CAENORHABDITIS ELEGANS AS A MODEL TO STUDY MOLECULAR MECHANISMS OF METHYLMERCURY TOXICITY

By

Kirsten Jeanne Helmcke

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacology

May, 2010

Nashville, Tennessee

Approved:

Professor Michael Aschner

Professor BethAnn McLaughlin

Professor Eugenia V. Gurevich

Professor Ariel Y. Deutch

Professor David M. Miller, III

ACKNOWLEDGEMENTS

Many individuals and organizations contributed to and made this work possible. I would like to thank the Vanderbilt Interdisciplinary Graduate Program, the Pharmacology Department, and the Training Program in Molecular Toxicology (T32 ES007028) for not only providing financial support but also for giving me the educational foundation on which I could build my research. Many individuals in these departments and programs contributed to my success, I would particularly like to thank the Director of Graduate Studies in Pharmacology, Joey Barnett, and the Educational Coordinator for Pharmacology, Karen Gieg, for their guidance and assistance.

My committee of Michael Aschner, BethAnn McLaughlin, Eugenia Gurevich, Ariel Deutch, and David Miller has been very helpful in guiding my research. As my committee chair, BethAnn provided me thorough meeting summaries outlining the necessary steps to further my project amazingly quickly after each meeting and was happy to discuss any questions or issues I had. While neither Eugenia nor Ariel is a toxicology expert, they both provided valuable insights and support. David was extremely accommodating in allowing for my development as a *C. elegans* biologist by providing a number of resources including attendance at his group meetings and the assistance of his lab members, whom I am grateful to for helping me to develop many of the methodologies that I used.

In addition to the Miller lab, the support of many collaborators who provided scientific input as well as technical expertise was required for completion of this work. Lars Evje in Tore Syversen's lab performed ICP-MS experiments. The Randy Blakely lab provided materials and equipment for conducting thrashometer experiments and I was able to conduct these studies with the help of Dawn Matthies and Shannon Hardie. Jiyang Cai provided materials, equipment, and assistance for HPLC experiments. I am appreciative of the time and resources that each one of these individuals put forth to assist me.

I am indebted to Michael Aschner for being a fantastic scientist, great teacher, and all-around wonderful person. Everyone in the Aschner lab also contributed to this work, whether directly or by helping with experiments, discussions, or friendship. In particular, I would like to thank the other worm researchers, including Catherine Au and George Jiang, who helped to set up the system and protocols in the lab, Alexandre Benedetto for helping to build the system in the lab, and Daiana Silva Avila, Margaret Adams, and Sudipta Chakraborty, for continuing with this work. I am also grateful to Alycia Buford for her administrative help in the lab.

I also would like to express my gratitude to my friends for helping keeping me grounded throughout this process. Finally, I would like to thank my parents for their unwavering love and support in all my endeavors and for being extraordinary role models.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Meaning
ADHD	attention deficit hyperactivity disorder
ADP	adenosine diphosphate
Al	aluminum
ANOVA	analysis of variance
As	arsenic
ATP	adenosine triphosphate
Ba	barium
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
C	cysteine
Ca	calcium
Cd	cadmium
Co	cobalt
Cr	chromium
CREB	cAMP response element binding
C. elegans	*Caenorhabditis elegans*
CGC	*Caenorhabditis* Genetics Center
Cu	copper
Cys	cystiene

dH2O ..... distilled water

DNA ..... deoxyribonucleic acid

dsRNA ..... double stranded RNA

EPA ..... Environmental Protection Agency

EtHg ..... ethylmercury

E. coli ..... *Escherichia coli*

FOXO ..... forkhead box O

γ-GluCys ..... gamma-glutamylcysteine

GABA ..... γ-aminobutyric acid

GFP ..... green fluorescent protein

GPx ..... glutathione peroxidase

GSH ..... reduced glutathione

GS-MeHg ..... glutathione-methylmercury complex

GSSG ..... oxidized glutathione

GST ..... glutathione s-transferase

GR ..... glutathione reductase

GRP78 ..... 78-kDa glucose-related protein

HNO_3 ..... nitric acid

HPLC ..... high performance liquid chromatography

HR-ICP-MS ..... High Resolution Inductively Coupled Plasma Mass Spectrometry

HSF ..... heat shock factor

HSP ..... heat shock protein

Hg ..... mercury

$HgCl_2$ ..... mercuric chloride

ICP-MS ..... Inductively Coupled Plasma-Mass Specrometry

Keap1 ..... kelch-like ECH-associated protein 1

KOH ..... potassium hydroxide

LAT-1 ..... large neutral amino acid transporter

LD_X ..... lethal dose for X% of organisms

L1 ..... first larval stage

L2 ..... second larval stage

L3 ..... third larval stage

L4 ..... fourth larval stage

MeHg ..... methylmercury

MeHgCl ..... methylmercury chloride

µg ..... microgram

mg ..... milligram

µL ..... microliter

mL ..... milliliters

mM ..... millimolar

mol/L ..... moles per liter

MT ..... metallothionein

MT-MeHg ..... metallothionein-methylmercury complex

M9 ..... *C. elegans* buffer

NFκB ..... nuclear factor kappa-light-chain-enhancer of activated B cells

ng ..... nanogram

NGM ..... nematode growth medium

Nrf2..... nuclear factor-erythroid 2-related factor 2

N2..... wild type *C. elegans*

OP50 .....*E. coli* strain

Pb ..... lead

ppm ..... parts per million

RNA.....ribonucleic acid

RNAi .....RNA interference

ROS..... reactive oxygen species

U..... uranium

VEGF..... vascular endothelial growth factor

WT..... wild type

Zn .....zinc

CHAPTER I

INTRODUCTION

Mercury

Mercury (Hg) is a heavy, silvery white, transition metal that exists in a liquid state at room temperature. Hg is found in different oxidation states, including the zero oxidation state (Hg^0), the first oxidation state as mercurous mercury (Hg^{1+}), and the second oxidation state as mercuric mercury (Hg^{2+}). Hg^0 is present in the metallic form or as vapor and, upon oxidation, is the source for the other forms in higher oxidation states. Electron loss yields Hg^{1+}, which is commonly found as calomel or mercurous chloride (as Hg_2^{2+}). Hg^{2+} is a major component of most organic and inorganic Hg-containing compounds, including methylmercury (MeHg) and ethylmercury (EtHg). Hg is found in a number of commonly used compounds, is released upon metabolism of organic Hg compounds, and is present in inhaled vapor, making an understanding of the effects of Hg on biological systems essential, particularly given that Hg has been identified as an important toxicant (Clarkson and Magos, 2006).

Hg, typically present in the liquid and vapor forms as Hg^0, undergoes the phenomenon of global cycling. The vapor is released from natural sources, such as volcanoes, soil, and water surfaces; and from man-made sources, such as coal-burning power stations and incinerators. The Hg^0 vapor can remain in the atmosphere for an extended period of time, allowing for vast dispersal around the

globe, during which it can be oxidized to Hg^{2+}. Due to its higher water solubility, Hg^{2+} accumulates in atmospheric moisture and falls to the earth as precipitation. Once it reaches the earth, Hg^{2+} can be reduced back to Hg^{0} and re-enter the atmosphere or be absorbed by vegetation. Alternatively, the Hg^{2+} that falls to earth can reach aquatic environments and come in contact with microorganisms, specifically sulfate-reducing bacteria that convert inorganic Hg to MeHg in a detoxification reaction. MeHg then travels up the food chain when fish that eat these bacteria are in turn eaten by larger fish (Fitzgerald and Clarkson, 1991; Mason *et al.*, 2005). During this process, bioaccumulation occurs to such an extent that sharks and carnivorous sea mammals end up having some of the highest levels of MeHg (4ppm), equivalent to a million-fold bioaccumulation (Clarkson and Magos, 2006).

Human exposure to mercury

Humans are exposed to Hg through three major routes: Hg vapor emitted from amalgam dental fillings, EtHg which is absorbed when it is used as a preservative in vaccines, and MeHg which is absorbed from seafood. Average daily intake of Hg has been measured at approximately 6.6 μg. Nearly 0.6 μg of this comes from MeHg in fish sources and approximately 4 μg comes from inorganic sources, most in the form of Hg^{0} vapor inhalation (WHO, 1990) from dental and occupational sources while atmospheric levels are negligible (Fitzgerald and Clarkson, 1991).

Depending on the form of exposure, Hg can produce effects in the body which involve various organ systems. Acrodynia, or painful extremities, is attributed to exposure to the Hg^{2+} used in agents such as laxatives and teething powders. Symptoms of Hg^0 inhalation through dental or occupational exposure include tremor, psychological disturbances, and renal toxicity (WHO, 1991; Clarkson and Magos, 2006). Due to the dissociation of Hg atoms, exposure to organic forms of Hg can result in symptoms similar to those observed with Hg^0 vapor exposure (WHO, 1991; Clarkson and Magos, 2006). Hg^{2+} is extremely toxic, with extensive exposure leading to renal failure as well as stomatitis and gastroenteritis, and even autoimmune disease (Pollard and Hultman, 1997; Clarkson and Magos, 2006).

The effects of Hg vapor emitted from dental fillings and EtHg found in vaccines on the health of organisms have been debated. While some research has found deleterious effects on the nervous system, these reports are countered by other studies finding no association between these toxicants and diseases. For example, Hg vapor has been associated with the induction of neurodegenerative diseases such as Alzheimer's Disease (Mutter *et al.*, 2004) and EtHg has been blamed for triggering autism (Geier and Geier, 2006). However, both of these causative relationships have come under scrutiny (Factor-Litvak *et al.*, 2003; Parker *et al.*, 2004; Thompson *et al.*, 2007). The use of Hg amalgams as dental fillings has fallen out of favor due to environmental concerns regarding Hg disposal and EtHg has been removed from most vaccines due to health concerns; therefore, these routes of Hg exposure are being

reduced. Conversely, in spite of the fact that the destructive properties of MeHg have been widely reported and accepted, consumption of this toxicant in seafood persists.

Methylmercury

Metabolism and bioaccumulation of MeHg

Organic Hg compounds are well-characterized with regard to distribution in the body and metabolism. The environmental protection agency (EPA) has established a reference dose of 0.1 µg/kg body weight/day, corresponding to a level of approximately 5.8 µg/L Hg in the blood or 1.0 µg/g in hair (EPA, 2001). Upon ingestion MeHg is well absorbed through the gastrointestinal tract. In the liver, MeHg can form a complex with reduced glutathione to be excreted in bile, which can be reabsorbed by the small intestine once broken down or can be metabolized by intestinal microflora to produce Hg^{2+}. Fecal excretion is the main route of elimination (Clarkson *et al.*, 1981; Patrick, 2002; Clarkson and Magos, 2006).

MeHg has high affinity for thiol groups, a property thought to contribute to its toxicity. This leads to the ability of MeHg to bind to proteins via their cysteine side chains. The MeHg-cysteine complex molecularly mimics methionine, allowing for its passage through the blood-brain and placental barriers and into cells via the large amino acid transporter, LAT1 (Kerper *et al.*, 1992; Simmons-Willis *et al.*, 2002; Yin *et al.*, 2008). MeHg accumulates in the brain at high levels, as much as five times the concentrations observed in blood (WHO, 1990).

Conversion to inorganic Hg occurs within the brain, and long-term studies have shown that years after exposure to MeHg, Hg accumulated in the brain in the inorganic form (Simmons-Willis *et al.*, 2002; Clarkson and Magos, 2006). Due to its passage through the blood-brain and placental barriers, MeHg in the brain of a newborn can reach levels as high as five times those seen in the mother (Cernichiari *et al.*, 1995; Clarkson, 2002; Clarkson and Magos, 2006).

Neurotoxicological effects of MeHg

The neurotoxicological effects of MeHg were revealed after unfortunate high-dose poisoning events, one due to local pollution of Minamata Bay in Japan and the subsequent high concentrations of MeHg in fish, and another event due to consumption of grain treated with a MeHg-fungicide in Iraq (Clarkson, 2002). Additional investigations of the neurotoxicity of MeHg were conducted in the seafood-consuming populations in the Seychelles (Davidson *et al.*, 1998; Myers *et al.*, 2009) and Faroe Islands (Grandjean *et al.*, 1997; Debes *et al.*, 2006).

High levels of MeHg exposure such as those encountered in the Minamata and Iraqi poisonings were manifested in a number of ways including sensory impairments, paralysis, hyperactive reflexes, cerebral palsy, and impaired mental development (National Research Council, 2000). In Minamata Bay, MeHg was released directly into the water by a chemical plant, leading to high MeHg content in marine samples (5.61-35.7 ppm). Consumption of these products led to Minamata disease, first discovered in 1956. MeHg levels in the 2252 officially recognized patients had hair MeHg levels as high as 705 ppm

(Harada, 1995) and umbilical cord samples of Minimata disease patients contained 1.60 ppm MeHg. In 1971-72 a MeHg poisoning event occurred in Iraq where approximately 6530 individuals were admitted to the hospital after eating grain treated with a MeHg fungicide. Levels of 240-480 ng Hg/mL blood were associated with increases in complaints of paresthesia (Clarkson *et al.*, 1976).

The neurotoxicological effects of MeHg on humans vary based on age at the time of exposure. Adults exposed to MeHg experience focal lesions, such as loss of cerebellar granular cells and occipital lobe damage (Clarkson and Magos, 2006), whereas younger individuals experience global alterations to the brain, including microcephaly and inhibition of neuronal migration, leading to distortion of cortical layers, cerebellar abnormalities, alterations in glial cells (such as decreased amino acid uptake (Aschner *et al.*, 1993)), and alterations in neurotransmitter systems.

Although some researchers observed delays in the achievement of developmental milestones upon low-level chronic MeHg exposure, a number of epidemiological studies conducted in populations exposed to MeHg through diet have been inconclusive as to the clinical effect of low-dose chronic exposure to MeHg through seafood consumption (Clarkson and Magos, 2006).

Two large studies have been conducted, one in the Seychelles Islands and one in the Faroe Islands (Grandjean *et al.*, 1997; Davidson *et al.*, 1998; Debes *et al.*, 2006; Myers *et al.*, 2009). In the Seychelles Islands, endpoints measured at 66 months largely revealed a positive association between MeHg exposure and developmental outcomes, revealed by the McCarthy Scales of

Children's Abilities-General Cognitive Index score, the Preschool Language Scale-Total Score, and the Woodcock-Johnson Applied Problems test (Davidson *et al.*, 1998). At 107 months, negative associations between MeHg exposure and performance were revealed by a decline in performance on Connor's Teacher Rating Scale ADHD Index, Wechsler Intelligence Scale for Children-Revised, the Grooved Pegboard with the non-dominant hand, and the Connor's Continuous Performance Task Risk Taking (Myers *et al.*, 2009). From the study in the Faroe Islands it was concluded that postnatal MeHg exposure produced no significant adverse effects when children were tested at 14 years of age (Debes *et al.*, 2006). However, endpoints tested at 7 years of age did reveal an adverse association between MeHg exposure and performance on Finger Tapping with both hands and the Reaction Time from the Continuous Performance Test (Grandjean *et al.*, 1997).

These differences have been attributed to differences in measurement techniques, the extent of MeHg exposure, and other confounding variables. Additionally, the health benefits of seafood consumption likely confound these results and lead to questions surrounding the costs and benefits of the consumption of seafood.

Mechanisms of MeHg action

While several neurological targets of MeHg have been identified, the specific mechanisms of cellular dysfunction are unknown. Microarray analyses have revealed many genes are altered upon exposure to MeHg and suggest an

effect of MeHg on transcription or RNA stability (McElwee *et al.*, 2007). A diverse range of potential targets, such as factors involved in cell cycle regulation, apoptosis, immune functioning, and G-protein signal transduction have been elucidated (Ayensu and Tchounwou, 2006). Some of the known effects of MeHg include an inhibition of DNA, RNA, and protein synthesis (Gruenwedel and Cruikshank, 1979); disruption of microtubules leading to mitotic alterations (Rodier *et al.*, 1984); and increases in intracellular calcium (Ca^{2+}) leading to alterations in neurotransmitter function, excitotoxicity, and oxidative stress (Sirois and Atchison, 1996). Disruption of Ca^{2+} by depolarization of the presynaptic membrane leads to alterations in dopamine, γ-aminobutyric acid (GABA), glycine, choline, and acetylcholine signaling (Dwivedi *et al.*, 1980; O'Kusky and McGeer, 1989; Levesque *et al.*, 1992; Aschner, 2000; Bemis and Seegal, 2000; Atchison, 2005; Kobayashi *et al.*, 2005; Basu *et al.*, 2006; Herden *et al.*, 2008; Sunol *et al.*, 2008; Dreiem *et al.*, 2009). A common theme of MeHg toxicity is targeted dysfunction of thiol groups, with its affinity for these groups being ten orders of magnitude higher than the affinity for oxygen-, chloride-, or nitrogen-containing ligands (West *et al.*, 2008). By binding to protein sulfhydryl groups, MeHg can indirectly alter the structure of DNA and RNA (Gruenwedel and Lu, 1970) and induce alterations in anabolic processes, enzyme function, and protein synthesis (Syversen, 1982; Myers *et al.*, 2009). For instance, MeHg interaction with microtubules is thought to be due to its ability to bind sulfhydryl groups (Vogel *et al.*, 1985). The inhibition of tubulin polymerization (Rodier *et al.*, 1984; Graff *et al.*, 1993) and microtubular fragmentation (Castoldi *et al.*, 2000) have

been shown to play a role in the toxicity of MeHg by disrupting various processes including mitosis and neuronal migration (Myers *et al.*, 2009).

On a molecular level, MeHg has been shown to be able to activate Nuclear factor-erythroid 2-related factor 2 (Nrf2). Nrf2 is able to activate the antioxidant response element/electrophile responsive element (ARE/EpRE) upon its release from Kelch-like ECH-associated protein 1 (Keap1) by binding to the ARE in the promoter region and activating gene expression (Itoh *et al.*, 1997). These induced genes include antioxidant proteins, phase II xenobiotic-metabolizing enzymes and phase III transporters, which allow for metabolism of xenobiotics. The activation of Nrf2 occurs via an interaction of MeHg with thiol groups on Keap1 which results in the release of Nrf2 from Keap1 (Toyama *et al.*, 2007).Additionally, increased expression of Nrf2 diminshes the toxicity of MeHg (Rand *et al.*, 2009; Wang *et al.*, 2009).

MeHg protective mechanisms

Studies have investigated the detoxification and removal of MeHg from biological systems, showing that a number of proteins are involved in the detoxification and excretion of MeHg; these include glutathione (GSH), heat shock proteins (HSPs), and metallothioneins (MTs). MeHg is also known to induce generation of reactive oxygen species (ROS) through alterations in mitochondrial respiration and the electron transport chain (Verity *et al.*, 1975; Yee and Choi, 1996) and the generation of hydroxyl radicals from the breakdown of hydrogen peroxide (Patrick, 2002). ROS can have a number of harmful effects

including DNA damage, lipid peroxidation, and amino acid oxidation. Anatomical brain regions with increased MeHg-induced ROS generation show increased damage, with toxic effects of MeHg mirroring the oxygen demands for the given cell type (Sarafian and Verity, 1991; Bondy, 1994; Yee and Choi, 1996). Although GSH, HSPs, and MTs have been implicated in resistance to MeHg toxicity, researchers have not fully elucidated their precise role in detoxification. However, many of their described roles involve protection through activation by or defense from ROS and the ability of these proteins to bind MeHg due to their Cys content. The potential mechanisms of protection afforded by these three systems are described in the following sections.

Glutathione

GSH is the major antioxidant within cells. It is a tripeptide consisting of glutamic acid, cysteine, and glycine and can exist in the reduced (GSH) or the oxidized (GSSG) state. It is formed when gamma glutamylcysteine (γ-GluCys) synthetase catalyzes the production of γ-GluCys from glutamic acid and cysteine (the rate-limiting component of the synthesis of GSH). GSH synthetase then catalyzes the production of GSH by combining γ-GluCys and glycine. Glutathione peroxidase (GPx) catalyzes the oxidation of GSH to GSSG in the presence of ROS. GSSG can then be converted back into GSH via glutathione reductase (GR) and the conversion of NADPH to NADP+ (Filomeni *et al.*, 2005). Alternatively, glutathione s-transferases (GSTs) can catalyze the conversion of

GSH to GS-, which can bind to various xenobiotics to facilitate excretion from the body (Hirata and Takahashi, 1981).

GSH has been shown to play a large part in MeHg toxicity. MeHg will readily bind to sulfhydryl groups. Since GSH is typically the sulfhydryl-containing compound in cells with the highest concentration, MeHg easily binds, forming a GS-MeHg complex. The binding of GSH to MeHg has two major effects. Firstly, binding the toxicant to GSH prevents it from damaging other proteins and tissues. Secondly, the GS-MeHg complex is excreted from the organism, both in bile (approximately 90% of MeHg excretion) and in urine (approximately 10% of MeHg excretion) and its existence in this form facilitates its excretion from the body (Patrick, 2002) (Figure 1A). The complex is also important in transport throughout the organism, particularly within the nervous system. Endothelial cells forming the blood-brain barrier excrete MeHg as a complex with GSH. Astrocytes, the first line of defense from toxicants in the brain (Tiffany-Castiglion and Qian, 2001) and a major depot for MeHg accumulation (Aschner *et al.*, 1990), also excrete the GS-MeHg complex. The addition of glutathione, glutathione stimulators, or glutathione precursors enhances this excretion and cell lines expressing five times the normal level of GSH do not readily accumulate MeHg and are resistant to its toxic effects (Patrick, 2002).
In addition to sequestering and eliminating the toxicant, GSH also plays a well-established role in the elimination of ROS (Figure 1A). GSH can react directly with radicals or, through the action of GPx, GSH can act as an electron donor to react with ROS, such as hydrogen peroxide, to form GSSG and water

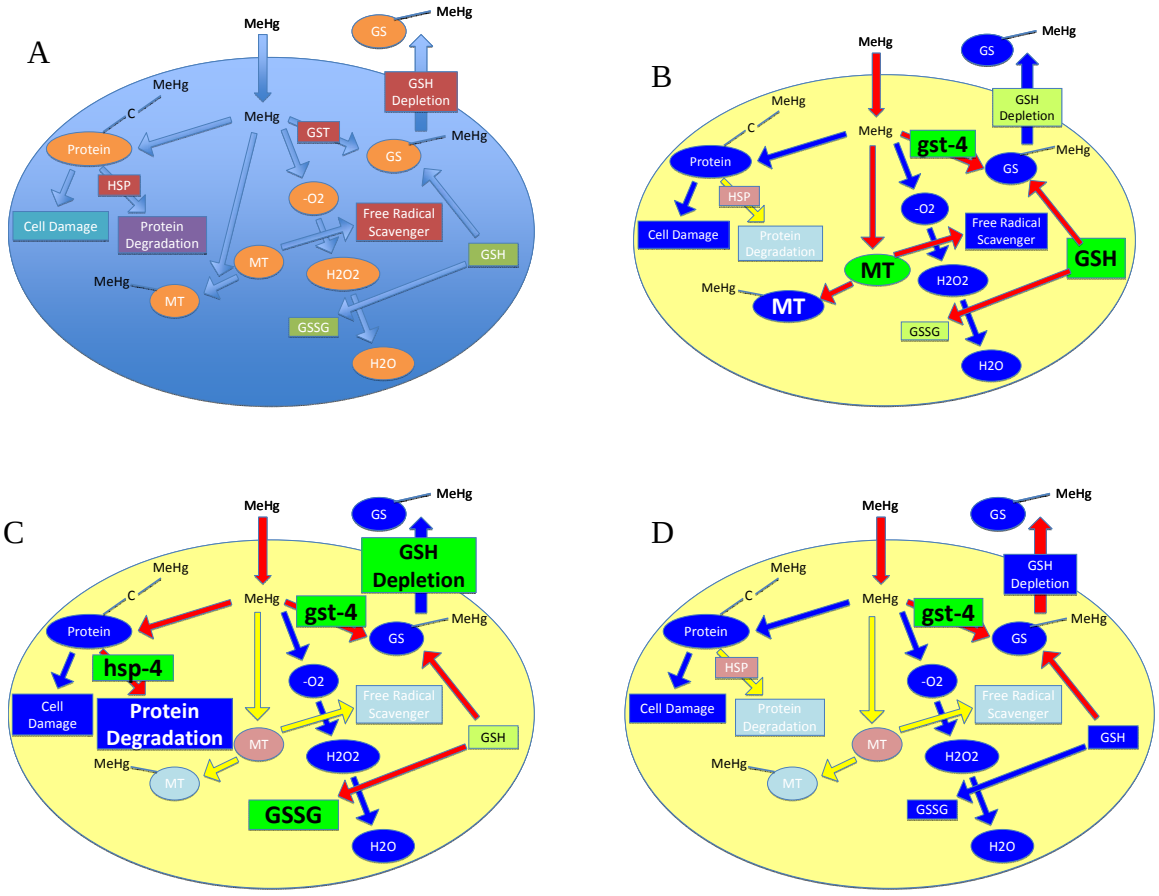

Figure 1. Model of the molecular mechanisms of MeHg toxicity. MeHg induces alterations in the cell by generating ROS and binding directly to Cys groups on proteins. These induce a number of downstream effects, including induction of HSPs to induce degradation of damaged proteins, MTs to bind free MeHg and reduce ROS, and GSH to reverse ROS damage and bind directly to MeHg for excretion (A). In L1 animals treated acutely with MeHg, MTs, *gst-4*, and GSH are all upregulated, assisting with MeHg detoxification (B). In L4 animals treated chronically with MeHg, levels of *hsp-4*, *gst-4*, and GSSG are increased, and GSH is depleted (C). In preconditioning, *gst-4* is increased. Due to the increase in *gst-4*, we suspect alterations in the GSH system, but these have not been assessed (D).

(Fonnum and Lock, 2004). Maintenance of GSH levels following MeHg exposure protects cells from oxidative injury (Kaur *et al.*, 2006). However, the excretion of MeHg in a complex with GSH causes levels of the antioxidant to decrease, thereby rendering cells vulnerable to damage induced by ROS (Fonnum and Lock, 2004). The excretion of the GS-MeHg complex inducing depletion in GSH can be further amplified by the ability of MeHg to block the uptake of cystiene and thus inhibit new GSH synthesis (Allen *et al.*, 2001). Together the decrease in GSH generation and increases in excretion have been shown to cause significant decreases in GSH in a mouse model of MeHg toxicity, with GSH levels being significantly lower (at post natal day 11 control animals contained approximately 3.8 nmol/mg protein while animals treated with 1, 3, or 10 mg/L contained approximately 3, 2.6, or 2.4 nmol/mg protein, respectively). Other contributors to the glutathione system were also significantly altered, with significant decreases in GPx and GR also being observed (Stringari *et al.*, 2008).

Due to the extensive research with GSH and its role in MeHg toxicity both by its direct conjugation with the toxicant for elimination and its protection from ROS generated by the toxicant (Figure 1A), we hypothesized that this system, along with others such as MTs, which also play a role in direct detoxification by binding Cys and play an antioxidant role, would be valuable targets to study further.

Metallothioneins

MTs are small, cysteine-rich metal binding proteins that are involved in metal detoxification and homeostasis and can protect cells from oxidative stress through this role and their role as antioxidants (Maret, 2008). Additionally, MTs can be involved in metal metabolism, cellular repair, regulation of gene expression, and are the source of Zn for enzymes (West *et al.*, 2008). Four MTs (MT-1, MT-II, MT-III, and MT-IV) exist in mammals, and two of these, MT-I and MT-II, have been best-characterized for their protection of the brain although they are ubiquitously expressed in all tissues (Penkowa, 2006). MT expression has been shown to increase upon exposure not only to various metals but also upon exposure to nonmetallic compounds (Sato and Bremner, 1993). Much like GSH, MTs are able to detoxify MeHg through binding and sequestering the toxicant and act as antioxidants to relieve the damage caused by ROS (Figure 1A).

Due to their high cysteine content, MTs have a high affinity for MeHg, resulting in the formation of a MT-MeHg complex that renders MeHg unable to damage other cellular targets. The ability of inorganic Hg to induce expression of MTs has been well-established (West *et al.*, 2008) and in some studies, MeHg has been shown to induce expression of MTs (Rising *et al.*, 1995; Tsui and Wang, 2005). However, a number of other studies have failed to establish a link between MT induction and MeHg exposure (Kramer *et al.*, 1996a; Kramer *et al.*, 1996b; Yasutake *et al.*, 1998; Gonzalez *et al.*, 2005). Although whether MeHg is able to induce expression of MTs is debated, the involvement of MTs in MeHg toxicity has been firmly established. For example, MeHg induces alterations in

behavior of MT-null animals (Yoshida *et al.*, 2008). Overexpression of MTs in primary rat astrocytes and astrocytoma cells can attenuate the toxicity of MeHg (Yao *et al.*, 1999; West *et al.*, 2008), induction of MTs by other metals decreases sensitivity to MeHg (Aschner *et al.*, 1998), and expression of MTs in MT-null cell lines affords protection against MeHg (Yao *et al.*, 2000).

MTs, which are known antioxidants (Maret, 2008), are free radical scavengers that have the ability to scavenge a variety of radicals including superoxide, hydroxyl, and organic radicals. MT-1 and MT-2 have been shown to be induced in response to oxidative stress (Bauman *et al.*, 1991; Sato and Bremner, 1993; Andrews, 2000) and MeHg exposure (Rising *et al.*, 1995). Zn, which often binds to the Cys groups on MTs, can be oxidized by ROS, which causes the release of Zn from MTs (Maret and Vallee, 1998; Krezel and Maret, 2007; Maret, 2008). Cellular systems lacking MTs have been shown to have a hypersensitivity to ROS (Lazo *et al.*, 1995) and levels of lipid peroxidation, protein nitrosylation, and DNA oxidation are increased in the brains of animals lacking MTs (Penkowa, 2006). Importantly, due to the ability of ROS to act as an intracellular messenger, the scavenging ability of MTs may be related to cellular signaling (Sato and Kondoh, 2002). Additionally, MTs have been indicated as contributors to the hormetic response, or the ability of a stressor to precondition the animal and blunt the effect of a subsequent stressor (Damelin *et al.*, 2000). The involvement of MTs in MeHg toxicity and in hormesis hinted that this toxicant might be able to induce a hormetic effect. The ability of MeHg to induce hormesis

would also indicate that other proteins, such as HSPs, are involved in its detoxification.

