldicarb when suppressing egg-laying (Bany et al. 2003). If cholinergic signaling is decreased, then are adult *gar-2(ok520)* animals resistant to aldicarb-induced paralysis? Are other acetylcholine receptors up-regulated when GAR-2 is mutated? Is GABAergic or serotonergic signaling altered in animals with in *gar-2(ok520)* mutants? Despites these caveats, the fact that a gene mutation of a neuronally-expressed protein resulted in significant protection from selenium exposure, suggesting that selenium is damaging neurons.

### 3.4.7 Incomplete rescue is at least partially due to increased apoptotic figures in the turn region

Serotonin treatment of selenium-treated animals partially rescued the observed egg-laying deficit. However, the rescue was not complete (Figure 15B). One possibility for the remaining deficits is that fewer eggs are being made as a result of increased apoptosis in the germ line, since, as stated previously, selenium exposure has been shown to increase apoptosis in a variety of cell types (Shen et al. 1999; Zhou et al. 2003; Goel et al. 2006; Xiao et al. 2006; Zhao et al. 2006; Zhao et al. 2006; Chen and Wong 2008). In Figure 5, adult hermaphrodites with mutations in genes involved in apoptosis were not protected from selenium exposure. However, this does not exclude the possibility that the germ line cell population is undergoing increased apoptosis as a result of selenium exposure.

*C. elegans* contains two gonads that connect to the uterus. The distal tip of each gonad contains the growth factor LAG-2, which promotes mitosis in neighboring germ cells (Lettre et

al. 2004). As the germ cells leave the distal tip, they enter meiosis and either develop into oocytes or undergo apoptosis as they travel through the turn region to enter the uterus (Lettre et al. 2004). Apoptosis can be measured in the germ line by detection of apoptotic bodies after feeding animals acridine orange-stained *E. coli* (Gumienny et al. 1999). If selenium treatment is causing increased apoptosis, an increased number of acridine orange-stained apoptotic bodies would be observed in germ line.

To test this hypothesis, developmentally synchronized animals were placed on NGM plates that were unsupplemented or supplemented with 5mM sodium selenite for 24 hours. Animals were then stained with acridine orange and examined for apoptotic bodies in the turn region of the gonad (Figure 17A-D). Animals treated for 24 hours with selenium were found to have approximately 3 times more apoptotic bodies than untreated animals (Figure 17E, $p<0.001$). This number is an estimate due to egg retention in selenium-treated animals making an accurate count difficult. However, the increased number of apoptotic bodies suggests that there is a decrease in the number of eggs being produced.

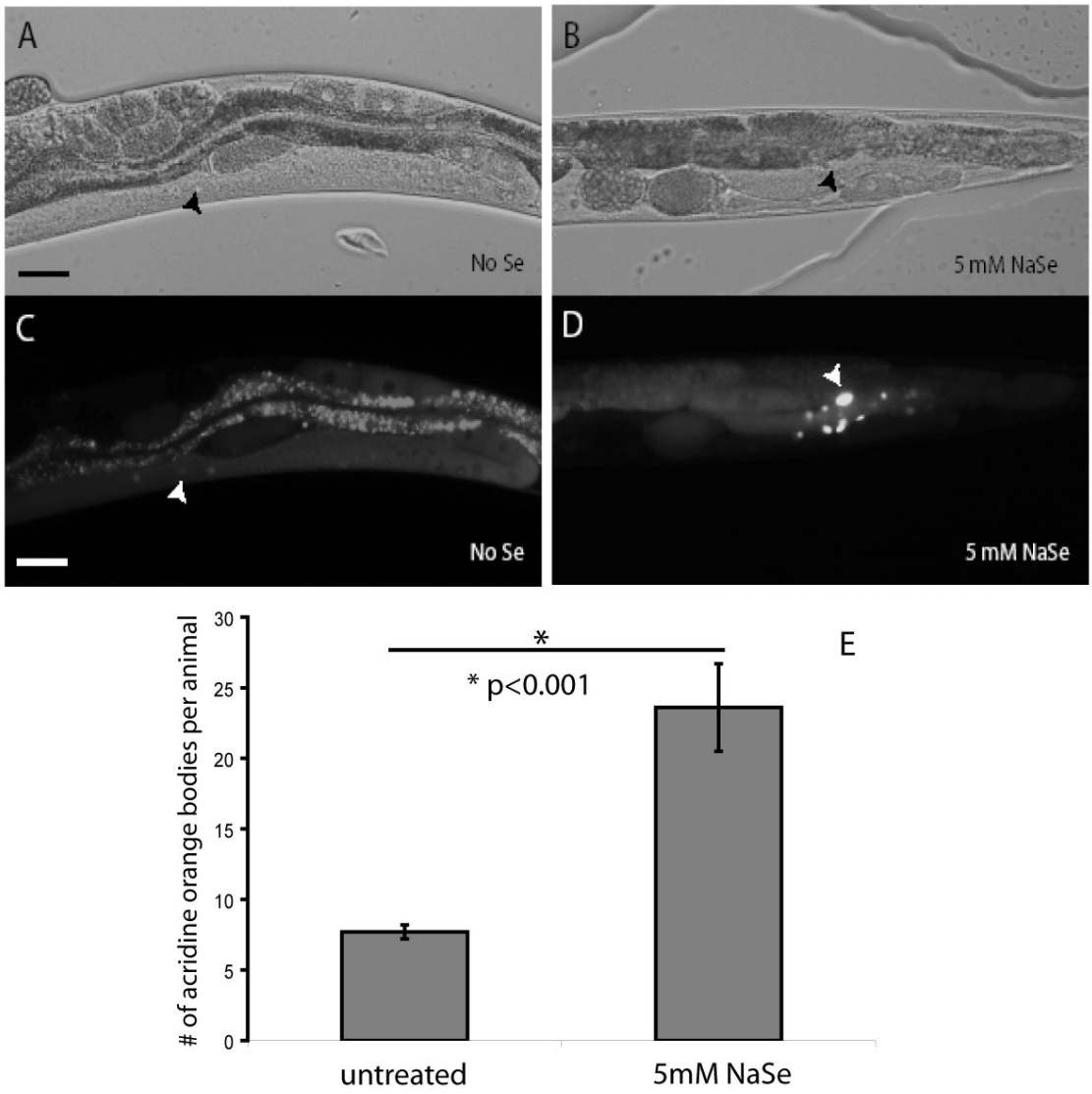



**Figure 17 Increased apoptosis observed in the germ line of selenium treated animals**

Increased apoptosis was observed in the germ line of selenium-treated animals. Animals were analyzed first using Nomarski optics to identify the distal tip/turn region in the uterus. **A** Untreated, **B** selenium-treated. Black arrows identify the region of interest). Animals were then analyzed by fluorescence microscopy in order to count fluorescent apoptotic bodies. **C** Untreated, **D** Selenium-treated. White arrows identify apoptotic bodies in the region of interest). **E** Graph representing the average number of apoptotic bodies in the uterine turn region of untreated and selenium treated animals. A two-tailed student t-test with unequal variance determined there to be a significant difference between untreated and selenium-treated

animals (n=20, $p<0.001$). Developmentally synchronous N2 hermaphrodites were placed on NGM plates treated with 5mM selenium or untreated. After 24 hours, untreated and selenium treated hermaphrodites were placed on new NGM plates treated with 0.02mg/ml acridine orange for 1 hour (Lettre et al. 2004). The animals were then transferred to a new untreated plate for an hour to clear their intestinal track and visualized by Nomarski optics and fluorescence microscopy.

## 3.5 DISCUSSION

This section provides significant evidence that selenium causes neuronal damage, specifically decreasing cholinergic signaling. This work also is important in establishing a model of neurodegeneration using selenium toxicity in *C. elegans* that differs from previous models. Neurodegenerative models for Alzheimer's disease and Huntington's disease in *C. elegans* both require expression of a human transgenic protein (Faber et al. 1999; Link et al. 2003; Link 2006).

The description of gross morphological changes resulting from selenium exposure is significant because it differs from the MEC-4(d) model of neurodegeneration. The MEC-4(d) model of neurodegeneration results from a dominant mutation in an amiloride sensitive Na+ channel that results in the swelling of a subset of neurons, which are involved in sensing touch along the animal's body wall (Driscoll and Chalfie 1991). Of note, MEC-4(d) neurodegeneration causes large vacuoles to form that are nearly half width of the body (Bianchi et al. 2004). This was never observed in animals exposed to selenium. MEC-4(d) vacuole formation is also suppressible by treatment with weak bases, a treatment that did not confer protection in selenium treated animals (Chapter 2, Figure 6) (Artal-Sanz et al. 2006). Further, mutations in calreticulin were found to suppress MEC-4(d) neurodegeneration, whereas we found that the *crt-1(rf)* mutation increased sensitivity to selenium (Chapter 2, Figure 5) (Xu et al. 2001). The differing morphology, effects of weak bases on survival, and survival with calreticulin mutations demonstrate that selenium induced neuronal damage is different from the MEC-4(d) model of neurodegeneration.

While there was no observable damage in gross muscle morphology as a result of selenium exposure, there was an increase in cytosolic protein turnover. This increase in protein catabolism was suppressible by treatment with the nicotinic agonist levamisole and the acetylcholinesterase inhibitor, aldicarb, suggesting that the increased protein catabolism is due to decreased cholinergic. This is significant, as ALS disease progression is associated with prominent muscle wasting that has been shown to involve catabolism of muscle proteins, a finding suggesting that loss of cholinergic motor neuron innervation leads to increased breakdown of muscle proteins (Corbett et al. 1982).

The decreased cholinergic signaling suggested by the increased muscle catabolism was confirmed when animals determined to be damaged by selenium (i.e. exhibited backing deficits) showed increased resistance to paralysis induced by the acetylcholinesterase inhibitor, aldicarb. This is intriguing, since decreases in cholinergic receptors have been reported in ALS tissue, although the etiology of this is unknown (Manaker et al. 1988; Berger et al. 1992). In addition, the muscles of severely selenium-damaged animals were retained the ability to hyper-contract when treated with the nicotinic agonist, levamisole. This is unsurprising, as selenium-treated animals were observed to be hyper-contracted or shorter when compared to untreated animals (personal observation and non-formally confirmed with the Biosorter in Chapter 2 where the "time-of-flight" was observed to be decreased in selenium-treated animals when compared to untreated animals, suggesting that selenium treated animals were shorter in length). Combined, these data suggest that high selenium exposure causes neurotoxicity that results in decreased cholinergic signaling, resulting in increased muscle catabolism. However, the studies in this dissertation do not address the possibility that neuronal neurotransmission in general is being affected, and not only cholinergic signaling.

This chapter also demonstrated that selenium causes damage to the egg-laying circuit. An irreversible decrease in egg-laying was observed after only 6 hours of selenium treatment. This decrease was significantly but incompletely rescued by treatment with serotonin and the incomplete rescue appears to be due to increased apoptosis in the germ line. Combined, these data demonstrate that the decreased egg-laying caused by selenium exposure is due to pre-synaptic (neuronal) damage, since the muscles can still respond to serotonin. What remains unknown is what neurotransmitter(s) is being affected. The most direct possibility is that serotonin signaling is being decreased, and that supplementation with exogenous serotonin rescues the deficit. Another school of thought is that egg-laying is controlled by cholinergic signaling and that serotonin is simply "priming" the muscles to for acetylcholine (Weinshenker et al. 1995). This introduces a subtle distinction in the role of neurotransmission control of *C. elegans* egg-laying. With either possibility, the data presented here demonstrates that the vulval muscles can still function and that neurotransmission (either serotonin and/or acetylcholine) is being decreased.

The increased resistance of the muscarinic cholinergic receptor mutant, *gar-2(ok520),* underscores the fact that selenium is damaging neurons. GAR-2 is only expressed in neurons, including ventral cord neurons (VCs) and hermaphrodite specific neurons (HSNs); however, more research is required to understand the protective role of *gar-2(ok520).* One potential hypothesis is that the decrease in cholinergic signaling may be a protective adaptation in response to selenium exposure. However, to date, the only known function for *gar-2* is an inhibitory role on egg-laying. If GAR-2 has other unknown inhibitory functions, this would suggest that cholinergic signaling is increased in *gar-2* mutants, and that the protection in motility is a result of maintaining higher levels of cholinergic signaling compared to wild-type

animals when exposed to selenium. Before addressing either of these possibilities, more information needs to be learned about *gar-2(ok520).* The mutant appears to be a knockout containing a 1.3kb deletion that encompasses part of the 5' UTR, as well as the first exon and intron. However, *C. elegans* contains two additional mAChR, GAR-1 and GAR-3, that, although less well characterized, could potentially compensate for *gar-2(ok520).*

This chapter primarily focused on changes in cholinergic signaling in response to selenium exposure. However, alterations in GABAergic signaling resulting from selenium toxicity are unknown at this time. Potential methods of investigating altered GABAergic signaling involve two possible assays. One involves touching the animals and scoring a "shrinker" phenotype (McIntire et al. 1993; McIntire et al. 1993). That is, causing the body wall muscles to contract simultaneously, causing the animal to temporally shrink. Decreased cholinergic signaling may be difficult to measure with this assay, as selenium-treated animals already appear to be shorter than untreated animals (personal observation). A second possible assay to test defects in GABA neurotransmission would be to measure defecation since the enteric muscle contractions resulting in expulsion of waste is controlled by GABA (McIntire et al. 1993; McIntire et al. 1993).

