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The putative multidrug resistance protein MRP-7 inhibits methylmercury-associated animal toxicity and dopaminergic neurodegeneration in *Caenorhabditis elegans*

Natalia VanDuyn and **Richard Nass***
From the Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA

Abstract

Parkinson's disease (PD) is the most prevalent neurodegenerative motor disorder worldwide, and results in the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta. Gene-environment interactions are believed to play a significant role in the vast majority of PD cases, yet the toxicants and the associated genes involved in the neuropathology are largely ill-defined. Recent epidemiological and biochemical evidence suggests that methylmercury (MeHg) may be an environmental toxicant that contributes to the development of PD. Here we report that a gene coding for the putative multidrug resistance protein MRP-7 in *Caenorhabditis elegans* (*C. elegans*) modulates whole animal and DA neuron sensitivity to MeHg. In this study we demonstrate that genetic knockdown of MRP-7 results in a 2-fold increase in Hg levels and a dramatic increase in stress response proteins associated with the endoplasmic reticulum, golgi apparatus, and mitochondria, as well as an increase in MeHg-associated animal death. Chronic exposure to low concentrations of MeHg induces MRP-7 gene expression, while exposures in MRP-7 genetic knockdown animals results in a loss of DA neuron integrity without affecting whole animal viability. Furthermore, transgenic animals expressing a fluorescent reporter behind the endogenous MRP-7 promoter indicate that the transporter is expressed in DA neurons. These studies show for the first time that a multidrug resistance protein is expressed in DA neurons, and its expression inhibits MeHg-associated DA neuron pathology.

Keywords

neurodegeneration; neurotoxicity; Parkinson's disease; methylmercury

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease that is characterized by the selective loss of dopamine (DA) neurons in the substantia nigra (Martin *et al*. 2011, Nass & Przedborski 2008). Hallmark symptoms of the disorder include resting tremors, bradykinesia, and muscle rigidity. The rare familial forms of PD have provided significant insight into possible molecular pathways involved in the more common idiopathic PD (IPD), and include those involved in mitochondrial function and regulating oxidative stress (Nass & Przedborski 2008). Although the etiology of IPD is largely unknown, it is likely that environmental components contribute significantly to the development of the disorder.

*To whom correspondence should be addressed: Richard Nass, Department of Pharmacology and Toxicology, 635 Barnhill Drive, MS 549, Indiana University School of Medicine, Indianapolis, Indiana 46202; USA. Phone (317) 278-8505; FAX (317) 274-7787; ricnass@iupui.edu.

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Methylmercury (MeHg) is a ubiquitous environmental toxicant that primarily targets the central nervous system (Clarkson & Magos 2006). Exposure to high levels of MeHg can result in motor and sensory deficits, mental retardation, and death. MeHg can diffuse relatively easily through cellular membranes, and the toxicant has been shown to disrupt a number of metabolic processes including respiration, calcium homeostasis, and redox balance (Atchison & Hare 1994, Rush *et al.* 2012). The toxicant has also been shown to induce the expression of glutathione S-transferases (GSTs) that conjugate xenobiotics to reduced glutathione (GSH) that are then exported from the cell (Di Simplicio *et al.* 1993, Yu *et al.* 2010). DA neurotransmission is also sensitive to MeHg as the toxicant can cause abnormal DA release, reuptake, and metabolism (Faro *et al.* 2002, Dreiem *et al.* 2009). MeHg has been shown to accumulate in the substantia nigra and striatum during chronic exposure through drinking water, and although the molecular mechanisms are not known, recent studies suggest that the toxicant may also contribute to the development of PD (Petersen *et al.* 2008, Moller-Madsen 1994).