Hormesis

Hormesis, also known as preconditioning or an adaptive stress response, is a process whereby a sublethal stressor renders an organism more resistant to subsequent injury. This has been demonstrated in a number of models ranging from cell cultures to humans under a variety of conditions, including lifestyle factors such as exercise (Kojda and Hambrecht, 2005; Gomez-Pinilla, 2008), dietary energy restriction (Masoro, 2005; Martin *et al.*, 2006) phytochemicals (Mattson, 2008a), or cognitive stimulation (Scarmeas and Stern, 2003); environmental exposure to toxicants (Damelin *et al.*, 2000; Calabrese, 2005), radiation (Upton, 2001), or temperature (Li *et al.*, 2002); and intrinsic factors such as ischemia (Yellon and Downey, 2003), endocrine status, or neurotransmitters (Marini *et al.*, 2007; Mattson, 2008b). This process has been necessary to allow organisms to adapt to changing environmental conditions (Mattson, 2008b). However, the therapeutic use of hormesis is extremely controversial due to a number of concerns including the generalizability of the phenomenon across conditions, the difficulty of ensuring exposure at a hormetic dose, and ethical considerations of exposing individuals to known pathogens (Elliott, 2008).

Many of the specific mechanisms of action of hormesis are still unknown. While hormesis typically refers to exogenous agents, it can be a part of normal physiological functioning, such as the ability of glutamate to cause energetic and

oxidative stress at low levels which can activate hormetic pathways and render a cell resistant to more severe stress, while a higher exposure to glutamate would result in excitotoxicity (Mattson, 2003; Marini *et al.*, 2007). Additionally, exposure to one stressor can often offer protection from exposure to another, resulting in cross-modal protective effects of exposure to low doses of these agents. Exposure to these stressors and agents cause stress and signaling events that can involve free radicals, ion fluxes, energy depletion, receptors, kinases, phosphatases, deacetylases, and transcription factors such as Nrf2 (Lee and Surh, 2005), FOXOs (Frescas *et al.*, 2005), CREB, and NF-κB (Carlezon *et al.*, 2005; Mattson and Meffert, 2006). Downstream of these, antioxidants such as superoxide dismutases, catalase, glutathione, and glutathione peroxidase; protein chaperones such as HSP70 and GRP78; growth factors such as BDNF, VEGF, and bFGF; and other effectors such as mitochondrial proteins and calcium-regulating proteins can promote the hormetic response (Mattson, 2008b).

Relating to MeHg exposure, HSPs of the HSP70 family and MTs were upregulated following exposure of cells to various heavy metals (Damelin *et al.*, 2000). Additionally, hormetic mechanisms have been implicated as a possible explanation of latency observed in cases of MeHg poisoning (Burbacher *et al.*, 1990). The involvement of HSPs in the hormetic response and their ability to potentially protect an animal from a MeHg insult led to the further investigation of these proteins.

Heat Shock Proteins

Under normal conditions, HSPs function as molecular chaperones, assisting with protein folding, directing proteins to proper organelles, assembly and disassembly of protein complexes, and inhibition of aggregation. Upon stress, for example in the presence of MeHg (Sacco *et al.*, 1997), these proteins function to assist in the refolding and repair of denatured proteins and can facilitate new protein synthesis (Hubbard and Sander, 1991) (Figure 1A). HSPs, particularly members of the HSP70 family, have been shown to be involved in hormesis. HSP70s are ATP-binding proteins; however, upon binding hydrophobic residues, HSP70's ATPase function is stimulated. When ATP is converted to ADP, HSP70 binds peptides to render them inactive and to prevent them from aggregating. Since oxidative stress can cause a reduction in cellular ATP levels due to the sensitivity of mitochondria to ROS (Lenaz, 1998), HSP70's ability to release ADP to bind ATP is hampered. This results in continued prevention of aggregation of damaged proteins (Mayer and Bukau, 2005). In cellular systems and in *Drosophilia melanogaster*, researchers have shown that HSP70 plays a role in hormesis. In cellular systems, induction of HSP70s upon stressors has been shown (Verbeke *et al.*, 2001) and overexpression of HSP70s has been shown to induce protection to stressors (Amin *et al.*, 1996; Plumier *et al.*, 1997). In *Drosophilia*, low-level heat stress, shown to induce HSP70, produced lifespan extension (Hercus *et al.*, 2003) and strains carrying an increase in copies of hsp70 genes displayed an increase in survival, which increased upon exposure to heat (Tatar *et al.*, 1997).

Due to the demonstrated and hypothesized involvement of GSH, MTs, and HSPs, we further explored these proteins relating to MeHg toxicity and how they interacted with each other to protect the animal from the toxicant.

Contributions of GSH, HSPs, and MTs to MeHg toxicity

Systems involving GSH, HSPs, and MTs have been shown to act in harmony to detoxify and excrete MeHg (Figure 1A). Upon MeHg entering a cell, a number of processes can be activated. The two major mechanisms through which MeHg wreaks havoc on a cell are MeHg binding directly to sulfhydryl groups on proteins, and the generation of ROS. Both of these processes can induce the activation of HSPs, which can assist to either repair or degrade the damaged proteins (Hubbard and Sander, 1991). MeHg can also induce the expression of MT-1 and MT-2 (Rising *et al.*, 1995), which in turn bind to and sequester MeHg and scavenge free radicals generated by MeHg. The GSH system has also been shown to play a role in MeHg toxicity through various pathways. Through the cycling of GSH with GSSG, GSH can reduce oxidized proteins to repair ROS damage, but it can also directly bind to MeHg due to its high cysteine content with the assistance of GSTs (Fonnum and Lock, 2004). As a complex with MeHg, GSH is eliminated from the system (Patrick, 2002).

A model system to examine the molecular mechanisms of MeHg toxicity: *Caenorhabditis elegans*

The task of fully elucidating mechanisms of MeHg toxicity has proven difficult due to the complexities of mammalian models and the inability of cell systems to demonstrate characteristics of an intact organism. Therefore, a model system lacking many of the complexities of mammalian systems and having some of the advantages of a cellular system while retaining the advantages of an intact organism would be highly beneficial. We used the model organism, *Caenorhabditis elegans*, to overcome these barriers. The simplicity of the *C. elegans* nervous system allows for the assessment of all 302 neurons within the system while retaining them within a single living organism. This organism has high homology with mammalian systems, and contains many of the proteins known to be involved in MeHg toxicity, including HSPs, GSH, and MTs.

C. elegans is a free-living, soil nematode naturally occurring in temperate climates (Hope, 1999). *C. elegans* has been used as a valuable biological model ever since Sydney Brenner's Nobel Prize-winning investigations used the nematode to perform genetic screens for the purpose of unveiling mutations that alter its movement (Brenner, 1974). Brenner (Brenner, 1974) demonstrated the usefulness of *C. elegans* as a model system for genetic analysis. Since then, *C. elegans* has been extensively used, with researchers citing its small size, transparency, rapid generation time, short lifespan, simple and measurable behavior, extensive biological characterization, and genetic tractability due to the high degree of conservation of gene sequence as advantages for its use (Hope,

1999). These advantages allow *C. elegans* the unique benefit of being used as an *in vivo* system while maintaining many beneficial characteristics of an *in vitro* system.

The physical features of *C. elegans* make it a particularly attractive biological model. With adult worms being approximately 1 mm in length, a large number of worms can be grown in a very small space, most often on agar plates containing *Escherichia coli* (*E. coli*), which *C. elegans* consume as food (Brenner, 1974). *C. elegans* are relatively easy and inexpensive to maintain and their transparency allows for the observation of cells and features within the entire organism without the need to kill or dissect the organism (Hope, 1999; WormAtlas, 2002-2009).

A number of features associated with the *C. elegans* life-cycle and behavior make research using these worms quite manageable. *C. elegans* proceed through their life cycle in approximately three days and have a lifespan of about three weeks. Adults lay eggs which hatch into the first larval stage (L1). Under normal conditions (when food is present and temperature is near 20°C), the worms proceed through a series of molts, entering the second, third, and fourth larval stages (L2, L3, and L4, respectively) before becoming adults capable of laying their own eggs (Byerley *et al.*, 1976; Hope, 1999). As the worms are mostly hermaphroditic (approximately 99%), one worm is able to generate approximately 300 progeny. However, rare males are present (less than 1%), an asset to conducting genetic experiments since various strains can be crossed with one another (Brenner, 1974). *C. elegans* can be frozen at -80°C

indefinitely in small vials allowing researchers to maintain large quantities of stocks with varying genetic backgrounds for long periods of time, thawing them only when a particular worm strain is desired (Brenner, 1974; Hope, 1999).

The large body of knowledge that is available to those using *C. elegans*, such as the mapping of cell lineages (Sulston and Horvitz, 1977), makes their use straightforward, particularly from a developmental and genetic standpoint. The mapping of cell lineages allows researchers to determine potential developmental defects while the ability to manipulate genes allows for the generation of mutants, which can be analyzed using a variety of methods such as behavioral tests, reproduction analysis, and lethality studies. Additionally, many resources are available to *C. elegans* researchers, such as libraries of various strains including the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, online resources such as www.wormbase.org and www.wormbook.org (Antoshechkin and Sternberg, 2007).

Since an appropriate biological model must have substantial similarities between the organism tested and the organism of interest, *C. elegans* must contain many similarities with other organisms, namely mammals. Conservation of physiological processes and signaling pathways make *C. elegans* a good biological model for mammals (National Research Council, 2000). The genome has been extensively studied and numerous *C. elegans* genes have high homology with mammalian genes. Homologues for 60-80% of human genes have been found in *C. elegans* (Kaletta and Hengartner, 2006).

Nervous system

The *C. elegans* nervous system is well-characterized and a complete wiring diagram is available (Sulston, 1983; Sulston *et al.*, 1983). It contains only 302 neurons of 118 subtypes (Chalfie and White, 1988; Hobert, 2005), 6393 chemical synapses, 890 electrical junctions, and 1410 neuromuscular junctions (Chen *et al.*, 2006). The presence of *C. elegans* strains expressing fluorescent proteins in specific neuronal subtypes allows for specific neuronal subtypes in the live worm to be observed. Additionally, the functions of many of these neurons have been determined by laser ablation and drug exposure studies allowing behavioral studies to reveal alterations in neuronal networks (Avery and Horvitz, 1989).

Well-characterized behaviors of *C. elegans* can be experimentally assessed for changes, e.g., disruptions in regular movement including alterations in typical sinusoidal movement or alterations in swimming behavior if placed in a drop of liquid; many of these assays have been automated to allow for higher throughput analysis. *C. elegans* typically move in a sinusoidal motion on agar plates as they consume bacteria. *C. elegans*' response to various stimuli can also be assessed by observing chemotaxis (Li *et al.*, 2009), learning (Zhang *et al.*), and mating behavior (Hope, 1999; Leung *et al.*, 2008; Peterson *et al.*, 2008).

Toxicological model

Due to its advantages as a research tool, *C. elegans* makes for a practical means for studying toxic compounds. Research to determine the relevance to

mammalian systems has been conducted, showing that the results obtained from tests measuring the dose at which 50% of *C. elegans* die (LD_{50}) and tests measuring the LD_{50} of mammals are comparable, making *C. elegans* a useful early model for toxicity testing (Williams and Dusenbery, 1988). Williams and Dusenbery (Williams and Dusenbery, 1987) outlined its potential use as a screening test for neurotoxicants, including metal species, using behavioral testing. The use of lethality, reproduction, and behavioral tests for determining toxicity has been investigated, resulting in the determination that lethality is the least sensitive endpoint but that behavior and reproduction were much more sensitive, and yielded similar results. More recently, researchers have used *C. elegans* to elucidate the mechanisms of toxicity and the potential for various toxicants to induce alterations in expression of particular genes. Analysis of testing conditions (such as developmental stage, food presence, and salt content) has shown that factors such as medium ionic concentration and pH impacted the results, while other factors such as age of the *C. elegans* and presence of *E. coli* as a food source did not have a significant effect on the results when testing for survival (Donkin, 1995).

As a toxicological model, *C. elegans* has been shown to be predictive of mammalian toxicity. Many studies have been conducted investigating the toxicity of various compounds including pesticides (Cole *et al.*, 2004), mitochondrial inhibitors (Ishiguro *et al.*, 2001; Braungart *et al.*, 2004; Ved *et al.*, 2005), and metals (Roh *et al.*, 2006). These studies showed that the LD_{50} values in worms correlate with the LD_{50} values found in rats and mice, with results demonstrating

that *C. elegans* is useful as a predictive model for neurological and developmental toxicity studies in mammalian species. Although LC_{50} levels of metallic salts in *C. elegans* (for example, Hg levels at 100 mg/L in the presence of bacteria), were higher than LD_{50} levels in mammalian systems (Hg levels at 7 mg/kg in rats and mice and an average of 15 mg/kg in all mammals), the relative order of toxicity of metals and other compounds was extremely similar in worms and mammalian systems (Williams and Dusenbery, 1988).

Many neurotoxicological endpoints have been investigated using the *C. elegans* model system, including behavioral abnormalities, assessment of alterations in specific molecular pathways, genetic screening, and specific damage to the *C. elegans* nervous system. To evaluate the cytotoxic potential of MeHg, we took a comprehensive approach to examine and understand the stress response and adaptation.

Tools for studying *C. elegans*: RNAi

Researchers made headway in determining the molecular consequences of toxicant exposure using the *C. elegans* model system. The availability and ability to generate knockout worms along with the availability of various techniques such as microarrays, RNAi, and GFP-tagging have greatly aided this effort. Most recently DNA microarray has been used to investigate the genomic gene expression of *C. elegans*, and it was used successfully in investigations of genes expressed differentially during development (Jiang *et al.*, 2001), aging (Lund *et al.*, 2002), and exposure to toxicants (Reichert and Menzel, 2005).

Using this technique, Reichert et al. (Reichert and Menzel, 2005) demonstrated that exposure to different xenobiotics leads to downregulation of certain genes and induction of those that codify detoxifying enzymes. For example, they found that of the compounds they tested, fluoranthene was able to induce the most genes, including those belonging to the cytochrome P450, and GST families.

RNAi in *C. elegans* is a very useful technique and in 2006 Andrew Fire and Craig Mello received the Nobel Prize in Physiology or Medicine for their work in this area (Fire *et al.*, 1998). RNAi can be effectively used to silence particular genes of interest by injecting, feeding, or soaking the worms in the interfering double-strand RNA (dsRNA). It can also be used as a screening tool to determine which genes may be necessary for *C. elegans* to mount an appropriate response to a toxicant to avoid an undesired outcome (death, movement defects, decrease in progeny generation, etc.). RNAi has emerged as one of the most powerful tools for functionally characterizing large sets of genomic data. Only recently has the technology advanced to a state where large scale screens can be performed and RNAi libraries covering approximately 90% of the genome are publicly available (Kamath and Ahringer, 2003; Fewell and Schmitt, 2006). The use of RNAi in *C. elegans* brought important advances to the toxicity field, e.g., in the research for mechanisms of action of toxicants, in the identification of new therapeutic targets, and to elucidate mechanisms of human diseases (Wolters and MacKeigan, 2008).

Tools for studying *C. elegans*: mutagenesis

The distinct advantages of *C. elegans* allow them to be quickly grown in large quantities and mutagenized using various mutagens to conduct forward genetic screens. Following mutagenesis, *C. elegans* can be tested on a variety of parameters including, for example, resistance or hypersensitivity to toxicants. Researchers can expose mutagenized worms to levels of toxicants known to be lethal to wild type worms, and, if the mutagenized worms are able to survive, these worms can be investigated to assess the identity of the mutation and understand how it makes them more resistant to the toxicant than the wild type worms. Once a resistant or hypersensitive mutant is identified, the mutation is located using 2- and 3-point mapping and confirmed using single gene rescue or RNAi phenocopying (Hodgkin, 1999). Forward genetics is efficient for studying *C. elegans* because mutants can include genes expressed in a variety of tissues. *C. elegans* are hermaphroditic, so homozygous mutant strains can be produced in the F2 generation via self-crossing.

Tools for studying *C. elegans*: behavioral analysis

Tests that examine various behavioral endpoints and alterations in neurons and neurotransmitter systems in *C. elegans* have been developed including those that examine feeding, locomotion, memory, and movement. Using toxicants, researchers have conducted many experiments to examine behavioral outcomes following exposure. Feeding alterations decreased upon exposure to some metals (Jones and Candido, 1999; Boyd *et al.*, 2003) and have

been examined in a high-throughput manner (Boyd *et al.*, 2007). Chemotaxis and altering behavior to avoid a toxicant have been observed upon exposure to some metals (Sambongi *et al.*, 1999; Hilliard *et al.*, 2005). Learning, the ability to associate a particular temperature with food and return to that temperature under starvation conditions, was also affected by toxicant exposure (Ye *et al.*, 2008). Many researchers have examined the ability of *C. elegans* to move properly following toxicant exposure, often using computer tracking systems to enable the high throughput assessment of many worms. Since the nervous system in *C. elegans* has been so well characterized, alterations in specific behaviors can be attributed to particular circuits and can lead to further investigation of those circuits. The locomotor neuronal network in *C. elegans* is formed by the A- and B-type motor neurons and the inhibitory D-type motor neurons that receive their input from the interneurons AVA, AVB, AVD, and PVC (Riddle, 1997). Tracking systems that examine alterations in movement can indicate alterations in these neurons or circuitry.

Tools for studying *C. elegans*: neuroanatomy

As previously noted, *C. elegans* has a very well characterized nervous system, allowing for the analysis of cell number and location as well as connectivity. Due to the availability of strains (from sources such as the CGC) expressing markers such as green fluorescent protein (GFP) in specific neuronal subsets, researchers can directly examine the appearance of the nervous system following toxicant insult to assess endpoints including alterations in location of

neurons, alterations in outgrowths, and degeneration. Although alterations in function are not assessed, the ability to view the nervous system in a live animal is extremely useful and predicted to be of high value to toxicologists studying agents thought to induce degeneration or alterations in nervous system architecture or wiring.

Metal toxicity testing in *C. elegans*

C. elegans has been used as a model system to elucidate the toxicity and toxicological mechanisms of various heavy metals, such as aluminum (Al), arsenic (As), barium (Ba), cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), uranium (U), and zinc (Zn) (Williams and Dusenbery, 1988). In general, these studies focused on various toxic endpoints, such as lethality, reproduction, lifespan, and protein expression. Some focus has also been directed to the effects of these metals on the nervous system by assessing behavior, reporter expression, and neuronal morphology (Dhawan *et al.*, 1999).

For instance, a defect in locomotion reflects an impairment of the neuronal network formed by the interneurons AVA, AVB, AVD, and PVC providing input to the A- and B-type motor neurons (responsible for forward and backward movement), and the inhibitory D-type motor neurons involved in the coordination of movement (Riddle, 1997). By recording short videos and subsequently analyzing them using computer tracking software, it has been possible to quantify the overall movement of *C. elegans* (distance traveled, directional change, etc.), body bends, and head thrashes upon metal treatments, allowing to further

correlate the data with damage to neuron circuitry. These computer-tracking studies showed that worms displayed a dose-dependent decrease in locomotory movement upon exposure to Pb (Johnson and Nelson, 1991; Anderson *et al.*, 2001; Anderson *et al.*, 2004) and Al (Anderson *et al.*, 2004), while an increase in locomotion was observed upon exposure to low concentrations of Hg as compared with Cu (Williams and Dusenbery, 1988). Another study showed that exposure to Ba impaired both body bend and head thrashing rates in a dose-dependent manner (Wang and Wang, 2008), corroborating mammalian data on the effect of Ba on the nervous system attributed to its ability to block potassium channels (Johnson and Nelson, 1991).

Feeding behavior has also been shown to be affected upon heavy metal exposure. Feeding requires a different neuronal circuitry including M3 (involved in pharyngeal relaxation), MC (control of pumping rate), M4 (control of isthmus peristalsis), NSM (stimulate feeding), RIP (ring/pharynx interneuron), and I (pharyngeal interneurons) neurons (Riddle, 1997). A decrease in feeding was observed when worms were exposed to Cd or Hg (Jones and Candido, 1999; Boyd *et al.*, 2003).

Behavioral research studying the effect of heavy metals on *C. elegans* has also taken the route of assessing the ability of the worm to sense the toxicant and alter its behavior accordingly, involving other neural circuitry, such as the amphid and phasmid neurons responsible for chemosensation (Riddle, 1997). By generating concentration-gradient containing plates, Sambongi et al. (Sambongi *et al.*, 1999) discovered that *C. elegans* was able to avoid Cd and Cu but not Ni,

and that the amphid ADL, ASE, and ASH neurons were responsible for this avoidance as their combined ablation eliminated the avoidance phenotype. Furthering the investigation into the role of ASH neurons, researchers found that a Ca^{2+} influx could be elicited upon exposing the *C. elegans* to Cu, which may provide insight into the mechanism of the ability of the worm to display avoidance behaviors (Hilliard *et al.*, 2005).

C. elegans exhibits both short-term and long-term learning-related behaviors in response to specific sensory inputs (Rankin *et al.*, 1990), which involve defined neuronal networks. As an example, thermosensation-associated learning and memory rely on the AFD sensory neuron sending inputs to the AIY and AIZ interneurons, whose signals are integrated by the RIA and RIB interneurons to command the RIM motorneuron (Mori *et al.*, 2007). When assessing the function of this circuitry, worms grown and fed at a defined temperature are moved to a food-deprived test plate exposed to a temperature gradient. The ability of the worms to find and remain in the area of the test plate corresponding to the feeding temperature reflects the functioning of the thermosensation learning and aforementioned memory network (Mori *et al.*, 2007). Interestingly, worms exposed to Al and Pb exhibit poor scores at this test, indicative of a significant reduction of the worms' learning ability (Ye *et al.*, 2008). This recapitulates the learning deficits observed in young patients overexposed to the same metals (Garza *et al.*, 2006; Goncalves and Silva, 2007).

While behavioral testing was indicative of the neuronal circuitries affected by heavy metals, additional experiments uncovered the molecular mechanisms

of their neurotoxic effects. For example, in the previously described study, after determining that Al and Pb induced memory deficits, the investigators showed that the antioxidant vitamin E effectively reversed these deficits, indicating that oxidative stress plays a role in Al and Pb neurotoxicity (Ye *et al.*, 2008). The involvement of oxidative stress in metal-induced toxicity was further confirmed when worms mutated in glutamylcysteine synthetase *(gcs-1)*, the rate-limiting enzyme in glutathione synthesis, exhibited hypersensitivity to As exposure when compared to wildtype animals (Liao and Yu, 2005). Studies conducted in mammalian models found that Hg is able to block Ca^{2+} channels. In neurons, this blockage can induce spontaneous release of neurotransmitters (Atchison, 2005). In *C. elegans*, the Ca^{2+} channel blocker was found to protect against Hg exposure, suggesting that Ca^{2+} signaling plays a role in the toxicity of Hg in this model organism as in mammals (Koselke *et al.*, 2007).

Observation of neuron morphology following heavy metal exposure was also performed using *C. elegans* strains expressing GFP in discrete neuronal populations. Tests using depleted U evoked no alterations in the dopaminergic nervous system of *C. elegans*, an observation corroborated with data from mammalian primary neuronal cultures (Jiang *et al.*, 2007). Meanwhile, *kel-8* (a Kelch-like protein involved in degradation of glutamate receptors (Schaefer and Rongo, 2006)) and *numr-1* (a nuclear-localized metal responsive element (Tvermoes and Freedman, 2008)), which are involved in resistance to Cd toxicity, were upregulated upon Cd exposure. In particular, GFP levels of KEL-8::GFP and NUMR-1::GFP were increased in the pharynx and the intestine in addition to

the constitutive expression observed in AWA neurons, indicating that these proteins may be involved in toxicity or protection (Freedman *et al.*, 2006; Jackson *et al.*, 2006; Cui *et al.*, 2007). Furthermore, *numr-1* was shown to be induced in response to heavy metals, such as Cd, Cu, Cobalt (Co), Chromium (Cr), Ni, As, Zn, and Hg, further indicating its involvement in the response to these metals. NUMR-1::GFP was localized to nuclei within the intestine and the pharynx and co-localized with the stress-responsive heat-shock transcription factor HSF-1::mCherry (Tvermoes and Freedman, 2008). This indicates that these particular genes were altered in response to heavy metals and demonstrates the utility of using GFP reporters to guage the involvement of various proteins in responses to toxicants. This type of research may aid in the understanding of the toxicity of or the protection against these and other agents.

Previous research has been conducted exposing *C. elegans* to Hg and measuring a variety of endpoints, including lethality (Williams, 1990; Donkin, 1995), induction of transgenes (Cioci, 2000), and movement (Williams and Dusenbery, 1987). However, this previous research has largely dealt with Hg in the form of $HgCl_2$, not organic forms of the metal. Lethality testing from other labs has shown that inorganic Hg is able to kill the nematode in a dose-dependent manner (Williams and Dusenbery, 1988; Williams, 1990; Donkin, 1995), and initial studies in our lab on organic forms of Hg, including MeHg and EtHg, have demonstrated the lethal effect of these compounds on *C. elegans*. However, to our knowledge, no other research has been conducted using *C. elegans* to determine the toxicity of MeHg. Due to its strength as a model system for the

dissection of genetic contributors to toxicity, use of *C. elegans* for these tests will permit elucidation of the potential mechanisms of the profound neurotoxicity observed upon developmental exposure to MeHg.

Proteins linked to MeHg toxicity in mammals are conserved in *C. elegans*

C. elegans displays high homology to mammalian systems, and contains many of the genes known to be involved in MeHg toxicity, including GSH, HSPs, and MTs and undergoes processes involved in toxicity such as hormesis. Additionally, *C. elegans* contains a homolog of Nrf2, *skn-1*. Previous work in *C. elegans* has shown *gst-4* upregulation in response to a variety of stressors, including paraquat (Tawe *et al.*, 1998), juglone (Kampkotter *et al.*, 2007; Kahn *et al.*, 2008), hyperbaric oxygen (Link and Johnson, 2002), progesterone (Custodia *et al.*, 2001), diethylstilbetrol (Reichert and Menzel, 2005), and acrylamide (Tawe *et al.*, 1998; Hasegawa *et al.*, 2008); *hsp-4* (a member of the HSP70 family) in response to heat, tunicamycin (Calfon *et al.*, 2002), and irradiation (Bertucci *et al.*, 2009); and *mtl-1* and *mtl-2* in response to cadmium, uranium and heat (Freedman *et al.*, 1993; Swain *et al.*, 2004; Jiang *et al.*, 2007). Hormesis has been previously observed in *C. elegans*. Following dietary restriction or exposure to sublethal heat stress, animals display an increase in lifespan and a resistance to exposure to a subsequent stressor (Cypser *et al.*, 2006). *C. elegans* express nearly 50 GSTs (van Rossum *et al.*, 2001), approximately 10 HSPs of the HSP70 family (Heschl and Baillie, 1989), and 2 MTs (Freedman *et al.*, 1993). We chose to examine *gst-4*, *hsp-4*, and both *mtl-1* and *mtl-2*. *gst-4* is highly homologous

with human glutathione-requiring prostaglandin D synthase (Wormbase). *hsp-4* has high homology with human heat shock 70 kDa protein 5 (HSPA5), a 78 kDa glucose-regulated protein precursor (GRP78) (Wormbase). In *C. elegans*, this protein has been shown to be induced upon accumulation of unfolded proteins (Shen *et al.*, 2001) and in hormesis induced by heat stress (Olsen *et al.*, 2006). Although the *C. elegans* MTs have marked differences from the mammalian MT genes, such as differences in their organization and in their coding and flanking sequences, they have many important shared elements, such as the high Cys content in the form of a Cys-X-Cys motif (Freedman *et al.*, 1993). *mtl-1* is most highly homologous with human MT-3 while *mtl-2* is most highly homologous with human keratin-associated protein 5-9 (Wormbase).

The proposed research program: use of *C. elegans* to assess MeHg cytotoxicity

Our initial hypothesis was that MeHg would induce structural changes in the *C. elegans* nervous system, and that we could glean information about the molecular mechanisms of these alterations by using the genetic tractability of this platform. The goal of our experiments was to determine the mechanisms of MeHg toxicity, specifically in the nervous system. Since the toxicity of MeHg had never before been tested on *C. elegans*, our first aim was to elucidate the overall effects of the toxicant on this model organism. Our initial experiments included the measurement of many endpoints including lethality, Hg accumulation, lifespan, brood size, growth, and behavior. MeHg did induce lethality and a

developmental delay, likely due to a decrease in pharyngeal pumping rate. However, in animals surviving the initial MeHg insult, at Hg concentrations equivalent to those found to cause neuronal abnormalities in mammalian systems (Falluel-Morel *et al.*, 2007; Helmcke *et al.*, 2009), it failed to induce alterations in lifespan (more than 24 hours after treatment), brood size, or thrashing rate. These results gave us a general understanding of the effect of MeHg on the organism as a whole. The lack of an obvious movement phenotype and the lack of MeHg-dependent alterations in lifespan and brood size were surprising given the known literature on the effects of other toxicants on *C. elegans* and the effects of MeHg in mammalian systems.

Due to the known effects of MeHg on the nervous system and the extensive characterization of the *C. elegans* nervous system, our second aim addressed the ability of MeHg to induce morphological alterations in the neurons of *C. elegans*, such as the ability of cells to reach the proper location and form proper outgrowths. Since data in mammalian systems have revealed gross morphological changes to the structure of the nervous system following exposure to MeHg, we hypothesized that the nervous system of *C. elegans* would also exhibit morphological alterations following exposure to the toxicant. Neither qualitative observations of a number of neuronal subtypes including dopaminergic, GABAergic, serotonergic, glutamatergic, and cholinergic nor in-depth quantitative analysis of dopaminergic and GABAergic neurons elucidated morphological changes in these neurons. These studies addressed the structure of the nervous system, but did not assess alterations in the functioning of the

neurons. The lack of changes in the structure of the nervous system was surprising; however, it is corroborated with the lack of alterations in behavioral assays. Since MeHg is a neurotoxicant in mammals which induces morphological changes, our results indicate that *C. elegans* may express unique mechanisms to cope with the MeHg insult, affording it increased resistance.

We had expected to observe alterations in the nervous system that might display as obvious movement phenotypes or morphological changes. The third and final aim of this work examined mechanisms of MeHg resistance to determine molecules and pathways involved in *C. elegans* resistance to MeHg. In these experiments, we assayed the involvement of GSH, HSPs, and MTs in MeHg toxicity while also examining the ability of MeHg to induce hormesis in *C. elegans.* Since each of these systems had shown involvement in both MeHg toxicity and preconditioning in other models, we expected to see alterations in their expression level and alterations upon exposing molecular mutants to the toxicant. Based on prior literature reports, we expected that MeHg would cause the generation of ROS and direct protein damage by binding to Cys groups on various proteins. We predicted that these effects would trigger a cascade of events that would result in a number of alterations including upregulation of HSPs, MTs, and GSTs, while depleting GSH through the generation of GSSG and the expulsion of the GS-MeHg complex (Figure 1A). We also hypothesized that if we used animals lacking any of those components, the animal would be more sensitive to MeHg than its wild-type counterpart. Additionally, we theorized that MeHg would be able to induce a hormetic effect in *C. elegans* and that the

proteins involved in MeHg detoxification noted above were involved in this response.