This chapter provides significant evidence that excess selenium exposure causes neuronal damage and could potentially be used as a model for sporadic ALS, the first model of sporadic ALS. However, further work needs to be done in investigating alterations in neurotransmitters other than acetylcholine.

# 4.0 THE ROLE OF THE INSULIN PATHWAY IN SELENIUM TOXICITY

## 4.1 ABSTRACT

The DAF-2-insulin-like pathway is a major stress response in *C. elegans* that controls gene expression of stress responsive genes, including superoxide dismutases (SOD), glutathione peroxidases, catalases, etc. Previously in Chapter 2, we have shown that increased oxidative stress contributes to selenium toxicity. Here, we show that mutations in genes from the DAF-2-insulin pathway alter sensitivity/resistance of animals to toxic selenium toxicity. Animals with mutations in the insulin-like receptor, *daf-2*, are known to have an extended life as well as altered metabolism. Previous studies have shown that tissue-specific expression of wild-type *daf-2* in *daf-2* mutants differentially rescues the long life and altered metabolism in the neurons and gut, respectively. We investigated whether tissue-specific expression of wild-type *daf-16* in the neurons, muscle, or gut, thus allowing a responsive wild-type insulin pathway, could restore resistance to selenium. Here, we show that addition of wild-type DAF-16 in any tissue can partially restore resistance to selenium toxicity, with the most protection provided by intestinal expression. A reduction-of-function mutation in the iron-manganese superoxide dismutase gene, *sod-2*, was found to partially restore selenium sensitivity to *daf-2(rf)* mutants.

## 4.2 INTRODUCTION

In chapter 2, we showed that selenium toxicity causes an increase in ROS (Figure 7). Further, movement deficits caused by selenium exposure could be alleviated by various antioxidants in wild-type *C. elegans* (Figure 8). To gain insight into potential mechanism(s) of selenium toxicity, common stress pathways were screened for sensitivity/resistance to selenium toxicity. Mutations in genes encoding members of the DAF-2-insulin signaling pathway provided the most strikingly altered sensitivity.

In a permissive growth environment, a single wild-type *C. elegans* will develop from an egg passing through four larval stages before reaching adulthood in 3.5 days at 20°C and live approximately two weeks (Brenner 1974). Under conditions of environmental stress, such as decreased food supply or overcrowding, *C. elegans* can enter an alternate third larval stage called dauer. Animals in the dauer larva stage can live for months and have increased resistance to heat, microbials, and others stresses (Cassada and Russell 1975; Albert et al. 1981; Riddle et al. 1981; Golden and Riddle 1984). Mutations in genes have been identified that alter an animal's ability to enter the dauer larva stage and are referred to as having abnormal *da*uer larva *f*ormation, or Daf. There are two classes of *daf* genes, dauer larva constitutive (*daf-c*) or dauer defective (*daf-d*). Daf-c animals enter the dauer larva stage at an increased rate when exposed to high temperature (Swanson and Riddle 1981). While Daf-d animals fail to enter the dauer larva state regardless of environmental conditions (Wood 1988). Both *daf-d* and *daf-c* mutations can be found in the DAF-2 insulin-like pathway.

Mutations in the gene encoding the insulin-like receptor, DAF-2, have been found to regulate dauer larva development, with temperature-sensitive reduction-of-function mutations causing dauer larvae formation in the absence of other environmental cues, like overcrowding or

starvation (Riddle 1977; Kenyon et al. 1993). Adult animals containing mutant *daf-2* share characteristics with dauer larvae, such as increased lifespan, resistance to oxidative stress, and themotolerance (Kenyon et al. 1993; Lithgow et al. 1995; Honda and Honda 1999). Under normal growth conditions, the DAF-2-insulin-like receptor initiates a signaling cascade leading to the subsequent phosphorylation and inactivation of the FOXO transcription factor DAF-16, preventing it from entering the nucleus (Dorman et al. 1995; Vanfleteren and De Vreese 1997; Paradis and Ruvkun 1998; Lin et al. 2001). When under environmental stress, these signaling events do not occur and DAF-16 constitutively localizes to the nucleus initiating the transcription of stress-related response genes.

Mutations in the *daf-2* gene and its downstream effector, the phosphoinositide 3-kinase, AGE-1, exhibit increased lifespan and levels of antioxidant enzymes (Kenyon et al. 1993; Dorman et al. 1995; Honda and Honda 1999; McElwee et al. 2003). Additionally, both *daf-2* and *age-1* mutations confer antimicrobial properties and decreased metabolism (Vanfleteren and De Vreese 1995; Garsin et al. 2003). Mutations in the gene encoding the major downstream target DAF-16 confer the opposite phenotypes, resulting in decreased lifespan, inability to form dauer larvae, and sensitivity to external stress (Gottlieb and Ruvkun 1994; Larsen et al. 1995; Murakami and Johnson 1996). A number of studies have been performed to identify downstream targets of the DAF-16 transcription factor. These have identified a large number of stress response genes, including catalases and iron-manganese superoxide dismutases (McElwee et al. 2003; Murphy et al. 2003; Oh et al. 2006).

Since discovery of the increased longevity and stress resistance of *daf-2* mutants, focus has turned to the targets of DAF-16 that are responsible for various phenotypes (Honda and Honda 1999; Ookuma et al. 2003; Lamitina and Strange 2005). A popular theory for aging

involves an accumulation of cellular damage from free radicals, leading to dysfunction and death (Harman 1960). DAF-16 targets that are involved in antioxidant responses, like iron manganese superoxide dismutases (SOD) and catalases, have been investigated for their potential roles in longevity. One Mn-SOD, SOD-3, has increased mRNA expression in both dauer larvae and *daf-2* mutants, suggesting that SOD-3 is important in extending lifespan (Honda and Honda 1999; Lee et al. 2003; McElwee et al. 2003; Murphy et al. 2003). However, individual reduction-of-function mutants for either *sod-3* or *sod-2* had no effect on stress resistance or longevity (Honda et al. 2008). Decreased expression of both Mn-SODs in the *daf-2* mutant background reversed the increased resistance to oxidative stress but did not affect longevity (Honda et al. 2008). Treatment of wild-type animals with superoxide dismutase and catalase mimetics has been found to have conflicting effects on *C. elegans* lifespan (Melov et al. 2000; Keaney et al. 2004). The evolving role of Mn-SODs suggests that their function is more complex than simply reducing oxidative stress.

Alterations in insulin signaling have been shown to exhibit different phenotypes depending upon the tissue in which it is expressed. Neuronal expression of wild type DAF-2 or AGE-1 in *daf-2* or *age-1* respective mutant backgrounds rescues the extended life span found in these mutants, while muscle-specific expression of DAF-2 and AGE-1 rescues the metabolism phenotype (Wolkow et al. 2000). Another study found that intestinal expression of DAF-16 in a *daf-16* mutant background partially restored the decreased lifespan phenotype of the mutant (Libina et al. 2003). This differential tissue-specific rescue of metabolism and lifespan poses similar questions as to which tissue the insulin pathway is functioning in to provide protection from toxic environmental exposures, such as with selenium.

Here, we show that disinhibiting the insulin pathway alters the organisms' sensitivity to high selenium exposure. Conversely, mutations in the gene encoding the insulin-like receptor DAF-2 and its downstream target AGE-1, which have been shown to confer increased resistance to oxidative stress, were also found to protect against selenium toxicity. Similarly, mutations in the downstream target, the FOXO transcription factor daf-16, confer sensitivity to selenium toxicity. Next, we investigated if there is a tissue specific requirement for insulin signaling with selenium toxicity. Over-expression of the FOXO transcription factor DAF-16 under the control of tissue specific promoters demonstrates that expression in any tissue confers protection while the greatest protection is provided by intestinal expression. Two targets of DAF-16 were also investigated, SOD-2 and SOD-3 for altered sensitivity to selenium exposure. Interestingly, mutations in the gene *sod-2* conferred sensitivity while mutations in gene *sod-3* provided resistance to selenium. A double *sod-2;sod-3* mutant revealed an epistatic relationship in which SOD-3 is epistatic to SOD-2. Introducing mutant *sod-2* into the *daf-2* mutant background was found to marginally, but significantly, increase sensitivity to selenium.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Strain maintenance and growth conditions

The following strains were obtained from the *Caenorhabditis* Genetics Center; wild-type Bristol N2, TJ1052: *age-1(hx546)II*, GR1310: *akt-1(mg144)V*, DR26: *daf-16 (m26)I*, CF1038: *daf-06(mu86)I*, DR1564: *daf-2(m42)III*, CB1370: *daf-2(e1370)III*, RB1072: *sod-2(ok1030)I*, VC433: *sod-3(gk235)X*, GR1333: *tph-1::GFP; rol-6(su1006)V*, CF1442: *daf-16(mu86)I;daf-2(e1370)III; muEx169 [unc-119p::GFP::daf-16 + rol-6(su1006)]*, CF1514 *daf-16(mu86)I;daf-2(e1370)III*; muEx211 [pNL213(ges-1p::GFP::daf-16) + rol-6(su1006)], CF1515 *daf-16(mu86)I;daf-2(e1370)III; muEx212 [pNL212(myo-3p::GFP::daf-16) + rol-6(su1006)]*.

Strains GA410: *wuIs56[sod-3::GFP;pRRF4 rol-6(su1006)]* indicated in the text as *sod-3::GFP* and GA429: *wuIs72[sod-2::GFP;pRRF4 rol-6(su1006)]* indicated in the test as *sod-2::GFP* were kinds gifts from David Gems in the Institute of Healthy Living at the University College of London. All strains used were maintained on standard NGM plates and experiments performed at 20°C unless otherwise stated without additional calcium as described in 2.3.1.

### 4.3.2 Developmentally synchronizing worm populations

Age synchronous animals were obtained as described in Section 2.3.2.

### 4.3.3 Selenium treatment

Sodium selenite was added to NGM plates (without additional calcium) to a final concentration of 5mM. Developmentally synchronized adult animals were then plated on 5mM sodium selenite supplemented plates and analyzed at 24-hour intervals for the behavioral deficits described in Section 2.3.4.

### 4.3.4 Movement assay

After plating developmentally synchronized adult animals on various conditions, animals were scored at 24-hour intervals for the following behaviors as described in Section 2.3.4. Animals were defined to be motile if they were able to move forward and reverse direction upon touching to the head and tail. Impaired backing was defined by the inability to reverse direction upon tactile stimulation to the head/tail. This test was performed twice to ensure that the behavior was reproducible for the animal. Animals had to fail this test twice to be considered as having impaired backing. Paralysis was defined as failure to complete one sinusoidal turn forward after a touch. Death was defined by lack of pharyngeal pumping. “Selenium-damaged animals” encompass those defined as “backing”, “paralyzed”, and “dead”.

### 4.3.5 Statistical analysis

Statistical analyses were performed as described in Section 2.3.9.

### 4.3.6 Isolation of genomic DNA

Genomic DNA used for genotyping mutants was obtained from a small number of animals by the following lysis procedure: lysis buffer contained the following chemical composition: 50mM KCl, 10mM Tris-HCl, pH 8.3, 2.5mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20 in autoclaved de-ionized water (Wicks et al. 2001). 100-250µl of lysis buffer was added to a separate microcentrifuge tube along with a fresh pinch of Proteinase K. Then, 20µl of lysis buffer was aliquoted into polymerase chain reaction (PCR) tubes and 20 well-fed F2 cross progeny were added to each tube. The PCR tubes containing the lysis buffer and worms were placed in the PCR machine (Eppendorf Mastercycler, Westbury, NY) running a program containing the following steps: 60°C for 60 minutes to lyse the worms, 95°C for 15 minutes to inactivate the Proteinase K, and 4°C for a continuous hold until the tubes were removed from the machine and the isolated DNA was then used for genotyping.

### 4.3.7 PCR genotyping

#### 4.3.7.1 *sod-2(ok1030)I*

RB1072 is the strain containing the mutant allele *sod-2(ok1030)I*. This mutant allele has a 900bp deletion and was created by the *C. elegans* Gene Knockout Consortium located at the Oklahoma Medical Research Foundation funded by the National Institutes of Health. The mutant has no observable phenotype and has to be identified through genotyping by the PCR. The primer sequence for genotyping was provided by the *C. elegans* Gene knockout Consortium.