Multidrug resistance proteins (MRPs) are a subset of the ATP-binding cassette genes that are phase III detoxification proteins involved in transporting exogenous and endogenous compounds across cellular membranes. The proteins typically have two nucleotide binding domains, and hydrolyze ATP to pump the substrates against a concentration gradient (Borst *et al.* 2000, Chen & Tiwari 2011). The nine MRP proteins found in vertebrates, categorized based on membrane topography, tissue distribution, and substrate specificity, have been extensively characterized in non-CNS tissues including the kidney, intestine, muscle, and skin (Borst et al. 2000, Chen & Tiwari 2011, Dean *et al.* 2001). The expression and function of MRPs in normal brain physiology have been contradictory and controversial, largely due to low expression levels that may be altered *in vitro* and interfering interactions with other biomolecules (Dallas *et al.* 2006). However, there is evidence that MRPs are expressed in neurons, likely play a role in modulating redox homeostasis and GSH efflux, and may contribute to the development of neurodegenerative diseases (Dallas et al. 2006, Furuno *et al.* 2002, Lam *et al.* 2001). Although MRPs have not been directly associated with the development of PD, transporter polymorphisms, both alone and in concert with pesticide exposures, have been correlated with an increased propensity to develop the disease (Lee *et al.* 2004, Dutheil *et al.* 2010). MRPs have not been shown to be expressed in DA neurons in humans or other mammalian systems, but they are known to transport MeHg conjugates out of cells and increase excretion in the kidney and liver (Madejczyk *et al.* 2007, Zalups & Bridges 2009).

The nematode *Caenorhabditis elegans* (*C. elegans*) is a useful genetic model to dissect the molecular components involved in DA neuron vulnerability and pathology (Nass & Blakely 2003, Nass *et al.* 2008). The nematode can easily be grown in microwell plates allowing for medium throughput whole genome screens that can identify genes involved in a potential phenotype. The *C. elegans* DA neurons contain the genes involved in DA signaling and metabolism, and expression of fluorescent proteins in the neurons allows for the *in vivo* analysis of neuronal morphology and integrity (Nass *et al.* 2002, Nass & Blakely 2003). The nematode also contains strong orthologues to vertebrate MRPs and cell death genes, and the DA neurons are sensitive to PD-associated toxicants including MeHg (Lakso *et al.* 2003, Ved *et al.* 2005, Vanduyn *et al.* 2013, Vistbakka *et al.* 2012, Sheps *et al.* 2004, Nass et al. 2008, Settivari *et al.* 2009, Vanduyn *et al.* 2010). In this study we asked if there were molecular transporters that may protect against MeHg-associated toxicity. Here we show that a previously uncharacterized *C. elegans* MRP, MRP-7, inhibits MeHg-induced GST and HSP gene expression and animal toxicity. We also show that the transporter modulates whole animal Hg levels, is expressed in DA neurons, and inhibits MeHg-associated DA neurodegeneration.

Materials and Methods

C. elegans strains and maintenance

C. elegans strains were obtained from the *Caenorhabditis elegans* Genetics Center: Bristol N2 wild-type (WT), NL2099 *rrf-3(pk1426).* The strain RJ928 was generated by a genetic cross between BY250 (P_{dat-1}::GFP) and NL2099 (Settivari et al. 2009). Genetic crosses were also performed between GFP reporter strains obtained from the CGC and NL2099 to allow the observation of other neuronal types: GABAergic – EG1285 x NL2099 [P_{unc-47}::GFP; *rrf-3(pk1426)*], glutamatergic – DA1240 x NL2099 [P_{eat-4}::GFP; *rrf-3(pk1426)*], cholinergic – LX929 x NL2099 [P_{unc-17}::GFP; *rrf-3(pk1426)*], serotonergic – GR1366 x NL2099 [P_{tph-1}::GFP; *rrf-3(pk1426)*]. Animals were cultured on NGM or 8P plates with lawns of OP50 or NA22 bacteria, respectively, at 20°C according to standard methods (Hope 1999, Brenner 1974).

RNAi reverse genetic screen

A RNAi screen in liquid was carried out using the Open Biosystems ORF RNAi feeding library (10,953 genes) (Thermo Fisher Scientific, Waltham, MA, USA) and a sub-library containing the clones from the Source BioScience (Ahringer) library (Nottingham, UK) that are absent in the Open Biosystems library (7,555 genes). RNAi bacteria cultures were prepared as described (Ahringer 2006). Approximately 40 synchronized L1 stage NL2099 worms were added to 50 μl of bacteria culture in a 96 well plate. The plates were incubated in a shaker at 20°C for 2 days, MeHg was added to each well to a final concentration of 5 μM, and the worms were grown 2 more days in the presence of MeHg. Bacteria clones that resulted in reduced viability in the liquid screen were confirmed with a live/dead assay on agar plates as previously described (Vanduyn et al. 2010). NL2099 L1 stage worms were grown on each RNAi clone for 48 h and assayed for viability following a 48 h exposure to 10 μM MeHg. Each clone was tested in triplicate (> 20 worms per replicate) and clones for which the average percent live worms were significantly different from WT were identified.

