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Characterization of the effects of methylmercury on *Caenorhabditis elegans*

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Abstract

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 (≤ 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.

Keywords

Caenorhabditis elegans; methylmercury

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Conflict of Interest Statement

None of the authors has a conflict of interest.

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Introduction

Mercury (Hg) is a toxicant to which humans are exposed regularly. Major routes of Hg exposure to humans include inhalation of Hg vapor released from amalgam dental fillings and consumption of seafood containing methylmercury (MeHg) (Clarkson, 2002; Clarkson and Magos, 2006). Thimerosal, which contains ethylmercury (EtHg), is used as a preservative in some vaccines and, although some limited evidence indicates that there may be a link between thimerosal and autism (Geier and Geier, 2006), this link has been largely discredited (Parker *et al.*, 2004; Thompson *et al.*, 2007). The presence of MeHg in seafood is caused by global cycling and bioaccumulation of the toxicant (Fitzgerald and Clarkson, 1991; Mason *et al.*, 2005). 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. 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, and alterations in neurotransmitter systems (Clarkson, 2002; Clarkson and Magos, 2006; Roh *et al.*, 2006). Although MeHg possesses high affinity for cysteine, allowing it to bind thiol groups, the specific molecular targets of MeHg are largely unknown (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. 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; Sulston *et al.*, 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 content, 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 500 μ L 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 demethylation 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% HNO₃ (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 HNO₃ 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 HNO₃, 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* hermaphrodites 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 1A). 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, $n=9$) statistically significantly ($p<0.001$) increased the toxicity to *C. elegans*, indicating that longer exposures are more lethal to *C. elegans* (Figure 1B).

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 2). 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 2). 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 1) 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 (Supplemental Figure 1). For example, average lifespan following a 30-minute treatment of L1 *C. elegans* (Supplemental Figure 1A) or a 15-hour treatment of L4 *C. elegans* was 13–15 days (Supplemental Figure 1B). 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 (Supplemental Figure 1C). None were significantly altered when using the log 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 (Supplemental Figure 2). 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 3). 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 4, Supplemental 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 had all reached the L2 stage while many worms treated at higher concentrations of MeHgCl remained L1s (4A). This trend continued 48 (4B) and 72 (4C) 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 (4D–E). Many control-treated animals reached the adult stage 24 hours after treatment while those treated at higher MeHgCl

had remained L4s (4D). 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 (4E) (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 5). 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 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* (Supplemental Figure 3). [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 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 (Supplemental Figure 4A) and 2 PDEs (Supplemental Figure 4B)] or ability of projections to travel from the nerve ring to the tip of the nose (Supplemental Figure 4C) in worms surviving MeHgCl insult (Figure 6A–B). GABAergic analysis also revealed no alteration in cell number in the head (Supplemental Figure 4D) or nerve cord (Supplemental Figure 4E), ability of projections to pass across the body (Supplemental Figure 4F), or number of breaks in the commissures (Supplemental Figure 4G) of *C. elegans* surviving MeHgCl treatment (Figure 4C–D).

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 1). As has been shown in other systems, MeHgCl was more toxic to younger as compared 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 2). 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 1), indicating that increased accumulation of Hg within *C. elegans* (Figure 2) 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). 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*. In our experiments, *C. elegans* did reach concentrations of Hg as high as those found in mammalian systems where deleterious alterations have been observed.

Neither *C. elegans* lifespan (Supplemental Figure 1) nor brood size (Supplemental Figure 2) 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 3) and a developmental delay following MeHgCl exposure was noted (Figure 4, Supplemental 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 (Bruinsma *et al.*, 2008). Although no alteration in thrashing behavior was noted (Supplemental Figure 3), the decreased pharyngeal pumping rate following MeHgCl exposure (Figure 5) 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 (Castoldi *et al.*, 2001), 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 *et al.*, 2000; Baraldi *et al.*, 2002), muscarinic cholinergic (Coccini *et al.*, 2000), and dopaminergic (Rossi *et al.*, 1997; Ong and Farooqui, 2005) 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 6, Supplemental Figure 4) 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

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

Figure 2.

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 3.

Body length of *C. elegans* was shorter following treatment with MeHgCl. After growth for 24 or 48 hours, animals treated at either L1 or L4 stages with the toxicant were significantly (***, $p < 0.001$, $n = 4$) shorter than control animals, 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.

Figure 4.

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.

Figure 5.

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 hours following treatment at 0.75 and 1 mM MeHgCl (**, $p < 0.01$, $n = 12$) and 72 hours following treatment at 1 mM MeHgCl (*, $p < 0.05$, $n = 12$). Exposure of L4 worms for 15 hours induced a decrease in pharyngeal pumping rate 24 hours 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 ($n = 8$).

Figure 6.

Representative dopaminergic and GABAergic *C. elegans* neurons following MeHgCl insult. Dopaminergic (A–B) and GABAergic (C–D) cells and projections are identical under control (A and C) and MeHgCl-treatment (B and D) conditions (at all concentrations observed, 0.1, 0.4, and 1 mM MeHg) following treatment at L1 for 30 minutes or L4 for 15 hours (n=4). Progeny of worms treated at L4 for 15 hours were also unaffected (n=4).