RB1072L (Internal Left) 5’ TCG AGG CTG GAA CTT CAA CT

RB1072R (Internal Right) 5’ CCC CTA ATA ACT GCA CCG AA

The PCR reaction for RB1072 was as follows: 12.5µl of GoTaq Green Master Mix from Promega (Cat # M7123), 15pmol of each primer, 1ul of worm lysis as prepared in Section 4.3.6, and brought to a final volume of 25µl with nuclease-free water. The PCR was run under the following conditions on an Eppendorf Master Cycler (Westbury, NY):

Step 1: 94°C for 2 minutes

Step 2: 94°C for 30 seconds

Step 3: 61°C for 30 seconds

Step 4: 72°C for 2 minutes and 30 seconds

Step 5: Go to Step 2 and repeat 30 times

Step 6: HOLD 4°C indefinitely

Amplified samples were run on a 1% agarose 0.5X TAE gel. The gels were then stained with SYBR Gold (1:10,000, Invitrogen, Carlsbad, CA) to stain the DNA. The gels were then visualized on a UV light box and a picture was taken for documentation. The wild-type band was 2320bp in length and the mutant band was 420bp in length.

#### 4.3.7.2 *sod-3(gk235)X*

VC433 is the strain containing the mutant allele *sod-3(gk235)X*. This mutant allele has a 390bp deletion and was created by *C. elegans* Gene Knockout Consortium at the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia. Like the mutant for *sod-2*, *sod-3(gk235)X* has no observable phenotype so PCR genotyping was utilized when creating double mutants.

PCR primers were designed using the bioinformatics program Primer Design which is part of the larger bioinformatics bundle, DNA STAR program (DNASTAR, Inc, Madison WI). s*od-3* genomic DNA sequence was obtained from the NCBI database. Primer Design delimited

the location for potential primers to sequences left and right of the deletion. The program was further delimited to create primer sets that generated a 800-1200bp wild-type gene product over the deletion location so that the mutant PCR product containing a 390bp deletion could easily be viewed on an agarose gel. The following primer set was chosen and ordered from IDT DNA Technologies, Inc (Coralville, IA).

VC433L 5’ CGG TTG CGG GGT AAG TC

VC433R 5’ CGA AGT CGC GCT GAA ACA A

The PCR reaction for VC433 was as follows: 12.5ul of GoTaq Green Master Mix from Promega (Cat # M7123), 15pmol of each primer per reaction, 1ul of worm lysis, and brought to volume with nuclease-free water to a final volume of 25ul. The PCR was run under the following conditions on an Eppendorf Master Cycler (Westbury, NY):

Step 1: 94°C for 2 minutes

Step 2: 94°C for 30 seconds

Step 3: 61°C for 30 seconds

Step 4: 72°C for 1 minute

Step 5: Go to Step 2 and repeat 30 times

Step 6: 72°C for 2 minutes

Step 7: HOLD 4°C indefinitely

Amplified samples were run on a 1% agarose 0.5X TAE gel. The gels were then stained with SYBR Gold (1:10,000, Invitrogen, Carlsbad, CA) to stain the DNA. The gels were then viewed on a UV light box and a picture was taken for documentation. The wild-type band was 952bp and the mutant band was 562bp in length.

### 4.3.8 Mutant phenotypic selection

#### 4.3.8.1 *daf-2(e1370)III* mutant selection

F1 cross progeny were grown at 25°C and F2 dauer larva were selected from unstarved plates. These animals exhibited the constitutive dauer phenotype, which confirms that they were homozygous for the *daf-2(e1370)* allele. The dauer larvae were allowed to recover at 15°C and PCR genotyping was used to confirm the presence of *sod-2(ok1030)*.

#### 4.3.8.2 Strains containing *rol-6(su1006)*

The following selection method was used for creating double mutant strains in which one gene was linked to *rol-6(su1006)*. This gene encodes cuticle collagen that when mutated alters the animals movement. The mutant allele *rol-6(su1006)* results in dominantly inherited right-handed rolling phenotype (Cox et al. 1980). Strains containing this mutant allele were created by crossing males containing the other mutation of interest into young hermaphrodites containing *rol-6(su1006)*. Hermaphrodite F1 progeny were clonally picked to new plates, allowed to lay eggs overnight, and removed. For each plate, the F2 progeny were analyzed for the rolling phenotype. Rollers from a plate containing a combination of rollers and non-rollers was expanded clonally, since the segregation of phenotypes in the F2 progeny demonstrated that the original cross was successful. F3 progeny were then tested for additional phenotypic analysis, as with *daf-2(e1370)III*, or by genotyping as described in Section 4.3.6. Clonal expansion of roller mutants was continued until the progeny of a single plate were homozygous for the rolling phenotype and the other mutant confirmed by phenotype or genotype.

### 4.3.9 Western blot analysis

Age-synchronous animals from *wuIs56[sod-3::GFP;pRRF4 rol-6(su1006)]*, *sod-2(ok1030)I;wuIs56[sod-3::GFP;pRRF4 rol-6(su1006)]*, *wuIs72[sod-2::GFP;pRRF4 rol-6(su1006)]*, *sod-3(gk235)V;wuIs72[sod-2::GFP;pRRF4 rol-6(su1006)]*, were treated with 5mM sodium selenite for 6 hours. After that time, 30 animals were transferred to a labeled microcentrifuge tube containing 20µl of de-ionized water. Samples were reduced by adding 7µl of 4X LDS sample buffer (cat# NP0007, Invitrogen, Carlsbad, CA) immediately before incubating the samples at 70°C for 10 minutes. During the 10 minute incubation, 3µl of 10X reducing agent (cat# NP0004, Invitrogen, Carlsbad, CA) was added to each sample tube, which was briefly agitated, and returned to 70°C for the remainder of the 10 minutes.

Samples were electrophoresed on a 3-8% acrylamide tris-acetate gel (cat# EA037ABOX, Invitrogen, Carlsbad, CA) using 1X tris-acetate running buffer (cat# LA0041, Invitrogen, Carlsbad, CA) under reducing conditions (addition of 500µl of antioxidant to inner chamber, cat# NP0005, Invitrogen, Carlsbad, CA). The gel was run for 1 hour at 150 volts (constant). Proteins in the gel were transferred to polyvinylidene fluoride (PVDF) membrane using 1X NuPage transfer buffer (cat# NP0006, Invitrogen, Carlsbad, CA) under reducing conditions (1:1000 antioxidant to transfer buffer). The gel was transferred at 30 volts (constant) for 1 hour. Transfer efficiency of the gel to the PVDF membrane was confirmed using SimplyBlue Safe Stain (cat# LC6060, Invitrogen, Carlsbad, CA).

The PVDF membrane containing the transferred proteins was blocked using 3% dry milk to prevent indiscriminate binding of the antibodies to the PVDF membrane. The blot was then incubated with rabbit polyclonal anti-GFP (cat# ab290-50, Abcam, Cambridge, MA) diluted to 1:4,000 in 3% milk for 2 hours at room temperature. The blot was washed twice for 5 minutes

each in tris-buffered saline, twice for 5 minutes each in tris-buffered saline with 0.1% tween-20, and again washed twice for 5 minutes each in tris-buffered saline. The blot was then incubated with goat anti-rabbit-HRP (horse radish peroxidase) at 1:5,000 (resuspended to 0.4 mg/ml, cat# 111-035-003, Jackson Immuno Research, Inc, West Grove, PA) for 2 hours at room temperature. The blot was again washed twice for 5 minutes each in tris-buffered saline, twice for 5 minutes each in tris-buffered saline with 0.1% tween-20, and again washed twice for 5 minutes each in tris-buffered saline. The blot was exposed to chemiluminescence (LumiGlo Chemiluminescent substrate kit, cat# 54-61-02, KPL, Gaithersburg, MD) to catalyze the oxidation of HRP that was conjugated to the secondary antibody that results in light emission that is then detected on film. Since protein from equal numbers of developmentally synchronized adult animals was used for each condition in the Western blot, a subsequent loading control blot was not performed.

## 4.4 RESULTS

### 4.4.1 Insulin pathway mutants have altered sensitivity to selenium

The *C. elegans* DAF-2 insulin pathway plays a major role in one stress response cascade that controls transcription of genes including glutathione S transferase, Mn-superoxide dismutases, catalases, and HSP20 (Honda and Honda 1999; Lee et al. 2003; Murphy et al. 2003; Oh et al. 2006). To determine whether the insulin pathway is altered in response to high selenium exposure, mutants for various proteins in the DAF-2 insulin pathway were tested for altered sensitivity/resistance to selenium. In Figure 18, a reduction-of-function mutant in the insulin-like receptor, *daf-2(m41)*, had an increased resistance to selenium exposure based on the behavioral assay described in Section 2.3.4 when compared to wild-type animals. This was expected, since in Chapter 2 it was demonstrated that oxidative stress is a contributing factor in selenium toxicity (Figures 7 and 8, and Table 1) and mutations in *daf-2* confer resistance to oxidative stress as evidenced by previous studies demonstrating increased expression of antioxidant proteins and longevity (Honda and Honda 1999; Lee et al. 2003; Oh et al. 2006).

A mutation in the PI-3 kinase downstream of DAF-2, *age-1(hx546)*, also confers increased resistance to selenium toxicity (Figure 18 $p<0.005$ at 48 hours and $p<0.05$ at 72 hours), which is consistent with the results observed with mutation in *daf-2*. Disruption of AKT-1 consensus phosphorylation sites on DAF-16 causes DAF-16 to accumulate in the nucleus (Paradis and Ruvkun 1998; Lin et al. 2001). A gain-of-function allele of the serine threonine kinase *akt-1(mg144)*, shown to increase phosphorylation of DAF-16, and therefore inhibiting it

from entering the nucleus, is shown in Figure 18 to have increased sensitivity to selenium ($p<0.05$ at 24 hours and $p<0.005$ at 48 and 72 hours) (Paradis and Ruvkun 1998). Mutations in *daf-16* result in decreased lifespan and increased sensitivity to stress (Kenyon et al. 1993; Murakami and Johnson 1996). A mutation in the FOXO transcription factor *daf-16(m26)* confers significantly increased sensitivity to selenium (Figure 18, $p<0.005$ at all time points). All of the mutations tested have altered sensitivities to selenium that correspond to their roles in responding to stress. Mutations in *daf-2* and *daf-16* have opposite responses to selenium exposure, similar to their divergent effects on lifespan and response to stress (Kenyon et al. 1993). The enhanced sensitivity of *akt-1(mg144),* which has previously been shown to prevent dauer arrest in multiple *age-1*alleles, suggests that DAF-16 function is responsible for the resistance observed in *daf-2* mutant (Morris et al. 1996; Paradis and Ruvkun 1998).

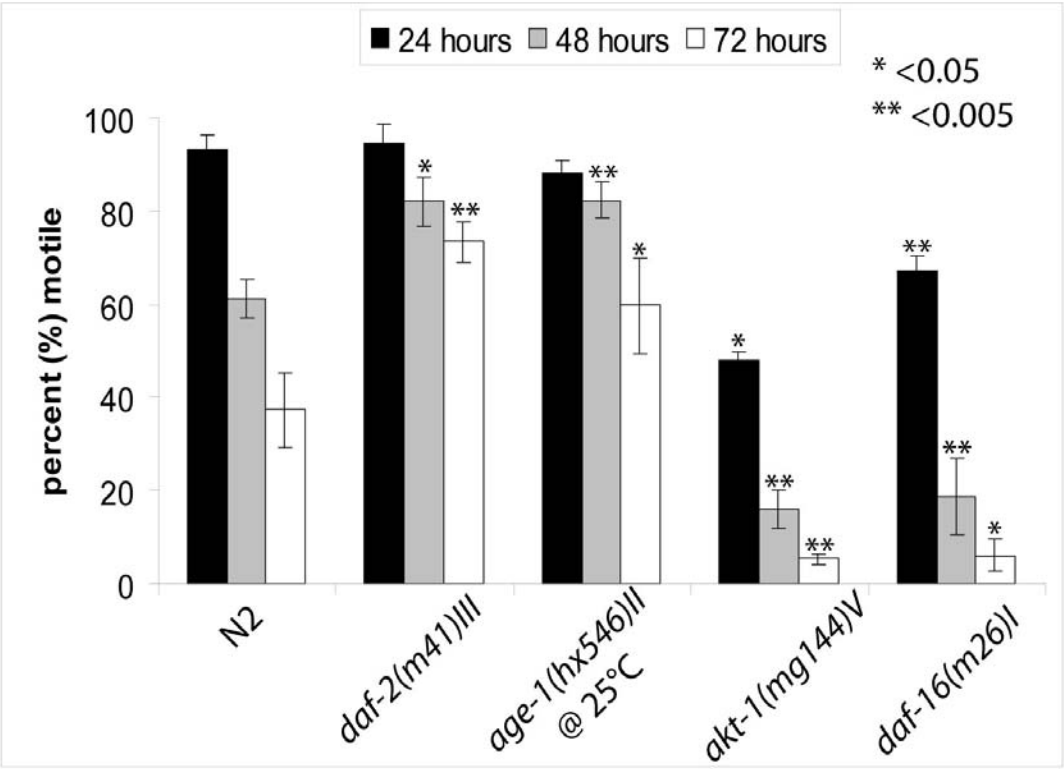


**Figure 18 Mutations in the insulin pathway have altered sensitivity to sodium selenite**

Animals containing mutations in the *daf-2* insulin pathway have altered sensitivity to selenium. *daf-2(m41)* was found to be resistant to selenium when compared to wild-type N2 at the 48 and 72 hour time points ($p<0.05$ and $p<0.005$, respectively). *age-1(hx546)* was also found to be resistant to selenium at the 48 and 72 hour time points ($p<0.005$ and $p<0.05$, respectively). *akt-1(mg144)* was found to have an increased sensitivity at all three time points (24hours: $p<0.05$, 48hours: $p<0.005$, 72hours: $p<0.005$). *daf-16(m26)* was also found to be more sensitive to selenium than wild type N2 at all three time points (24hours: $p<0.005$, 48hours: $p<0.005$, 72hours: $p<0.05$). Twenty developmentally synchronous adult hermaphrodites were placed on NGM plates supplemented with 5mM sodium selenite and scored at 24 hour intervals for movement deficits as described in Section 2.3.4. Mutants were compared to wild-type N2 animals using a one-tailed student t-test with unequal variance as described in Section 2.3.9. Non-selenium-treated animals had a greater than 90% normal movement at 72 hours (data not shown). The graph represents the average of 3 populations (n=60). Similar results were obtained in repeated experiments (data not shown).