RNAi clone sequencing

The RNAi feeding clone in well III-6J08 of the Source BioScience library was grown overnight in LB broth + 50 μg/ml ampicillin and a miniprep kit was used to isolate plasmid DNA (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany). Sequencing services were carried out by ACGT, Inc. (Wheeling, IL, USA) using their provided M13F (−20) primer. The resulting nucleotide sequence was mapped to the *C. elegans* genome using the BLASTN feature at WormBase.org.

Sequence alignment

A BLAST search of the *C. elegans* MRP-7 protein sequence revealed that 14 human proteins match with an E value of 0.0, with identity scores ranging from 31–43%. MRP1 (ABCC1) shares 43% identity and 61% similarity with MRP-7, the highest of any of the MRPs, so a sequence alignment was prepared between these proteins. The human MRP1 protein sequence (accession NP_004987.2) was obtained from the NCBI Protein database and the *C. elegans* MRP-7 protein sequence was obtained from WormBase.org. ClustalW was used to align the sequences. Fully conserved residues were highlighted in dark gray and residues that are similar between the two sequences were highlighted in light gray (similar includes both strongly and weakly similar residues as defined by ClustalW). The transmembrane helices of MRP1 were indicated by a thick black line and the Walker A, Walker B and the ABC signature sequences of MRP1 were indicated in boxes.

Whole animal viability assay

RNAi by feeding was carried out on RNAi plates (NGM agar with 1 mM isopropyl β-d-thiogalactoside (IPTG) and 100 μg/ml ampicillin) seeded with HT115(DE3) bacteria (Kamath & Ahringer 2003). HT115 was transformed with the L4440 empty vector for a control. To target *mrp-7*, the clone labeled C51G7.a located in well III-6J08 of the RNAi feeding library from Source BioScience was used. Nematodes were exposed to RNAi for two generations to ensure sufficient gene knockdown. For synchronization gravid adult NL2099 strain nematodes were lysed with hypochlorite solution and the embryos were incubated for 18 h in M9 buffer. L1 stage worms were washed three times in H_2O and put onto the RNAi plates. Worms (P0) were grown to adulthood and synchronized again. L1 larvae (F1) were placed on RNAi plates and grown for 24 h at 20°C (to the L2/L3 stage) and then transferred to RNAi plates containing various concentrations of MeHg (CH_3HgCl, 442534, Sigma-Aldrich, St. Louis, MO). After a 48 h incubation at 20°C, the number of live worms was determined; worms were considered alive when a touch on the nose with a wire pick caused movement. At least 20 worms were counted for each experiment and the experiment was repeated 3 times. Results are presented as mean ± SEM.

RT-PCR analysis

NL2099 worms were exposed to MeHg under two different conditions before being collected for RNA isolation. L1 stage worms were exposed to 400 nM MeHg for 4 days on *mrp-7* or empty vector RNAi bacteria (Novillo *et al.* 2005, Vanduyn et al. 2010). Second generation *mrp-7* RNAi or WT worms were grown to the L4 stage then exposed to 25 μM MeHg for 4 h. After exposure, nematodes were washed from the plates with water and TRIzol reagent (Life Technologies, Grand Island, NY, USA) was added to the pellet of nematodes. Samples were frozen at −80°C and later thawed for processing, or RNA was isolated immediately as previously described. The RNA was dissolved in nuclease-free H_2O and the concentration was measured using a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA). RNA samples were stored at −80°C. cDNA synthesis was carried out using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions with 1 μg of total RNA as the template. cDNA was quantified using a ND-1000 spectrophotometer and stored at −20°C. RT-PCR primers were designed using Primer3 software to be gene specific and exon spanning to avoid amplification of contaminating genomic DNA. Glyceraldehyde-3-dehydrogenase (GAPDH) was selected as the gene for normalization in the qRT-PCR because its expression does not change as a result of MeHg treatment (Vanduyn et al. 2010). Primer sequences are listed in Supplementary Table 1. Real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and the StepOnePlus Real-Time PCR System (Life Technologies, Grand Island, NY, USA). 25 μg of cDNA was used as the template, reactions were performed in triplicate and each experiment was repeated with three independent cDNA samples. Primer specificity and the formation of a single product were verified by a melting curve. Relative fold change in expression of each gene was calculated using the ΔΔCt method, normalized to GAPDH.