To test our hypotheses, we used GFP reporter strains to quantify changes in *gst-4*, *hsp-4*, and *mtl-1* in response to MeHg exposure. We further examined the role of GSH by measuring GSH and GSSG, which varied based on exposure duration and stage at exposure.

MeHg caused a hormetic response in *C. elegans*, suggesting that MeHg could evoke a protective pathway in this organism. Although our data indicate that *gst-4* induction plays a robust role in the hormetic response, other preconditioning-associated proteins including *hsp-4*, *mtl-1*, and *mtl-2* were unchanged.

Taken together, our results indicate that MeHg does not induce overt morphological alterations in the *C. elegans* nervous system, yet the nematode is a valuable model organism for neurotoxicological research. The continuation of this line of experimentation can provide insights into the mechanisms by which *C. elegans* is able to protect itself from MeHg insult, for example, by metabolism of the metal or efficient repair mechanisms. These results could provide investigators with tools necessary to enable them to prevent or minimize MeHg toxicity in humans.

CHAPTER II

CHARACTERIZATION OF THE EFFECTS OF MEHG ON *C. ELEGANS*

Summary

The rising prevalence of methylmercury (MeHg) in seafood and in the global environment provides an impetus for delineating the mechanism of the toxicity of MeHg. Deleterious effects of MeHg have been widely observed in humans and in other mammals, the most striking of which occur in the nervous system. Here we test the model organism, *Caenorhabditis elegans (C. elegans),* for MeHg toxicity. The simple, well-defined anatomy of the *C. elegans* nervous system and its ready visualization with green fluorescent protein (GFP) markers facilitated our study of the effects of methylmercuric chloride (MeHgCl) on neural development. Although MeHgCl was lethal to *C. elegans,* induced a developmental delay, and decreased pharyngeal pumping, other traits including lifespan, brood size, swimming rate, and nervous system morphology were not obviously perturbed in animals that survived MeHgCl exposure. Despite the limited effects of MeHgCl on *C. elegans* development and behavior, intracellular mercury (Hg) concentrations ($\leq$ 3 ng Hg/mg protein) in MeHgCl-treated nematodes approached levels that are highly toxic to mammals. If MeHgCl reaches these concentrations throughout the animal, this finding indicates that *C. elegans* cells, particularly neurons, may be less sensitive to MeHgCl toxicity than mammalian cells. We propose, therefore, that *C. elegans* should be a useful

model for discovering intrinsic mechanisms that confer resistance to MeHgCl exposure.

Introduction

For a thorough review of Hg toxicology, please see dissertation introduction. MeHg is of particular concern due to its ability to pass through the blood-brain and placental barriers where it molecularly mimics methionine and enters cells via the large amino acid transporter, LAT1 (Kerper *et al.*, 1992; Simmons-Willis *et al.*, 2002; Yin *et al.*, 2008) allowing MeHg to accumulate in both the brain and the fetus. MeHg has varying effects on the nervous system based on age at exposure. Although MeHg possesses high affinity for cysteine, allowing it to bind thiol groups, and can accumulate within astrocytes, the specific molecular targets of MeHg are largely unknown (Aschner *et al.*, 1990; Kerper *et al.*, 1992; Simmons-Willis *et al.*, 2002).

Despite many years of investigation, numerous questions surround the mechanisms of MeHg toxicity in mammals. Investigators have taken various approaches to study MeHg toxicity using many model systems including rat, mouse, zebrafish, and cell culture. However, these systems are limited by their complexity or removal from an intact organism. To address this, we have adopted an alternative approach of using the model organism, *Caenorhabditis elegans (C. elegans)*, to study MeHg toxicity.

C. elegans has been used extensively in biological research and provides many advantages, including its small size, rapid life cycle, self-fertilization, and

ready genetic manipulation; the *C. elegans* nervous system has been mapped, and its genome fully sequenced (Sulston and Horvitz, 1977; Sulston, 1983; White *et al.*, 1986; Wood, 1988; *C. elegans* sequencing consortium, 1998). Earlier studies of toxicity in *C. elegans* have revealed high predictive value for mammalian systems (Williams and Dusenbery, 1988; National Research Council, 2000; Cole *et al.*, 2004; Leung *et al.*, 2008). In addition to measurements investigating effects on the overall health of *C. elegans* (lethality, life span, brood size, behavior, etc.), some assessments included determination of gene induction using reporter strains and protection afforded by a particular gene through the use of knockout, over-expression strains, RNAi, or mutagenesis experiments (Leung *et al.*, 2008).

We used *C. elegans* to study MeHg toxicity and tested several different endpoints including lethality, Hg accumulation, lifespan, brood size, body length, overall development, swimming behavior, and pharyngeal pumping rate. We also used green fluorescent protein (GFP) markers for specific neuronal populations to assess the development and appearance of the nervous system following methylmercuric chloride (MeHgCl) insult.

Our studies revealed that Hg approached levels (≤3 ng Hg/mg protein) in *C. elegans* tissues that are highly toxic to mammals (for example, in rat brain, 0.05 ppm resulted in significant structural alterations (Falluel-Morel *et al.*, 2007)). Although exposure to MeHgCl induced dose-dependent developmental delay and lethality, surviving animals were surprisingly unaffected. The absence of observable defects in development or morphology in the *C. elegans* nervous

system is particularly noteworthy given the sensitivity of mammalian neurons to MeHg. Our results indicate that *C. elegans* may exhibit unique mechanisms for detoxifying, trafficking, or metabolizing MeHgCl that render its nervous system resistant or inaccessible to MeHg.

Methods

C. elegans Maintenance

C. elegans were grown on plates containing nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 as previously described (Brenner, 1974). Unless otherwise noted, hermaphroditic wildtype N2 Bristol strain was used for all experiments. Transgenic lines expressing promoter GFP reporters used in this study were: NW1229 *F25B3.3::GFP* (a marker of Ras1 guanine nucleotide exchange factor, pan-neuronal GFP expression) (Altun-Gultekin *et al.*, 2001), LX929 *unc-17::GFP* (a marker of a synaptic vesicle acetylcholine transporter, labels cholinergic neurons) (Chase *et al.*, 2004), CZ1200 *unc-25::GFP* (a marker of glutamic acid decarboxylase, labels GABAergic neurons) (Huang *et al.*, 2002), EG1285 *unc-47::GFP* (a marker of a transmembrane vesicular GABA transporter, labels GABAergic neurons) (McIntire *et al.*, 1997), TL8 *cat-1::GFP* (a marker of a synaptic vesicular monoamine transporter, labels catecholaminergic neurons) (Colavita and Tessier-Lavigne, 2003), GR1333 *tph-1::GFP* (a marker of tryptophan hydroxylase, labels serotonergic neurons) (Sze *et al.*, 2000), DA1240 *eat-4::GFP* (a marker of vesicular glutamate transporter, labels glutamatergic neurons)

(Asikainen *et al.*, 2005) (all obtained from the *Caenorhabditis* Genetics Center, Minneapolis, MN) BY250 *dat-1::GFP* (a marker of the dopamine transporter, labels dopaminergic neurons) (Nass *et al.*, 2001), and *F49H12.4::GFP* (labels PVD neurons) (Watson *et al.*, 2008).

MeHgCl Treatments

Animals were treated with an alkaline bleach solution to obtain a synchronous population prior to treatment with MeHgCl (Stiernagle, 1999) and synchronized populations of selected larval stages (either L1 or L4) were treated. Treatment was conducted by combining larvae (2500 L1s or 300 L4s), concentrated OP50, the appropriate volume of MeHgCl dissolved in water, and M9 buffer to a volume of 500µL in 1.7 mL siliconized tubes. Following the desired treatment duration (30 minutes to 15 hours), animals were washed twice with deionized water by centrifugation and placed on OP50-containing NGM plates.

Lethality

Following MeHgCl treatment and washing, animals were transferred (approximately 300 per plate) to 60 mm NGM plates seeded with OP50 and allowed to grow for 24 hours. Animals were then counted and scored as dead or alive. Viability was scored based on appearance and ability to move in response to poking with a platinum wire (Bischof *et al.*, 2006; Roh *et al.*, 2007).

Determination of Hg Content

C. elegans larvae were treated with MeHgCl as described above. After 24 hours of culture on OP50-containing NGM plates, both live and dead animals were collected and washed twice with deionized water. For L1 treatments, approximately 10,000 animals were pooled and assessed, for L4 treatments, approximately 900 animals were pooled and assessed. As expected, protein content was higher in samples treated with lower concentrations of MeHgCl. Average protein content per sample was approximately 110 mg, ranging from 16 mg to 254 mg. The pelleted pool of live and dead worms was sonicated and a small aliquot was used for protein measurement; the remainder of the sample was used for inductively coupled plasma-mass spectrometry (ICP-MS) analysis of Hg content. Although it is possible that some demethylation occurred during the study, it is unlikely that an appreciable amount of inorganic Hg was formed. This would be an interesting extension of this research, however due to small sample size, information regarding the potential demethylmation of MeHg could not be collected in this study. Protein content was determined following manufacturer instructions for a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford IL). Preparation of the sample for ICP-MS involved addition of nitric acid followed by heat digestion and dilution of the samples with water. The samples were digested in PP tubes (352059, BD) in a block heater after addition of 65% HNO_3 (Merck, Suprapur). The samples were transferred to Teflon tubes and digested in an UltraClave (Milestone). After digestion the samples were diluted directly in the Teflon tubes with ultrapure water (PURLAB Ultra Analytic,

Elga) to achieve a final acid concentration of 0.6 mol/L. High Resolution-Inductively Coupled Plasma-Mass Spectrometry (HR-ICP-MS) analysis was performed using a Thermo (Finnigan) model Element 2 instrument (Bremen, Germany). The RF power was 1400 W. The sample was introduced using an SC-2 with SC-FAST option auto sampler (ESI, NE, USA) with a peristaltic pump (pump speed 0.25 mL/min). The instrument was calibrated using 0.6 mol/L HNO_3 solutions of multielement standards at appropriate concentrations. Internal standards were not used. To check for possible drift in the instrument, a standard solution with known elemental concentrations was analyzed for every 10 samples. In addition, blank samples (0.6 mol/L HNO_3, Suprapur) were analyzed for approximately every 10 samples. The samples were analyzed in random order, and the analyst was not aware of the identity of the samples. Hg was determined in the low resolution mode ($M/\Delta m=300$).

Lifespan and Brood Size Analysis

For lifespan assays, 40 live *C. elegans* hermaprodites from each MeHgCl concentration group were picked to a fresh NGM plate 24 hours following treatment. On each succeeding day, worms were counted and scored as live or dead. Live *C. elegans* were picked to fresh plates every day during egg-laying and every other day once they ceased laying eggs until no live *C. elegans* remained. The experiment was carried out in quadruplicate.

For brood size analyses, one live *C. elegans* was placed on each of four NGM plates per treatment concentration 24 hours after MeHgCl exposure. Every

24 hours, this animal was transferred to a new NGM plate until no new progeny were generated in a 24-hour period. The progeny on each of the fresh plates were counted and the experiment was carried out in quadruplicate. This approach allowed the measurement of the overall number of progeny generated and the interval between MeHgCl exposure at different concentrations and progeny generation.

Measurement of Size and Developmental Progress

Following treatment and washing, *C. elegans* were imaged on a Nikon Eclipse 80i microscope. Body length was measured using Nikon Element software to trace the body contour from the posterior bulb of the pharynx to the anus. Twenty worms per treatment were also assessed for their development through the larval stages using the following criteria: L1s had 4 or fewer gonadal cells, L2s had more than 4 gonadal cells and the gonad had begun to extend along the length of the animal, L3 worms had undergone further extension of the gonad and vulval morphogenesis had begun to occur, L4s displayed dorsal rotation of the gonad, and adults had observable eggs.

Behavioral Analysis: Pharyngeal Pumping and Thrashing Rates

Pharyngeal pumping rate was assessed using a Leica MZ16FA microscope following MeHgCl treatment and washing. Pumps per minute were manually counted following treatment with MeHgCl. To test thrashing rates, *C. elegans* were placed in 10uL of water on a Pyrex Spot Plate and their behavior

was videotaped through a microscope for three minutes, as previously described (Matthies *et al.*, 2006). Briefly, AVI movies were generated using a frame grabber Piccolo graphics card (Ingenieur Helfrich) and VidCap32 AVI capture application (Microsoft, Redmond, CA). The movies were analyzed using a script written in MatLab 7.0.1 (MathWorks, Natick, MA) to determine the position of the worm in each frame using motion detection and selection of a pixel designating the centroid of the worm (available upon request). Worm oscillation over time was displayed following calculation of movement in Hz. Four worms per treatment were tested in each behavioral analysis.

Microscopic Observation of Neurons

GFP-reporter strains were treated with MeHg as described above (30-minute treatment of L1 and 15-hour treatment of L4 animals followed by washing and culture on OP50-containing NGM plates). *C. elegans* treated at the L1 or L4 stage and the progeny of those worms treated at the L4 stage were observed using a Nikon Eclipse 80i microscope. Quantitative analysis of *dat-1::GFP* worms involved counting the number of head neurons (4 CEPs and 2 ADEs), projections from CEP neurons to the tip of the nose, and neurons in the *C. elegans* body (2 PDEs). Quantitative analysis of *unc-25::GFP* worms involved counting the number of head neurons (4 RMEs), the number of neurons along the ventral nerve cord (13 VDs and 6 DDs), the number of commissures traveling across the body, and whether there were any breaks in the commissures or the nerve cord. Other GFP strains (*F25B3.3::GFP, unc-17::GFP, unc-47::GFP, cat-1::GFP, tph-*

1::GFP, eat-4::GFP, F49H12.4::GFP) were examined to assess for obvious changes in overall structures of the labeled neurons.

Statistics

GraphPad Prism 4 was used to assess significance, for dose response, Hg content, brood size, pharyngeal pumping rate, thrashing rate, body length, and neuronal quantification, ANOVA with Bonferroni's Multiple Comparison Test was applied, for lifespan, log rank test was applied. When p-values were lower than 0.05, groups were considered significantly different, higher than 0.05 were not considered significantly different.

Results

C. elegans larvae are sensitive to MeHgCl

Dose-response curves were generated to test for dose-dependent toxicity of MeHg to *C. elegans*. L1 and L4 larval stages were selected to coincide with developmental processes in the worm (L1) and of the germ line of the worm (L4). Worms treated for 30 minutes with MeHg at the L1 stage [LC_{50}=1.08 mM, n=10 (throughout document, each 'n' is one separate experiment, usually conducted at least in triplicate)] were significantly ($p<0.001$) more sensitive to MeHg compared with worms treated at the L4 stage (LC_{50}=4.51 mM, n=6) (Figure 2A). Additionally, increasing the duration of MeHg exposure in L4 worms from 30 minutes to 6 hours (LC_{50}=0.57 mM, n=6) and 15 hours (LC_{50}=0.33 mM, n=9)

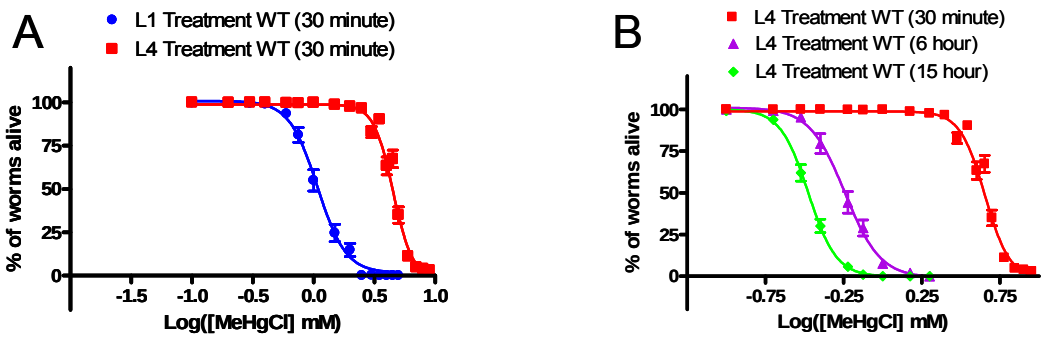

Figure 2. Dose–response curve of lethality of MeHgCl to *C. elegans*. Worms treated at L1 (LC_{50}=1.08±0.02, n=10 p<0.001) were more sensitive to the toxicant than worms treated at the L4 stage (LC_{50}=4.51±0.01, n=6) (A). Toxicity increased as exposure duration increased, L4 worms were treated for 15 h (LC_{50}=0.33±0.01, n=9), for 6 h (LC_{50}=0.57±0.01, n=6), and for 30 min (LC_{50}=4.51±0.01, n=6) (B).

statistically significantly ($p<0.001$) increased the toxicity to *C. elegans*, indicating that longer exposures are more lethal to *C. elegans* (Figure 2B).

Hg accumulates in a dose-dependent manner in animals treated with MeHgCl

Hg content was measured for selected exposures for different treatments, including L1 treatment for 30 minutes and L4 treatment for 30 minutes, 6 hours, and 15 hours (n=3 for each treatment). Exposures tested were selected to represent a range of doses that corresponded to a low concentration (LC_0), at least one medium concentration (LC_{20}-LC_{80}), and at least one high concentration (LC_{100}) for each of the conditions tested. Hg content was not tested when dose-response curves indicated death of all worms. The resulting values indicate that there is an increase in Hg content with increased MeHgCl exposure (Figure 3). Comparing the animals treated for 30 minutes and for 15 hours at L4, Hg content was significantly higher following a treatment at 0.1 and 0.4 mM MeHgCl ($p<0.05$) with the longer exposure. As the MeHgCl concentration to which the worms were exposed increased, Hg content also increased. After treatment of L1 animals for 30 minutes, worms treated at 1 mM MeHgCl contained significantly more Hg than control-treated worms (*, $p<0.05$). After treatment of L4 worms for 30 minutes, worms treated at all MeHgCl concentrations had significantly higher Hg content than control-treated worms (*, $p<0.05$). Additionally, animals treated with 1 and 10 mM MeHgCl contained significantly more Hg than those treated with 0.1 or 0.4 mM MeHg (*, $p<0.05$). Following a 6-hour treatment, L4 worms

treated with 0.4 mM MeHg contained significantly more Hg than the control-treated worms (*, $p<0.05$). Following 15-hour treatment at L4 stage, control worms (0 mM MeHgCl) contain an average of 0.02 ng Hg/mg protein, whereas those treated at 0.1 and 0.4 mM MeHgCl contain an average of 0.45 and 3.34 ng Hg/mg protein, respectively ($p<0.001$ vs. controls). As duration of exposure increases, Hg accumulation in *C. elegans* significantly increased in a time-dependent manner (Figure 3). For instance, when L4s were treated for 30 minutes at 0.4 mM MeHgCl, the average Hg content was 0.29 ng Hg/mg protein; when the duration of exposure increased to 6 hours and 15 hours, average Hg content increased to 0.81 and 3.34 ng Hg/mg protein, respectively ($p<0.01$). A comparison of the Hg content of L1s and L4s treated for 30 minutes revealed that L1s had significantly lower levels of Hg ($p<0.01$). This finding indicates that L1s may be more sensitive to Hg than the dose-response curves (Figure 2) revealed, as they are killed at lower levels of internal Hg than are L4 animals with comparable Hg content.

MeHgCl does not alter lifespan or brood size of *C. elegans*

For animals that survive exposure to MeHgCl, longevity did not seem to correlate with exposure dose (Figure 4). For example, average lifespan following a 30-minute treatment of L1 *C. elegans* (Figure 4A) or a 15-hour treatment of L4 *C. elegans* was 13-15 days (Figure 4B). Additionally, we tested the lifespan of the progeny of L4 *C. elegans* treated for 15 hours, which had an average lifespan of 15-17 days (Figure 4C). None were significantly altered when using the log

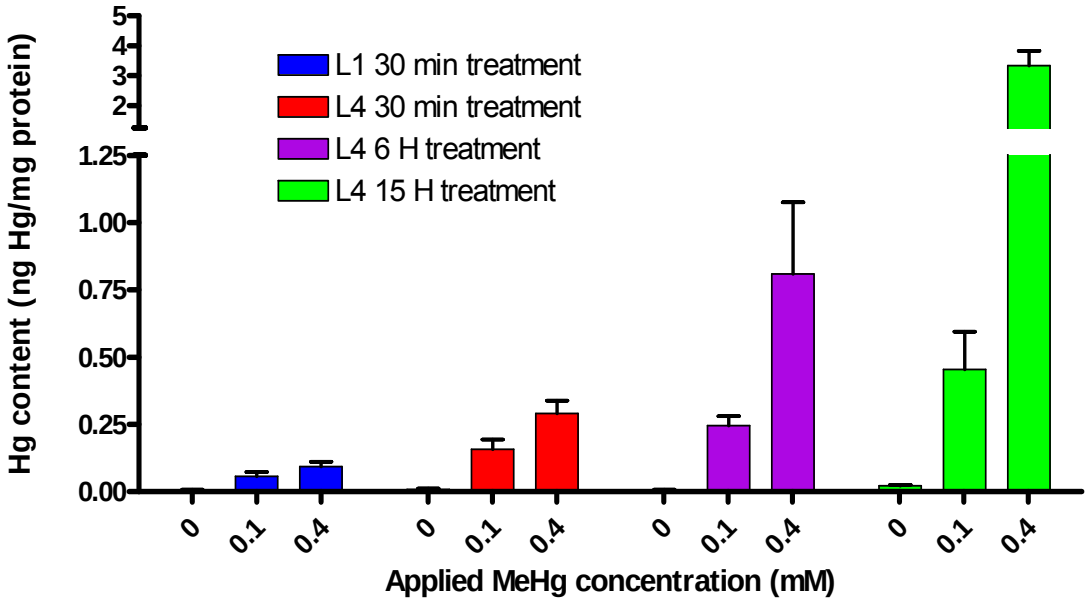

Figure 3. Hg content in *C. elegans* following MeHgCl exposure. Hg content was measured as a function of sample protein content (n=3). Hg content significantly increased as the duration of exposure to MeHgCl increased and as the MeHgCl treatment concentration increased.

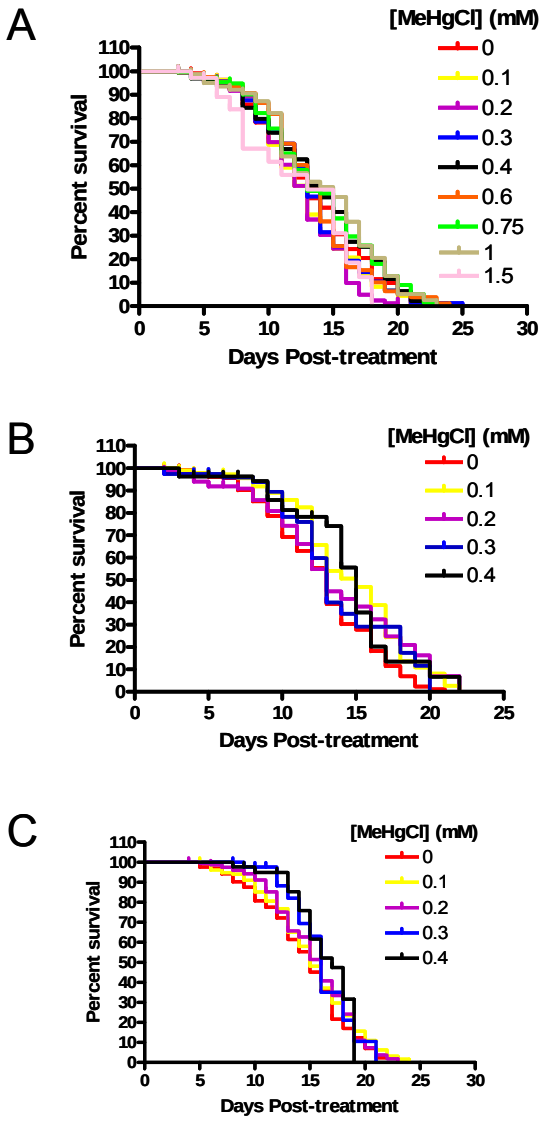

Figure 4. Lifespan is unaltered following MeHgCl in *C. elegans*. Animals treated for 30 min at L1 stage (A) and 15 h at L4 stage (B) had an average lifespan of 13–15 days following treatment while progeny of animals treated for 15 h at L4 stage (C) had an average lifespan of 15–17 days (n=5).

rank test to compare the control and MeHg-treatment groups (n=5). In measuring brood size, the same three populations (L1 30-minute treatment, L4 15-hour treatment, and progeny of L4 treatment) were tested (Figure 5). Animals that underwent 15-hour L4 treatment had an overall decrease in brood size (progeny generation of L1 30-minute treated worms at 0 mM MeHgCl was 279±14, of L4 15-hour treated worms was 221±11, and of progeny of L4 15-hour treated worms was 243±13). However, the only significant MeHg-dependent alteration in brood size occurred when L1 30-minute treated worms were exposed to 1 mM MeHgCl (187±21 progeny generated compared to 279±14 progeny generated under control conditions, $p<0.001$). There were no other statistically significant alterations in brood size (n=6).

MeHgCl treatment retards *C. elegans* larval development

Following treatment with MeHgCl, *C. elegans* length was altered in a dose-dependent manner, with higher MeHgCl doses correlating with shorter length (Figure 6). This observation prompted an investigation into a potential developmental delay of *C. elegans* following MeHgCl treatment. This study detected a corresponding dose-dependent developmental delay (Figure 7, Table 1, n=5 experiments). Under normal conditions at 20°C, *C. elegans* embryogenesis takes 14 hours, and then the worm undergoes a series of molts at 29, 38, 47, and 59 hours post fertilization (Hope, 1999). Retarded development occurred in both the worms treated at the L1 stage for 30 minutes and those treated at L4 for 15 hours. After growth for 24 hours, control-treated L1 larvae

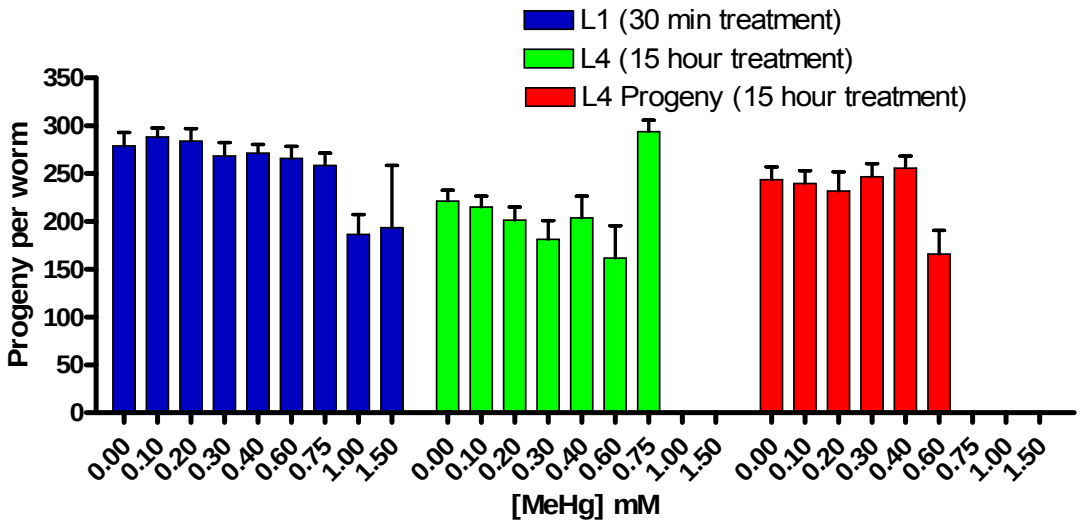

Figure 5. *C. elegans* brood size is unaltered following MeHgCl exposure. Animals treated for 30 min at L1 stage, 15 h at L4 stage, and progeny of those treated for 15 h at L4 stage demonstrated no dose-dependent alteration in brood size (n=6).

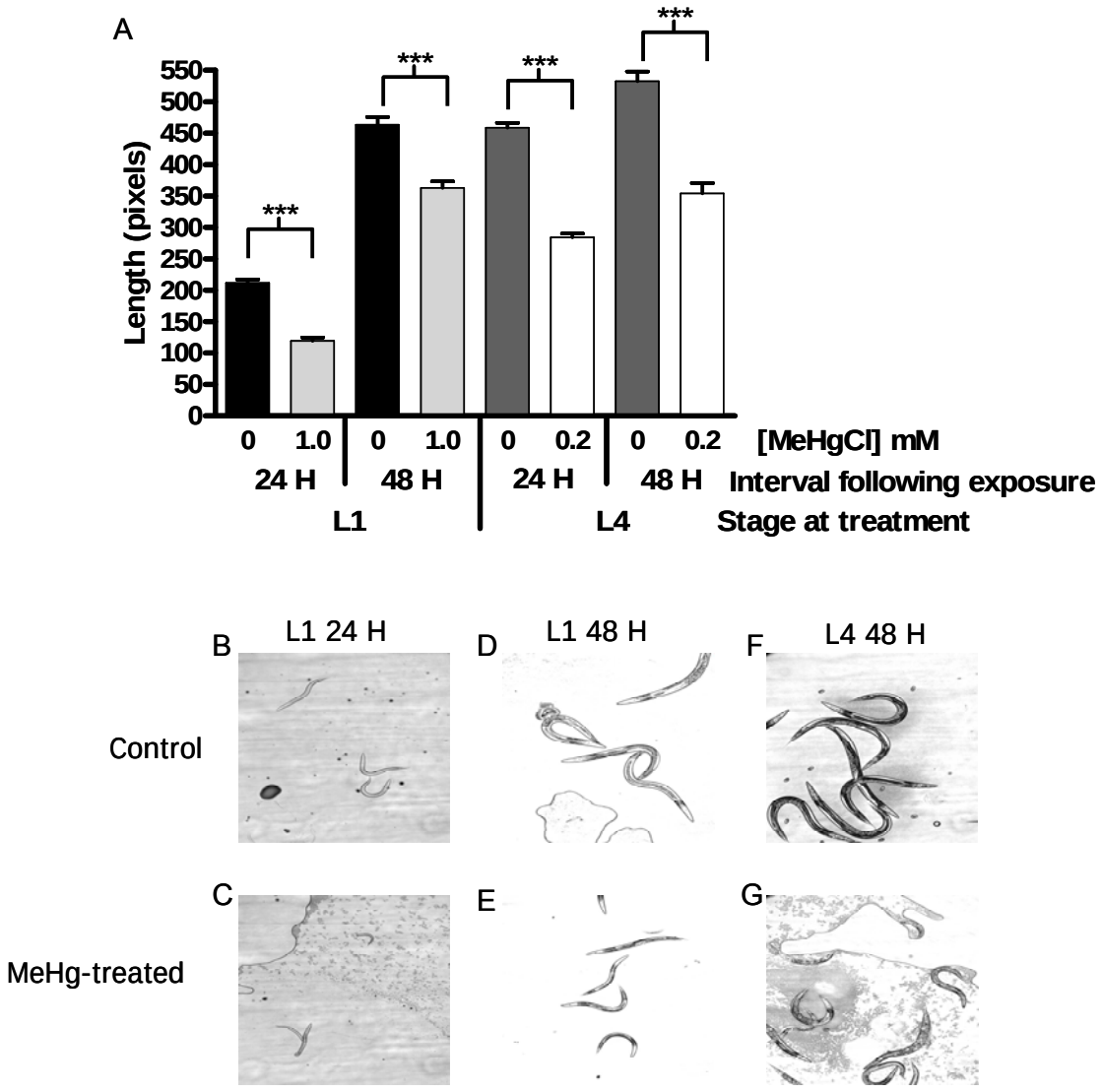

Figure 6. Body length of *C. elegans* was shorter following treatment with MeHgCl. After growth for 24 or 48 h, animals treated at either L1 (C and E) or L4 (G) stages with the toxicant were significantly (***$p < 0.001$, n=4) shorter than control animals (B, D, and F), as measured using the Nikon Element software to measure their length in pixels (arbitrary units) according to their body contour from the posterior bulb of the pharynx to the anus (A).