### 4.4.2 Selenium resistance of *daf-2(e1370)I* is due to the function of DAF-16

Different alleles were tested to ensure that the *daf-2(m41)* resistance to selenium and *daf-16(m26)* sensitivity to selenium observed in Figure 18 were not allele-specific. In Figure 19, a second reduction-of-function allele *daf-2(e1370)* also displayed an increased resistance to selenium when compared to wild type N2 ($p<0.001$ at all four time points). Another reduction of function allele *daf-16(mu86)* was found to be sensitive at 24 hours and 48 hours when compared to N2 (Figure 19, $p<0.05$ and $p<0.001$, respectively). These results demonstrate that the observed resistance of *daf-2(m41)* and sensitivity of *daf-16(m26)* in Figure 18 were not allele-specific phenotypes.

While DAF-2 is known to regulate a number of genes though control of DAF-16, it also has less understood roles that are independent of DAF-16 function. Increasing protein catabolism via the autophagic process is essential for dauer larva formation, resulting in increased stress resistance and longevity in *daf-2* mutants, although bioinformatics, chip arrays, and chromatin immunoprecipitation of DAF-16 targets have not identified any known autophagic genes (Lee et al. 2003; McElwee et al. 2003; Melendez et al. 2003; Murphy et al. 2003; Oh et al. 2006). In the *daf-2(e1370)* background, autophagy rates did not change when *daf-16(mu86)* was also included in the background, again suggesting that the increased autophagy is not controlled by DAF-16 (Hansen et al. 2008). Autophagy was shown to be partially regulated by another FOXO transcription factor which is not a known target of DAF-16, PHA-4 (Hansen et al. 2008). Therefore, it is critical to determine whether *daf-2* resistance to selenium is due to the function of DAF-16 or another downstream regulator.

To determine whether the selenium resistance observed in the animals carrying mutations for insulin-like receptor, DAF-2, are due to the function of the downstream effector, the FOXO transcription factor DAF-16, a double mutant, *daf-2(e1370);daf-16(mu86)* was screened for altered sensitivity/resistance to selenium exposure. When *daf-16(mu86)I* was included in the *daf-2(e1370)III* background, sensitivity to selenium was restored (Figure 19; $p<0.001$). This demonstrates that the resistance observed in the *daf-2* backgrounds is due to the function of DAF-16.

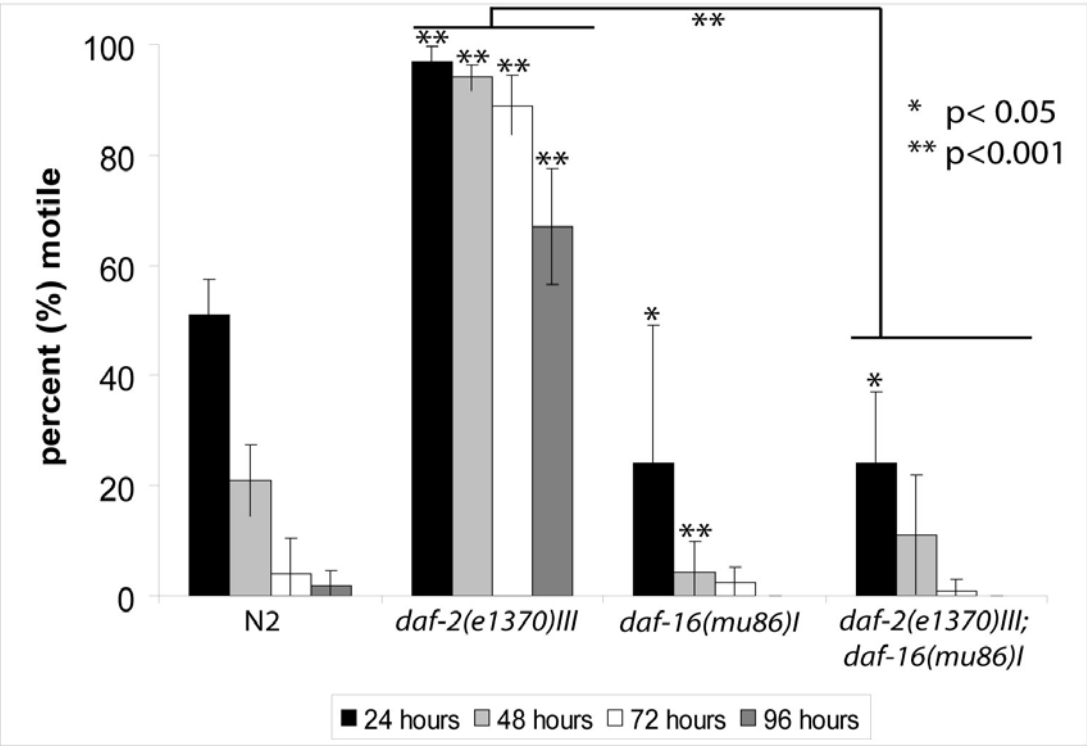



**Figure 19 *daf-2* mutant resistance is due to function of the FOXO-transcription factor DAF-16**

Different alleles of *daf-2* and *daf-16* retained altered sensitivity to selenium and *daf-2* resistance depends on the function of DAF-16. *daf-2(e1370)* was found to be significantly more resistant to selenium than wild-type N2 at all four time points ($p<0.001$). *daf-16(mu86)I* was found to be significantly more sensitive at the 24 hour and 48 hour time points ($p<0.05$ and $p<0.001$, respectively). *daf-2(e1370); daf-16(mu86)* was found to be more sensitive than N2 at 24 hours ($p<0.05$). *daf-2(e1370); daf-16(mu86)* was also found to be significantly more sensitive than *daf-2(e1370)* ($p<0.001$ at all four time points). Twenty developmentally synchronous adult hermaphrodites were placed on NGM plates supplemented with 5mM sodium selenite and scored at 24 hour intervals for movement deficits as described in Section 2.3.4. Statistical analysis was preformed using a one-tailed student's t-test with unequal variance as described in Section 2.3.9. On the graph, significance is indicated as * ($p<0.05$) or ** ($p<0.001$). Mutant populations were compared to wild-type N2 unless indicated by bars. Non-selenium-treated animals had greater than 90% normal motility at 96 hours (data not shown).

### 4.4.3 Temporal expression of daf-2 affects sensitivity to selenium

A variety of phenotypes can be observed when examining animals containing a gene mutation. However, since the mutation is present during development, viability can be achieved by causing compensatory alterations in other signaling cascades. In mammalian systems, this problem can be addressed by using conditional knockouts, where a gene's expression can be controlled through pharmacologic treatments. Conditional knockouts exist in *C. elegans* as temperature-sensitive alleles, where altering the temperature alters the function of the product of the gene mutation. While multiple alleles of *daf-2* are temperature-sensitive, one allele, *m41*, is a "true" temperature sensitive allele. When grown at 15°C, *daf-2(m41)* animals have a lifespan and thermotolerance similar to wild-type animals (Gems et al. 1998). When grown at 25°C, animals have a significantly longer lifespan than wild-type animals, similar to other *daf-2* alleles (Gems et al. 1998). These phenotype combinations suggest that *m41* synthesis or function is thermolabile (Gems et al. 1998). Shifting the temperature at which the animals are incubated allows the insulin receptor to be "turned off" acutely to determine whether the *daf-2* mutant acquires selenium resistance due to changes during development or if acutely turning the receptor off can confer protection from selenium exposure.

To determine whether turning off DAF-2 acutely in young adults can still confer selenium resistance, the mutant strain carrying the temperature sensitive allele, *m41*, and another allele, *e1370,* for DAF-2, along with wild type N2 were grown at 15°C until they were adults. The allele *e1370* is not considered a "true" temperature sensitive allele because it retains the increased lifespan and thermotolerance when grown at 15°C (Gems et al. 1998). The animals were then transferred to selenium supplemented and untreated plates as adults and shifted to 25°C, "turning off" the DAF-2 receptor function in animals containing the *m41* allele. The

animals were then scored for movement deficits as previously described in Section 2.3.4. The non-temperature-sensitive allele *(e1370)* retained an increased resistance to selenium when compared to wild-type N2 through the temperature shift which had been observed in Figure 18 and Figure 19 (Figure 20; $p<0.005$ at all time points). The temperature sensitive allele, *m41*, however, was found to have a statistically significant increase in sensitivity, not only to *e1370* (Figure 20; $p<0.005$ at all time point) but also to N2 (Figure 20; $p<0.005$ at 24 and 48 hours). This suggests that the increased resistance in the DAF-2 mutant background is due to developmental effects and cannot be protected acutely. However, no time was allowed for dephosphorylation of DAF-16 and transcription/translation of new proteins. While the results are intriguing, the experiment would need to be performed again after determining the time period required after "turning off" the receptor for the downstream consequences to occur.

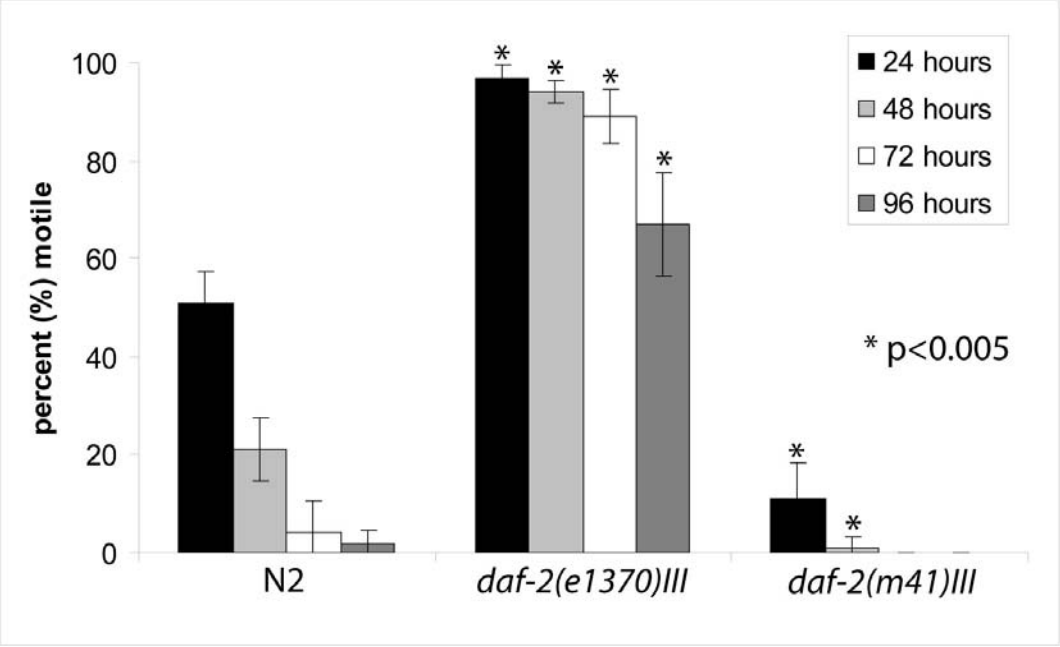



**Figure 20 Controlling temporal function of DAF-2 using a "true" temperature sensitive allele**

Temporal function of DAF-2 alters *daf-2* resistance to selenium. *daf-2(e1370)* animals were significantly more resistant to selenium when compared to wild-type N2 ($p<0.005$ at all time points). *daf-2(m41)* animals were more sensitive to selenium when compared to wild type N2 ($p<0.005$ at 24 and 48 hours). *daf-2(m41)* was found to be significantly more sensitive than *daf-2(e1370)* at all time points ($p<0.001$, not shown on graph). Animals were grown at 15°C until adulthood. Twenty developmentally synchronous adult hermaphrodites were placed on NGM plates supplemented with 5mM sodium selenite and shifted to 25°C for the remainder of the experiment. Animals were then scored for movement deficits as described in Section 2.3.4 Non-selenium-treated animals had a greater than 90% normal motility at 96 hours (data not shown). Statistical analysis was performed using a one-tailed student t-test with unequal variance as described in 2.3.9 comparing mutant populations to N2. The graph above represents 3 populations of 20 animals per population (n=60). The experiment was repeated tree times with similar results.