DA neurodegeneration assay

RNAi by feeding was carried out as described above using the strain RJ928. Gravid adult RJ928 worms were lysed with hypochlorite solution and embryos were incubated for 18 h in M9 buffer. L1 stage worms were washed three times in H_2O and transferred onto RNAi plates containing various concentrations of MeHg (0–0.5 μM) and control or *mrp-7* RNAi bacteria. Worms were incubated at 20°C for 4 days and then scored for DA neuron degeneration as previously described (Vanduyn et al. 2010, Settivari et al. 2009). Briefly,

50–60 worms were immobilized on a 2% agarose pad with 2% sodium azide and the GFP-expressing DA neurons were observed using a fluorescent microscope (Leica MZ 16 FA, Leica Microsystems, Heerbrugg, Switzerland). To illustrate the degeneration, a Z-stack of images was captured using the FV1000-MPE Confocal/Multiphoton Microscope (Olympus America, Center Valley, PA, USA) and the Max Intensity Z projection image was created in ImageJ (Rasband 1997–2012). The results are presented as the percent of worms with normal CEPs. The experiment was repeated at least 3 times and bars represent the mean ± SEM.

Sample preparation for Hg level determination

NL2099 worms grown on RNAi plates seeded with the appropriate RNAi bacteria to the gravid adult stage (P0) were lysed with hypochlorite solution, embryos were incubated in M9 solution for 18 h, and synchronized L1s (F1) were placed onto RNAi plates. Worms were grown for 3 days to the adult stage then transferred to RNAi plates containing 1 μM MeHg and seeded with the appropriate RNAi bacteria. Worms were grown on the RNAi + MeHg plates for the indicated amount of time at 20°C. After exposure, worms were collected in pre-weighed metal-free 1.5 ml tubes and the worm pellets were washed three times with ice cold H_2O. Since L1s were present on the plate, the adult worms were allowed to settle by gravity on ice and the L1s were removed during the washes to ensure that the Hg measurement reflected only the content in adult worms. After the final wash, the H_2O was removed and samples were frozen at −20°C until further processing. Frozen worm pellets were thawed, excess H_2O was removed, and the weight was recorded. Samples were transferred into Teflon vessels with 50% HNO_3 and digested using the MARSXpress microwave digestion system (CEM, Matthews, NC, USA).

ICP-MS analysis

The digested worm samples were diluted with H_2O to 2% acid. Samples for introduction into the instrument included the worm sample diluted to 0.1 mg/ml based on the wet weight of the original pellet, an internal standard mixture containing Bi, In, Li, Sc, Tb, Y (IV-ICPMS-71D, Inorganic Ventures, Christiansburg, VA, USA) at 1 ppb, and Au (MSAU-10PPM, Inorganic Ventures, Christiansburg, VA, USA) at 200 ppb. Samples were introduced into the X Series ICP-MS (Thermo Fisher Scientific, Waltham, MA, USA) and ^{202}Hg was monitored with Y and Bi as the internal standards. The [Hg] in ng/ml (ppb) was divided by the concentration of worms in the sample and the data was expressed as ng Hg/mg worms (wet weight) or ppm Hg. Four samples were collected for each strain at each time point and bars represent the mean ± SEM.

Plasmid construction and generation of transgenic animals

For co-expression studies, CFP and YFP were used as these fluorophores have minimal overlap in their excitation and emission spectra and are frequently used together for double-labeling experiments (Miller *et al.* 1999). To express YFP in the DA neurons, the GFP in pRN200 (Nass et al. 2002) was replaced with YFP from the vector L4817 (Fire Lab Vector Kit, Addgene, Cambridge, MA, USA). pRN200 and L4817 were digested with AgeI and ApaI which flank the GFP or YFP regions of the plasmids. The YFP fragment was ligated into the pRN200 vector using ExpressLink T4 DNA Ligase (Invitrogen, Carlsbad, CA, USA) and the construct was confirmed with PCR. A transcriptional fusion for *mrp-7* was created by amplifying a 3466 bp region 5′ of the start codon from genomic DNA based on primers designed by The Genome BC *C. elegans* Gene Expression Consortium (Hunt-Newbury *et al.* 2007): F – GTCGACTAGAGGATCCTTTAAAATCTCGTCGACATCACT, R– CCAATCCCGGGGATCCCTAATTTTTGGAGTTTGTGTT. The In-Fusion HD cloning