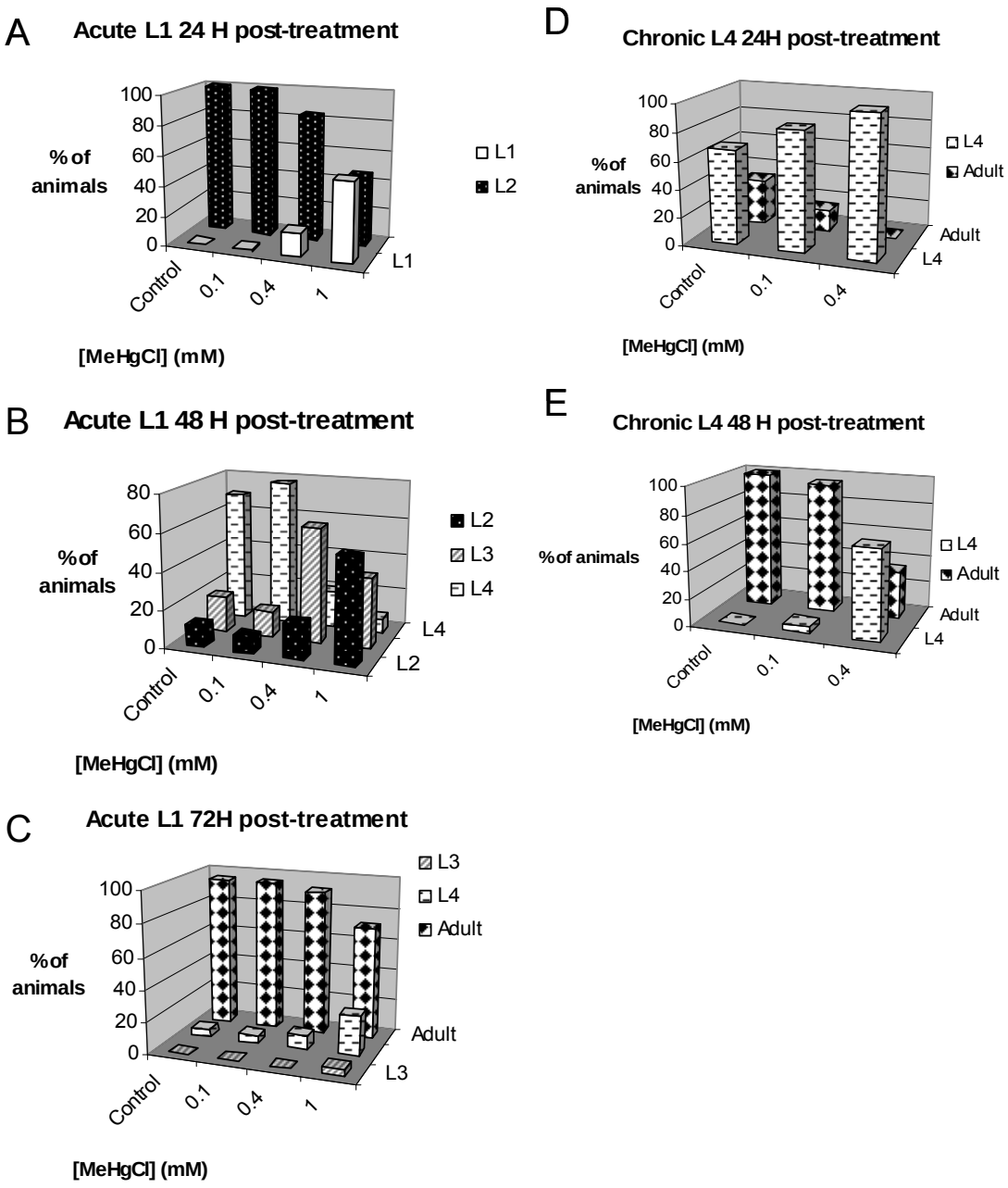

Figure 7. *C. elegans* larvae were developmentally delayed following exposure to MeHgCl. Animals treated at higher concentrations of MeHgCl took longer to develop through the larval stages and into adults following a 30-minute exposure at L1 stage (A–C) or a 15-hour exposure at L4 stage (D-E).

Table 1. MeHgCl developmentally delays *C. elegans*. Following control or MeHgCl treatment, life stages of worms were assessed. Percentages of worms at each larval stage or at adult stage are indicated (n=5).

	Acute L1 24H post			Acute L1 48H post				Acute L1 72H post		
mM MeHgCl	L1	L2		L2	L3	L4		L3	L4	Adult
0	0.00	100.00		11.25	18.75	70.00		0.00	5.00	95.00
0.1	1.00	99.00		8.75	13.75	77.50		0.00	5.00	95.00
0.4	16.00	84.00		18.75	61.25	20.00		0.00	9.00	91.00
1	53.00	47.00		55.00	37.50	7.50		4.00	25.00	71.00

	Chronic L4 24H post			Chronic L4 39H post	
mM MeHgCl	L4	Adult		L4	Adult
0	67.50	32.50		0.00	100.00
0.1	85.00	15.00		5.00	95.00
0.4	100.00	0.00		65.00	35.00

had all reached the L2 stage while many worms treated at higher concentrations of MeHgCl remained L1s (Figure 7A). This trend continued 48 (Figure 7B) and 72 (Figure 7C) hours after treatment, when most worms had reached the adult stage. This trend also occurred in animals treated for 15 hours at the L4 stage (Figure 7D-E). Many control-treated animals reached the adult stage 24 hours after treatment while those treated at higher MeHgCl had remained L4s (Figure 7D). At 48 hours after treatment, all control-treated worms had reached the adult stage, while only some of those treated with higher MeHgCl concentrations had reached the adult stage (Figure 7E) (n=5 experiments).

Pharyngeal pumping decreases following MeHgCl exposure, thrashing is unaffected

Pharyngeal pumping rates were significantly decreased in a dose-dependent manner following 15-hour treatment of L4 *C. elegans* with MeHgCl (control-treated worms pumped at a rate of 230±6 pumps per minute 24 hours following treatment while worms treated at 0.1 and 0.4 mM MeHgCl pumped at 168±9 and 69±11 pumps per minute, respectively, $p<0.001$, Figure 8). Other researchers have demonstrated that at the L4 stage, *C. elegans* typically pump at a rate of 150-200 pumps per minute. The rate increases as they mature into adults and peaks 2 days later at 300-350 pumps per minute before declining as the worm ages (Huang *et al.*, 2004). Since the pumping rates observed in our experiments were lower than expected even for L4 *C. elegans*, we do not attribute this decrease to the developmental delay. A similar trend was observed

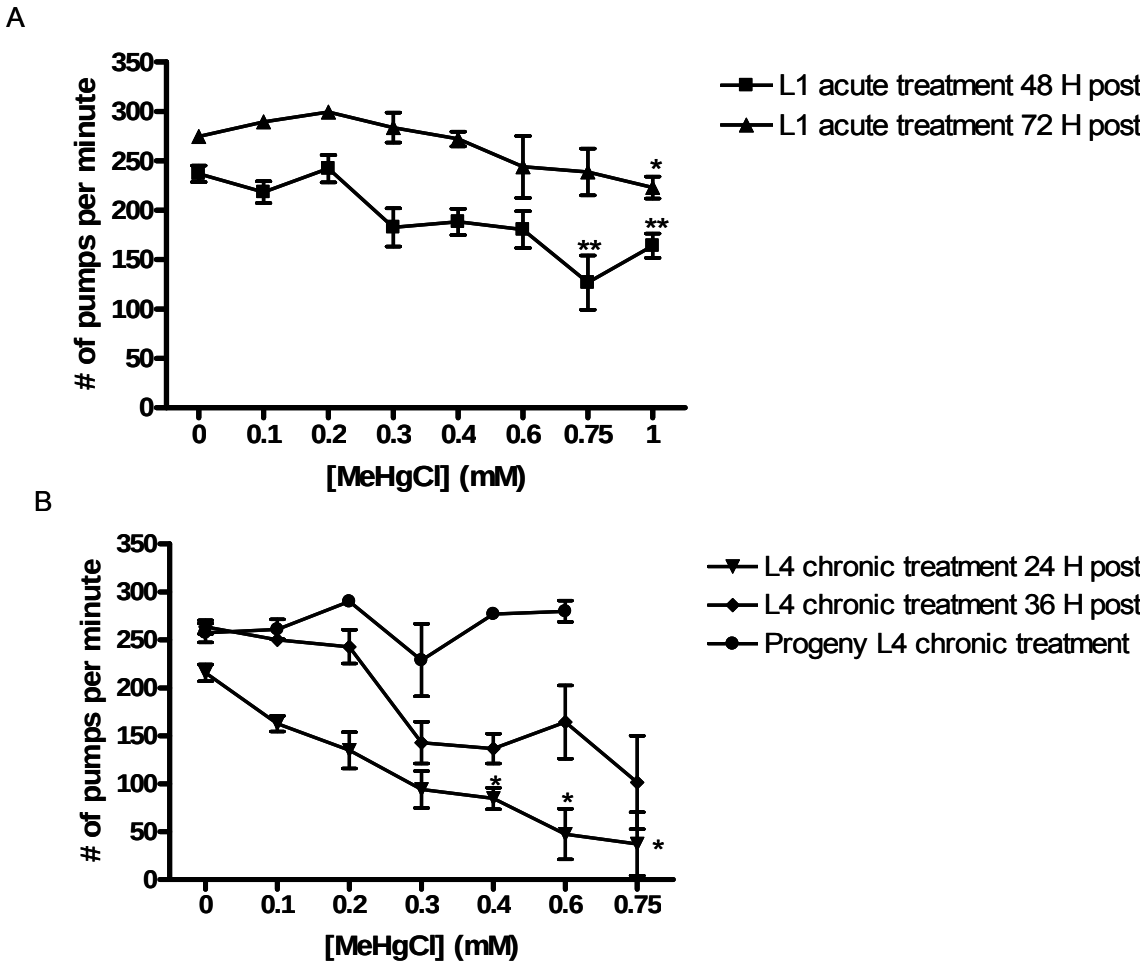

Figure 8. Pharyngeal pumping rates of *C. elegans* decrease following MeHgCl exposure. Number of pharyngeal pumps per minute significantly decreased in a dose-dependent manner following 30 minute MeHgCl exposure of L1 worms 48 h following treatment at 0.75 and 1 mM MeHgCl (**p<0.01, n=12) and 72 h following treatment at 1 mM MeHgCl (*p<0.05, n=12). Exposure of L4 worms for 15 h induced a decrease in pharyngeal pumping rate 24 h following exposure at 0.4, 0.6, and 0.75 mM MeHg (*p<0.05, n=11). No alteration in pharyngeal pumping rate was noted in progeny of L4-treated animals (n=8).

in animals treated at the L1 stage for 30 minutes, and significant differences were noted between control worms and those treated at 0.4 and 1 mM MeHgCl ($p<0.05$). The decreased pumping rate induced by MeHgCl could contribute to the decreased rate of development in worms. No alterations were seen in the pumping rate of the progeny of *C. elegans* treated for 15 hours at the L4 stage at any concentration tested (0.1, 0.2, 0.3, and 0.4 mM MeHgCl, n=7). Thrashing data showed no trends in MeHgCl-dependent alterations on the swimming behavior of *C. elegans* (Figure 9). [There was one outlier among worms treated as L4s for 15 hours at 0.1 mM MeHgCl 24 hours following treatment. Mean thrashing rate was 0.27 ($p<0.05$) while thrashing means for all other groups ranged from 0.38 to 0.65 and were not statistically significantly different from each other (n=6, data not shown)].

Alterations in neuronal morphology were not observed in worms that survived MeHgCl exposure

GFP markers were used to observe cholinergic, glutamatergic, serotonergic, dopaminergic, and GABAergic neuronal populations for potential alterations following MeHgCl insult. Animals were treated with 0, 0.1, 0.4, and 1 mM MeHgCl for a 30-minute treatment at the L1 stage, and a 15-hour treatment at the L4 stage. Live worms treated at the L1 stage were observed 24, 48, and 72 hours following treatment and worms treated at the L4 stage were observed 24 and 48 hours following treatment. Additionally, progeny of L4-treated animals were observed once they reached the L4 stage. No obvious phenotypes were

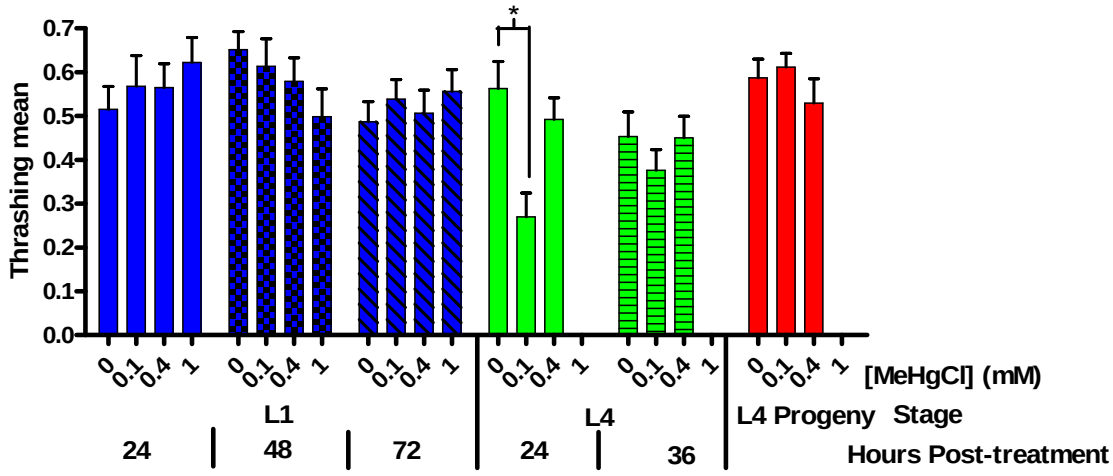

Figure 9. Thrashing rate of animals treated as L1s for 30 min or as L4s for 15 h and progeny of L4s treated for 15 h was not altered in a MeHgCl-dose-dependent manner (n=6).

observed in these neuronal populations under any of the treatment paradigms.

Due to ease of measurement because of a low cell number and readily available GFP markers, dopaminergic and GABAergic neuronal populations were quantitatively investigated. Analysis of the dopaminergic system revealed no alteration in cell number [6 head neurons (Figure 10C) and 2 PDEs (Figure 10D)] or ability of projections to travel from the nerve ring to the tip of the nose (Figure 10E) in worms surviving MeHgCl insult (Figure 10A-B). GABAergic analysis also revealed no alteration in cell number in the head (Figure 11C) or nerve cord (Figure 11D), ability of projections to pass across the body (Figure 11E), or number of breaks in the commissures (Figure 11F) of *C. elegans* surviving MeHgCl treatment (Figure 11A-B).

Discussion

Here we describe our first experiments to probe the neurotoxicity of MeHgCl in the model organism, *C. elegans*. No neuronal alterations were observed upon MeHgCl exposure, indicating that the *C. elegans* nervous system may possess unique mechanisms for dealing with the insult of this toxicant. However, the possibility does exist that MeHg is metabolized, excreted, or sequestered from neurons, resulting in minimal exposure to these cells. Other results (lethality, pharyngeal pumping, etc.) demonstrate MeHgCl toxicity to *C. elegans* and begin to reveal some of the alterations that occur following exposure to this metal. Lethality was observed at high MeHgCl doses (Figure 2). As has been shown in other systems, MeHgCl was more toxic to younger as compared

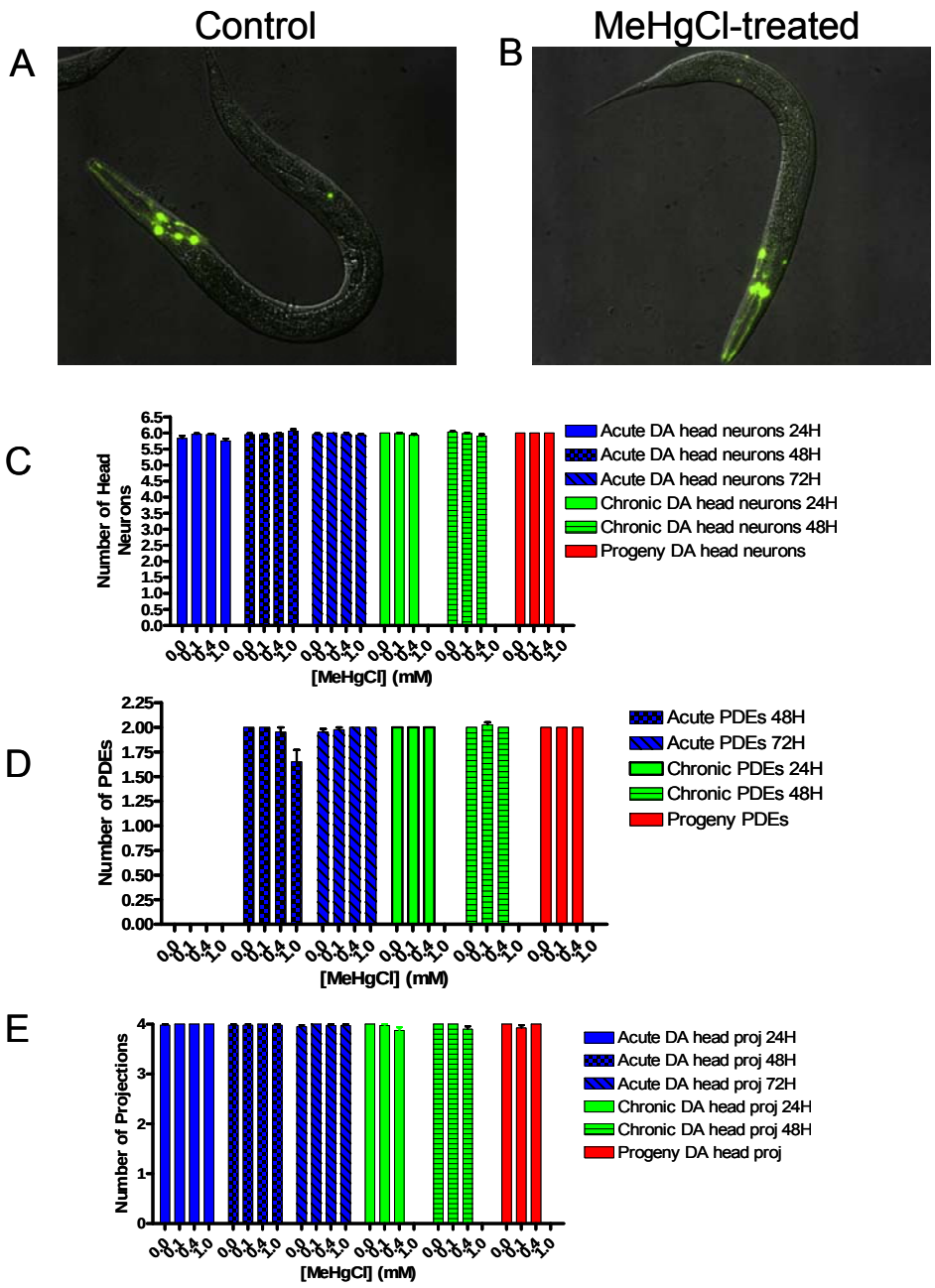

Figure 10. Representative dopaminergic *C. elegans* neurons following MeHgCl insult. Cells and projections are identical under control (A) and MeHgCl treatment (B) conditions (at all concentrations observed, 0.1, 0.4, and 1 mM MeHg) following treatment at L1 for 30 min or L4 for 15 h (n=4). Progeny of worms treated at L4 for 15 h were also unaffected (n=4). Upon quantification of dopaminergic head neurons (CEPs and ADEs) (C), dopaminergic body neurons (2 PDEs) (D), dopaminergic projections from the head to the anterior tip of the worm (CEPs) (E), no significant MeHgCl concentration-dependent alterations were detected (n=4).

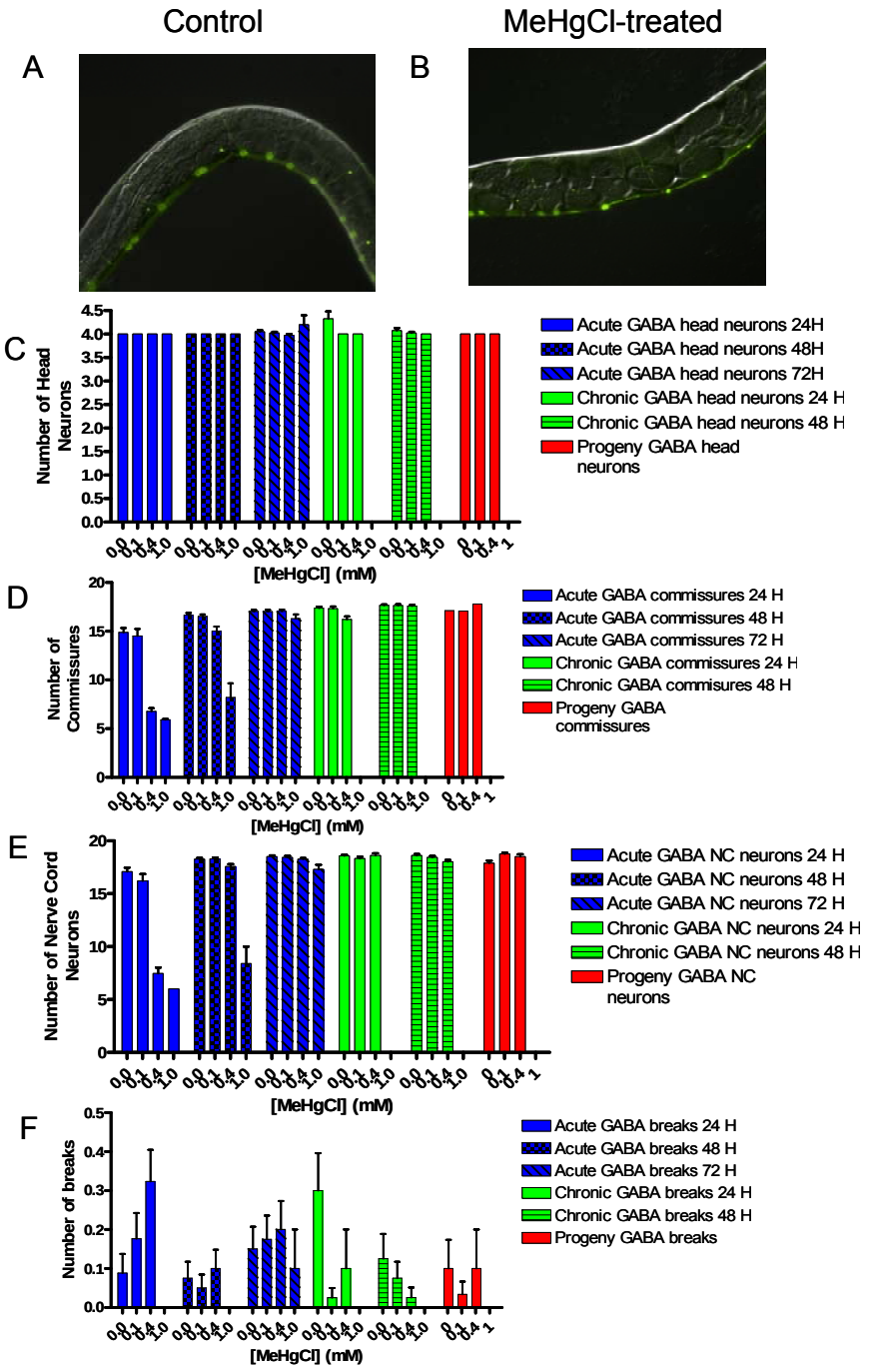

Figure 11. Representative GABAergic *C. elegans* neurons following MeHgCl insult. Cells and projections are identical under control (A) and MeHgCl treatment (B) conditions (at all concentrations observed, 0.1, 0.4, and 1 mM MeHg) following treatment at L1 for 30 min or L4 for 15 h (n=4). Progeny of worms treated at L4 for 15 h were also unaffected (n=4). Upon quantification GABAergic head neurons (C), GABAergic commisures (D), GABAergic nerve cord neurons (E), or beaks in GABAergic projections (F), no significant MeHgCl concentration-dependent alterations were detected (n=4).

to older individuals (Clarkson and Magos, 2006). However, this result cannot be explained by increased accumulation of MeHgCl in younger *C. elegans* as the young (L1) worms accumulated less Hg than their older (L4) counterparts (Figure 3). Instead, the enhanced sensitivity of L1 stage larvae may be due to inhibition of essential developmental pathways including, for example, mechanisms for detoxifying MeHgCl that could be in place in the more mature L4 larvae. Mammalian systems have displayed an inability to demethylate MeHg until after birth, indicating that in mammals, the processes involved in demethylation as a form of detoxification do not develop until later in life (Dock *et al.*, 1994). Furthermore, MeHgCl displayed increased toxicity as duration of exposure increased (Figure 2), indicating that increased accumulation of Hg within *C. elegans* (Figure 3) may be responsible for this increased toxicity instead of an increased duration of exposure to the toxicant.

Although our studies required exposure of *C. elegans* to relatively high external doses of MeHgCl, Hg accumulation within *C. elegans* is not excessively high when compared to levels observed in the brains of mammals exposed to MeHgCl. In our studies, the levels observed in worms ranged from 0-3.3 ng Hg/mg protein. A number of studies have investigated Hg levels in mammalian brain following MeHgCl treatment. In human autopsy studies, brain levels of Hg between 1913 and 1970 decreased, from an average level of 34 parts per million (ppm) to an average level of 1.3 ppm (1 ppm equals 1 ng/mg) (Kevorkian *et al.*, 1972). Examples of determination of Hg content following MeHgCl exposure include rats treated with MeHgCl, registering Hg levels of 0-8 ppm, depending on

dosage and duration (Newland *et al.*, 2006) and mice treated with MeHgCl having 0-3 ppm when pregnant mice were exposed and their pups tested at various postnatal days (Stringari *et al.*, 2008). A single dose of 5 µg/g body-weight MeHgCl in rat pups, resulting in brain Hg levels of approximately 0.05 ppm, produced extensive alterations in the brain, including reduced hippocampal size and cell number as well as deficits in learning (Falluel-Morel *et al.*, 2007). Furthermore, Hg levels in Minimata disease patients have been measured at 1.60 ppm in umbilical cord samples (Harada, 1995). Alterations in the *C. elegans* nervous system would have been expected due to the body of literature indicating that alterations are seen in the nervous system of other organisms at the concentrations observed in *C. elegans*. For example, in rodent studies, damage to the nervous system has been observed at 4.5 ppm for neuropathological damage and 0.5 ppm for neurobehavioral alterations (Castoldi *et al.*, 2008), and in children the threshold for clinical effects is 1 ppm in the brain (Burbacher *et al.*, 1990). Comparing these to our experiments, where levels reached 3.3 ppm in *C. elegans*, concentrations of Hg as high as those found in mammalian systems where deleterious alterations have been observed were measured. Additionally, brain levels in human brains have been assessed from individuals in the Seychelles (1.475 ngHg/mg protein) (Lapham *et al.*, 1995), in Minimata patients (2 ngHg/mg protein) (Takeuchi, 1985), and in Iraqi patients (68.5 ng/mg protein) (Choi *et al.*, 1978). Although levels in Iraqi patients were much higher than those we measured, the presence of toxicity in Minimata patients and some evidence of alterations in individuals in the Seychelles

suggest that, at the levels we measured in *C. elegans* (0-3.3 ng Hg/mg protein), neuronal abnormalities would be expected.

Neither *C. elegans* lifespan (Figure 4) nor brood size (Figure 5) was altered upon MeHgCl exposure possibly indicating that essential reproductive processes are resistant to the effects of MeHgCl and that the aging process in *C. elegans* is not accelerated by exposure to MeHgCl. Hg concentrations were not tested more than 24 hours following treatment, but Hg may be excreted at a high rate, decreasing the effect of MeHg after a number of days. Stress factors or detoxification may also be induced following toxicant exposure, allowing *C. elegans* to cope with MeHgCl following the initial insult much more efficiently compared to mammalian systems. Additionally, the *C. elegans* reproductive system may be less sensitive to MeHgCl toxicity. However, a decrease in *C. elegans* size (Figure 6) and a developmental delay following MeHgCl exposure was noted (Figure 7, Table 1). Taken together, these results indicate that *C. elegans* may have a mechanism for stunting development when stressed with MeHgCl and returning to normal development once more favorable conditions are encountered. Developmental delay in *C. elegans* is not unique to MeHgCl exposure, as researchers investigating other chemicals have observed similar outcomes. Some toxicants have had more dramatic effects, for example, exposure to antipsychotic compounds led to larval arrest and dauer formation (Donohoe *et al.*, 2006) whereas exposure to ethanol led to a decrease in brood size and life span in addition to a developmental delay (Davis *et al.*, 2008). Although no alteration in thrashing behavior was noted (Figure 9), the decreased

pharyngeal pumping rate following MeHgCl exposure (Figure 8) indicates that *C. elegans* may consume less bacteria following exposure. It is possible that decreased feeding could be an adaptive response to limit MeHgCl intake as well as delay development until a less toxic environment is attained. Although gross morphological alterations in neurons were not noted, MeHgCl may have specific effects on the neurons of the pharyngeal nervous system, leading to the alterations in pharyngeal pumping rate. Further investigation of the pharyngeal nervous system morphology or functioning should reveal insights into the mechanism of the decreased pumping rate.

Extensive research investigating alterations in mammalian brain and mammalian cell lines following exposure to MeHgCl has revealed mitotic arrest in the cerebellum (Rodier *et al.*, 1984), necrosis and apoptosis (Castoldi *et al.*, 2001), disruption of microtubules (Castoldi *et al.*, 2001), alterations in calcium levels and signaling, oxidative stress (Castoldi *et al.*, 2001), and alterations in neurotransmitter systems (Sobotka *et al.*, 1974; Castoldi *et al.*, 2001), specifically in the glutamatergic (Brookes, 1992; Aschner, 2000; Baraldi *et al.*, 2002), muscarinic cholinergic (Coccini *et al.*, 2000), and dopaminergic (Rossi *et al.*, 1997; Faro *et al.*, 2002) systems. Although a major target of MeHg toxicity in mammals is the nervous system (Clarkson and Magos, 2006), surprisingly, alterations in the nervous system of *C. elegans* were not observed.