### 4.4.4 Any tissue-specific expression of DAF-16 partially rescues from a *daf-16*-deficient selenium sensitivity

Previous work has shown that neuronal tissue-specific expression of wild-type DAF-2 and AGE-1, in their corresponding mutant backgrounds, rescued their mutant extended lifespan phenotype, while specific muscle expression is sufficient to rescue their metabolic defects (Wolkow et al. 2000). Additionally, tissue-specific expression of DAF-16 in the intestine of *daf-16(-)* animals rescues their decreased longevity (Libina et al. 2003).

To determine in which tissue DAF-16 is functioning to provide protection from selenium toxicity, animals expressing tissue-specific *daf-16+* constructs were used. In addition to expressing a translational fusion of DAF-16::GFP under control of various tissue-specific promoters (*unc-119p* for the nervous system, *ges-1p* for the intestine, and *myo-3p* for the muscles), these animals also contained a *daf-2(e1370);daf-16(mu86)* mutant background. The

*daf-16(mu86)* background allowed for wild-type DAF-16 expression only in the tissue-of-interest while the *daf-2(e1370)* background ensured that the product of the DAF-16::GFP tissue-specific transgene would localize to the nucleus. Additional controls were added to this experiment, since the strains containing the tissue-specific DAF-16 transgenes also contained the *rol-6(su1006)* reporter. Previous observations with rollers and selenium exposure noted increased sensitivity in rollers when compared to non-rollers (data not shown). For this reason, strains containing *daf-2(e1370)* and *daf-16(mu86)* were crossed into GR1333, a strain containing a *rol-6(su1006),* to be used as controls.

When the GR1333 strain was included in the *daf-2(e1370)* background, animals were found to have a significant increase in resistance to selenium when compared to wild-type animals (GR1333) (Figure 21; $p<0.005$, at all time points, not indicated on graph). Conversely, when GR1333 was crossed into the *daf-16(mu86),* the animals were found to be significantly more sensitive to selenium toxicity than wild-type animals (Figure 21; $p<0.005$ at 24 and 48 hours, not indicated on graph). Also, when *daf-16(mu86)* was introduced into the *daf-2(e1370)* background, animals were found to be more sensitive to excess selenium when compared to wild-type animals (Figure 21; $p<0.005$, not indicated on graph). These results demonstrate that *rol-6(su1006)* did not alter the relative sensitivity to selenium toxicity of *daf-2(e1370)* and *daf-16(mu86)* to wild-type animals.

Tissue-specific DAF-16 expressing animals were compared to GR;*daf-2(e1370)*;*daf-16(mu86)* since the DAF-16 transgenes are expressed in this background. Expression of DAF-16 in any tissue was found to provide significant protection from selenium exposure, with the greatest protection provided by DAF-16 expression in the intestine (Figure 21; $p<0.005$ at 24 and 48 hours and $p<0.05$ at 72 and 96 hours).

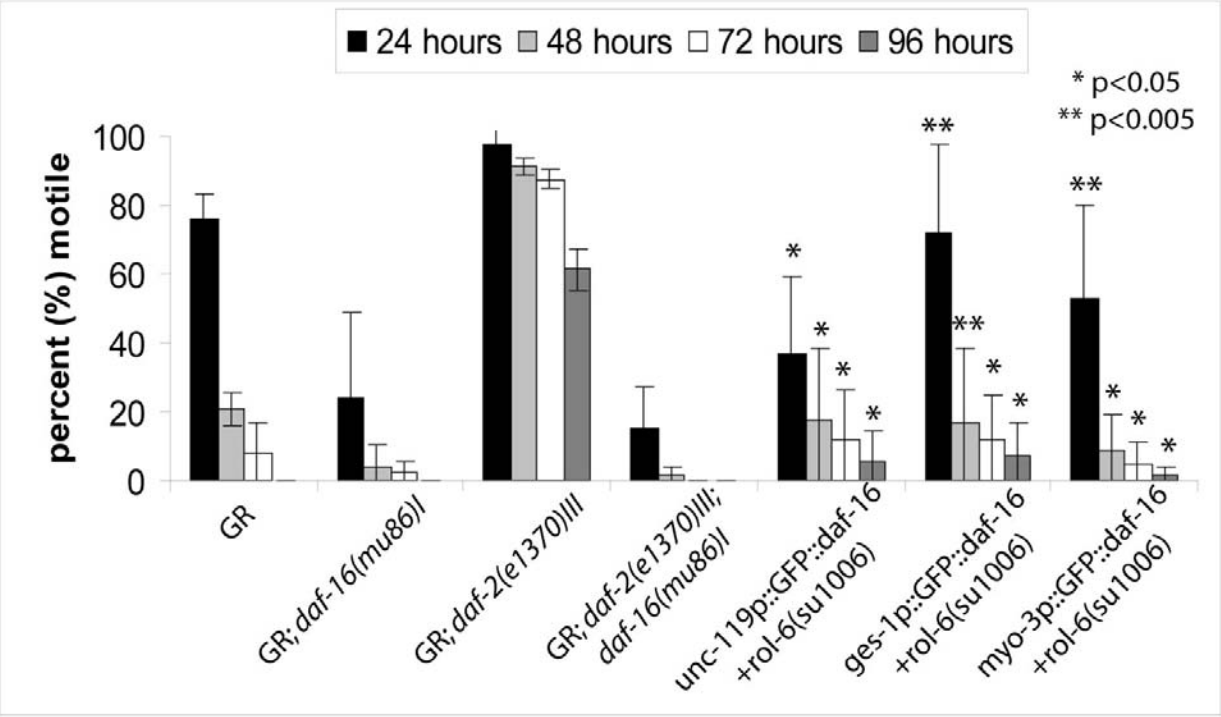



**Figure 21 The effect of tissue-specific expression of DAF-16 on selenium sensitivity**

As observed previously, GR;*daf-16(mu86)* was more sensitive to selenium than "wild-type" GR ($p<0.005$ at 24 and 48 hours-not indicated on graph). GR;*daf-2(e1370)* was found to be resistant at all time points ($p<0.005$-not indicated on graph). GR;*daf-2(e1370);daf-16(mu86)* was found to be sensitive to selenium ($p<0.005$ at 24 and 48 hours-not indicated on graph). DAF-16 tissue-specific strains were then compared to GR;*daf-2(e1370);daf-16(mu86)* as indicated by * or ** on graph. All DAF-16 tissue-specific expressing strains had a statistically significant increased resistance to selenium at all time points as labeled on the graph. Statistical analysis of the DAF-16 tissue specific strains was performed by comparing the results from each tissue-specific expressing stain to GR; *daf-16(mu86);daf-2(e1370).* Significance was determined using a one-tailed student t-test with unequal variance as described in Section 2.3.9. Non-selenium-treated animals had a greater than 90% survival after 96 hours (data not shown). Twenty developmentally synchronous animals were placed on NGM plates supplemented with 5mM sodium selenite and scored as for movement deficits as described in Section 2.3.4. Graphed above is the average of 10 populations of 20 animals per population (n=200). GR is GR1333: *tph-1::gfp;rol-6(su1006)* was used as a control for this experiment due to expression of a *rol-6(su1006)* reporter in the DAF-16 tissue-specific expressing animals. Animals that contain rollers are more sensitive to selenium than non-rollers (data not shown).

### 4.4.5 Investigating DAF-16 targets that potentially contribute to selenium resistance

In Chapter 2, increased ROS were detected in selenium-treated wild-type animals along with oxidized amino acids when *C. elegans* were exposed to selenium (Figure 7). In addition, three antioxidants: reduced glutathione, quercetin, and α-tocopherol (vitamin e), all partially, but significantly, suppressed the movement deficits caused by excess selenium exposure (Figure 8A-C). Also, DAF-16 has also been shown to be activated by quercetin exposure (Kampkotter et al. 2008). These data are consistent with selenium toxicity involving a response to oxidative stress. In Figure 19, the *daf-2* mutant phenotype of relative selenium resistance was demonstrated to be controlled by the FOXO transcription factor DAF-16. During stressed conditions, DAF-16 is de-phosphorylated and re-locates into the nucleus, where it is involved in transcriptional regulation of a variety of stress response genes (McElwee et al. 2003; Murphy et al. 2003; Oh et al. 2006). Additionally, targets of DAF-16 that are involved in oxidative stress response have been identified, including 2-iron-manganese superoxide dismutases and 3-catalases. These function in a stepwise fashion. First, superoxide dismutase converts the superoxide anion into hydrogen peroxide, while catalases catalyze the conversion of hydrogen peroxide into water and oxygen. Here, we focused on the two-iron-manganese superoxide dismutases (MnSOD, SOD-2 and SOD-3), since they are involved in the first step of reducing the superoxide anion.

Age-synchronous mutants for *sod-2* and *sod-3* were therefore tested for altered sensitivity to selenium. *sod-2(ok1030),* which contains a 900bp deletion from a gene whose wild-type size is 1285bp, had an expected statistically significant increase in sensitivity to selenium (Figure 22A; $p<0.005$ at 24 hours). Unexpectedly, *sod-3(gk235),* which contains a 390base pair deletion in a 1.5kb gene was highly resistant to selenium exposure (Figure 22A, $p<0.05$). Since SOD-2 and SOD-3 are both iron-manganese superoxide dismutases, it is reasonable to consider that

when one MnSOD (SOD-2) is mutated, that the other MnSOD (SOD-3) could compensate. To address this possibility, the *sod-2(ok1030);sod-3(gk235)* double mutant was created and confirmed by PCR genotyping (since the double mutants, like the single mutants, have no observable phenotype). These animals were found to have increased sensitivity to selenium when compared to *sod-3(gk235)* and *sod-2(ok1030),* suggesting that the resistance observed in *sod-3(gk235)* is due to SOD-2 (Figure 22A, $p<0.005$). However, it should also be noted that the *sod-2(ok1030);sod-3(gk235)* double mutant was significantly more sensitive that *sod-2(ok1030)* which was more sensitive than N2.

One possibility for the increased resistance to selenium observed in *sod-3(gk235)* animals is that SOD-2 protein levels increased in this mutant background. To examine this possibility, a Western analysis was performed using transgenic strains expressing SOD-2::GFP or SOD-3::GFP in the reciprocal mutant background (*sod-3(rf)* and *sod-2(rf)*, respectively) and analyzed for relative changes in GFP protein levels (Figure 22B). There was no significant difference in the GFP levels observed between the *sod-2(ok1030)*;SOD-3::GFP and the reciprocal mutant/GFP construct. Also, selenium exposure did not result in an increase in SOD-2::GFP or SOD-3::GFP protein expression. This suggests that *sod-3(gk235)* mutant resistance is not due to an increase in SOD-2 protein expression, but rather an epistatic interaction.

Another *C. elegans* group has been investigating the role of iron-manganese superoxide dismutases in terms of longevity. They found that mutations in *sod-2* and *sod-3*, both separately and together, had no effect on lifespan when compared to wild-type animals in unstressed conditions (Honda et al. 2008). Under hyperoxic conditions or paraquot treatment, single mutations in *sod-2* and *sod-3* had lifespans similar to wild-type animals whereas the double mutant was sensitive, but had no effect on longevity (Honda et al. 2008). This differs from our

finding in that *sod-2(ok520)* was hyper-sensitive to selenium treatment, while *sod-3(gk235)* was resistant. Our results parallel the hyperoxia/paraquot findings in that the double *sod-2;sod-3* mutant was highly sensitive to selenium exposure (Honda et al. 2008). It should also be noted that different SOD gene alleles were used between the two labs.