system (Clontech, Mountain View, CA, USA) was used to insert the putative *mrp-7* promoter into a vector created by replacing the GFP in pPD95.73 with CFP from L4816 (as described above) and linearized with BamHI. Transgenic animals expressing the transcriptional fusion were generated by injecting N2 worms with P_{mrp-7}::CFP at 75 ng/μl, P_{dat-1}::YFP at 50 ng/μl, pRF4 [*rol-6(su1006)*] at 50 ng/μl and pUC19 at 25 ng/μl. Transgenic animals were identified by the roller phenotype.

Confocal microscopy analysis

Primary cell cultures were created using embryos from the new P_{mrp-7}::CFP strain (RJ1089) as previously described (Bianchi & Driscoll 2006, Vanduyn et al. 2010). Worm cultures were started by picking rollers to enrich for transgenic animals and grown to the gravid adult stage. Hypochlorite synchronization solution was added to lyse the adults and the eggs were collected and washed with egg buffer (118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES). Sucrose flotation (60%) was used to separate the eggs from debris, and chitinase (C6137, Sigma, St. Louis, MO, USA) was used to digest the eggshells. The embryonic cells were dissociated using a syringe and a 21-gauge needle. Cells were grown in L-15 medium (containing 10% FBS and 1% pen/strep), on polylysine-coated cover slips at 20°C. After a 72 h incubation, cells were fixed with 4% paraformaldehyde and mounted onto slides with VECTASHIELD HardSet Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Confocal images were obtained using the Nikon Eclipse Ti with NIS Elements AR software (Nikon Instruments Inc., Melville, NY, USA) and 440 nm and 514 nm lasers. Z-stacks were created in ImageJ.

Statistics

All results are presented as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A student's t-test was used to analyze the difference between two groups. One-way ANOVA with a Bonferroni post-hoc test was performed to compare the expression levels of each gene as measured by RT-PCR between the different exposure conditions. Two-way ANOVA with a Bonferroni post-hoc test was performed to compare WT and RNAi animals under different exposure conditions.

RESULTS

The putative multidrug resistance transporter MRP-7 inhibits MeHg-induced animal death

MeHg is a potent environmental neurotoxicant that has been associated with disruption of microtubule integrity, neuronal axon guidance, and cell death (Guo *et al.* 2013, Sager & Syversen 1984, Ceccatelli *et al.* 2010). The genetic pathways and mediators of the MeHg-induced defects are largely ill-defined. In order to identify genes whose expression inhibits MeHg-associated toxicity, we utilized RNAi to knock down gene expression of over 90% of the genes in the *C. elegans* genome, and identified a gene designated by the primer pair G51G7.a from the Ahringer RNAi library whose genetic repression dramatically increases MeHg-associated animal sensitivity. Sequencing of the designated gene fragment indicated the gene is not *wah-1* as annotated in the Ahringer library, but the gene Y43F8C.12, which codes for the putative multidrug resistance protein MRP-7. A BLAST search with the human genome and a sequence alignment using ClustalW2 suggest that this previously uncharacterized protein is most homologous to the human MRP1 protein (Fig. 1). The *C. elegans* MRP-7 protein is highly conserved with human MRP1 (61% similarity and 43% amino acid identity). The putative transporter is predicted to have 3 transmembrane domains (TMDs) and, consistent with vertebrates, 17 transmembrane helices, Walker A, ABC signature, and Walker B consensus sequences between TMD 11 and TMD 12, and within the C-terminus (Bakos *et al.* 1996, Frelet & Klein 2006).