There are a number of possible explanations for this observation. The experiments described here assess the overall function of selected behavioral circuits (thrashing and pharyngeal pumping) but do not assay the function of

specific individual neurons which therefore could be selectively inactivated by MeHgCl treatment. Although we did not observe significant changes in neuron morphology, the neuron-specific GFP markers used in our study would not have revealed functional defects in synaptic activity. MeHg may not reach sufficiently high concentrations in *C. elegans* to have a deleterious effect on the nervous system before the animal is affected in some other way, i.e., another tissue is damaged, leading to lethality. Another possible explanation is that *C. elegans* neurons utilize mechanisms to overcome the toxicity of MeHg that are not similarly activated in mammalian neurons. The elucidation of such mechanisms may reveal pathways that could be exploited in cases of MeHg poisoning in humans. Interestingly, quantification of alterations in the nervous system (Figure 10-11) did not reveal any alterations in appearance of neurons although, as indicated by dose-response curves, some animals were likely sick or dying.

Dead animals could not be assessed since autofluorescence within the entire animal makes the GFP reporter indistinguishable from the rest of the animal. However, *C. elegans* were observed at time points during exposure and at various time points after exposure, no trends existed indicating that neurons were affected before death of the animal. This result shows that these worms are most likely not dying due to perturbations within the nervous system but via alternative mechanisms that do not affect the nervous system. We propose that *C. elegans* may exhibit a potent adaptive response, such as the involvement of glutathione or metallothioneins, allowing the neurons to survive MeHgCl insult.

Taken together, our experiments show that while MeHgCl is toxic to *C. elegans*, the nervous system of this model organism does not appear to be as sensitive to MeHg as mammalian neurons. Therefore, additional studies in *C. elegans* may reveal unique mechanisms of MeHg handling, allowing us to glean important information by making use of many advantages that *C. elegans* provides as a model organism with resistance to MeHg neuronal toxicity.

CHAPTER III

HORMETIC EFFECT OF MEHG ON *C. ELEGANS*

Summary

Extensive research has demonstrated some of the toxic effects of methylmercury (MeHg), yet the molecular mechanisms underlying its toxicity remain largely unknown. *C. elegans* offers a unique biological model to explore the mechanism of MeHg toxicity given the many advantages associated with its ease of use and genetic power. Since our previous studies indicated that *C. elegans* is resistant to MeHg neurotoxicity, the present study was designed to examine the molecular mechanisms associated with this resistance. We hypothesized that since glutathione (GSH), heat shock proteins (HSPs), and metallothioneins (MTs) have shown involvement in MeHg toxicity, the toxicant would induce expression of *gst-4::GFP*, *hsp-4::GFP*, *mtl-1::GFP*, and *mtl-2::GFP* in *C. elegans*. Our studies demonstrated a modest, but significant increase in fluorescence in *gst-4::GFP* and *mtl-1::GFP* strains at an acute, low MeHgCl exposure at the L1 stage, while a chronic MeHgCl exposure at the L4 stage induced increases in *gst-4::GFP* and *hsp-4::GFP*. Knockout *gst-4* animals showed no alterations in MeHgCl response compared to wildtype animals while *mtl* knockouts displayed increased sensitivity to MeHgCl exposure. GSH levels were increased in acute MeHgCl exposure and depleted in chronic exposure. We also demonstrated the ability of MeHgCl to induce hormesis, an adaptive

phenotype whereby a sublethal exposure to MeHgCl rendered *C. elegans* resistant to a subsequent exposure to the organometal. The involvement of *gst-4*, *hsp-4*, *mtl-1*, and *mtl-2* in the hormetic response was examined. An increase in *gst-4::GFP* expression after a low-dose acute exposure to MeHgCl indicated that *gst-4* is critical for this response. Our results implicate GSH, HSPs, and MTs in protecting *C. elegans* from MeHg toxicity and show that *gst-4* is involved in MeHgCl-induced hormesis.

Introduction

See the introduction of this dissertation for a detailed review of MeHg toxicity and the use of *C. elegans* as a model system. We designed experiments in *C. elegans* to determine the molecular mechanisms of proteins previously demonstrated to play a role in MeHg detoxification in an effort to better understand the adverse health effects of MeHg in humans.

Prior work described in this dissertation indicated that there are no appreciable morphological alterations in GABAergic, dopaminergic, cholinergic, glutamatergic, or serotonergic neuronal subtypes in response to MeHg insult, showing that the *C. elegans* nervous system demonstrates resistance to the toxicant at concentrations equivalent to those known to be detrimental to the mammalian nervous system (Helmcke *et al.*, 2009). A number of proteins are involved in the detoxification and excretion of MeHg; these include glutathione (GSH), heat shock proteins (HSPs), and metallothioneins (MTs).

GSH is a tripeptide consisting of glutamic acid, cysteine, and glycine and can exist in the reduced (GSH) or the oxidized (GSSG) state. GSH is oxidized to form GSSG in the presence of reactive oxygen species (ROS). GSSG can then be converted back into GSH via glutathione reductase (GR) and the conversion of NADPH to NADP+ (Filomeni *et al.*, 2005). Alternatively, glutathione s-transferases (GSTs) can catalyze the conversion of GSH to GS-, which can bind to MeHg and facilitate its excretion from the body (Figure 12)(Hirata and Takahashi, 1981). Under normal conditions, HSPs function as molecular chaperones, assisting with protein folding, directing proteins to proper organelles, assembly and disassembly of protein complexes, and inhibition of aggregation. Upon stress, for example in the presence of MeHg (Sacco *et al.*, 1997), these proteins function to assist in the refolding and repair of denatured proteins and can facilitate new protein synthesis (Hubbard and Sander, 1991).

MTs are small, cysteine-rich metal binding proteins that are involved in metal detoxification and homeostasis and can protect cells from oxidative stress through this role and as their role as antioxidants (Maret, 2008). Due to their high cysteine content, they have a high affinity for MeHg. Additionally, MeHg has been shown to induce expression of these MTs (Rising *et al.*, 1995; Tsui and Wang, 2005) and alterations in behavior of MT-null animals (Yoshida *et al.*, 2008). Although GSH, HSPs, and MTs have been implicated in resistance to MeHg toxicity, researchers have yet to elucidate their precise role in detoxification.

We chose *C. elegans* as the preferred experimental model system because of the resistance of its nervous system to MeHgCl. To determine

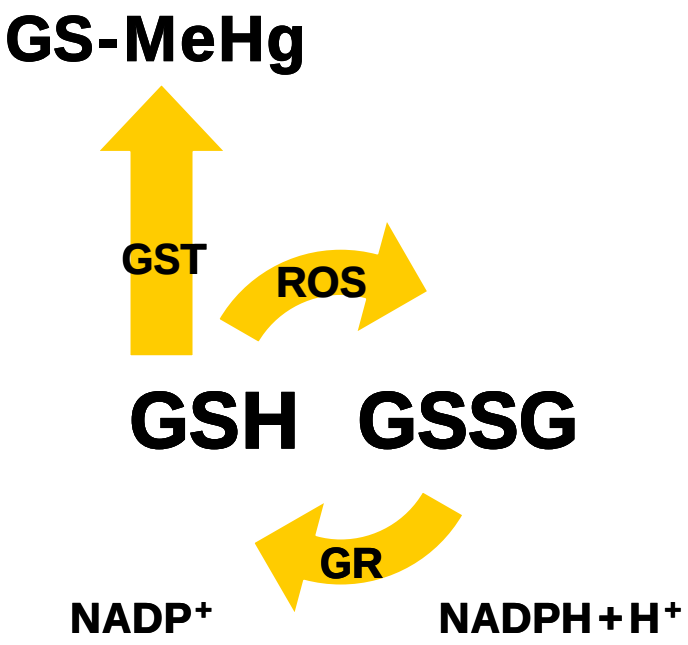

Figure 12. Glutathione cycle. GSH is converted to GSSG upon exposure to ROS. GR can convert GSSG back to GSH while converting NADPH to NADP+. GSTs can assist with the conjugation of GSH to MeHg for excretion from the system.

whether GSTs, HSPs, or MTs play a role in MeHgCl detoxification, we examined their expression through the use of GFP reporter strains. We observed induction of *gst-4* and *hsp-4*, and *mtl-1* following exposure to MeHgCl.

Hormesis refers to a process whereby a sublethal stressor renders an organism more resistant to subsequent stress. This has been demonstrated in a number of models, ranging from cell cultures to humans under a variety of stress conditions, including dietary restriction, exercise, radiation, exposure to various chemicals including metals, and heat (Damelin *et al.*, 2000; Cypser *et al.*, 2006; Mattson, 2008b; Bourg, 2009). Although the precise mechanisms of hormesis are unknown, previous research has identified proteins and elucidated mechanisms that may be involved, including HSPs of the HSP70 family and MTs, both of which can be upregulated following exposure to heavy metals (Damelin *et al.*, 2000). Additionally, a time lag is often observed between MeHg exposure and the appearance of symptoms of toxicity. Hormetic mechanisms have been implicated as a possible explanation of this latency (Weiss *et al.*, 2002). We designed studies to assess the ability of MeHgCl to induce a hormetic response in *C. elegans*. Hormesis has been previously observed in *C. elegans*. Following dietary restriction or exposure to sublethal heat stress, animals display an increase in lifespan and a resistance to exposure to a subsequent stressor (Cypser *et al.*, 2006). We examined the role of *gst-4*, *hsp-4*, and *mtl-1* and *mtl-2* in the observed hormetic response by assessing the ability of *C. elegans* to display a hormetic response following exposure to sublethal concentrations of

MeHgCl. We hypothesized that MeHgCl would induce expression of these proteins and cause increases in GFP expression.

The overall goal of our studies, therefore, was to address the mechanism of action of MeHgCl toxicity and gain a better understanding into the resistance of the *C. elegans* nervous system to MeHgCl. We set to explore several proteins previously shown to be involved in MeHg resistance with a secondary objective of linking them to the hormetic effect of MeHg.

Materials and Methods

C. elegans maintenance and strains

C. elegans were cultured on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50 as previously described (Brenner, 1974). In addition to the wildtype N2 Bristol strain, transgenic lines expressing GFP reporters used in this study were: CL2166 *gst-4::GFP* (Link and Johnson, 2002), SJ4005 *hsp-4::GFP* (Calfon *et al.*, 2002) (obtained from the *Caenorhabditis* Genetic Center (CGC), Minneapolis, MN), *mtl-1::GFP*, and *mtl-2::GFP* (both gifts of the lab of Dr. Jonathan Freedman). The following knockout strains were also used: RB1823 *gst-4* (*ok2358*), VC128 *mtl-2* (*gk125*) (both obtained from the CGC, Minneapolis, MN), *mtl-1* (*tm1770*), and double *mtl-1/2* (*zs1*) knockouts (gifts from Hughes and Sturzenbaum) (Hughes and Sturzenbaum, 2007). With the exception of the *hsp-4::GFP* strain, which was kept at 15°C throughout experimentation due to induction of the heat shock

proteins at higher temperatures, animals were kept at 20-23°C throughout experimentation, and hermaphroditic worms were used in all experiments.

MeHgCl treatments

Animals were treated as previously described (Helmcke *et al.*, 2009). Briefly, animals were treated with an alkaline bleach solution prior to treatment with MeHgCl to obtain a synchronous population (Stiernagle, 1999). *C. elegans* were exposed to MeHgCl in one of two treatment paradigms. In the first paradigm, animals were treated for 30 minutes at 18-24 hours after synchronization, at the L1 stage. In the second paradigm, animals were allowed to grow to the L4 stage following synchronization. They were then treated at the L4 stage for 15 hours. All treatments involved combining larvae, (2500 L1s or 300 L4s), concentrated OP50, the appropriate volume of MeHgCl dissolved in water, and M9 buffer to a volume of 500µL in 1.7 mL siliconized tubes. Following treatment, animals were washed twice with deionized water by centrifugation and placed on OP50-containing NGM plates. For hormesis experiments, animals were subjected to a combination of both treatments (Figure 17A). Animals were treated under control, 0.3, or 0.6 mM MeHgCl conditions at the L1 stage for 30 minutes, washed, allowed to recover and grow to the L4 stage on OP50-containing NGM plates, and then treated at the L4 stage for 15 hours, and washed again. Dose-response curves were generated for each treatment condition.

Hg content

C. elegans larvae were treated with MeHgCl as described above. For L1 treatments, approximately 10,000 animals were pooled and assessed; for L4 treatments, approximately 900 animals were pooled and assessed. To separate live animals from dead animals, a sucrose floatation method was used. After treating and washing animals as described above, they were allowed to recover for 24 hours on OP50-containing NGM plates. They were then washed off the plate and into tubes with cold M9 buffer, centrifuged, and washed again with cold M9. After washing, a cold 30% sucrose solution was added to the worms, and they were centrifuged again. Worms floating on the top of the sucrose solution were live worms and were collected and washed an additional 3 times with M9. These samples were then sonicated and analyzed. Protein content was determined following manufacturer instructions for a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford IL). The remainder of the sample was used for inductively coupled plasma-mass spectrometry (ICP-MS) analysis of Hg content. Preparation of the sample for ICP-MS involved addition of nitric acid followed by heat digestion and dilution of the samples with water. The samples were digested in PP tubes (352059, BD) in a block heater after addition of 65% HNO_3 (Merck, Suprapur). The samples were transferred to Teflon tubes and digested in an UltraClave (Milestone). After digestion the samples were diluted directly in the Teflon tubes with ultrapure water (PURLAB Ultra Analytic, Elga) to achieve a final acid concentration of 0.6 mol/L. High Resolution-Inductively Coupled Plasma-Mass Spectrometry (HR-ICP-MS) analysis was performed using a Thermo

(Finnigan) model Element 2 instrument (Bremen, Germany). The RF power was 1400 W. The sample was introduced using an SC-2 with SC-FAST option auto sampler (ESI, NE, USA) with a peristaltic pump (pump speed 0.25 mL/min). The instrument was calibrated using 0.6 mol/L HNO_3 solutions of multielement standards at appropriate concentrations. Internal standards were not used. To check for possible drift in the instrument, a standard solution with known elemental concentrations was analyzed for every 10 samples. In addition, blank samples (0.6 mol/L HNO_3, Suprapur) were analyzed for approximately every 10 samples. The samples were analyzed in random order, and the analyst was not aware of the identity of the samples. Hg content was determined in the low resolution mode ($M/\Delta m=300$).

Lethality

Following treatment, wild type or knockout animals were placed on 60 mm NGM plates seeded with OP50 and allowed to grow for 24 hours. Animals were scored as dead or alive based on appearance and ability to move in response to being poked with a platinum wire (Bischof *et al.*, 2006; Roh *et al.*, 2007).

Measurement of fluorescence intensity

For each strain, control animals were imaged first. The imaging settings, including exposure time, were determined based on control animals. A Zeiss LSM 510 META upright confocal microscope was used for the imaging of *hsp-4::GFP*, *mtl-1::GFP*, and *mtl-2::GFP* strains. Autofluorescence was subtracted

from each image, allowing for the analysis of GFP intensity using Metamorph software. For hormesis experiments and all experiments using the *gst-4::GFP* strain, *C. elegans* were imaged on a Nikon Eclipse 80i microscope. These images were analyzed using NIS-Element Basic Research Software to determine the fluorescence intensity of the animals.

Glutathione quantification

A scaled-up version of the treatment paradigms described above was conducted. *C. elegans* were treated in 50-mL conical tubes to a volume of 25 mL, using 125,000 animals for L1 treatments and 15,000 animals for L4 treatments. Following treatment, a pooled sample of live and dead animals were washed 3x with dH2O, and duplicates of L4 treatments were pooled to yield 30,000 worms for glutathione analysis while L1 treatments were not pooled, yielding 125,000 animals for glutathione analysis. Immediately following washing, equal volumes of *C. elegans* and 10% perchloric acid/0.2 M boric acid/10 µM γ-glutamylglutamate were combined with approximately 500 mL of 1.0 mm zirconia beads in a 2 mL microtube. Samples were placed in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) and beat for 20 seconds, then quickly placed in an ice water bath for 1 minute. This cycle was repeated 7 times before the samples were centrifuged for 30 minutes at 4°C. The supernatant was removed and frozen at -80°C until being subjected to further processing. For glutathione measurement, 300 µL sample was combined with 60 µL iodoacetic acid solution (14.8 mg/mL H2O). The pH was adjusted to 9.0±0.2 using KOH in saturated

potassium tetraborate. After 20-minute incubation, 300 µL dansylchloride solution was added (20 mg/mL acetone), samples were mixed and allowed to incubate 24 hours in the dark. Following this incubation, 500 µL chloroform was added, samples were mixed and centrifuged, and aqueous layer was collected for injection to obtain HPLC results. HPLC separation was conducted as previously described (Reed *et al.*, 1980), using an 80% methanol solution as solvent A, an acetate buffered methanol solution as solvent B, and a propylamine column (Custom LC, Huston, TX), with detection performed with a fluorescence detector with excitation maximum at 335 nm and emission at 515 nm. HPLC results were analyzed on a per-worm basis, assuming 125,000 animals per L1 treatment and 30,000 animals per L4 treatment.

Statistics

GraphPad Prism 4 was used to assess significance. For all experiments, ANOVA with Bonferroni's Multiple Comparison Test was applied to raw data. When p-values were less than 0.05, groups were considered significantly different, groups with p-values greater than 0.05 were not considered significantly different. For each experiment discussed, 'n' indicates the number of experiments, not number of animals.

Results

Hg accumulates in live animals following MeHgCl treatment

Hg content was measured in animals that survived a 15 hour exposure at the L4 stage at 0, 0.1, and 0.4 mM MeHgCl. Exposures tested were selected to represent a range of doses that corresponded to a low concentration (LC_0), a medium concentration (LC_{20}-LC_{80}), and at a high concentration (LC_{100}). As the MeHgCl concentration to which the worms were exposed increased, Hg content in the samples analyzed also increased, with Hg levels at 0.015±0.006 ng Hg/mg protein for samples treated with 0 mM MeHgCl, 0.297±0.136 ng Hg/mg protein for samples treated with 0.1 mM MeHgCl, and 3.775±1.231 ng Hg/mg protein (Figure 13).

Increased expression of *gst-4*, *hsp-4*, *mtl-1*, and *mtl-2* following MeHgCl exposure

Immediately following treatment of L1 *C. elegans* for 30 minutes and of L4 *C. elegans* for 15 hours with MeHgCl, fluorescence intensities of *gst-4::GFP*, *hsp-4::GFP*, *mtl-1::GFP* and *mtl-2::GFP* strains were measured. *hsp-4::GFP* and *mtl-2::GFP C. elegans* displayed no alteration in fluorescence after 30-minute treatment at the L1 stage at 0.2 or 0.4 mM MeHgCl. Under this treatment paradigm, however, a significant increase was noted in fluorescence of *gst-4::GFP* following treatment at 0.2 mM MeHgCl (Figure 14A, $p<0.01$) and *mtl-1::GFP* following treatment at 0.4 mM MeHgCl (Figure 14C, $p<0.05$) (n=5). These data indicate that a short, low exposure to MeHgCl at the L1 stage can induce

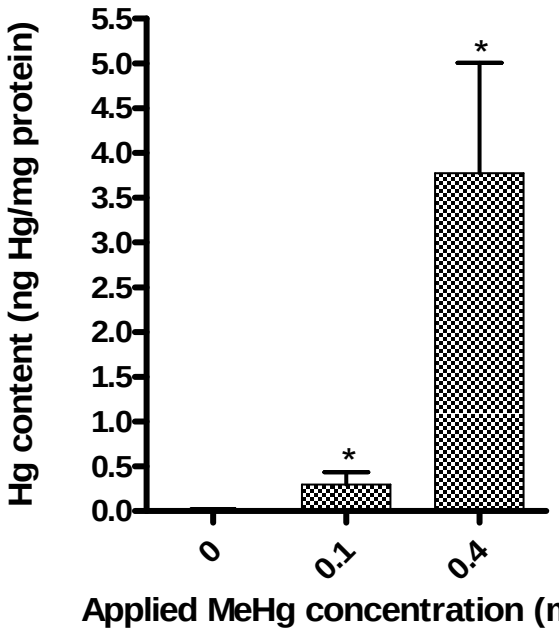

Figure 13. Concentration of Hg in live animals following MeHgCl exposure.

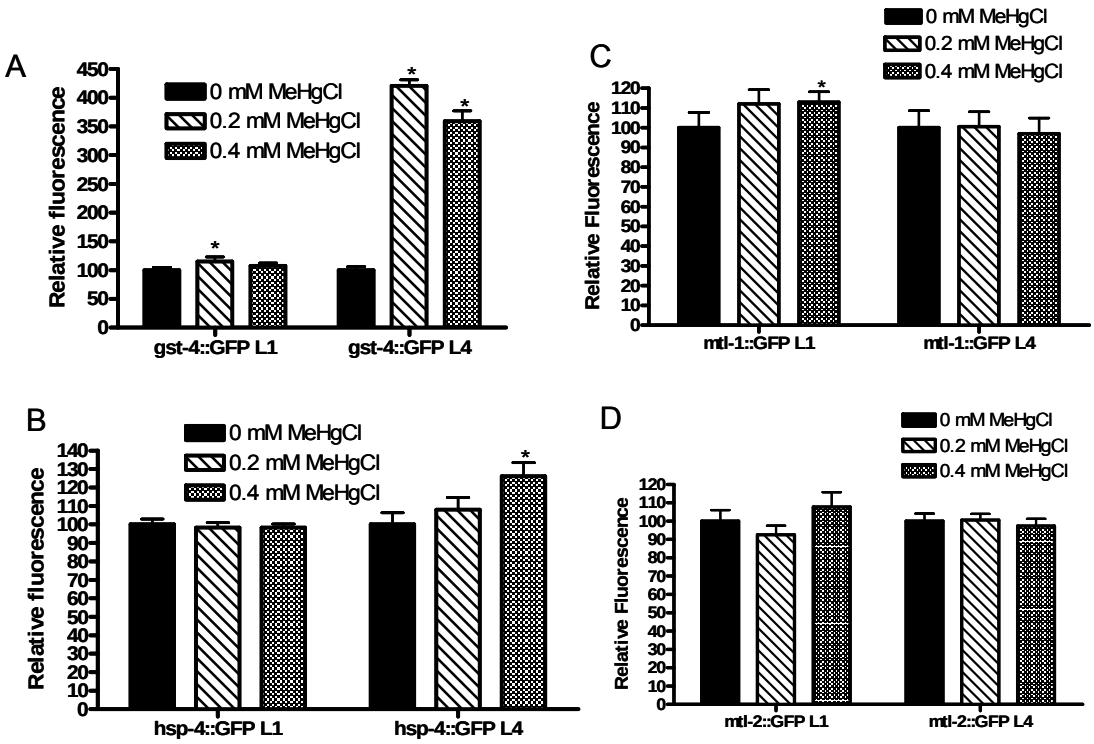

Figure 14. Treatment of *gst-4::GFP* (A)*, hsp-4::GFP* (B), *mtl-1::GFP* (C) and *mtl-2::GFP* (D) *C. elegans* with MeHgCl induces increases in GFP fluorescence. After an acute treatment at the L1 stage, increases in fluorescence were observed in *gst-4::GFP* (A, $p<0.01$) and *mtl-1::GFP* (C, $p<0.05$) strains (n=5). Chronic treatment at the L4 stage induced in increases in fluorescence in *gst-4::GFP* (A, $p<0.001$) and *hsp-4::GFP* (B, $p<0.05$) strains (n=5).

expression of *gst-4* and *mtl-1*; however, expression of these proteins is not induced at a higher concentration of MeHgCl.

Following a 15-hour treatment at the L4 stage, a significant increase in fluorescence was noted in *gst-4::GFP* [for which a large (4 fold) increase in fluorescence was seen (Figure 14A, $p<0.001$)] and *hsp-4::GFP* (Figure 14B, $p<0.05$) while *mtl-1::GFP* and *mtl-2::GFP* worms displayed no changes in fluorescence intensity (Figure 14C-D) (n=5).

mtl but not *gst-4* knockouts display increased sensitivity to MeHgCl

Lethality tests of L1 animals treated acutely and L4 animals treated chronically were conducted on *mtl-1*, *mtl-2*, *mtl-1/2*, and *gst-4* knockouts and compared to the lethality of wildtype animals. No significant shifts in dose-response curves were observed for animals treated acutely at the L1 stage (N2 LC_{50}=1.08±0.02, *mtl-1* LC_{50}=0.78±0.02, *mtl-2* LC_{50}=1.15±0.1, *mtl-1/2* LC_{50}=1.12±0.05, *gst-4* LC_{50}=0.99±0.01, n=4) (Figure 15A-B) or in *gst-4* knockout animals treated chronically at the L4 stage (N2 LC_{50}=0.33±0.01, *gst-4* LC_{50}=0.33±0.02, n=4) (Figure 15C). However, all three *mtl* knockout strains were significantly more sensitive to MeHgCl than the wildtype strain (*mtl-1* LC_{50}=0.18±0.05, *mtl-2* LC_{50}=0.22±0.02 and *mtl-1/2* LC_{50}=0.17±0.02, n=6, $p<0.05$) (Figure 15D).

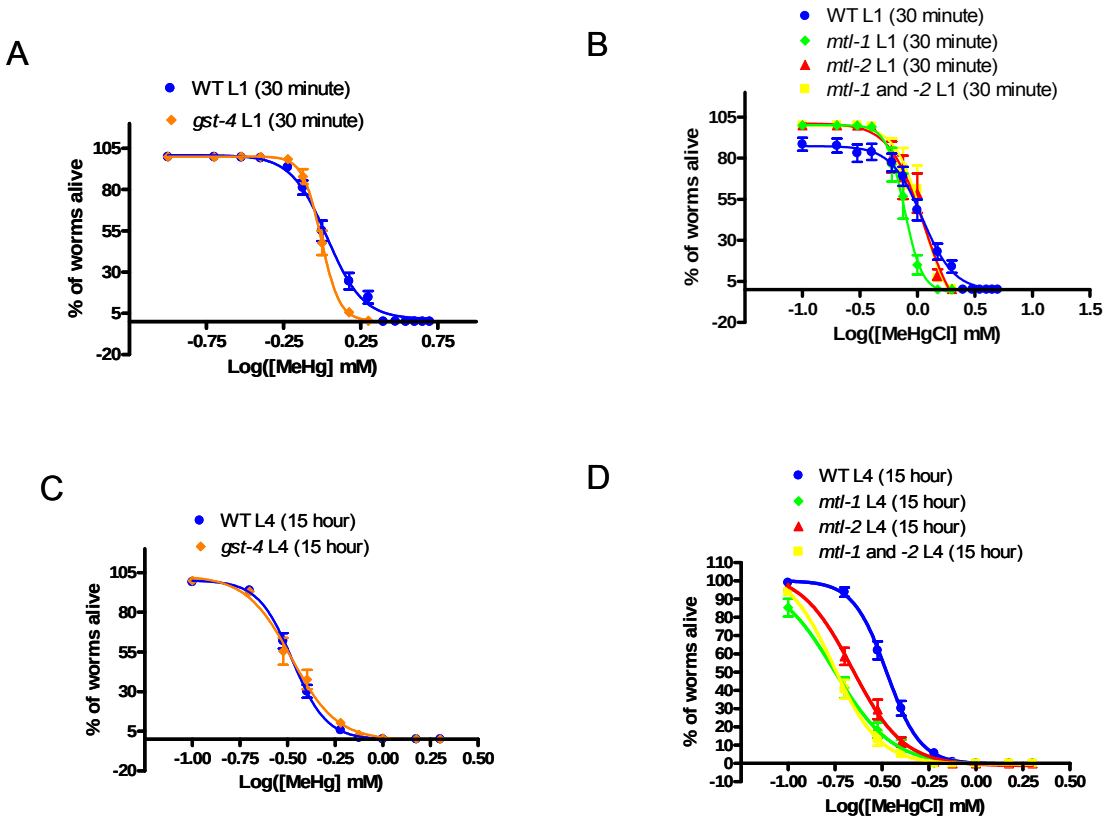

Figure 15. Treatment of knockout animals reveals increased sensitivity in *mtl* null animals following chronic exposure to MeHgCl. Dose response curves following acute treatment at the L1 stage did not reveal shifts in *gst-4* (LC_{50}=0.99±0.01) (A), *mtl-1* (LC_{50}=0.78±0.02), *mtl-2* (LC_{50}=1.15±0.1), or *mtl-1/2* (LC_{50}=1.12±0.05) (B) strains when compared to wild type (LC_{50}=1.08±0.02) (n=4). Chronic exposure of L4s did not induce a shift in *gst-4* animals (LC_{50}=0.33±0.02) (C) as compared to wild type (LC_{50}=0.33±0.01) but did induce a significant shift in *mtl-1* (LC_{50}=0.18±0.05), *mtl-2* (LC_{50}=0.22±0.02), and *mtl-1/2* (LC_{50}=0.17±0.02) strains (D, $p<0.05$) (n=4).

MeHgCl induces hormesis in wild-type *C. elegans*

We tested the ability of an acute exposure at the L1 stage to shift the dose-response curve of a subsequent exposure to MeHgCl. Animals treated under control conditions (e.g. 0 mM MeHgCl) for the initial MeHgCl exposure (LC_{50}=0.15±0.004 mM MeHgCl) were significantly ($p<0.05$) more sensitive to the subsequent exposure to MeHgCl than those treated at the 0.3 (LC_{50}=0.19±0.005 mM MeHgCl) or 0.6 (LC_{50}=0.20±0.004 mM MeHgCl) mM MeHgCl conditions when LC_{50} values were compared (Figure 16). *C. elegans* with prior exposure to MeHgCl were more resistant to the subsequent MeHgCl exposure, as indicated by the rightward shift in the dose-response curve.