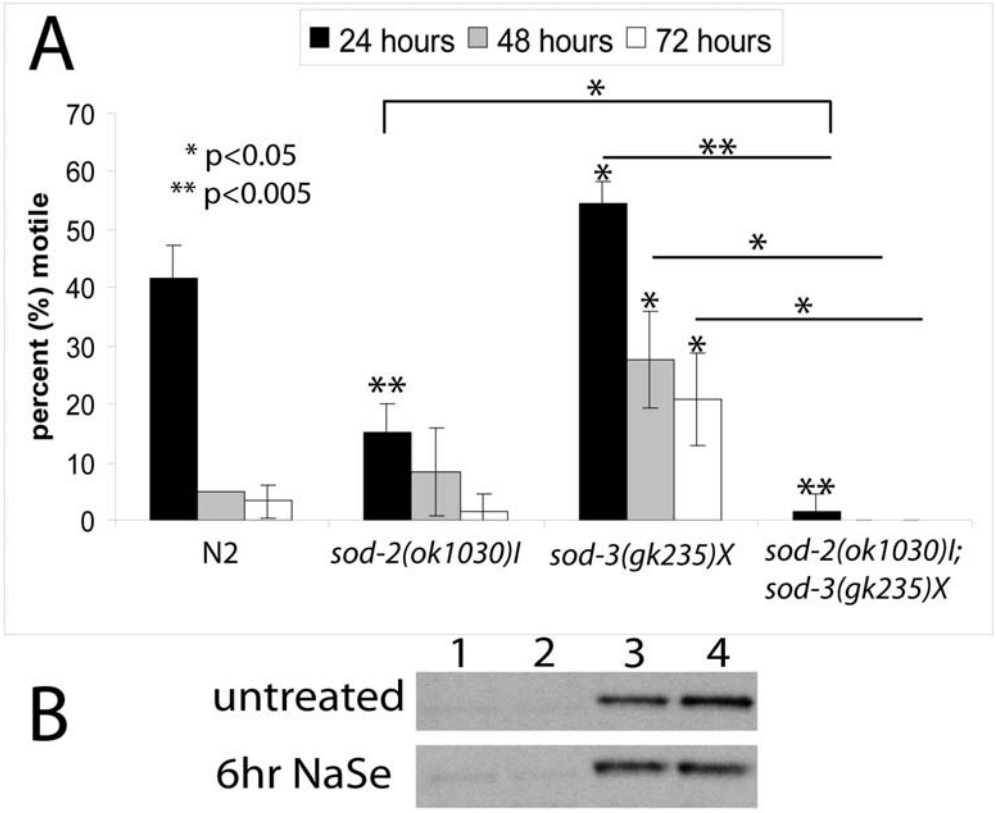



**Figure 22 Targets of DAF-16, *sod-2(ok1030)1* and *sod-3(gk235)X* have differing sensitivity/resistance to selenium**

**(A)** Two Mn-SOD mutants have different sensitivities to selenium. *sod-2(ok1030)* was found to be significantly more sensitive than N2 at the 24 hour time point ($p<0.05$). *sod-3(gk235)* was more resistant to N2 at all three time points ($p<0.05$). This resistance was eliminated when *sod-2(ok1030)* was crossed into the *sod-3(gk235)* mutant background. *sod-2(ok1030);sod-3(gk235)* was found to be significantly more sensitive than N2 at 24 hours ($p<0.005$). *sod-2(ok1030);sod-3(gk235)* was also found to be more sensitive than *sod-3(gk235)* ($p<0.005$ at 24 hours and $p<0.05$ at 48 and 72 hours) and *sod-2(ok1030)* ($p<0.05$ at 24

hours). Twenty developmentally synchronous animals were placed on NGM plates supplemented with 5mM sodium selenite and scored as for movement deficits as described in Section 2.3.4. Untreated plates had greater than 90% survival after 72 hours (data not shown). Statistics were performed using a one-tailed student's t-test with unequal variance as described in Section 2.3.9 comparing mutant populations to N2, unless indicated by the bars on the graph. Above is an average of 3 plates containing 20 animals (n=60). The experiment was performed 3 times with similar results. **(B)** Western immunoblot for anti-GFP demonstrates that reciprocal expression of protein does not increase in mutant backgrounds and that selenium exposure does not induce expression. Developmentally synchronized adult animals were placed on NGM plates supplemented with 5mM sodium selenite for 6 hours. Samples were collected and Western blot analysis was performed as described in Section 4.3.9 using SOD::GFP fusion constructs. The predicted size of *sod-2::GFP* was 51.9kD and the expected size for *sod-3::GFP* was 51.6kD. Lane 1: SOD-2::GFP, Lane 2: *sod-3(gk235)*;SOD-2::GFP, Lane 3: SOD-3:GFP, Lane 4: *sod-2(ok1030)*;SOD-3::GFP.

### 4.4.6 Does *sod-2(rf)* restore sensitivity to selenium in *daf-2(e1370)* background?

We have shown that mutations in the insulin-like receptor DAF-2 confer significant resistance to selenium exposure (Figures 18 and 19). Two targets of DAF-2, the 2-iron-manganese superoxide dismutases, SOD-2 and SOD-3, were found to have differing sensitivities to selenium exposure (Figure 22). A mutation in SOD-2, *sod-2(ok1030),* was found to confer significant sensitivity to selenium exposure when compared wild-type animals (Figure 22, $p<0.005$). This is unsurprising, assuming the deletion confers a reduction-of-function to the mutant (wild-type *sod-2* is 1121bp and the mutant has a 900bp deletion). Next, we asked whether if reducing *sod-2* function in the *daf-2(e1370)* background could re-sensitize the animals to selenium exposure.

To determine whether the resistance observed in *daf-2(e1370)* is due to the function of SOD-2, an assumed reduction-of-function allele for *sod-2*, *sod-2(ok1030)* was crossed into the

*daf-2(e1370)* background. Homozygosity for *sod-2(ok1030)* was confirmed by PCR and *daf-2(e1370)* was confirmed by selecting for the constitutive dauer phenotype at 25°C. When these animals were tested for altered sensitivity to selenium, *daf-2(e1370);sod-2(ok1030)* exhibit a marginal, but statistically significant, increase in sensitivity to selenium when compared to *daf-2(e1370)* (Figure 23, $p<0.05$ at 48 hours and 96 hours and $p<0.005$ at 72 hours and 120 hours). However, *daf-2(e1370);sod-2(ok1030)* animals are still more resistant to selenium when compared to N2 (Figure 23, $p<0.005$ at all time points). This suggests that SOD-2 contributes to protection from selenium exposure, but is not the only factor involved.

This finding corresponds to the role of SOD-2 in longevity (Honda et al. 2008). Interestingly, including *sod-3(rf)* in the *daf-2* mutant background increased longevity (Honda et al. 2008). When oxidative stress was induced by paraquot, knockouts of either Mn-SOD, *sod-2* or *sod-3*, in the *daf-2(rf)* background has no effect on survival, but the double mutant, *sod-2;sod-3*, was found to be sensitive (Honda et al. 2008). While this experiment was not performed in terms of selenium exposure, it is a likely outcome in that both mutant *daf-2* and *sod-3* have increased resistance to selenium. Similarly, it is also likely that a triple mutant of *sod-2;sod-3,daf-2* would be significantly more sensitive to selenium toxicity than the *sod-2;daf-2* double reduction-of-function mutant tested in Figure 23.

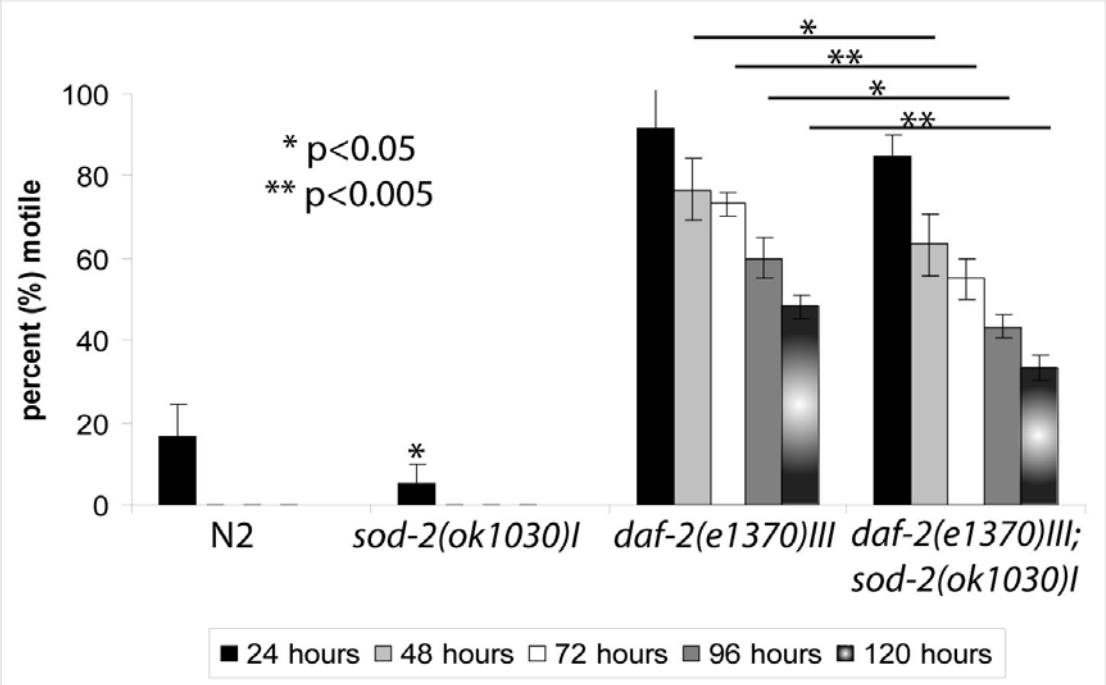



**Figure 23 *sod-2(ok1030)I* partially restores selenium sensitivity to *daf-2(e1370)III***

*sod-2(ok1030)* was found to marginally, but significantly, restore selenium sensitivity to *daf-2(e1370)*. *sod-2(ok1030)* was sensitive to selenium when compared to N2 ($p<0.05$). Both *daf-2(e1370)* and *daf-2(e1370);sod-2(ok1030)* had a significant resistance to selenium when compared to N2 ($p<0.005$, not indicated on the graph). When compared to *daf-2(e1370)*, *daf-2(e1370);sod-2(ok1030)* animals were more sensitive except at the 24 hour time point (at 48 hours $p<0.05$, at 72 hours $p<0.005$, at 96 hours $p<0.05$, and 120 hours $p<0.005$). Twenty developmentally synchronous animals were placed on NGM plates supplemented with 5mM sodium selenite and scored as for movement deficits as described in Section 2.3.4. Statistical analysis was performed as described in Section 2.3.9 using a one-tailed student's t-test with unequal variance. On the graph, significance is indicated by * ($p<0.05$) or ** ($p<0.005$) comparing mutant populations to N2, unless indicated by bars on the graph. Untreated animals had greater than 90% survival at 120 hours (data not shown).

## 4.5 DISCUSSION

The DAF-2-insulin-like pathway has been and continues to be the focus of research on stress response and longevity in *C. elegans*. Here, we find that animals containing mutations in genes of the insulin pathway have altered sensitivity to high selenium exposure (5mM sodium selenite). Mutations in the insulin-like receptor, *daf-2*, and the downstream PI-3 kinase, *age-1*, confer increased resistance to selenium exposure consistent with their effects on stress and longevity (Kenyon et al. 1993; Murakami and Johnson 1996). Correspondingly, a gain-of-function allele for *akt-1*, which results in increased phosphorylation of the FOXO-transcription DAF-16, is sensitive to selenium exposure (Paradis and Ruvkun 1998; Lin et al. 2001). Similarly, reduction-of-function mutations in *daf-16,* which have shortened lifespan and increased sensitivity to stress, also have increased sensitivity to selenium (Figure 18).

Here, we also confirmed that the selenium resistance observed in DAF-2 mutants was due to the function of DAF-16 (Figure 19). This is significant, since autophagy is up-regulated in *daf-2* mutants, but has yet to be shown as a target of DAF-16 (McElwee et al. 2003; Melendez et al. 2003; Murphy et al. 2003; Oh et al. 2006). This was further demonstrated when *daf-16(mu86)* was crossed into the *daf-2(e1370)III* background and no difference in autophagy was observed (Hansen et al. 2008). Yet, the role of autophagy is likely context dependent, having roles ranging from protection to cell death. Inducing autophagy has been found to be protective in models of Parkinson's disease by reducing the accumulation of alpha-synuclein insoluble inclusions (Ferrucci et al. 2008). Autophagy is highly active in healthy neurons but disrupted in neurons of Alzheimer's mice (Boland et al. 2008). However, inactivation of the autophagic gene *bec-1* leads to apoptotic cell death in *C. elegans* (Takacs-Vellai et al. 2005). It is likely that autophagy is protective in the *daf-2* mutant background, and while autophagic genes are not known target(s)

of DAF-16, they likely interact with DAF-16 target(s), including a BCL-2 interacting protein (Oh et al. 2006). BEC-1 and CED-9 (ortholog to mammalian BCL-2) have been found to be in complex, and BEC-1 depletion triggers caspase-dependent cell death (Takacs-Vellai et al. 2005). Thus, the physiologic output of autophagy is likely altered depending on the genetic context. It could be hypothesized that in a *daf-16*(-) background, the BEC-1 and CED-9 complex is disrupted due to a decrease in the BCL-2 interacting protein, leaving the animal susceptible to caspase-dependent cell death.

Further, we have shown that acutely "turning off" the insulin-receptor in adult animals does not protect animals from selenium exposure (Figure 20). This is surprising, since DAF-16 is known to target several antioxidant enzymes and antioxidants have been shown to protect from selenium exposure in both *C. elegans* (Figure 8) and in an vertebrate model of selenium toxicity (McElwee et al. 2003; Murphy et al. 2003; Deore et al. 2005; Oh et al. 2006). Since a developmental adaptation is implied, further experiments could be designed to determine at which larval stage "turning off" the DAF-2 receptor confers protection to selenium toxicity. This can be done by shifting developmentally synchronized *m41* animals to the non-permissive temperature at various larval stages, transferring adults to selenium treating plates and scoring for movement deficits. This experiment would provide insight as to what other signaling cascades may be interacting with the DAF-2-insulin pathway to confer resistance to selenium.