Our previous studies in *C. elegans* indicate that MeHg confers concentration-dependent animal death with an LC50 of 95 μM (Vanduyn et al. 2010). Vertebrate MRPs have been shown to confer increased resistance to toxic substrates in a dose-dependent manner, largely by pumping substances out of the cell (Leslie *et al.* 2004). In order to determine if a reduction in MRP-7 would significantly reduce the LC50, nematodes were first grown on RNAi bacteria to reduce expression of *mrp-7*; the reduction in mRNA levels of *mrp-7* was confirmed by quantitative RT-PCR (Supplementary Fig. 1). Animals were then exposed to various concentrations of MeHg ranging from 0–10 μM, and the number of live animals was determined 48 h later. We find that a decrease in *mrp-7* gene expression results in a dramatic reduction in the LC50 to approximately 5 μM (Fig. 2). Furthermore we utilized RNAi to evaluate whether any of the other MRPs may contribute to MeHg-associated vulnerability, and found that no other gene affected animal viability (data not shown). These results indicate that MRP-7 inhibits MeHg-induced animal death in a dose dependent manner, and suggests that the protein may inhibit toxicity through limiting Hg accumulation within the cell.

MRP-7 expression reduces whole-animal Hg levels

Expression of MRP1 confers resistance to a variety of toxicants, and has been shown to actively transport metalloids as well as glutathione (GSH) and GSH-conjugated xenobiotics (Dallas et al. 2006). In vertebrates MeHg has been shown to induce the expression of glutathione S-transferases (GSTs) that conjugate the toxicant to GSH allowing for cellular excretion through phase III proteins. Considering the high homology between MRP1 and MRP-7, and that MRP1 has been shown to play a role in phase III detoxification, we asked whether MRP-7 could be involved in limiting cellular Hg levels. To determine if MRP-7 expression can modulate whole animal Hg levels, we grew WT or *mrp-7* RNAi adult animals on 1 μM MeHg for up to 48 h and determined whole animal Hg concentrations by ICP-MS. We find that *mrp-7* RNAi animals have approximately 2-fold greater levels of Hg than WT at the later time points of chronic MeHg exposure (Fig. 3). These results indicate that MRP-7 may transport Hg out of the cell to reduce MeHg-associated cellular stress.

MRP-7 expression reduces MeHg-associated endoplasmic reticulum, mitochondria, and Golgi apparatus stress response

Exposure to MeHg has been shown to increase cellular ROS and stress response gene expression in both vertebrates and in nematodes (Yu et al. 2010, Limke *et al.* 2004, InSug *et al.* 1997, Goering *et al.* 2000, Vanduyn et al. 2010, Rudgalvyte *et al.* 2013). In order to determine if the expression of MRP-7 may modulate MeHg-associated cellular stress, particularly in specific intracellular compartments, we evaluated gene expression levels of several glutathione S-transferases (GSTs) and heat shock proteins (HSPs) following both acute and chronic toxicant exposures in both WT and *mrp-7* genetic knockdown animals. GST-38 is expressed within the intestines and nervous system and has been shown to affect whole animal vulnerability to numerous toxicants and other stressors, including MeHg (Vanduyn et al. 2010, Liao & Freedman 1998). HSP-6 and HSP-16.1 are expressed exclusively in the mitochondria and Golgi apparatus, respectively, with the latter involved in maintaining Ca^{2+} homeostasis in the Gogi by functionally interacting with the Ca^{2+} and Mn^{2+} transporter PMR-1 (Kourtis *et al.* 2012). HSP-4 is a member of the hsp70 family of molecular chaperones that has been shown to be upregulated in response to ER stress (Ient *et al.* 2012). A 4-hr exposure to MeHg results in over 30-fold increase in *gst-38* and *hsp-16.1* gene expression, suggesting as in vertebrate studies that the toxicant perturbs redox homeostasis, protein folding, and Ca^{2+} regulation (Fig. 4a and b) (Kourtis et al. 2012, Lee & Lee 2013). We do not find a significant change in gene expression of either of these proteins (or the other tested stress response proteins) in a *mrp-7* genetic knockdown background compared to WT following acute exposure, consistent with lower cellular Hg levels found in

both genetic backgrounds (Fig. 3). In contrast, following a chronic 96-hr exposure to MeHg, there is a significant increase in *hsp-4*, *hsp-6*, and *hsp-16.1* gene expression in *mrp-7* RNAi animals relative to WT animals, suggesting that an increase in cellular Hg levels disrupts protein homeostasis or function within the endoplasmic reticulum, mitochondria, and Golgi complex, respectively (Fig. 4d) (Kourtis et al. 2012, Ient et al. 2012, Lee & Lee 2013). We have also recently shown that the PD-associated GST-π homologue GST-1 in *C. elegans* is expressed in DA neuron and modulates toxicant-induced DA neurodegeneration (Settivari *et al.* 2013). MRP-7 RNAi animals chronically exposed to MeHg show a small but significant increase in *gst-1* expression (Fig. 4c) suggesting that cells that express *gst-1* are also sensitive to MeHg. These results suggest that MRP-7 may also be expressed in DA neurons and play a role in modulating MeHg-associated DA neuron vulnerability.