Contribution of *gst-4*, *hsp-4*, *mtl-1*, and *mtl-2* to hormesis

We conducted experiments to assess the involvement of *gst-4*, *hsp-4*, *mtl-1*, and *mtl-2* in hormesis. Fluorescence of wild-type *gst-4::GFP*, *hsp-4::GFP*, *mtl-1::GFP* and *mtl-2::GFP* strains was measured following exposure to hormesis conditions. Animals were imaged immediately before and immediately following their second exposure (chronic L4) to MeHgCl. Images collected before the second MeHgCl treatment, after an acute L1 treatment followed by washing and growth to the L4 stage in the absence of MeHgCl, revealed minimal changes in fluorescence intensity. No significant alterations in fluorescence intensity were noted in wild-type, *mtl-1::GFP*, or *mtl-2::GFP* animals. A significant decrease in fluorescence was observed in *gst-4::GFP* animals treated at both 0.3 and 0.6 mM MeHgCl and *hsp-4::GFP* animals treated at 0.6 mM MeHgCl (Figure 17B,

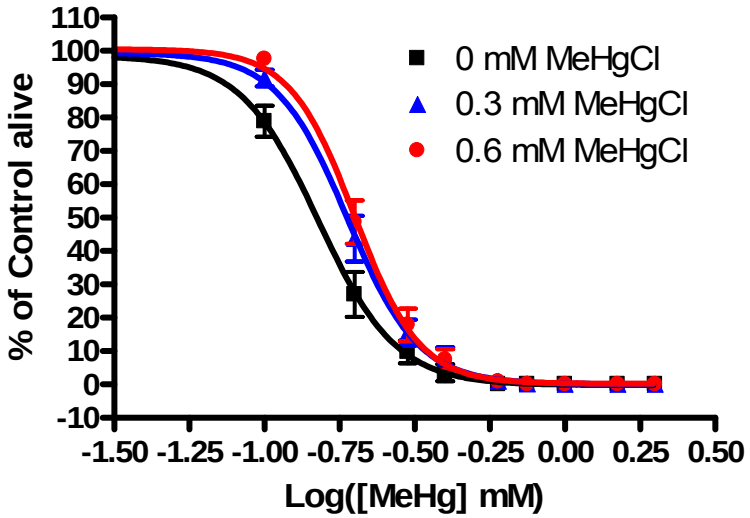

Figure 16. Pretreatment with MeHgCl renders *C. elegans* more resistant to a subsequent exposure to the toxicant. Dose-response curves were significantly shifted rightward under 0.3 (LC_{50}=0.19±0.005 mM MeHgCl) or 0.6 (LC_{50}=0.20±0.004 mM MeHgCl) mM MeHgCl ($p<0.05$) from control (LC_{50}=0.15±0.004 mM MeHgCl) pretreatment conditions.

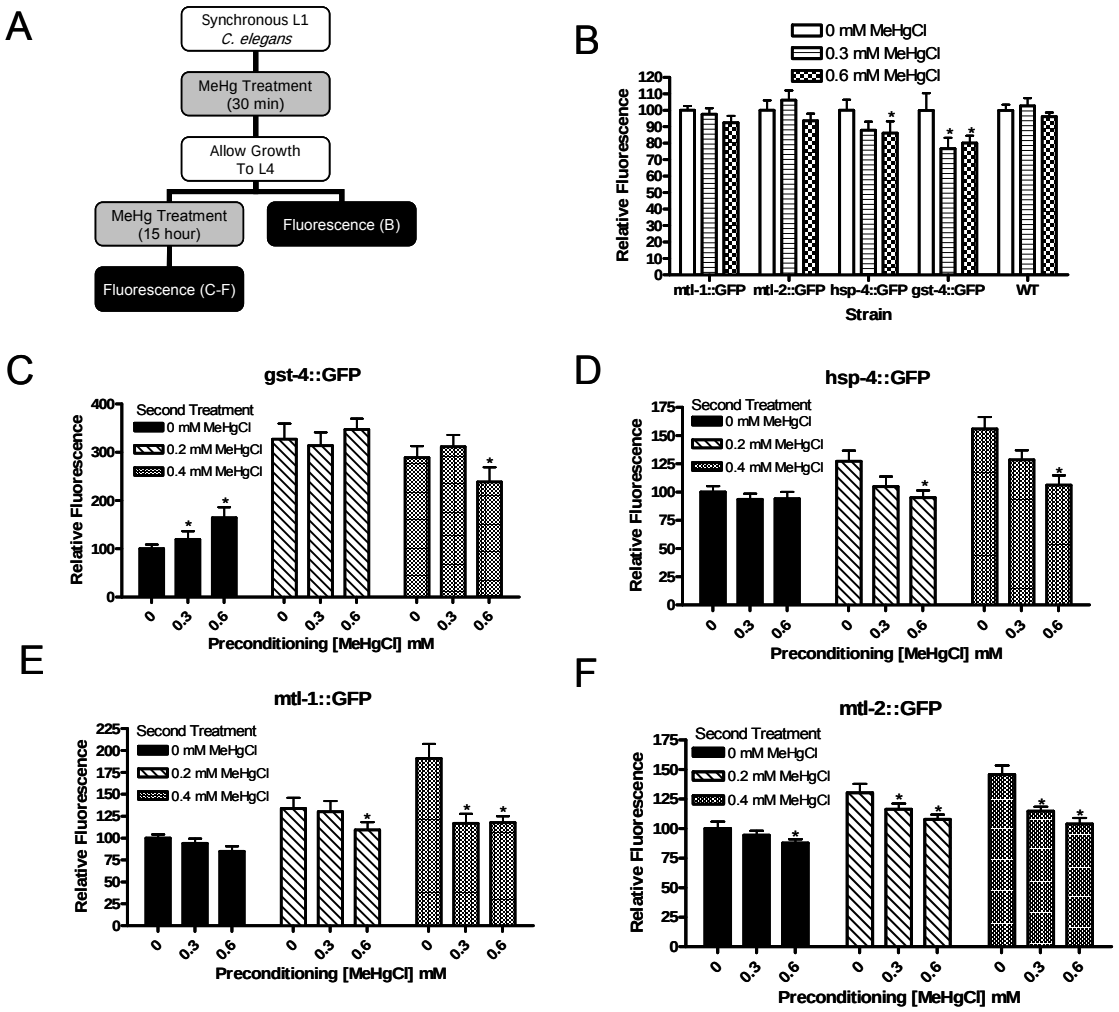

Figure 17. Fluorescence of *gst-4::GFP*, *hsp-4::GFP*, *mtl-1::GFP*, and *mtl-2::GFP* strains following hormesis treatments. Treatment paradigm includes animals treated at the L1 stage for 30 minutes, allowed to grow to the L4 stage, assessed for fluorescence or treated again and assessed for fluorescence after second treatment (A). Decreases were noted in fluorescence in *gst-4::GFP* and *hsp-4::GFP* animals after a single treatment and recovery (B, n=4). After an initial treatment with MeHgCl and subsequent exposure to control treatment conditions, *gst-4::GFP* animals showed an increase in fluorescence ($p<0.05$). At higher subsequent MeHgCl levels and in all conditions of *hsp-4::GFP*, *mtl-1::GFP*, and *mtl-2::GFP* worms only decreases in fluorescence at increasing MeHgCl concentrations were noted (C-F, n=4).

p<0.05). These findings were surprising given the previous findings of increases in fluorescence when the hormesis model was not used.

After the second treatment of L4 larvae, alterations in fluorescence were more dramatic and similar to the trends observed following a single L4 chronic treatment. A slight decrease in baseline fluorescence of *hsp-4*::GFP (Figure 17D), *mtl-1::GFP* (Figure 17E), and *mtl-2::GFP* (Figure 17F) strains was noted as the initial, acute MeHgCl concentration increased, and this trend continued in animals treated at higher MeHgCl concentrations (p<0.05). However, when the chronic treatments varied and the acute treatment was kept constant, an overall increase in fluorescence was noted with increasing MeHgCl concentrations (p<0.05). In *gst-4::GFP* L4 animals, dramatic increases in fluorescence were observed following chronic treatments with increasing MeHgCl concentrations (Figure 17C, p<0.01).

By analyzing these data in another way i.e. comparing fluorescence of the animals exposed to increasing chronic treatments within each acute paradigm (instead of comparing fluorescence of animals exposed to increasing acute treatment within each chronic treatment, as described above) significant increases in fluorescence are observed (Figure 18). With the exception of *hsp-4::GFP* (Figure 18C) animals treated at the 0.6 mM MeHgCl acute exposure level, significant increases in fluorescence occur in each of the treatment groups. In *hsp-4::GFP* (Figure 18 C) and *gst-4::GFP* (Figure 18D) animals, these findings confirm the previous results described in this paper, whereby a chronic exposure to MeHgCl induces an increase in fluorescence in these animals. However, our

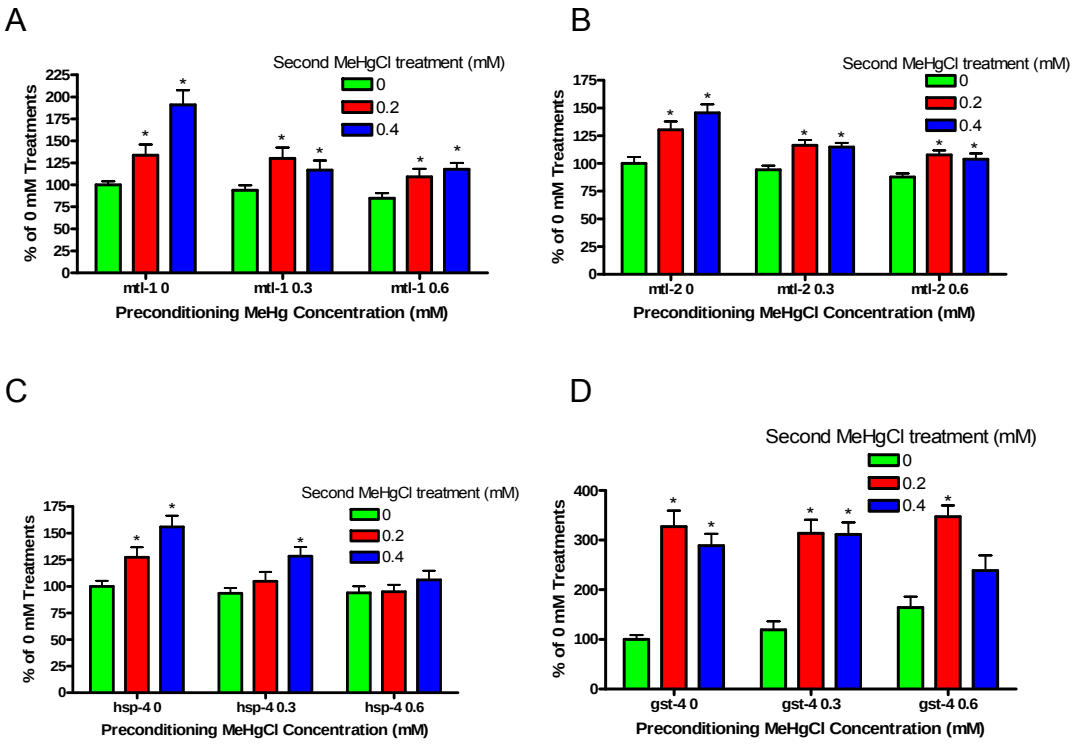

Figure 18. Re-analysis of *mtl-1::GFP*, *mtl-2::GFP*, *hsp-4::GFP*, and *gst-4::GFP* fluorescence following hormesis treatments. Acute treatment at the L1 stage with MeHgCl is indicated along the Y-axis, chronic treatment at the L4 stage with MeHgCl is indicated in the colors/legend. Significant increases ($p<0.05$) are observed in fluorescence of *mtl-1::GFP* and *mtl-2::GFP* in each of the preconditioning paradigms as the subsequent concentration of MeHgCl increases (A-B). When pretreatment is 0.3 mM MeHgCl, *hsp-4::GFP* fluorescence is only increased at the highest (0.4 mM) MeHgCl concentration and does not significantly increase when acute treatment is at 0.6 mM MeHgCl (C). Large increases in fluorescence were observed in *gst-4::GFP* animals with increased chronic MeHgCl concentrations except at 0.6 mM MeHgCl pretreatment and 0.4 mM MeHgCl subsequent treatment (D).

previous data presented here did not reveal an increase in fluorescence in *mtl-1::GFP* (Figure 14C) or *mtl-2::GFP* (Figure 14D) animals. The results from these hormesis data, therefore, reveal that *mtl-1* and *mtl-2* can be induced upon a second exposure to MeHgCl, but not a single chronic exposure.

MeHgCl induces alterations in glutathione levels

Glutathione profiles were different between animals acutely and chronically exposed to MeHgCl. After an acute exposure to MeHgCl, a trend of increasing GSH and GSH/GSSG ratio were noted while no changes were observed in total glutathione levels (Figure 19A). Chronic exposure to MeHgCl induced decreases in GSH, GSH/GSSG ratio, and total glutathione levels (Figure 19B).

Discussion

The studies reported here represent the first experiments in *C. elegans* to address the mechanism of action of MeHgCl toxicity in *C. elegans* and provide insights into the unique resistance of the *C. elegans* nervous system to MeHgCl. For the most part, specific molecular mechanisms of MeHgCl resistance are unknown. Previous data from our lab (Helmcke *et al.*, 2009) showed that while lethality, pharyngeal pumping, growth, and development were affected in *C. elegans* exposed to MeHgCl, brood size, lifespan, thrashing rate, and nervous system morphology were largely unaffected.

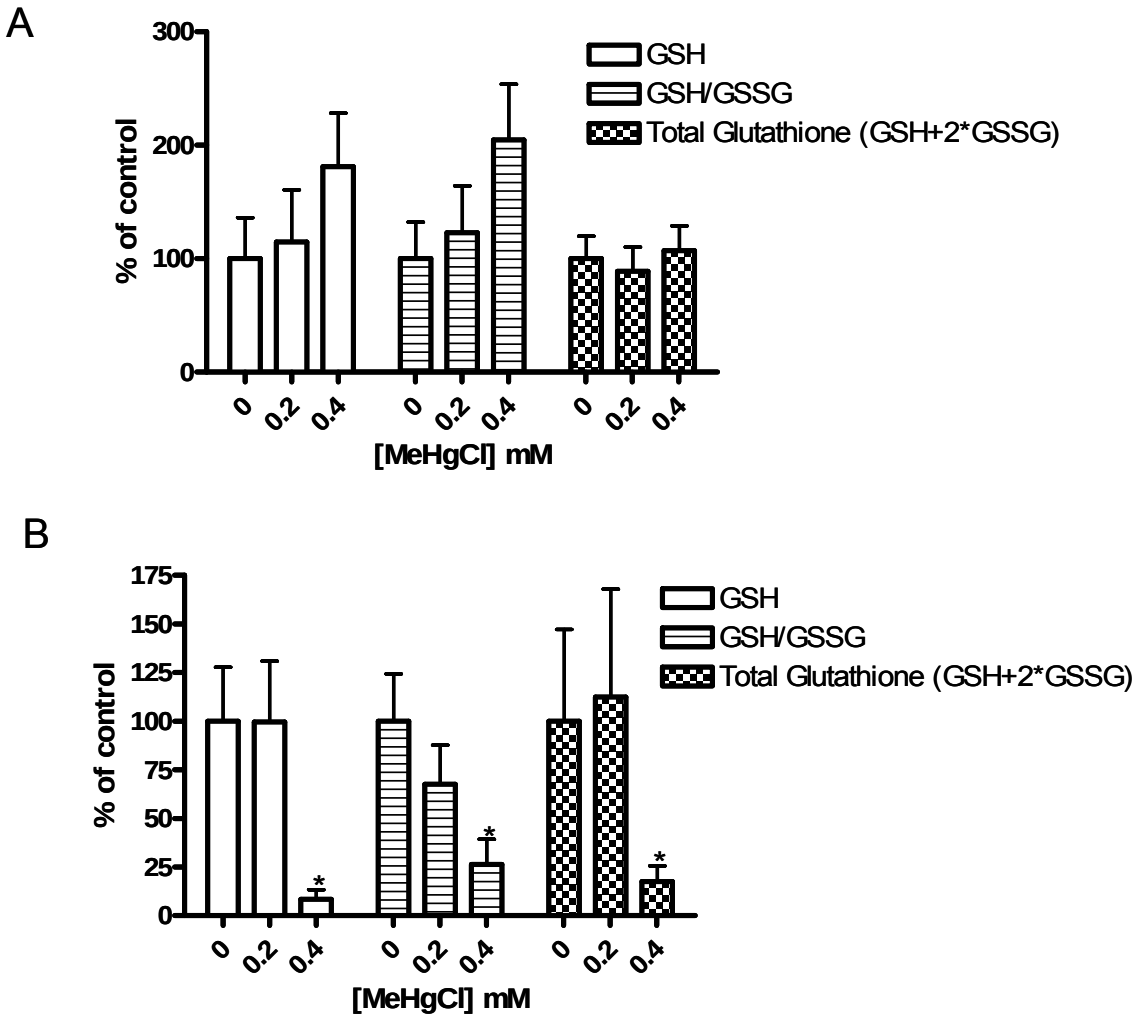

Figure 19. Glutathione levels in *C. elegans* treated with MeHgCl. Levels of GSH and GSSG/GSH ratio increased after acute exposure (A) while these values along with total glutathione level decreased after chronic exposure (B).

The current studies showed that in *C. elegans*, MeHgCl exposure resulted in increasing levels of Hg accumulation in animals that survived exposure to the toxicant. MeHgCl induced alterations in the expression of MT, GST, and HSP, all of which have been implicated in resistance to the organometal. These data show that expression of *gst-4* and *hsp-4* is induced by a long exposure time of L4 animals to MeHgCl while expression of *mtl-1* and *gst-4* is induced by a short exposure time of L1 animals to MeHgCl. These results indicate expression of these proteins is upregulated following exposure to MeHgCl. Even though no increases in *mtls* were noted following a chronic exposure at the L4 stage, a lack of these proteins conferred increased sensitivity in *C. elegans*, indicating that while *mtls* are not induced by the toxicant, they are involved in protection and detoxification.

Interestingly, despite the induction of *gst-4* upon exposure to MeHgCl, no shift in the lethality dose-response curve was observed of the *gst-4* knockout strain, indicating that its absence does not increase the sensitivity of the animal to the toxicant. One possible explanation for this result is that while *gst-4* is involved in the response to the toxicant, other mechanisms are able to compensate in its absence. *C. elegans* express nearly 50 GSTs (van Rossum *et al.*, 2001), approximately 10 HSPs of the HSP70 family (Heschl and Baillie, 1989), and 2 MTs (Freedman *et al.*, 1993). Previous work in *C. elegans* has shown that *gst-4* is upregulated in response to a variety of stressors, including paraquat (Tawe *et al.*, 1998), juglone (Kampkotter *et al.*, 2007; Kahn *et al.*, 2008), hyperbaric oxygen (Link and Johnson, 2002), progesterone (Custodia *et*

al., 2001), diethylstilbetrol (Reichert and Menzel, 2005), and acrylamide (Tawe *et al.*, 1998; Hasegawa *et al.*, 2008); *hsp-4* in response to heat, tunicamycin (Calfon *et al.*, 2002), and irradiation (Bertucci *et al.*, 2009); and *mtl-1* and *mtl-2* in response to cadmium and heat (Freedman *et al.*, 1993; Swain *et al.*, 2004). These proteins are involved in the nematode's response to the toxicants and may be mediating detoxification processes. Our results indicate that some of the same mechanisms are involved in detoxification in *C. elegans* as have been identified in other model systems (Sacco *et al.*, 1997; Schlawicke Engstrom *et al.*, 2008; Yoshida *et al.*, 2008).

MeHg induces the generation of ROS, mediators of MeHg toxicity in glial and neuronal cell culture (Sarafian and Verity, 1991; Yee and Choi, 1996). MTs are free radical scavengers and are induced in response to oxidative stress (Bauman *et al.*, 1991; Maret, 2008) and also in response to MeHg exposure (Rising *et al.*, 1995). The role of GSH in ROS elimination has been well-established and maintenance of GSH levels following MeHg exposure protects cells from oxidative injury (Kaur *et al.*, 2006). Our results demonstrate increases in *gst-4*, GSH, *hsp-4* and *mtl-1*, which implicate the induction of oxidative stress by MeHg, corroborating results in mammalian systems (Garg and Chang, 2006; Reardon, 2007).

Most parameters examined after the L1 acute treatment both in these studies and in previous studies (Helmcke *et al.*, 2009) demonstrated slight or no changes. It is however possible that the 30-minute exposure may be too short to induce alterations large enough to be quantified in our assays. Although the

investigated proteins may contribute to the resistance of the *C. elegans* nervous system to MeHgCl, further investigation of these mechanisms, including the use of knockouts or RNAi can be used to further establish the mechanisms involved.

The hormesis phenomenon has been established in many systems upon exposure to various stressors. Given the effects of MeHgCl on *gst-4*, *hsp-4*, *mtl-1*, and *mtl-2*, we examined whether these proteins play a role in hormesis and whether changes in expression levels afford protection to *C. elegans*. We observed that animals with prior early exposure to MeHgCl showed a significant increase in resistance to a subsequent exposure to MeHgCl. Although this was the first time MeHgCl was shown to have this hormetic effect on *C. elegans,* this phenomenon is not unique to MeHgCl and has been observed when *C. elegans* were exposed to stressors, such as dietary restriction and heat-stress (Cypser *et al.*, 2006). The hormesis effect is also not unique to *C. elegans* and has been shown in systems ranging from cells to humans upon exposure to a variety of stressors, including Hg and Cd exposure to cells (Damelin *et al.*, 2000), radiation exposure to rodents (Zhang *et al.*, 2009), and exercise and caloric restriction to humans (Calabrese, 2005; Mattson, 2008b).

Our results indicate that adaptation takes place in animals exposed to MeHgCl which renders them better-equipped to deal with a second exposure to the same stressor. There are many potential explanations and candidate proteins responsible for this phenomenon, including the upregulation of proteins involved in detoxification, upregulation of proteins involved in excretion or downregulation of proteins involved in uptake, only one of which we elucidated here. Further

investigation, such as microarray experiments will be valuable for identifying the other specific proteins involved, potentially other GSTs or HSPs, in MeHgCl resistance, and could perhaps enable humans to more effectively deal with poisoning events.

Here, we assessed whether MTs, a GST, or a HSP could contribute to the hormetic response of *C. elegans*. Since these proteins are not upregulated prior to the second insult of MeHgCl, these data cannot explain the hormetic phenotype observed in the lethality experiments. However, expression of *gst-4::GFP* increased as the acute exposure concentration of MeHgCl increased while chronic exposure conditions remained at baseline. Large increases in *gst-4::GFP* fluorescence are consistent with the proposal that GST levels are elevated in response to MeHgCl toxicity. However, the involvement of *gst-4* in the hormetic response remains unclear. While increases in fluorescence were noted in groups with increasing initial exposure concentrations when the subsequent treatment was 0 mM MeHgCl, this same trend was not observed upon exposure to higher chronic concentrations of MeHgCl. This lack of a further increase in fluorescence could be attributed to a ceiling effect or an inability to differentiate very bright fluorescence. The increase in fluorescence, even at control conditions does provide some evidence that *gst-4* could play a role in hormesis, since initial exposure to increasing MeHgCl concentrations induces an increase in fluorescence in subsequent stressful treatment conditions. We also observed increases in *mtl-1* and *mtl-2* expression when animals were preconditioned with MeHgCl. These results were inconsistent with our previous observations

demonstrating the lack of an increase in fluorescence in these strains upon a single chronic exposure to MeHCl. However, as this trend occurred in both animals treated under control conditions and animals treated with higher concentrations of MeHgCl, we hypothesize that these proteins are not responsible for the shift in the lethality dose-response curve upon hormesis.

The lack of an increase and the presence of a decrease in fluorescence in the other proteins examined (*hsp-4*, *mtl-1*, and *mtl-2*) was surprising, particularly given the data outlined earlier in the paper demonstrating some increases in fluorescence and sensitivity to knockouts of strains carrying GFP reporters or knockouts of these proteins. These data indicate that a mechanism that we did not examine plays a significant role in hormesis, and perhaps its involvement in this process renders the examined proteins less-important in this response.

After observing the involvement of *gst-4*, we further explored the contribution of GSH to the toxicity of MeHgCl. MeHgCl induced an increase in GSH and the GSH/GSSG ratio upon acute L1 exposure. Results showed that total GSH levels remained unchanged. These results indicate that acute MeHgCl increases production of GSH, assisting with the detoxification of MeHgCl. Increasing the duration of MeHgCl exposure in *C. elegans* at L4 to 15 hours resulted in a vastly different glutathione profile. At 0.2 mM MeHgCl, the GSH levels remained constant while the GSH/GSSG ratio decreased, caused by an increase in the amount of GSSG, presumably due to an accumulation of reactive oxygen species. At 0.4 mM MeHgCl, GSH, the GSH/GSSG ratio and the total glutathione levels all significantly decreased, indicating that GSH is both

converted to GSSG due to the presence of reactive oxygen species and excreted in a complex with MeHg. Taken with the *gst-4::GFP* data, these results confirm the involvement of glutathione in MeHgCl toxicity in *C. elegans*. These data suggest that the increase in *gst-4* successfully catalyzes the conjugation of MeHg to GSH, which is then excreted, causing the GSH and total glutathione levels to decrease. These data corroborate what has been found in mammalian systems, with MeHg being excreted as a complex with glutathione (Hirata and Takahashi, 1981). Additionally, alterations in the glutathione cycle can cause alterations in MeHg metabolism, such as a depletion of GSH leading to a decreased rate of conjugation to MeHg and a decrease in MeHg excretion (Schlawicke Engstrom *et al.*, 2008). These findings indicate that independent lowering of GSH level would sensitize animals to MeHg while increasing GSH level may be protective. Taken together, our findings of alterations in GSH, *gst-4*, *mtl-1,* and *mtl-2* confirm the involvement of ROS in MeHgCl toxicity and the ability of these proteins to confer resistance as shown by studies in other systems.

While previous researchers have shown the toxicity of MeHg and have identified some mechanisms involved in detoxification, we furthered their work by examining the role of GSH, HSP, and MTs in protection from MeHgCl, specifically by examining their role in hormesis. Our work begins to elucidate potential mechanisms of MeHgCl toxicity and neuroprotection in *C. elegans*, however, many other pathways are likely involved. Future studies should confirm our results based on GFP reporter expression as examined by fluorescence by conducting quantitative PCR experiments. To further extend this work,

experiments should be designed to reveal the pathways responsible for the hormetic response. Induction or supplementation of components of these pathways could afford a better understanding of resistance to toxicants, stress, or aging. Hormesis, in cases where exposure to very low doses may be beneficial while the slightest increase could have deleterious effects, needs to be better understood. Due to its ease of use and the genetic advantages associated with its use, *C. elegans,* especially with respect to the availability of knockout and GFP-tagged strains, is an ideal model for future studies. Techniques such as microarray experiments can be used to identify candidate genes involved in hormesis, which can be further investigated using the immense genetic advantages that the *C. elegans* model provides.

CHAPTER IV

CONCLUSION

Summary

MeHg is a toxicant known to induce nervous system damage in humans although the mechanisms through which it causes this damage remain poorly understood. Since humans are regularly exposed to MeHg through consumption of seafood and can be exposed through poisoning events as occurred in Minimata Bay and Iraq, an understanding of the molecular mechanisms of MeHg toxicity and protection are essential for discovering potential therapeutics. A model such as *C. elegans*, featuring a simple nervous system in an intact organism was an ideal model for this research as it could be used as a high throughput system to assess death and protection to assist with the discovery of molecules involved in MeHg toxicity and protection.

Although a number of endpoints we measured indicated a sensitivity of *C. elegans* to MeHg, we were not able to demonstrate alterations in the morphology of the nervous system following exposure to MeHg as expected. Due to the lack of alterations induced in the nervous system of this model, *C. elegans* may be a poor model for studying MeHg-induced neurotoxicity. However, our model can provide valuable insights into the mechanisms of MeHg toxicity and protection of this model system and its nervous system. Previous experiments in other systems have indicated the involvement of pathways in MeHg toxicity and

detoxification. For example, relating to the systems we examined, GSH acts as an antioxidant and binds directly to MeHg, facilitating its elimination; MTs can also act as antioxidants and can bind to MeHg, sequestering it in the system to prevent binding to other targets; and HSPs can reduce or prevent damage caused by MeHg by acting as a molecular chaperone to assist with the degradation or repair of proteins damaged by MeHg. Our investigations revealed alterations in some pathways contributing to MeHg detoxification upon exposure to the toxicant which differ depending on the age of the animal treated and the duration of the exposure (Figure 1A). In L1 animals treated for 30 minutes, we observed increases in *gst-4*, GSH, and MT expression, implicating the involvement of these pathways in detoxification of the toxicant following a short-duration exposure at a young age (Figure 1B). Following a 15-hour treatment of L4 animals, we observed an increase in *gst-4* and *hsp-4*, but no alteration in MT expression although MT knockout animals were more sensitive to the toxicant under this treatment paradigm. We also observed an increase in GSSG levels and a depletion of GSH, indicating that the glutathione system could not compensate to protect the older organisms over a long-duration exposure (Figure 1C). Neither *hsp-4* nor MTs played a role in preconditioning, while *gst-4* was upregulated (Figure 1D).