This experiment has another caveat, in that no time was allowed for de-phosphorylation of DAF-16 and subsequent transcription and translation of target genes. To confirm that there is a developmental effect, further experiments would need to be performed to determine how long after the shift to the non-permissive temperature is required for the consequences of "turning off" the receptor to be observed (like increase in lifespan when compared to N2 animals). The

experiment could then be repeated, allowing for the appropriate time at the non-permissive temperature, before transferring the animals to selenium supplemented plates.

Tissue-specific expression of DAF-16 demonstrated that expression in the intestinal tract provides the most protection from selenium exposure. These results are not unexpected, as the animals are exposed to selenium through their food source. The intestinal track is therefore the primary exposure site and deals with absorbing/extruding selenium from food. Somewhat surprising is the finding that neuronal expression of DAF-2 in a *daf-2* reduction-of-function mutant background rescued the extended lifespan (Wolkow et al. 2000). These findings are difficult to interpret, since tissue-specific overexpression of DAF-16 in neurons and intestine has been shown to upregulate expression of the DAF-16 target, SOD-3, in muscle, demonstrating that "tissue-specific" expression of DAF-16 has effects on neighboring tissues (Libina et al. 2003). However, the majority of the tissue-specific expression occurs in the tissue in which it is expressed. Together, these results demonstrate that the DAF-2 insulin signaling pathway is involved in protecting wild-type animals from selenium exposure, regardless of tissue expression.

Next, we identified a target of DAF-16, the iron-manganese superoxide dismutase, *sod-2*, that plays a role in protection from excess selenium exposure. Reduction-of-function mutations in the two iron-manganese superoxide dismutases, *sod-2* and *sod-3*, had differing sensitivity to selenium, while a double *sod-2;sod-3* reduction-of-function mutant was relatively sensitive to selenium (Figure 22). Our finding agrees with another labs findings that *sod-2* is epistatic to *sod-3* (Honda et al. 2008). The role of superoxide dismutases has long been considered as free radical scavengers that are thought to protect animals from ROS accumulated with age (Saltzman and Fridovich 1973). In line with this, overexpression of MnSOD in *Drosophila* resulted in an

increased lifespan (Sun et al. 2002). However, heterozygous mouse knockouts for MnSOD were found to sustain increased oxidative damage but lived a normal lifespan, separating the role of oxidative stress from aging (Van Remmen et al. 2003). Our data, combined with these studies, support a growing field investigating superoxide anion/hydrogen peroxide as a signaling molecule rather than their previously described roles as a free radicals causing cellular damage (Veal et al. 2007). As an example of this, MnSOD has been found to signal matrix metalloproteinases via $H_2O_2$ in a human fibrosarcoma cell line (Ranganathan et al. 2001).

However, the answer may not be that simple. *C. elegans* is an unusual organism in that it has two iron-manganese superoxide dismutases, whereas most species have only one (Landis and Tower 2005). MnSODs localize to the mitochondria and are responsible for protecting the organelle from the superoxide anion produced as a byproduct of aerobic respiration. MnSOD knockout mice die within 10 days of birth (Li et al. 1995). However, knockouts of MnSOD in *C. elegans*, both individually and combined, are viable and have a normal lifespan (Honda et al. 2008). Other considerations include the status of other superoxide dismutases, like the cytoplasmic copper/zinc superoxide dismutase. In baker's yeast, a fraction of Cu/Zn SOD-1 localizes to the mitochondrial intermembrane space (Sturtz et al. 2001). In a murine ALS model, mitochondrial localization mutant SOD-1 has been found to trigger caspase-dependent cell death (Takeuchi et al. 2002). One potential hypothesis to explain the *C. elegans* data is that knockouts or reduction-of-function mutants of MnSODs may lead to increased SOD-1 localization to the mitochondria, making it susceptible to cell death triggers. The opposite findings from the reduction-of-function of *sod-2* and *sod-3* to selenium exposure raise interesting questions as to their function(s). Are their roles as free radical scavengers their primary roles? What roles might they have in cellular signaling? Is expression/localization of any of the Cu/Zn SODs (SOD-1,4,5)

altered as a result of knocking out either/both MnSOD? Viable MnSOD reduction-of–function mutants in *C. elegans* provide a useful tool for understanding potential signaling mechanism(s) and provide insight into basic SOD biology.

Another consideration for investigating SOD regulation and expression is another *C. elegans* FOXO transcription factor, *pha-4,* since DAF-16 and PHA-4 have similar consensus sites (DAF-16: TTGTTTAC and PHA-4; T(A/G)TT(T/G)(A/G)(T/C) ) (Gaudet and Mango 2002; Lee et al. 2003). In a model of dietary restriction, *pha-4* was shown to regulate *sod-2,* contributing to lifespan extension (Panowski et al. 2007). At this time, it is unknown what effect selenium exposure has on *pha-4* and its targets.

This chapter demonstrates that the *daf-2* insulin-like pathway is protective in response to excess selenium exposure. One target of DAF-16, *sod-2,* was found to contribute to this protection. However, our data also suggests that protection from selenium toxicity is more complicated than simply increasing expression of SOD-2, as SOD-2 expression was not induced by selenium exposure. Further evidence that Mn-SOD expression is more complex than previously thought, is that knockouts of both Mn-SODs in *C. elegans* result in viable animals with normal longevity (Honda et al. 2008). In addition to analyzing expression and localization of non-Mn-SODs, further work is required using RNAi for *pha-4,* to determine whether regulation of SOD expression could involve multiple transcription factors in response to selenium exposure.

# 5.0 OVERALL DISCUSSION

*C. elegans* has proven to be a useful system for studying selenium toxicity. Specifically, it provides a laboratory model for understanding observations from environmental studies. As observed in vertebrates, both organic and inorganic sources of selenium were found to be toxic in adult *C. elegans* (Figure 1) (Panter et al. 1996; Usami and Ohno 1996). Manipulation of the environmental conditions in the system has provided further insight into the mechanisms of toxicity. For the first time, we show that increased temperature, which increases metabolism, resulted in exacerbating selenium toxicity. This suggests that selenium is causing an increase in oxidative stress (Figure3) (Klass 1977). Increased oxidative stress resulting from selenium exposure was later confirmed (Figure 7) and the resulting movement deficits and mortality could be suppressed by treatment with antioxidants (Figure 8), similar to results in a vertebrate model (Deore et al. 2005). The antioxidant studies replicate what had been observed in vertebrate models, further demonstrating the usefulness *C. elegans* to study excess selenium exposure.

Further, the difference between minimal and physiological levels of calcium, as originally established by Brenner, altered sensitivity to selenium, suggesting that calcium signaling is involved in signaling for death as a result of selenium exposure. Further investigations will involve testing animals with mutations in genes responsible for calcium signaling for altered sensitivities to selenium. While calcium levels have been observed as a result of selenium toxicity in cultured rabbit lenses, these ongoing and future experiments will

for the first time provide insight into the role of calcium signaling as a result of excess selenium exposure (Hightower and McCready 1989).

This dissertation concentrates on the effects of selenium toxicity in adult animals, although we do establish that selenium causes a decrease in the number of eggs being laid, which is also observed in environmental studies (Figure 15) (Ohlendorf et al. 1986; Lemly 1997; Lemly 2004). Further work needs to be done to investigate the effects of selenium on various larval stages. While not formally tested, observations of eggs laid from animals placed on selenium as adults suggests that they undergo developmental arrest in the late first or early second larval stage (data not shown). Further insight into this phenotype can be gained by investigating a reduction-of-function mutation in *pdk-1*. The protein product from this gene is involved in the *daf-2*-insulin-like cascade and adult animals tested on selenium were found to have no difference in sensitivity when compared to wild-type animals (data not shown). However, this was the only mutation where eggs laid on selenium developed to adults (personal observation).

## 5.1 MECHANISMS OF CELL DEATH RESULTING FROM SELENIUM TOXICITY

High selenium exposure results in the death of adult *C. elegans,* but the mechanism remain unclear. Neither canonical mechanisms of necrosis or apoptosis appear to be involved (Figures 5 and 6). This is significant, as it differs from other models of neurodegeneration in *C. elegans,* in that reduction-of function mutations in genes implicated in cell death (both apoptosis and necrosis) do not protect animals from from paralysis and death due to excess selenium exposure (Driscoll and Chalfie 1991; Syntichaki et al. 2002; Driscoll and Gerstbrein 2003; Artal-Sanz et

al. 2006). In fact, they sensitize animals. However, it is possible that reducing the function of either cell death mechanism could supraphysiologically upregulate the other pathway, resulting in sensitivity to selenium. One way to address this issue would be to create a *cad-1(rf);ced-3(rf)* double mutant and test it for altered sensitivity to selenium. However, reducing the function of the two-main cell death pathways could lead to unknown developmental problems. Another approach for this experiment would be to test *cad-1(rf)* animals on *ced-3* RNAi. However, this experiment comes with another series of caveats. In *C. elegans*, RNAi penetrates well into the intestine but poorly into the neurons (Kamath et al. 2001). Results from such an experiment could therefore be difficult to interpret if signaling events are neuronally controlled.

Apoptotic and necrotic mechanisms are not independent, and are connected to one another through another cell death pathway, autophagy. Normally, autophagy is involved in protein and organelle turnover, and inducing autophagy is protective in models of Parkinson's disease by reducing the accumulation of alpha-synuclein insoluble inclusions (Ferrucci et al. 2008). Further, autophagy has been found to be highly active in healthy neurons, but disrupted in neurons of Alzheimer's mice (Boland et al. 2008). It is likely that autophagy is protective against selenium-induced damage, as suggested by the sensitivity of *cad-1(rf)* (Figure 5) and ongoing experiments in the lab (data not shown).

Other experiments to investigate the role of autophagy with selenium toxicity might involve treating animals with autophagy-promoting drugs, such as rapamycin, geldanamycin, and 6-aminonicotinamide and comparing altered sensitivity/resistance to autophagy-inhibiting drugs such as cycloheximide, LY294002, and 3-methyladenine. Additionally, RNAi for autophagy genes like *bec-1* can also be used to check animals for altered sensitivity to selenium, with the caveats as described previously for RNAi in *C. elegans*. To confirm that autophagy is

being altered with the various RNAi/drug/and selenium treatments, rates of autophagy can be monitored using animals expressing *lgg-1::GFP*, which is a vacuolar protein that is incorporated into the autophagosome (Hansen et al. 2008).

## 5.2 NEURONAL DAMAGE

Chapter 3 focused on neuronal damage caused by selenium exposure. Specifically, the movement deficits and paralysis resulting from selenium exposure are due to neuronal damage. Selenium toxicity causes a decrease in cholinergic signaling that was demonstrated by resistance to an acetylcholinesterase inhibitor (aldicarb) and sensitivity to a nicotinic agonist (levamisole). Future screens of mutants can be tested with a levamisole/aldicarb assay to separate mutations whose altered resistance is due to effects on muscle rather than neurons. However, the limitations of this assay must be recognized. Animals containing mutations for genes involved in cholinergic signaling will be resistant to aldicarb before selenium exposure. Also, animals containing mutations in nicotinic acetylcholine receptor will be resistant to levamisole.

This dissertation provides provocative evidence that selenium toxicity can be used to model motor neuron death, as evidenced by decreased cholinergic signaling and an increase in cytosolic muscle protein catabolism. It also strengthens the hypothesis that selenium toxicity can be used as a model for sporadic ALS. This is significant as it could provide the first model of sporadic ALS. Previous studies have implicated metals in sporadic ALS, but this is the first time that an environmental toxin was shown to cause decreased cholinergic signaling (Figure 11 and 12) (Kasarskis et al. 1995; Vinceti et al. 2002). We have identified a susceptibility factor for

sensitivity to selenium, reduction-of-function in the FOXO transcription factor DAF-16 in Chapter 4. SNPs of the human ortholog in this gene were found associated with an increased risk of developing sporadic ALS, potentially identifying a significant parallel between sporadic ALS and selenium neurotoxicity in *C. elegans*. Table 2 lists factors identified in ALS patients and similarities to selenium toxicity that we have identified to date. While not everyone exposed to high environmental selenium develops ALS, our data suggests that certain genetic factors cause susceptibility to the environmental toxin. This is exciting since the parallels observed suggests our model is relevant environmental toxin neurotoxicity that induces sporadic ALS.