Chronic MeHg exposure induces MRP-7 gene expression

Cellular resistance to hormones, xenobiotics, and heavy metals has been correlated with an increase in MRP expression (Deeley & Cole 2006, Magnarin *et al.* 2004, James & Davey 2009). Inorganic Hg has been shown to induce MRPs in mammalian and fish cell lines that results in increases in toxicant efflux and cellular resistance (Della Torre *et al.* 2012, Aleo *et al.* 2005). We have shown that genetic knockdown of *mrp-7* results in an increase in the cellular stress response and whole animal sensitivity to MeHg indicating that MRP-7 plays an integral role in reducing cellular toxicity and suggests that as in vertebrates, MRP-7 expression may be induced to facilitate cellular excretion of the toxicant (Fig. 2 and 4). In order to determine if MRP-7 gene expression may be modulated by chronic exposure to MeHg, we utilized RT-PCR to evaluate *mrp-7* gene expression levels in control and animals exposed to 400 nM MeHg for 96 h. As can be seen in Fig. 5, the exposure group has an approximately 1.7-fold increase *mrp-7* mRNA levels relative to unexposed animals. We find no change in *mrp-7* expression levels in acute exposure conditions, consistent with the relatively low level of MeHg-associated stress (data not shown). These results indicate that MeHg induces *mrp-7* gene expression following chronic exposures to the toxicant and suggests that a concomitant increase MRP-7 protein may contribute to the cellular resistance.

MRP-7 is expressed in DA neurons

Vertebrate studies have shown that MeHg confers DA neuronal dysfunction and cell death (Gotz *et al.* 2002, Tiernan *et al.* 2013). Our prior studies have demonstrated that low chronic exposure to MeHg also confers DA neurodegeneration in *C. elegans* (Vanduyn et al. 2010). Considering that MRP-7 inhibits MeHg-associated animal toxicity and Hg accumulation, we asked whether MRP-7 is expressed in DA neurons which could facilitate cellular Hg efflux. To determine if MRP-7 may be expressed in *C. elegans* DA neurons, we first generated transgenic animals (RJ1089) co-expressing yellow fluorescent protein (YFP) behind the endogenous dopamine transporter (DAT-1) promoter and cyan fluorescent protein (CFP) behind the endogenous MRP-7 promoter (Fig. 6a–d) (Hunt-Newbury et al. 2007, Nass et al. 2002). *In vivo* analysis of the expression pattern of the MRP-7 reporter fusion indicates that the protein is expressed in head neurons (as well as several other cells in the body), but the expression pattern within the head made it difficult to determine whether the protein is specifically expressed in the DA neurons (Fig. 6a–d). We therefore generated primary cultures from the RJ1089 animals, and observed MRP-7-associated CFP fluorescence in all cells expressing YFP, indicating that the approximately 3.5 kb promoter of MRP-7 is sufficient to drive the expression of the MRP protein in DA neurons (Fig. 6e–h). The expression of MRP-7 in other cell types is also consistent with the differential expression patterns of MRP1 in vertebrates (Dallas et al. 2006). These results indicate that MRP-7 is likely expressed in DA neurons and suggests that the transporter may inhibit MeHg-associated DA neurodegeneration.