One explanation for the lack of alterations in many parameters including altered sensitivity of knockouts and alterations in GFP reporter fluorescence intensity upon MeHg exposure in L1 animals treated for 30 minutes may be that the exposure duration is too short to induce alterations in protein expression or

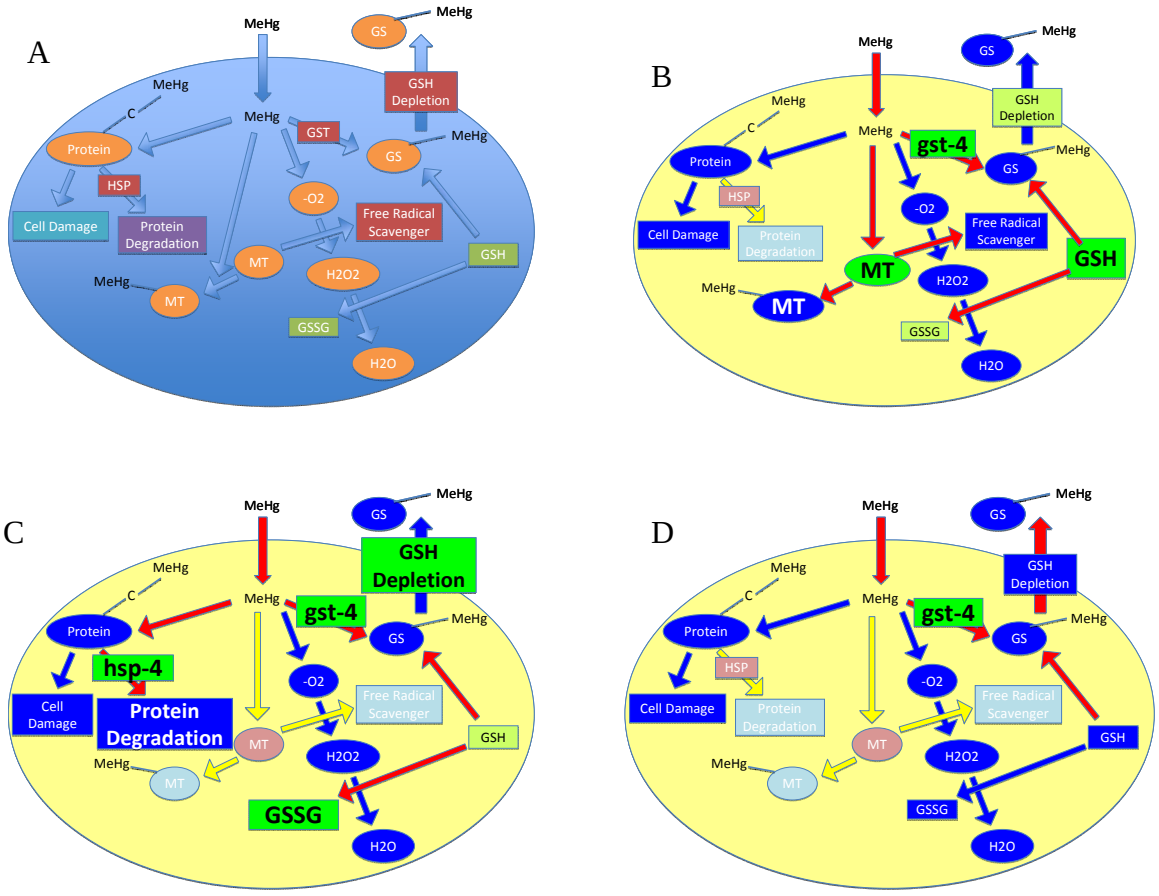

Figure 1. Model of the molecular mechanisms of MeHg toxicity. MeHg induces alterations in the cell by generating ROS and binding directly to Cys groups on proteins. These induce a number of downstream effects, including induction of HSPs to induce degradation of damaged proteins, MTs to bind free MeHg and reduce ROS, and GSH to reverse ROS damage and bind directly to MeHg for excretion (A). In L1 animals treated acutely with MeHg, MTs, *gst-4*, and GSH are all upregulated, assisting with MeHg detoxification (B). In L4 animals treated chronically with MeHg, levels of *hsp-4*, *gst-4*, and GSSG are increased, and GSH is depleted (C). In preconditioning, *gst-4* is increased. Due to the increase in *gst-4*, we suspect alterations in the GSH system, but these have not been assessed (D).

nervous system morphology. Additionally, the Hg levels that were measured in these animals were quite low, possibly below the level required to induce alteration in levels of the proteins we tested. In young (L1) animals, Hg levels were on the order of three times lower than levels in older (L4) animals treated under the same conditions. This could occur due to a lack of protective mechanisms in young *C. elegans*, for example their development later in the animal's life cycle, or exposure to the toxicant that induces damage at a critical window of development that cannot be repaired early in the animal's life cycle. Despite the low accumulation of Hg in these animals, MeHg displayed higher toxicity in these younger animals than in the older animals, indicating that MeHg may be deleterious to the developmental processes in the young animal or that compensatory mechanisms of MeHg toxicity are not yet established in L1 worms. MeHg might induce death via some pathway unrelated to those we examined at low concentrations in these young animals, not allowing accumulation of the toxicant to levels high enough to induce dramatic alterations in the systems that we did examine.

The animals treated chronically at the L4 stage do display more of the expected effects such as increased expression of *hsp-4*, GSH depletion, and shifts in the lethality curves of *mtl-1* and *mtl-2* knockout animals, however, these alterations did not occur in all of the systems that we examined. The emergence of these expected effects may be due to higher accumulation of Hg within the animals and a longer duration of exposure to the toxicant. The lack of an effect on nervous system morphology may be explained by the fact that the nervous

system is already largely in place by the L4 stage, so that exposing animals at this time point would not induce developmental disruptions. A lack of alterations in some of the other systems we tested may be attributable to differences between *C. elegans* and mammalian models or redundancy within the system. Alternate proteins may take over the role of others in knockout models. It is possible that a suite of proteins, each having a small alteration in their expression level, is induced, with the change in each protein being small and statistically undetectable by our methods.

While many of the endpoints that we measured, including lifespan, brood size, and thrashing behavior, did not reveal a MeHg dose-dependent alteration, we did observe alterations in size, development, and pharyngeal pumping rate. One explanation for the presence of alterations in these specific endpoints is that *C. elegans* mounts an adaptive response to MeHg by preventing the uptake of additional MeHg by reducing pharyngeal pumping rate and stunting growth and development until more favorable conditions are encountered.

We did not observe obvious alterations in the nervous system of *C. elegans* upon exposure to MeHg. This is potentially due to the presence of novel pathways that are able to protect the *C. elegans* nervous system from the toxicant. Our behavioral experiments indicated that there may be some alteration in the pharyngeal nervous system upon exposure to MeHg, but our thrashing assays indicated that there is not an effect of the toxicant on overall movement of the animal. However, we did not directly test neuronal function, and while

morphology appeared unaltered, it is possible that the neurons have alterations in different parameters such as in signal transduction or neurotransmitter content.

In the hormesis model, we noted a potential role of *gst-4*. Further experiments, such as an experiment with the *gst-4* KO would be needed to confirm the role of this protein in hormesis since the increase in fluorescence was only observed under control conditions. This could be attributed to a ceiling effect, or the inability to detect greater increases in fluorescence due to the dramatic increase in fluorescence and consequent excessive brightness of these animals. Additionally, the Hg levels in animals treated with MeHg under the hormesis paradigm are unknown. Prior exposure to MeHg could alter the ability of Hg to accumulate within the worm by altering pathways related to uptake, including pharyngeal pumping, or excretion, which could lead to a decrease in Hg content of the animals previously exposed to MeHg.

In our experiments, we noted a dramatic increase in expression of some proteins (*gst-4* under L4 chronic conditions), more subtle increases in other proteins (*gst-4* under L1 acute conditions, *hsp-4* and *mtl-1* under L4 chronic conditions), and no increase in other proteins (*mtl-2* under L4 chronic conditions, *mtls* and *hsp-4* under L1 acute conditions). Although we initially expected to see more dramatic increases in expression, our results indicate that in response to MeHg, *C. elegans* is able to upregulate specific proteins. This provides important evidence that MeHg is not simply inducing the expression of many proteins involved in the stress response, but that it selectively induces upregulation of proteins that might be involved explicitly in the protection of the organism from

the particular toxicant. Upregulation was less dramatic in young worms, which may be due to a smaller effect in these worms, but could also be explained by the shorter duration of exposure to the toxicant. In mammalian systems, researchers have observed similar effects, such as upregulation of antioxidant genes. Additional experiments, such as microarray experiments, have demonstrated the specificity of gene up- or down-regulation upon MeHg exposure in cellular or mammalian systems (Hwang and Naganuma, 2006; Padhi *et al.*, 2008; Glover *et al.*, 2009).

The most dramatic molecular alterations that we observed were in the glutathione system, pointing to the primary involvement of glutathione in the response to and protection from MeHg toxicity. This could blunt the effect of MeHg on other secondary systems, leading to a decreased response in the presence of glutathione. Without the protection from the glutathione system, perhaps the *mtls* and *hsp-4* would play a more prominent role in the detoxification of MeHg from the *C. elegans* system.

Though we did reveal these potential mechanisms of protection from MeHg, we did not elucidate the mechanism of death from MeHg toxicity in *C. elegans*. Since we did not observe obvious morphological alterations in the nervous system, we hypothesize that death is occurring via a mechanism unrelated to the nervous system. A decrease in pharyngeal pumping rate was observed upon exposure to MeHg. Since this contributes to the ability of the worm to eat, lowering or completely ceasing pharyngeal pumping action could cause damage to the worm, however, due to the lack of dauer formation of

animals treated with MeHg, this mechanism of death seems unlikely. MeHg could induce necrosis or apoptosis of *C. elegans* cells essential for life. Mechanisms for protecting animals from MeHg insult could also be essential for other functions within the worm. When these systems are monopolized by MeHg, they are not able to exert their normal function and lead to death of the animal. Further experimentation on systems that could lead to MeHg-induced death in *C. elegans* are required to elucidate the mechanism of death.

Future Directions

These experiments have laid an excellent foundation for the discovery of targets of MeHg toxicity and protection, however, further experimentation will confirm and further our results. While we observed no changes in the structure of the nervous system, our studies do not address the functioning of the nervous system beyond gross behavioral experiments. Further experiments could include a more in-depth observation of potential effects of neurotransmitter systems that were not quantitatively assessed in these studies, such as assessing animals treated with MeHg for resistance to aldicarb, an acyetylcholinesterase inhibitor, which could reveal alterations in synaptic transmission (Nonet *et al.*, 1998) or behavioral assays aimed at assessing the functioning of specific neuronal circuits. Our experiments assessed L1s treated acutely and L4s treated chronically. Experiments changing these paradigms, such as treating L1s for a longer duration may yield interesting findings regarding the ability of a lower

concentration of MeHg to induce alterations in the nervous system of young worms treated over a long duration.

The lack of a neuronal phenotype is unique when comparing *C. elegans* to mammalian systems and future research should investigate the mechanisms that *C. elegans* employs for neuroprotection. For example, a microarray experiment comparing control-treated to MeHg-treated animals would provide valuable insights into potential candidate genes that demonstrate altered expression upon MeHg exposure that contribute to the protection of the nervous system or to the hormetic phenomenon we observed. One candidate to test is *skn-1*, the *C. elegans* homolog of Nrf2, a protein demonstrated to confer resistance to MeHg in cellular and *Drosophila* models (Rand *et al.*, 2009; Wang *et al.*, 2009). The described experiments only examined one *hsp* and *gst*. Other candidates, such as *hsp-16*, also shown to be upregulated in a hormesis model (Olsen *et al.*, 2006) should be examined. Our research indicated that while GSTs, HSPs, and MTs may be involved in these processes, it is likely that other mechanisms of defense may also play a role. After identification of potential contributing factors, this model system could be employed to conduct experiments using knockout or overexpression strains. Testing alterations in the response to these strains either in the organism or specifically in the nervous system (such as an increased sensitivity of knockouts or a decreased sensitivity of overexpression strains) would be crucial for determining the involvement of specific proteins in protection of *C. elegans* from MeHg.

Additional experiments should address the contributions of GSTs, HSPs, and MTs to MeHg toxicity in *C. elegans*. For example, quantitative PCR experiments should be used to confirm our results based on GFP reporter fluorescence. Our experiments only tested the increase in fluorescence or a reporter for the genes described. We did not assess protein level nor the activity level of these proteins. While a knockout of glutathione is embryonically lethal in *C. elegans* (*gcs-1*, the rate-limiting enzyme in glutathione synthesis, RNAi treatment induces embryonic lethality (WormBase)), experiments using agents known to alter glutathione levels could answer questions not only regarding the involvement of glutathione in MeHg toxicity, but also those related to the involvement of secondary pathways that might be masked by the involvement of glutathione in detoxification of MeHg. Although *gst-4* does demonstrate increased expression upon MeHg exposure, the lack of *gst-4* does not change the dose-response effect of MeHg on the organism. Knocking out all of the *gst*s could help to reveal the importance of these proteins and the GSH system in MeHg protection and detoxification, however, as *C. elegans* contains approximately 50 of these proteins, this work could be difficult and could lead to an embryonic lethal strain.

An interesting extension of this work would be to determine the role of *gst-4* in MeHg toxicity and whether other *gsts* or alternate proteins are able to compensate for *gst-4* loss. Conversely, *mtls* demonstrated little to no alterations in their expression upon MeHg exposure but their lack rendered L4 animals treated chronically more sensitivity to the toxicant. One extension of this finding

would be to determine how *mtl*s are able to afford protection to MeHg without being upregulated and whether there are alterations in MeHg accumulation in *mtl* knockout animals. For example, one such experiment would examine the glutathione profile of *mtl* knockout animals following treatment with MeHg.

The ability of MeHg to induce hormesis and the mechanisms involved in this process should also be further examined. Future experiments could assess the ability of Hg to accumulate in *C. elegans* preconditioned with MeHg. A decrease in the level of Hg in preconditioned animals would help to describe mechanisms of detoxification through decreased uptake or increased excretion, which contribute to hormesis. Additionally, experiments could be directed toward determining whether hormesis is time-dependent, i.e., whether the initial exposure must occur during some critical window or whether hormesis can be induced by MeHg exposure at any point in the *C. elegans* life cycle. Further investigations into the effects of preconditioning on GSH and GSSG levels as well as the ability of MeHg to induce hormesis in *gst-4* knockouts will help to reveal the mechanisms of this phenomenon.

The mechanism of death and the protection of the morphology of the nervous system should also be further examined. By examining systems required for life in *C. elegans* but that aren't involved in endpoints such as lifespan and brood size (since no alterations were observed in these parameters), researchers can discover the mechanisms of death upon exposure to MeHg. One important finding of this research was the decrease in pharyngeal pumping rate, which likely led to a developmental delay. One way to test a potential contribution of

pharyngeal pumping rate to MeHg toxicity would be to expose animals with alterations in pharyngeal pumping rate (such as *eat* mutants) to MeHg to determine whether MeHg acts synergistically with the mutation and causes greater death. An important control to consider in these experiments would be to determine the Hg content of these animals under various MeHg concentrations since they may accumulate MeHg at a decreased rate due to a decrease in pumping. Experiments testing the potential contribution of necrosis or apoptosis should also be conducted. Mutant animals, for example those with a suppressed ability to induce necrotic-like pathways (such as calreticulin mutants (Xu *et al.*, 2001)) could be used to determine whether necrosis plays an important role in MeHg toxicity. If it does play an important role, protection would be afforded in such a mutant. Research using markers of necrosis and apoptosis would also be valuable since these studies would reveal not only a mechanism of death but also in which cells this process may be important.

Additional experiments should also examine the sensitivity of *C. elegans* neurons to MeHg. Although we measured Hg content in *C. elegans*, the specific content in the environment of *C. elegans* neurons was not assessed. Therefore, experiments such as *C. elegans* neuronal cell cultures will be instrumental for discovering at which MeHg concentration alterations in *C. elegans* neurons are observed.

Implications

Our research has a number of implications for toxicology research and human health. We demonstrated the usefulness of the *C. elegans* model system in toxicology research. Although it displays high homology with mammalian systems, the processes and mechanisms are not always identical in the two systems. We demonstrated this in our data revealing that the *C. elegans* nervous system morphology was largely unaffected by MeHg while previous researchers had shown major alterations in the architecture of the mammalian nervous system upon exposure to the same toxicant. While this could signify an inability to use this system as a model for studying MeHg toxicity, or even toxicity research in general, we used this to our advantage. Revealing these alterations is as important as revealing similarities, as these alterations could provide hints and directions for the pursuit of therapies to protect or heal damage caused by toxicants. Our experiments demonstrate the advantages of using *C. elegans* as a model for toxicology research, even in cases of divergent responses from mammalian systems, such as we found with MeHg toxicity. Follow-up studies furthering our research could reveal genes that confer resistance to MeHg and could assist in the identification of pharmacological interventions aimed at preventing or repairing damage caused by MeHg toxicity.

REFERENCES

Allen, J. W., Shanker, G., and Aschner, M. (2001). Methylmercury inhibits the in vitro uptake of the glutathione precursor, cystine, in astrocytes, but not in neurons. *Brain Res* **894**, 131-140.

Altun-Gultekin, Z., Andachi, Y., Tsalik, E. L., Pilgrim, D., Kohara, Y., and Hobert, O. (2001). A regulatory cascade of three homeobox genes, ceh-10, ttx-3 and ceh-23, controls cell fate specification of a defined interneuron class in *C. elegans*. *Development* **128**, 1951-1969.

Amin, V., Cumming, D. V., and Latchman, D. S. (1996). Over-expression of heat shock protein 70 protects neuronal cells against both thermal and ischaemic stress but with different efficiencies. *Neurosci Lett* **206**, 45-48.

Anderson, G. L., Boyd, W. A., and Williams, P. L. (2001). Assessment of sublethal endpoints for toxicity testing with the nematode *Caenorhabditis elegans*. *Environ Toxicol Chem* **20**, 833-838.

Anderson, G. L., Cole, R. D., and Williams, P. L. (2004). Assessing behavioral toxicity with *Caenorhabditis elegans*. *Environ Toxicol Chem* **23**, 1235-1240.

Andrews, G. K. (2000). Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem Pharmacol* **59**, 95-104.

Antoshechkin, I., and Sternberg, P. W. (2007). The versatile worm: genetic and genomic resources for *Caenorhabditis elegans* research. *Nat Rev Genet* **8**, 518-532.

Aschner, M. (2000). Astrocytic swelling, phospholipase A2, glutathione and glutamate: interactions in methylmercury-induced neurotoxicity. *Cell Mol Biol (Noisy-le-grand)* **46**, 843-854.

Aschner, M., Conklin, D. R., Yao, C. P., Allen, J. W., and Tan, K. H. (1998). Induction of astrocyte metallothioneins (MTs) by zinc confers resistance against the acute cytotoxic effects of methylmercury on cell swelling, Na+ uptake, and K+ release. *Brain Res* **813**, 254-261.

Aschner, M., Du, Y. L., Gannon, M., and Kimelberg, H. K. (1993). Methylmercury-induced alterations in excitatory amino acid transport in rat primary astrocyte cultures. *Brain Res* **602**, 181-186.

Aschner, M., Eberle, N. B., Goderie, S., and Kimelberg, H. K. (1990). Methylmercury uptake in rat primary astrocyte cultures: the role of the neutral amino acid transport system. *Brain Res* **521**, 221-228.

Asikainen, S., Vartiainen, S., Lakso, M., Nass, R., and Wong, G. (2005). Selective sensitivity of *Caenorhabditis elegans* neurons to RNA interference. *Neuroreport* **16**, 1995-1999.

Atchison, W. D. (2005). Is chemical neurotransmission altered specifically during methylmercury-induced cerebellar dysfunction? *Trends Pharmacol Sci* **26**, 549-557.

Avery, L., and Horvitz, H. R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron* **3**, 473-485.

Ayensu, W. K., and Tchounwou, P. B. (2006). Microarray analysis of mercury-induced changes in gene expression in human liver carcinoma (HepG2) cells: importance in immune responses. *Int J Environ Res Public Health* **3**, 141-173.

Baraldi, M., Zanoli, P., Tascedda, F., Blom, J. M., and Brunello, N. (2002). Cognitive deficits and changes in gene expression of NMDA receptors after prenatal methylmercury exposure. *Environ Health Perspect* **110 Suppl 5**, 855-858.

Bauman, J. W., Liu, J., Liu, Y. P., and Klaassen, C. D. (1991). Increase in metallothionein produced by chemicals that induce oxidative stress. *Toxicol Appl Pharmacol* **110**, 347-354.

Bertucci, A., Pocock, R. D., Randers-Pehrson, G., and Brenner, D. J. (2009). Microbeam irradiation of the *C. elegans* nematode. *J Radiat Res (Tokyo)* **50 Suppl A**, A49-54.

Bischof, L. J., Huffman, D. L., and Aroian, R. V. (2006). Assays for toxicity studies in *C. elegans* with Bt crystal proteins. *Methods Mol Biol* **351**, 139-154.

Bondy, S. C. (1994). Chapter 20: Induction of oxidative stress in the brain by neurotoxic agents. In *Principles of neurotoxicology* (L. W. Chang, Ed.), pp. 563-582. Marcel Dekker, Inc., New York.

Bourg, E. L. (2009). Hormesis, aging and longevity. *Biochim Biophys Acta*.

Boyd, W. A., Cole, R. D., Anderson, G. L., and Williams, P. L. (2003). The effects of metals and food availability on the behavior of *Caenorhabditis elegans*. *Environ Toxicol Chem* **22**, 3049-3055.

Boyd, W. A., McBride, S. J., and Freedman, J. H. (2007). Effects of genetic mutations and chemical exposures on *Caenorhabditis elegans* feeding: evaluation of a novel, high-throughput screening assay. *PLoS ONE* **2**, e1259.

Braungart, E., Gerlach, M., Riederer, P., Baumeister, R., and Hoener, M. C. (2004). *Caenorhabditis elegans* MPP+ model of Parkinson's disease for high-throughput drug screenings. *Neurodegener Dis* **1**, 175-183.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Brookes, N. (1992). In vitro evidence for the role of glutamate in the CNS toxicity of mercury. *Toxicology* **76**, 245-256.

Burbacher, T. M., Rodier, P. M., and Weiss, B. (1990). Methylmercury developmental neurotoxicity: a comparison of effects in humans and animals. *Neurotoxicol Teratol* **12**, 191-202.

Byerley, R. C., Cassada, R. C., and Russell, R. L. (1976). The life cycle of the nematode *Caenorhabditis elegans*. *Dev Biol* **51**, 23-33.

C. elegans sequencing consortium. (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**, 2012-2018.

Calabrese, E. J. (2005). Cancer biology and hormesis: human tumor cell lines commonly display hormetic (biphasic) dose responses. *Crit Rev Toxicol* **35**, 463-582.

Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**, 92-96.

Carlezon, W. A., Jr., Duman, R. S., and Nestler, E. J. (2005). The many faces of CREB. *Trends Neurosci* **28**, 436-445.

Castoldi, A. F., Barni, S., Turin, I., Gandini, C., and Manzo, L. (2000). Early acute necrosis, delayed apoptosis and cytoskeletal breakdown in cultured cerebellar granule neurons exposed to methylmercury. *J Neurosci Res* **59**, 775-787.

Castoldi, A. F., Coccini, T., Ceccatelli, S., and Manzo, L. (2001). Neurotoxicity and molecular effects of methylmercury. *Brain Res Bull* **55**, 197-203.

Castoldi, A. F., Onishchenko, N., Johansson, C., Coccini, T., Roda, E., Vahter, M., Ceccatelli, S., and Manzo, L. (2008). Neurodevelopmental toxicity of methylmercury: Laboratory animal data and their contribution to human risk assessment. *Regul Toxicol Pharmacol* **51**, 215-229.

Cernichiari, E., Brewer, R., Myers, G. J., Marsh, D. O., Lapham, L. W., Cox, C., Shamlaye, C. F., Berlin, M., Davidson, P. W., and Clarkson, T. W. (1995). Monitoring methylmercury during pregnancy: maternal hair predicts fetal brain exposure. *Neurotoxicology* **16**, 705-710.

Chalfie, M., and White, J. (1988). The Nervous System. In *The Nematode Caenorhabditis elegans* (W. B. Wood, Community of *C. elegans* Researchers, Ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Chase, D. L., Pepper, J. S., and Koelle, M. R. (2004). Mechanism of extrasynaptic dopamine signaling in *Caenorhabditis elegans*. *Nat Neurosci* **7**, 1096-1103.

Chen, B. L., Hall, D. H., and Chklovskii, D. B. (2006). Wiring optimization can relate neuronal structure and function. *Proc Natl Acad Sci U S A* **103**, 4723-4728.

Choi, B. H., Lapham, L. W., Amin-Zaki, L., and Saleem, T. (1978). Abnormal neuronal migration, deranged cerebral cortical organization, and diffuse white matter astrocytosis of human fetal brain: a major effect of methylmercury poisoning in utero. *J Neuropathol Exp Neurol* **37**, 719-733.

Cioci, L. K., L. Qui, and J.H. Freedman (2000). Transgenic strains of the nematode *Caenorhabditis elegans* as biomonitors of metal contamination. *Environmental Toxicology and Chemistry* **19**, 2122-2129.

Clarkson, T. W. (2002). The three modern faces of mercury. *Environ Health Perspect* **110 Suppl 1**, 11-23.

Clarkson, T. W., Amin-Zaki, L., and Al-Tikriti, S. K. (1976). An outbreak of methylmercury poisoning due to consumption of contaminated grain. *Fed Proc* **35**, 2395-2399.

Clarkson, T. W., and Magos, L. (2006). The toxicology of mercury and its chemical compounds. *Crit Rev Toxicol* **36**, 609-662.

Clarkson, T. W., Magos, L., Cox, C., Greenwood, M. R., Amin-Zaki, L., Majeed, M. A., and Al-Damluji, S. F. (1981). Tests of efficacy of antidotes for removal of methylmercury in human poisoning during the Iraq outbreak. *J Pharmacol Exp Ther* **218**, 74-83.

Coccini, T., Randine, G., Candura, S. M., Nappi, R. E., Prockop, L. D., and Manzo, L. (2000). Low-level exposure to methylmercury modifies muscarinic cholinergic receptor binding characteristics in rat brain and lymphocytes: physiologic implications and new opportunities in biologic monitoring. *Environ Health Perspect* **108**, 29-33.

Colavita, A., and Tessier-Lavigne, M. (2003). A Neurexin-related protein, BAM-2, terminates axonal branches in *C. elegans*. *Science* **302**, 293-296.

Cole, R. D., Anderson, G. L., and Williams, P. L. (2004). The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity. *Toxicol Appl Pharmacol* **194**, 248-256.

Cui, Y., Boyd, W. A., McBride, S. J., and Freedman, J. H. (2007). Toxicogenomic analysis of cadmium responsive transcription in *Caenorhabditis elegans* reveals novel genes and pathways involved in heavy metal resistance. SOT meeting 2007.

Custodia, N., Won, S. J., Novillo, A., Wieland, M., Li, C., and Callard, I. P. (2001). *Caenorhabditis elegans* as an environmental monitor using DNA microarray analysis. *Ann N Y Acad Sci* **948**, 32-42.

Cypser, J. R., Tedesco, P., and Johnson, T. E. (2006). Hormesis and aging in *Caenorhabditis elegans*. *Exp Gerontol* **41**, 935-939.

Damelin, L. H., Vokes, S., Whitcutt, J. M., Damelin, S. B., and Alexander, J. J. (2000). Hormesis: a stress response in cells exposed to low levels of heavy metals. *Hum Exp Toxicol* **19**, 420-430.

Davidson, P. W., Myers, G. J., Cox, C., Axtell, C., Shamlaye, C., Sloane-Reeves, J., Cernichiari, E., Needham, L., Choi, A., Wang, Y., Berlin, M., and Clarkson, T. W. (1998). Effects of prenatal and postnatal methylmercury exposure from fish consumption on neurodevelopment: outcomes at 66 months of age in the Seychelles Child Development Study. *Jama* **280**, 701-707.

Davis, J. R., Li, Y., and Rankin, C. H. (2008). Effects of developmental exposure to ethanol on *Caenorhabditis elegans*. *Alcohol Clin Exp Res* **32**, 853-867.

Debes, F., Budtz-Jorgensen, E., Weihe, P., White, R. F., and Grandjean, P. (2006). Impact of prenatal methylmercury exposure on neurobehavioral function at age 14 years. *Neurotoxicol Teratol* **28**, 363-375.

Dhawan, R., Dusenbery, D. B., and Williams, P. L. (1999). Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode *Caenorhabditis elegans*. *Journal of toxicology and environmental health* **58**, 451-462.

Dock, L., Rissanen, R. L., and Vahter, M. (1994). Demethylation and placental transfer of methyl mercury in the pregnant hamster. *Toxicology* **94**, 131-142.

Donkin, S. G. a. P. L. W. (1995). Influence of developmental stage, salts, and food presence on various end points using *Caenorhabditis elegans* for aquatic toxicity testing. *Environmental Toxicology and Chemistry* **14**, 2139-2147.

Donohoe, D. R., Aamodt, E. J., Osborn, E., and Dwyer, D. S. (2006). Antipsychotic drugs disrupt normal development in *Caenorhabditis elegans* via additional mechanisms besides dopamine and serotonin receptors. *Pharmacol Res* **54**, 361-372.

Elliott, K. (2008). Hormesis, ethics, and public policy: an overview. *Hum Exp Toxicol* **27**, 659-662.

EPA (2001). Methymercury (MeHg) (CASRN 22967-92-6). *Integrated Risk Information System*.

Factor-Litvak, P., Hasselgren, G., Jacobs, D., Begg, M., Kline, J., Geier, J., Mervish, N., Schoenholtz, S., and Graziano, J. (2003). Mercury derived from dental amalgams and neuropsychologic function. *Environ Health Perspect* **111**, 719-723.

Falluel-Morel, A., Sokolowski, K., Sisti, H. M., Zhou, X., Shors, T. J., and Dicicco-Bloom, E. (2007). Developmental mercury exposure elicits acute hippocampal cell death, reductions in neurogenesis, and severe learning deficits during puberty. *J Neurochem* **103**, 1968-1981.

Faro, L. R., do Nascimento, J. L., Alfonso, M., and Duran, R. (2002). Mechanism of action of methylmercury on in vivo striatal dopamine release. Possible involvement of dopamine transporter. *Neurochem Int* **40**, 455-465.

Fewell, G. D., and Schmitt, K. (2006). Vector-based RNAi approaches for stable, inducible and genome-wide screens. *Drug Discov Today* **11**, 975-982.

Filomeni, G., Rotilio, G., and Ciriolo, M. R. (2005). Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. *Cell Death Differ* **12**, 1555-1563.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Fitzgerald, W. F., and Clarkson, T. W. (1991). Mercury and monomethylmercury: present and future concerns. *Environ Health Perspect* **96**, 159-166.

Fonnum, F., and Lock, E. A. (2004). The contributions of excitotoxicity, glutathione depletion and DNA repair in chemically induced injury to neurones: exemplified with toxic effects on cerebellar granule cells. *J Neurochem* **88**, 513-531.

Freedman, J. H., Chen, M., Coughlan, S., and Boyd, W. A. (2006). Changes in gene expression associated with exposure to environmental toxicants. Abstract No. 1178. SOT meeting 2006.

Freedman, J. H., Slice, L. W., Dixon, D., Fire, A., and Rubin, C. S. (1993). The novel metallothionein genes of *Caenorhabditis elegans*. Structural organization and inducible, cell-specific expression. *J Biol Chem* **268**, 2554-2564.

Frescas, D., Valenti, L., and Accili, D. (2005). Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. *J Biol Chem* **280**, 20589-20595.

Garg, T. K., and Chang, J. Y. (2006). Methylmercury causes oxidative stress and cytotoxicity in microglia: attenuation by 15-deoxy-delta 12, 14-prostaglandin J2. *J Neuroimmunol* **171**, 17-28.

Garza, A., Vega, R., and Soto, E. (2006). Cellular mechanisms of lead neurotoxicity. *Med Sci Monit* **12**, RA57-65.

Geier, D. A., and Geier, M. R. (2006). A meta-analysis epidemiological assessment of neurodevelopmental disorders following vaccines administered from 1994 through 2000 in the United States. *Neuro Endocrinol Lett* **27**, 401-413.

Glover, C. N., Zheng, D., Jayashankar, S., Sales, G. D., Hogstrand, C., and Lundebye, A. K. (2009). Methylmercury speciation influences brain gene expression and behavior in gestationally-exposed mice pups. *Toxicol Sci* **110**, 389-400.

Gomez-Pinilla, F. (2008). The influences of diet and exercise on mental health through hormesis. *Ageing Res Rev* **7**, 49-62.

Goncalves, P. P., and Silva, V. S. (2007). Does neurotransmission impairment accompany aluminium neurotoxicity? *J Inorg Biochem* **101**, 1291-1338.