Further work needs to be performed to determine if other forms of neurotransmission are being decreased in response to selenium exposure. Serotonin signaling was shown to be significantly decreased by experiments showing partial rescue of the egg-laying deficit caused by selenium (Figure 15B). However, the concentration of serotonin used in egg-laying causes animals to hyper-contract. Further experiments could determine whether lower concentrations of serotonin could suppress the development of movement deficits and mortality. Similarly, GABAergic transmission could also be investigated, as described in Chapter 3, by examining the "shrinker" phenotype (McIntire et al. 1993; McIntire et al. 1993). GABAergic signaling is also likely to be altered in response to selenium exposure, given our preliminary observations that selenium-treated animals appeared shorter than untreated animals (data not shown, also observed with the BioSorter in experiment 7C).

One of the benefits of this system can also be a viewed as a weakness. The simplicity of the *C. elegans* makes it easy to work with experimentally. However, with simplicity, complexity of higher organisms is lost. While the ventral cord in *C. elegans* in roughly analogous to a spinal cord in mammals, we lose the supporting cells (glial cells and astrocytes) that protect neurons in

mammals as well as the higher organization (including sections of the spinal cord like cervical/lumbar/etc as well as motor neuron organization in various tracks). To truly model motor neuron loss, future experiments will need to be performed in a more complex model, like the mouse. However, *C. elegans* allows for relatively easy and fast identification of candidate signaling pathways of interest that would then be studied in a more complex model. For example, alterations in a FOXO transcription factor have been implicated as a susceptibility factor in both selenium toxicity and sporadic ALS (Dunckley et al. 2007). Initial mouse studies would include treating wild-type and knockout mice for a FOXO transcription factor with selenium and examining the spinal cord, post-mortem for alterations in neurons of the spinal cord.

**Table 2 Comparison of pathological findings between ALS and high selenium exposure**

(+) indicates a positive association to the pathological finding. (ND) indicates that the association has *N*ot been *D*etermined.

| **Pathological Findings** | **ALS** | **High Selenium** |
|---|---|---|
| Increased Oxidative Stress | + | +<br>(Figures 7 and 8) |
| DNA Damage | + | ?<br>(suggested by Figure 9D) |
| Mitochondrial Damage | + | +<br>(data not shown by Miguel Estevez) |
| Cholinergic Denervation | + | +<br>(Figure 11 by Nate Szewczyk) |
| Muscle Protein Catabolism | + | +<br>(Figure 11 by Nate Szewczyk) |
| SOD1 effects | + | ND |
| Bax-dependent apoptosis | + | ND |
| Alsin involvement | + | ND |
| Dynactin Involvement | + | ND |

## 5.3 INSULIN PATHWAY AND AMYOTROPHIC LATERAL SCLEROSIS

The insulin pathway was initially investigated in response to selenium since it is a major, well-defined, stress response pathway in *C. elegans*. One target of the insulin pathway, *sod-2*, was found to partially restore sensitivity in the *daf-2* mutant background (Figure 23), although our testing of targets was not exhaustive. Other targets of DAF-16 that should be tested for the ability to rescue the increased resistance in the *daf-2* mutant background include the 3-catalases, since they are responsible for converting hydrogen peroxide to water and oxygen. Hydrogen peroxide is previously generated by reduction of the superoxide anion by superoxide dismutase. This will be a challenge, since all 3 catalases lay sequentially in an 18kb region on chromosome 2 (Petriv and Rachubinski 2004). Again, it is likely that knocking out any individual catalase could result in compensation by another catalase and that the tight linkage makes creating double or triple mutants impossible. One possible solution is to create a small chromosomal deletion encompassing the 3 catalases. That may prove to cause embryonic lethality. In this situation, a non-specific RNAi could be constructed that recognizes all three catalases.

It should also be noted that knockout of Fe-Mn superoxide dismutase in mice is lethal within days (Li et al. 1995). Yet, *sod-2(ok1030);sod-3(gk235),* a worm MnSOD knockout, was viable with no grossly observable phenotype and no decrease in lifespan (Honda et al. 2008). This leads to intriguing questions about basic SOD biology between species. Do other SODs compensate by changing expression/localization in response to individual or double knockouts? This can be addressed using GFP reporter constructs for SOD-1, SOD-4, and SOD-5 in various combinations of SOD mutant backgrounds. In our lab, we have unpublished observations that SOD-1, which is diffusely expressed in the cytoplasm in unstressed conditions, changes localization in a hypoxic environment into punctate granules, which appear to be mitochondria,

although this has not been confirmed. What roles do SODs play in cellular signaling? While the genetics suggest that SOD-3 selenium resistance was due to function of SOD-2, Western data analysis suggests that the resistance is not due to changes in SOD-2 protein levels. This highlights the possibility that superoxide dismutases have more functions than simply scavenging the superoxide anion.

Data from the insulin pathway and selenium exposure also tie into ALS literature. DAF-16 is a FOXO transcription factor and a FOXO transcription factor has been identified as an ALS susceptibility factor from SNP analysis in a genome wide scan of sporadic ALS patients (Dunckley et al. 2007). Further, the FOXO3a transcription factor has been found to protect quiescent cells from oxidative stress by regulating expression of MnSOD (Kops et al. 2002). In a mutant SOD-1 transgenic mouse model of ALS, decreased MnSOD exacerbates motor neuron loss (Andreassen et al. 2000). In our model, decreased MnSOD has been found to be sensitive to selenium exposure (Figure 22). Decreased MnSOD has also been shown to exacerbate glutamate toxicity in rat cortical neurons (Li et al. 1998). While we have yet to demonstrate that decrease in MnSOD causes an increase in excitatory signaling, this is an intriguing parallel, since glutamate is an excitatory neurotransmitter, and excitotoxicity is one of the pathological findings in ALS. Glutamate is also the suspected target of riluzole, the only drug that has been shown to prolong life in ALS patients (Shaw and Ince 1997).

## 5.4 OTHER STRESS RESPONSE PATHWAYS

The DAF-2 insulin pathway was the only stress response pathway examined in this thesis. Other stress response pathways are also well-defined in *C. elegans,* including mitogen activated protein kinase (MAPK) pathways and the transforming growth factor beta (TGFβ) pathway. MAPK are likely also to be involved in responding to selenium toxicity, as selenium is a metalloid and MAPK have been implicated in response to the metal cadmium in *C. elegans* (Wang et al. 2008). TGFβ is another stress response pathway that might be investigated for a role in response to selenium toxicity. Preliminary experiments have demonstrated that reduction-of-function mutation in the TGFβ receptor, DAF-4, is highly sensitive (data not shown). Further experiments for each of these pathways involve testing reduction-of-function mutations for various proteins for altered sensitivity to selenium.

## 5.5 OVERALL IMPLICATIONS

This dissertation provides a large body of work that establishes *C. elegans* as a model for studying selenium toxicity. This work also demonstrates that exposure to high selenium can act as a motor neuron toxin and potentially as a model for human sporadic ALS. During this course this of investigation; we identified multiple genes where reduction-of-function mutation resulted in increasing an animal's sensitivity to selenium. Interestingly, SNP's for a human orthlog for

one of these genes (the FOXO transcription factor, *daf-16*) have been associated with sporadic ALS in a genome wide scan for SNP's associated with risk of developing sporadic ALS patients (Dunckley et al. 2007).What our data suggest is that certain genetic factors leave individuals susceptible to selenium exposure, which might explain why clusters of ALS cases have occurred in high selenium environments since genetically related individuals may reside in the same area. This also suggests that these genes could be investigated in ALS patients as a "susceptibility profile" and can be used to identify unexposed or unaffected individuals expected to be selenium sensitive.

# APPENDIX A

## ABBREVIATIONS

| Abbreviation | Meaning |
|---|---|
| 5-HT | 5-hydroxytryptamine hydrochloride or serotonin |
| ACh | Acetylcholine |
| ALS | Amyotrophic Lateral Sclerosis |
| $Ca^{2+}$ | calcium |
| CeMM | *Caenorhabditis elegans* Maintenance Media |
| Daf-c | dauer defective |
| Daf-d | dauer constitutive |
| Elg-c | Egg-laying constitutive |
| Egl-d | Egg-laying defective |
| gf | gain-of-function |
| HRP | Horse Radish Peroxidase |
| lf | loss-of-function |
| MnSOD | Iron/Manganese Superoxide Dismutase |
| NaSe | Sodium Selenite |

| | |
|---|---|
| NGM | Nematode Growth Media |
| PCR | Polymerase Chain Reaction |
| PVDF | Polyvinylidene Fluoride |
| rf | reduction-of-function |
| ROS | Reactive Oxygen Species |
| Se | Selenium |
| SNP | single nucleotide polymorphism |
| SOD | Superoxide Dismutase |

# APPENDIX B

# HPLC ANALYSIS OF SELENIUM TREATED *C. ELEGANS*

## B.1 RATIONALE FOR USING HPLC DETECTION OF OXIDIZED DAMAGE FROM SELENIUM EXPOSURE

ROS are known to cause many types of cellular damage, including oxidation of lipids, DNA, and proteins resulting in carbonyl formation, among others (Martin et al. 1996). Many of these oxidative damages can be detected by antibodies. Attempts to detect oxidative damage resulting from selenium exposure in *C. elegans* included single-stranded DNA antibodies and ubiquitin staining, were unsuccessful (data not shown). However, using antibodies for detection in *C. elegans* is problematic, especially in the nervous system. Permeablizing the animals results in them being susceptible to breaking and losing the structure of interest. Even with permeabilization, accessing the nervous system remains problematic, as it is protected from the exterior by the cuticle and from the interior by the gut lumen. Lack of staining, therefore, does not necessarily mean that the protein/damage detected by the antibody is not there, rather that the antibody cannot access a particular area. For these reasons, high performance liquid chromatography (HPLC) analysis was performed since it provides the benefit of detecting oxidative damage although we lose the advantages of studying an intact organism.

## B.2 METHODS FOR HPLC SAMPLE COLLECTION

Worms were developmentally synchronized by treatment with NaOH/Bleach (see 2.3.2) and grown to adults without starving after being plated. Adult worms were plated in the absence and presence of 5mM NaSe on large plates and in the absence and presence of 800ug/ml quercetin. 24 hours after plating on the various conditions, worms were collected in 15ml conicals with M9 The worms were washed 3 times with M9, using ~10ml for the washes. The worms were allowed to sediment between washes. Sedimentation was important with the NaSe samples in order to remove carcasses of dead animals as they do not sediment. The washes lasted for at least 30minutes, combined, in order to allow the animals to clear their intestine of food and waste. The last wash for each condition was saved to be used as a control. The worms were transferred to 2ml microcentrifuge tubes and homogenized (setting at 11) with a Poly-tron (PT-2100) for 15-30 seconds. The tip of the poly-tron was cleaned between samples, first with ethanol and then water (homogenized for 30seconds to a minute with each wash). Samples were centrifuged to remove carcasses and the supernatants were transferred to new tubes. Soluble samples, pellets, and the final washes were snap-frozen and stored at -80°C until taken to Dr Yao for analysis.

## B.3 RESULTS

To examine differences between untreated and selenium-treated animals, large, age synchronous populations of animals were placed as adults on untreated NGM plates or NGM plates treated with 5mM sodium selenite for 24 hours and samples were collected as described in 2.3.7. In

addition, subpopulations were co-treated with 800μg/ml quercetin (the antioxidant in Figure 8A that best maintained normal motility in the presence of NaSe) to determine whether quercetin could suppress the potential oxidative damage detected by HPLC. Table 3 contains the data collected by HPLC analysis in collaboration with Dr. Yao. Specifically, we were looking for changes in 3-nitrotyrosine, since increased levels have been observed in both familial and sporadic ALS (Beal et al. 1997). While no significant damage was found comparing untreated and selenium animals, what was observed was an increase in 3-nitrotyrosine in the washes of both selenium-treated and selenium-treated animals that were co-treated with quercetin, suggesting that the cells were sufficiently damaged that they were leaking their cellular contents prior to homogenization of the animal (Table 3).

**Table 3 HPLC comparison of selenium treated animals**

Mass numbers of developmentally synchronized N2 animals were grown to adulthood without starvation. Animals were then transferred to new plates that were untreated, treated with 5mM NaSe, 800ug/ml quercetin, and 800ug/ml quercetin + 5mM NaSe. Animals were treated for 24 hours before processing for HPLC analysis as described in the Methods.

| ***C. elegans*** **(ng/mg dry weight, n=4)** | **Tyrosine** | **Tryptophan** | **Guanine** | **3-NT** |
|---|---|---|---|---|
| Control | 1074±692 | 184±73 | 773±217 | 272±154 |
| Quercetin | 415±114 | 200±45 | 881±161 | 203±78 |
| 5mM NaSe | 1479±664 | 166±55 | 682±254 | 94±37 |
| 5mM NaSe +800ug/ml quercetin | 1044±298 | 220±130 | 604±169 | 105±27 |
| **Washes (ng/ml, n=4)** | | | | |
| Control | 21±42 | 0 | 108±132 | 32±18 |
| Quercetin | 1±2 | 0 | 32±16 | 35±34 |
| 5mM NaSe | 1±1 | 0 | 17±7 | 153±25 |
| 5mM NaSe +800ug/ml quercetin | 3±5 | 0 | 26±24 | 184±34 |

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