Gonzalez, P., Dominique, Y., Massabuau, J. C., Boudou, A., and Bourdineaud, J. P. (2005). Comparative effects of dietary methylmercury on gene expression in liver, skeletal muscle, and brain of the zebrafish (Danio rerio). *Environ Sci Technol* **39**, 3972-3980.

Graff, R. D., Philbert, M. A., Lowndes, H. E., and Reuhl, K. R. (1993). The effect of glutathione depletion on methyl mercury-induced microtubule disassembly in cultured embryonal carcinoma cells. *Toxicol Appl Pharmacol* **120**, 20-28.

Grandjean, P., Weihe, P., White, R. F., Debes, F., Araki, S., Yokoyama, K., Murata, K., Sorensen, N., Dahl, R., and Jorgensen, P. J. (1997). Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. *Neurotoxicol Teratol* **19**, 417-428.

Gruenwedel, D. W., and Cruikshank, M. K. (1979). Effect of methylmercury (II) on the synthesis of deoxyribonucleic acid, ribonucleic acid and protein in HeLa S3 cells. *Biochem Pharmacol* **28**, 651-655.

Gruenwedel, D. W., and Lu, D. S. (1970). Changes in the sedimentation characteristics of DNA due to methylmercuration. *Biochem Biophys Res Commun* **40**, 542-548.

Harada, M. (1995). Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. *Crit Rev Toxicol* **25**, 1-24.

Hasegawa, K., Miwa, S., Isomura, K., Tsutsumiuchi, K., Taniguchi, H., and Miwa, J. (2008). Acrylamide-responsive genes in the nematode *Caenorhabditis elegans*. *Toxicol Sci* **101**, 215-225.

Helmcke, K. J., Syversen, T., Miller, D. M., 3rd, and Aschner, M. (2009). Characterization of the effects of methylmercury on *Caenorhabditis elegans*. *Toxicol Appl Pharmacol*.

Hercus, M. J., Loeschcke, V., and Rattan, S. I. (2003). Lifespan extension of Drosophila melanogaster through hormesis by repeated mild heat stress. *Biogerontology* **4**, 149-156.

Heschl, M. F., and Baillie, D. L. (1989). Characterization of the hsp70 multigene family of *Caenorhabditis elegans*. *DNA* **8**, 233-243.

Hilliard, M. A., Apicella, A. J., Kerr, R., Suzuki, H., Bazzicalupo, P., and Schafer, W. R. (2005). In vivo imaging of *C. elegans* ASH neurons: cellular response and adaptation to chemical repellents. *Embo J* **24**, 63-72.

Hirata, E., and Takahashi, H. (1981). Degradation of methyl mercury glutathione by the pancreatic enzymes in bile. *Toxicol Appl Pharmacol* **58**, 483-491.

Hobert, O. (2005). Specification of the nervous system. *WormBook*, 1-19.

Hodgkin, J. (1999). Conventional Genetics. In *C. elegans: A Practical Approach* (I. A. Hope, Ed.), pp. 245-270. Oxford University Press, New York.

Hope, I. A. (1999). Background on *Caenorhabditis elegans*. In *C. elegans: A Practical Approach* (I. A. Hope, Ed.), pp. 1-15. Oxford University Press, New York.

Huang, C., Xiong, C., and Kornfeld, K. (2004). Measurements of age-related changes of physiological processes that predict lifespan of *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **101**, 8084-8089.

Huang, X., Cheng, H. J., Tessier-Lavigne, M., and Jin, Y. (2002). MAX-1, a novel PH/MyTH4/FERM domain cytoplasmic protein implicated in netrin-mediated axon repulsion. *Neuron* **34**, 563-576.

Hubbard, T. J., and Sander, C. (1991). The role of heat-shock and chaperone proteins in protein folding: possible molecular mechanisms. *Protein Eng* **4**, 711-717.

Hughes, S., and Sturzenbaum, S. R. (2007). Single and double metallothionein knockout in the nematode *C. elegans* reveals cadmium dependent and independent toxic effects on life history traits. *Environ Pollut* **145**, 395-400.

Hwang, G. W., and Naganuma, A. (2006). DNA microarray analysis of transcriptional responses of human neuroblastoma IMR-32 cells to methylmercury. *J Toxicol Sci* **31**, 537-538.

Ishiguro, H., Yasuda, K., Ishii, N., Ihara, K., Ohkubo, T., Hiyoshi, M., Ono, K., Senoo-Matsuda, N., Shinohara, O., Yosshii, F., Murakami, M., Hartman, P. S., and Tsuda, M. (2001). Enhancement of oxidative damage to cultured cells and *Caenorhabditis elegans* by mitochondrial electron transport inhibitors. *IUBMB Life* **51**, 263-268.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* **236**, 313-322.

Jackson, D., Lewis, J., Anderson, S., Gehman, E., Szilagyi, M., and Clegg, E. (2006). A nematode model to elucidate mechanisms of developmental toxicity. SOT meeting 2006.

Jiang, G. C., Tidwell, K., McLaughlin, B. A., Cai, J., Gupta, R. C., Milatovic, D., Nass, R., and Aschner, M. (2007). Neurotoxic potential of depleted uranium effects in primary cortical neuron cultures and in *Caenorhabditis elegans*. *Toxicol Sci* **99**, 553-565.

Jiang, M., Ryu, J., Kiraly, M., Duke, K., Reinke, V., and Kim, S. K. (2001). Genome-wide analysis of developmental and sex-regulated gene expression profiles in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **98**, 218-223.

Johnson, T. E., and Nelson, G. A. (1991). *Caenorhabditis elegans*: a model system for space biology studies. *Exp Gerontol* **26**, 299-309.

Jones, D., and Candido, E. P. (1999). Feeding is inhibited by sublethal concentrations of toxicants and by heat stress in the nematode *Caenorhabditis elegans*: relationship to the cellular stress response. *J Exp Zool* **284**, 147-157.

Kahn, N. W., Rea, S. L., Moyle, S., Kell, A., and Johnson, T. E. (2008). Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in *Caenorhabditis elegans*. *Biochem J* **409**, 205-213.

Kaletta, T., and Hengartner, M. O. (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nat Rev Drug Discov* **5**, 387-398.

Kamath, R. S., and Ahringer, J. (2003). Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* **30**, 313-321.

Kampkotter, A., Pielarski, T., Rohrig, R., Timpel, C., Chovolou, Y., Watjen, W., and Kahl, R. (2007). The Ginkgo biloba extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in *Caenorhabditis elegans*. *Pharmacol Res* **55**, 139-147.

Kaur, P., Aschner, M., and Syversen, T. (2006). Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. *Neurotoxicology* **27**, 492-500.

Kerper, L. E., Ballatori, N., and Clarkson, T. W. (1992). Methylmercury transport across the blood-brain barrier by an amino acid carrier. *Am J Physiol* **262**, R761-765.

Kevorkian, J., Cento, D. P., Hyland, J. R., Bagozzi, W. M., and Van Hollebeke, E. (1972). Mercury content of human tissues during the twentieth century. *Am J Public Health* **62**, 504-513.

Kojda, G., and Hambrecht, R. (2005). Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *Cardiovasc Res* **67**, 187-197.

Koselke, L., Sam, C., Hajela, R., and Atchison, B. (2007). Protective effects of verapamil on mercury toxicity in *C. elegans*. Abstract No. 98. SOT meeting 2007.

Kramer, K. K., Liu, J., Choudhuri, S., and Klaassen, C. D. (1996a). Induction of metallothionein mRNA and protein in murine astrocyte cultures. *Toxicol Appl Pharmacol* **136**, 94-100.

Kramer, K. K., Zoelle, J. T., and Klaassen, C. D. (1996b). Induction of metallothionein mRNA and protein in primary murine neuron cultures. *Toxicol Appl Pharmacol* **141**, 1-7.

Krezel, A., and Maret, W. (2007). Different redox states of metallothionein/thionein in biological tissue. *Biochem J* **402**, 551-558.

Lapham, L. W., Cernichiari, E., Cox, C., Myers, G. J., Baggs, R. B., Brewer, R., Shamlaye, C. F., Davidson, P. W., and Clarkson, T. W. (1995). An analysis of autopsy brain tissue from infants prenatally exposed to methymercury. *Neurotoxicology* **16**, 689-704.

Lazo, J. S., Kondo, Y., Dellapiazza, D., Michalska, A. E., Choo, K. H., and Pitt, B. R. (1995). Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. *J Biol Chem* **270**, 5506-5510.

Lee, J. S., and Surh, Y. J. (2005). Nrf2 as a novel molecular target for chemoprevention. *Cancer Lett* **224**, 171-184.

Lenaz, G. (1998). Role of mitochondria in oxidative stress and ageing. *Biochim Biophys Acta* **1366**, 53-67.

Leung, M. C., Williams, P. L., Benedetto, A., Au, C., Helmcke, K. J., Aschner, M., and Meyer, J. N. (2008). *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. *Toxicol Sci* **106**, 5-28.

Li, F., Mao, H. P., Ruchalski, K. L., Wang, Y. H., Choy, W., Schwartz, J. H., and Borkan, S. C. (2002). Heat stress prevents mitochondrial injury in ATP-depleted renal epithelial cells. *Am J Physiol Cell Physiol* **283**, C917-926.

Li, Y., Ye, H., Du, M., Zhang, Y., Ye, B., Pu, Y., and Wang, D. (2009). Induction of chemotaxis to sodium chloride and diacetyl and thermotaxis defects by microcystin-LR exposure in nematode *Caenorhabditis elegans*. *J Environ Sci (China)* **21**, 971-979.

Liao, V. H., and Yu, C. W. (2005). *Caenorhabditis elegans* gcs-1 confers resistance to arsenic-induced oxidative stress. *Biometals* **18**, 519-528.

Link, C. D., and Johnson, C. J. (2002). Reporter transgenes for study of oxidant stress in *Caenorhabditis elegans*. *Methods Enzymol* **353**, 497-505.

Lund, J., Tedesco, P., Duke, K., Wang, J., Kim, S. K., and Johnson, T. E. (2002). Transcriptional profile of aging in *C. elegans*. *Curr Biol* **12**, 1566-1573.

Maret, W. (2008). Metallothionein redox biology in the cytoprotective and cytotoxic functions of zinc. *Exp Gerontol* **43**, 363-369.

Maret, W., and Vallee, B. L. (1998). Thiolate ligands in metallothionein confer redox activity on zinc clusters. *Proc Natl Acad Sci U S A* **95**, 3478-3482.

Marini, A. M., Jiang, X., Wu, X., Pan, H., Guo, Z., Mattson, M. P., Blondeau, N., Novelli, A., and Lipsky, R. H. (2007). Preconditioning and neurotrophins: a model for brain adaptation to seizures, ischemia and other stressful stimuli. *Amino Acids* **32**, 299-304.

Martin, B., Mattson, M. P., and Maudsley, S. (2006). Caloric restriction and intermittent fasting: two potential diets for successful brain aging. *Ageing Res Rev* **5**, 332-353.

Mason, R. P., Abbott, M. L., Bodaly, R. A., Bullock, O. R., Jr., Driscoll, C. T., Evers, D., Lindberg, S. E., Murray, M., and Swain, E. B. (2005). Monitoring the response to changing mercury deposition. *Environ Sci Technol* **39**, 14A-22A.

Masoro, E. J. (2005). Overview of caloric restriction and ageing. *Mech Ageing Dev* **126**, 913-922.

Matthies, D. S., Fleming, P. A., Wilkes, D. M., and Blakely, R. D. (2006). The *Caenorhabditis elegans* choline transporter CHO-1 sustains acetylcholine synthesis and motor function in an activity-dependent manner. *J Neurosci* **26**, 6200-6212.

Mattson, M. P. (2003). Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Med* **3**, 65-94.

Mattson, M. P. (2008a). Diet-induced hormesis and longevity. *Ageing Res Rev* **7**, 43-48.

Mattson, M. P. (2008b). Hormesis defined. *Ageing Res Rev* **7**, 1-7.

Mattson, M. P., and Meffert, M. K. (2006). Roles for NF-kappaB in nerve cell survival, plasticity, and disease. *Cell Death Differ* **13**, 852-860.

Mayer, M. P., and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* **62**, 670-684.

McElwee, M. K., Snyder, D. W., Rice, J. R., Boyd, W. A., and Freedman, J. H. (2007). Molecular and Toxicological Responses to Mercurials in *C. elegans*. Society of Toxicology.

McIntire, S. L., Reimer, R. J., Schuske, K., Edwards, R. H., and Jorgensen, E. M. (1997). Identification and characterization of the vesicular GABA transporter. *Nature* **389**, 870-876.

Mori, I., Sasakura, H., and Kuhara, A. (2007). Worm thermotaxis: a model system for analyzing thermosensation and neural plasticity. *Curr Opin Neurobiol* **17**, 712-719.

Mutter, J., Naumann, J., Sadaghiani, C., Walach, H., and Drasch, G. (2004). Amalgam studies: disregarding basic principles of mercury toxicity. *Int J Hyg Environ Health* **207**, 391-397.

Myers, G. J., Thurston, S. W., Pearson, A. T., Davidson, P. W., Cox, C., Shamlaye, C. F., Cernichiari, E., and Clarkson, T. W. (2009). Postnatal exposure to methyl mercury from fish consumption: a review and new data from the Seychelles Child Development Study. *Neurotoxicology* **30**, 338-349.

Nass, R., Miller, D. M., and Blakely, R. D. (2001). *C. elegans*: a novel pharmacogenetic model to study Parkinson's disease. *Parkinsonism Relat Disord* **7**, 185-191.

National Research Council, U. S. (2000). *Scientific Frontiers in Developmental Toxicology and Risk Assessment (NRC, US)*. The National Academies Press, Washington, DC.

Newland, M. C., Reed, M. N., LeBlanc, A., and Donlin, W. D. (2006). Brain and blood mercury and selenium after chronic and developmental exposure to methylmercury. *Neurotoxicology* **27**, 710-720.

Nonet, M. L., Saifee, O., Zhao, H., Rand, J. B., and Wei, L. (1998). Synaptic transmission deficits in *Caenorhabditis elegans* synaptobrevin mutants. *J Neurosci* **18**, 70-80.

Olsen, A., Vantipalli, M. C., and Lithgow, G. J. (2006). Lifespan extension of *Caenorhabditis elegans* following repeated mild hormetic heat treatments. *Biogerontology* **7**, 221-230.

Padhi, B. K., Pelletier, G., Williams, A., Berndt-Weis, L., Yauk, C., Bowers, W. J., and Chu, I. (2008). Gene expression profiling in rat cerebellum following in utero and lactational exposure to mixtures of methylmercury, polychlorinated biphenyls and organochlorine pesticides. *Toxicol Lett* **176**, 93-103.

Parker, S. K., Schwartz, B., Todd, J., and Pickering, L. K. (2004). Thimerosal-containing vaccines and autistic spectrum disorder: a critical review of published original data. *Pediatrics* **114**, 793-804.

Patrick, L. (2002). Mercury toxicity and antioxidants: Part 1: role of glutathione and alpha-lipoic acid in the treatment of mercury toxicity. *Altern Med Rev* **7**, 456-471.

Penkowa, M. (2006). Metallothioneins are multipurpose neuroprotectants during brain pathology. *Febs J* **273**, 1857-1870.

Peterson, R. T., Nass, R., Boyd, W. A., Freedman, J. H., Dong, K., and Narahashi, T. (2008). Use of non-mammalian alternative models for neurotoxicological study. *Neurotoxicology* **29**, 546-555.

Plumier, J. C., Krueger, A. M., Currie, R. W., Kontoyiannis, D., Kollias, G., and Pagoulatos, G. N. (1997). Transgenic mice expressing the human inducible Hsp70 have hippocampal neurons resistant to ischemic injury. *Cell Stress Chaperones* **2**, 162-167.

Pollard, K. M., and Hultman, P. (1997). Effects of mercury on the immune system. *Met Ions Biol Syst* **34**, 421-440.

Rand, M. D., Dao, J. C., and Clason, T. A. (2009). Methylmercury disruption of embryonic neural development in Drosophila. *Neurotoxicology* **30**, 794-802.

Rankin, C. H., Beck, C. D., and Chiba, C. M. (1990). *Caenorhabditis elegans*: a new model system for the study of learning and memory. *Behav Brain Res* **37**, 89-92.

Reardon, A. M., Bhat, H.K. (2007). Methylmercury neurotoxicity: Role of oxidative stress. *Toxicological and Environmental Chemistry* **89**, 535-554.

Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. (1980). High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* **106**, 55-62.

Reichert, K., and Menzel, R. (2005). Expression profiling of five different xenobiotics using a *Caenorhabditis elegans* whole genome microarray. *Chemosphere* **61**, 229-237.

Riddle, D. L., Blumenthal, T., Meyer, B. J., and Preiss, J. R. (1997). *C. elegans II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Rising, L., Vitarella, D., Kimelberg, H. K., and Aschner, M. (1995). Metallothionein induction in neonatal rat primary astrocyte cultures protects against methylmercury cytotoxicity. *J Neurochem* **65**, 1562-1568.

Rodier, P. M., Aschner, M., and Sager, P. R. (1984). Mitotic arrest in the developing CNS after prenatal exposure to methylmercury. *Neurobehav Toxicol Teratol* **6**, 379-385.

Roh, J. Y., Jung, I. H., Lee, J. Y., and Choi, J. (2007). Toxic effects of di(2-ethylhexyl)phthalate on mortality, growth, reproduction and stress-related gene expression in the soil nematode *Caenorhabditis elegans*. *Toxicology* **237**, 126-133.

Roh, J. Y., Lee, J., and Choi, J. (2006). Assessment of stress-related gene expression in the heavy metal-exposed nematode *Caenorhabditis elegans*: a potential biomarker for metal-induced toxicity monitoring and environmental risk assessment. *Environ Toxicol Chem* **25**, 2946-2956.

Rossi, A. D., Ahlbom, E., Ogren, S. O., Nicotera, P., and Ceccatelli, S. (1997). Prenatal exposure to methylmercury alters locomotor activity of male but not female rats. *Exp Brain Res* **117**, 428-436.

Sacco, M. G., Zecca, L., Bagnasco, L., Chiesa, G., Parolini, C., Bromley, P., Cato, E. M., Roncucci, R., Clerici, L. A., and Vezzoni, P. (1997). A transgenic mouse model for the detection of cellular stress induced by toxic inorganic compounds. *Nat Biotechnol* **15**, 1392-1397.

Sambongi, Y., Nagae, T., Liu, Y., Yoshimizu, T., Takeda, K., Wada, Y., and Futai, M. (1999). Sensing of cadmium and copper ions by externally exposed ADL, ASE, and ASH neurons elicits avoidance response in *Caenorhabditis elegans*. *Neuroreport* **10**, 753-757.

Sarafian, T., and Verity, M. A. (1991). Oxidative mechanisms underlying methyl mercury neurotoxicity. *Int J Dev Neurosci* **9**, 147-153.

Sato, M., and Bremner, I. (1993). Oxygen free radicals and metallothionein. *Free Radic Biol Med* **14**, 325-337.

Sato, M., and Kondoh, M. (2002). Recent studies on metallothionein: protection against toxicity of heavy metals and oxygen free radicals. *Tohoku J Exp Med* **196**, 9-22.

Scarmeas, N., and Stern, Y. (2003). Cognitive reserve and lifestyle. *J. Clin. Exp. Neuropsychol.* **25**, 625-633.

Schaefer, H., and Rongo, C. (2006). KEL-8 is a substrate receptor for CUL3-dependent ubiquitin ligase that regulates synaptic glutamate receptor turnover. *Mol Biol Cell* **17**, 1250-1260.

Schlawicke Engstrom, K., Stromberg, U., Lundh, T., Johansson, I., Vessby, B., Hallmans, G., Skerfving, S., and Broberg, K. (2008). Genetic variation in glutathione-related genes and body burden of methylmercury. *Environmental health perspectives* **116**, 734-739.

Shen, X., Ellis, R. E., Lee, K., Liu, C. Y., Yang, K., Solomon, A., Yoshida, H., Morimoto, R., Kurnit, D. M., Mori, K., and Kaufman, R. J. (2001). Complementary signaling pathways regulate the unfolded protein response and are required for *C. elegans* development. *Cell* **107**, 893-903.

Simmons-Willis, T. A., Koh, A. S., Clarkson, T. W., and Ballatori, N. (2002). Transport of a neurotoxicant by molecular mimicry: the methylmercury-L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2. *Biochem J* **367**, 239-246.

Sirois, J. E., and Atchison, W. D. (1996). Effects of mercurials on ligand- and voltage-gated ion channels: a review. *Neurotoxicology* **17**, 63-84.

Sobotka, T. J., Cook, M. P., and Brodie, R. E. (1974). Effects of perinatal exposure to methyl mercury on functional brain development and neurochemistry. *Biol Psychiatry* **8**, 307-320.

Stiernagle, T. (1999). Maintenance of *C. elegans*. In *C. elegans: A Practical Approach* (I. A. Hope, Ed.). Oxford University Press, New York.

Stringari, J., Nunes, A. K., Franco, J. L., Bohrer, D., Garcia, S. C., Dafre, A. L., Milatovic, D., Souza, D. O., Rocha, J. B., Aschner, M., and Farina, M. (2008). Prenatal methylmercury exposure hampers glutathione antioxidant system ontogenesis and causes long-lasting oxidative stress in the mouse brain. *Toxicol Appl Pharmacol* **227**, 147-154.

Sulston, J. E. (1983). Neuronal cell lineages in the nematode *Caenorhabditis elegans*. *Cold Spring Harb Symp Quant Biol* **48 Pt 2**, 443-452.

Sulston, J. E., and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol* **56**, 110-156.

Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol* **100**, 64-119.

Swain, S. C., Keusekotten, K., Baumeister, R., and Sturzenbaum, S. R. (2004). *C. elegans* metallothioneins: new insights into the phenotypic effects of cadmium toxicosis. *J Mol Biol* **341**, 951-959.

Syversen, T. L. (1982). Effects of repeated dosing of methyl mercury on in vivo protein synthesis in isolated neurones. *Acta Pharmacol Toxicol (Copenh)* **50**, 391-397.

Sze, J. Y., Victor, M., Loer, C., Shi, Y., and Ruvkun, G. (2000). Food and metabolic signalling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature* **403**, 560-564.

Takeuchi, T. (1985). Human effects of methylmercury as an environmental neurotoxicant. In *Neurotoxicology* (K. Blum, and L. Manzo, Eds.), pp. 345-367. Marcel Dekker, New York.

Tatar, M., Khazaeli, A. A., and Curtsinger, J. W. (1997). Chaperoning extended life. *Nature* **390**, 30.

Tawe, W. N., Eschbach, M. L., Walter, R. D., and Henkle-Duhrsen, K. (1998). Identification of stress-responsive genes in *Caenorhabditis elegans* using RT-PCR differential display. *Nucleic Acids Res* **26**, 1621-1627.

Thompson, W. W., Price, C., Goodson, B., Shay, D. K., Benson, P., Hinrichsen, V. L., Lewis, E., Eriksen, E., Ray, P., Marcy, S. M., Dunn, J., Jackson, L. A., Lieu, T. A., Black, S., Stewart, G., Weintraub, E. S., Davis, R. L., and DeStefano, F. (2007). Early thimerosal exposure and neuropsychological outcomes at 7 to 10 years. *N Engl J Med* **357**, 1281-1292.

Tiffany-Castiglion, E., and Qian, Y. (2001). Astroglia as metal depots: molecular mechanisms for metal accumulation, storage and release. *Neurotoxicology* **22**, 577-592.

Toyama, T., Sumi, D., Shinkai, Y., Yasutake, A., Taguchi, K., Tong, K. I., Yamamoto, M., and Kumagai, Y. (2007). Cytoprotective role of Nrf2/Keap1 system in methylmercury toxicity. *Biochem Biophys Res Commun* **363**, 645-650.

Tsui, M. T., and Wang, W. X. (2005). Influences of maternal exposure on the tolerance and physiological performance of Daphnia magna under mercury stress. *Environ Toxicol Chem* **24**, 1228-1234.

Tvermoes, B., and Freedman, J. H. (2008). *Caenorhabditis elegans* gene, Numr-1, assembles into nuclear stress granules after cadmium treatment. SOT meeting 2008.

Upton, A. C. (2001). Radiation hormesis: data and interpretations. *Crit Rev Toxicol* **31**, 681-695.

van Rossum, A. J., Brophy, P. M., Tait, A., Barrett, J., and Jefferies, J. R. (2001). Proteomic identification of glutathione S-transferases from the model nematode *Caenorhabditis elegans*. *Proteomics* **1**, 1463-1468.

Ved, R., Saha, S., Westlund, B., Perier, C., Burnam, L., Sluder, A., Hoener, M., Rodrigues, C. M., Alfonso, A., Steer, C., Liu, L., Przedborski, S., and Wolozin, B. (2005). Similar patterns of mitochondrial vulnerability and rescue induced by genetic modification of alpha-synuclein, parkin, and DJ-1 in *Caenorhabditis elegans*. *J Biol Chem* **280**, 42655-42668.

Verbeke, P., Clark, B. F., and Rattan, S. I. (2001). Reduced levels of oxidized and glycoxidized proteins in human fibroblasts exposed to repeated mild heat shock during serial passaging in vitro. *Free Radic Biol Med* **31**, 1593-1602.

Verity, M. A., Brown, W. J., and Cheung, M. (1975). Organic mercurial encephalopathy: in vivo and in vitro effects of methyl mercury on synaptosomal respiration. *J Neurochem* **25**, 759-766.

Vogel, D. G., Margolis, R. L., and Mottet, N. K. (1985). The effects of methyl mercury binding to microtubules. *Toxicol Appl Pharmacol* **80**, 473-486.

Wang, D. Y., and Wang, Y. (2008). Phenotypic and behavioral defects caused by barium exposure in nematode *Caenorhabditis elegans*. *Arch Environ Contam Toxicol* **54**, 447-453.

Wang, L., Jiang, H., Yin, Z., Aschner, M., and Cai, J. (2009). Methylmercury toxicity and Nrf2-dependent detoxification in astrocytes. *Toxicol Sci* **107**, 135-143.

Watson, J. D., Wang, S., Von Stetina, S. E., Spencer, W. C., Levy, S., Dexheimer, P. J., Kurn, N., Heath, J. D., and Miller, D. M., 3rd (2008). Complementary RNA amplification methods enhance microarray identification of transcripts expressed in the *C. elegans* nervous system. *BMC Genomics* **9**, 84.

Weiss, B., Clarkson, T. W., and Simon, W. (2002). Silent latency periods in methylmercury poisoning and in neurodegenerative disease. *Environ Health Perspect* **110 Suppl 5**, 851-854.

West, A. K., Hidalgo, J., Eddins, D., Levin, E. D., and Aschner, M. (2008). Metallothionein in the central nervous system: Roles in protection, regeneration and cognition. *Neurotoxicology* **29**, 489-503.

White, J. G., Southgate, E., Thompson, J. N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond.* **B**, 1-340.

WHO (1990). Environmental Health Criteria 101: Methylmercury. *Geneva: International Program on Chemical Safety, World Health Organization*.

WHO (1991). Inorganic Mercury. *Environmental Health Criteria* **118**.

Williams, P. L., and Dusenbery, D. B. (1987). Screening test for neurotoxins using *Caenorhabditis elegans*. *Prog Clin Biol Res* **253**, 163-170.

Williams, P. L., and Dusenbery, D. B. (1988). Using the nematode *Caenorhabditis elegans* to predict mammalian acute lethality to metallic salts. *Toxicol Ind Health* **4**, 469-478.

Williams, P. L. a. D. B. D. (1990). Aquatic toxicity testing usiing the nematode, *Caenorhabditis elegans*. *Environmental Toxicology and Chemistry* **9**, 1285-1290.

Wolters, N. M., and MacKeigan, J. P. (2008). From sequence to function: using RNAi to elucidate mechanisms of human disease. *Cell Death Differ* **15**, 809-819.

Wood, W. B. (1988). Introduction to *C. elegans* Biology. In *The Nematode Caenorhabditis elegans* (W. B. Wood, Community of *C. elegans* Researchers, Ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

WormAtlas (2002-2009). http://www.wormatlas.org. (Z. F. Altun, Herndon, L.A., Crocker, C., Lints, R. and Hall, D.H., Ed.).

WormBase web site, http://www.wormbase.org, release WS204 date 29 Jul 2009.

Xu, K., Tavernarakis, N., and Driscoll, M. (2001). Necrotic cell death in *C. elegans* requires the function of calreticulin and regulators of Ca(2+) release from the endoplasmic reticulum. *Neuron* **31**, 957-971.

Yao, C. P., Allen, J. W., Conklin, D. R., and Aschner, M. (1999). Transfection and overexpression of metallothionein-I in neonatal rat primary astrocyte cultures and in astrocytoma cells increases their resistance to methylmercury-induced cytotoxicity. *Brain Res* **818**, 414-420.

Yao, C. P., Allen, J. W., Mutkus, L. A., Xu, S. B., Tan, K. H., and Aschner, M. (2000). Foreign metallothionein-I expression by transient transfection in MT-I and MT-II null astrocytes confers increased protection against acute methylmercury cytotoxicity. *Brain Res* **855**, 32-38.

Yasutake, A., Nakano, A., and Hirayama, K. (1998). Induction by mercury compounds of brain metallothionein in rats: Hg0 exposure induces long-lived brain metallothionein. *Arch Toxicol* **72**, 187-191.

Ye, H., Ye, B., and Wang, D. (2008). Trace administration of vitamin E can retrieve and prevent UV-irradiation- and metal exposure-induced memory deficits in nematode *Caenorhabditis elegans*. *Neurobiol Learn Mem* **90**, 10-18.

Yee, S., and Choi, B. H. (1996). Oxidative stress in neurotoxic effects of methylmercury poisoning. *Neurotoxicology* **17**, 17-26.

Yellon, D. M., and Downey, J. M. (2003). Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* **83**, 1113-1151.

Yin, Z., Jiang, H., Syversen, T., Rocha, J. B., Farina, M., and Aschner, M. (2008). The methylmercury-L-cysteine conjugate is a substrate for the L-type large neutral amino acid transporter. *J Neurochem* **107**, 1083-1090.

Yoshida, M., Shimizu, N., Suzuki, M., Watanabe, C., Satoh, M., Mori, K., and Yasutake, A. (2008). Emergence of delayed methylmercury toxicity after perinatal exposure in metallothionein-null and wild-type C57BL mice. *Environ Health Perspect* **116**, 746-751.

Zhang, Y., Li, S., Liang, Y., Wen, C., Guo, Q., and Su, B. (2009). Potential mechanisms of neuroprotection induced by low dose total-body gamma-irradiation in C57 mice administered with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Neurosci Lett* **450**, 106-110.

Zhang, Y., Ye, B., and Wang, D. Effects of Metal Exposure on Associative Learning Behavior in Nematode *Caenorhabditis elegans*. *Arch Environ Contam Toxicol*.