MRP-7 inhibits MeHg-induced DA neurodegeneration *in vivo*

MRPs inhibit cellular toxicity by transporting substrates out of the cell. Considering that MRP-7 inhibits MeHg-associated animal toxicity and the MRP-7 transcriptional fusion suggests the transporter is expressed in DA neurons, we asked if MRP-7 expression may inhibit MeHg-associated DA neurodegeneration. L1 animals were grown on plates containing various concentrations of MeHg and seeded with RNAi bacteria targeting *mrp-7* or containing an empty vector. After 4 days, the DA neurons were evaluated under a fluorescent dissecting microscope. Chronic exposure of *mrp-7* RNAi animals to 400 nM MeHg results in approximately 60% of the animals displaying significant DA neurodegeneration, while none of the WT animals show DA neuronal death (Fig. 7c and e). Importantly, there does not appear to be any gross morphological differences, or differences in growth and development between WT and *mrp-7* RNAi animals for this exposure condition, suggesting that the toxicant is not causing large scale cell death (Fig. 7a–d). Also, we did not see any morphological changes in neurons in WT or *mrp-7* knockdown animals expressing a GFP reporter in serotonergic, cholinergic, glutamatergic, or GABAergic neurons following toxicant exposure or any other apparent change (data not shown), suggesting that *mrp-7* does not play a role in affecting MeHg-associated vulnerability within these cells. Also, considering that vertebrate MRPs can have broad substrate specificities (Deeley & Cole 2006), and we have shown that the nematode's DA neurons are vulnerable to the PD-associated toxicants Mn^{2+} and Al^{3+} (Settivari et al. 2013, Vanduyn et al. 2013), we evaluated if MRP-7 may play a role DA neuron vulnerability to these metals. Our preliminary studies do not show a difference in whole animal or DA neuron vulnerability in WT and *mrp-7* genetic knockdown animals (data not shown), suggesting that MRP-7 is not involved Mn^{2+}- or Al^{3+}-associated toxicity, although other MRPs could theoretically compensate for the loss of the transporter. Overall these studies indicate that MRP-7 is expressed in DA neurons, and MRP-7 expression is sufficient to inhibit MeHg-induced DA neurodegeneration.

DISCUSSION

MeHg is a potent environmental neurotoxicant for which exposures can result in motor and sensory deficits, mental retardation and death (Clarkson & Magos 2006). Exposures to MeHg may also be a contributing factor in the development of PD, a neurological disorder resulting in the loss of DA neurons in the substantia nigra (Petersen et al. 2008). When exposed to MeHg, *C. elegans* recapitulates fundamental hallmarks of toxicant-associated animal pathology and neurodegeneration, yet as in its vertebrate counterparts, the molecular pathways and transporters involved in the cell death are ill-defined (Vanduyn et al. 2010). In this study we incorporated an unbiased, whole genome reverse genetic screen to identify genes for which expression inhibits MeHg-associated toxicity. Here we show that the putative ABC transporter MRP-7 is expressed in the DA neurons and inhibits MeHg-associated DA neurodegeneration. This study shows for the first time that an MRP is expressed *in vivo* in DA neurons, and suggests that the transporter may help mediate environment-associated neuropathology.

MRPs play an integral role in transporting xenobiotics out of non-neuronal cells and are a major contributor to multidrug resistance in cancer, but remarkably few studies have targeted MRPs to specific neuronal types or associated neuronal expression with function (Dallas et al. 2006). Although numerous vertebrate MRPs have been identified in the CNS, most notably within the blood-brain and blood-cerebrospinal barriers, microglia, and astrocytes, studies of MRP expression and function in the brain have often yielded contradictory results (Dallas et al. 2006). Mrp1, Mrp3, Mrp4, and Mrp5 transcripts have been identified in primary cultures of embryonic rat brain neurons and oligodendrocytes,

and immunohistochemical studies in human brain sections suggest expression of MRP4, MRP5, and MRP8, but neuron-specific expression and functional studies are lacking (Hirrlinger *et al.* 2002, Nies *et al.* 2004, Bortfeld *et al.* 2006). The relatively high number of isoforms (9) and overlapping specificities and tissue expressions, both in normal and pathological states, have likely contributed to the experimental discrepancies and inhibited more mechanistic studies (Dallas et al. 2006).

Several recent studies have implicated vertebrate MRP2 in modulating inorganic Hg levels in the GI tract and kidney and possibly in other cell types. MRP2 expression in liver and kidney has been shown to eliminate Hg from the organs, likely through Cys-S-conjugates with glutathione (GSH) (Bridges *et al.* 2011). MeHg may also be oxidized to inorganic Hg and excreted similarly (Norseth & Clarkson 1970). Furthermore, MRP2 may reduce fetal and placental burden of MeHg by transport through the placenta (Bridges *et al.* 2012). Recently Lobner and colleagues reported that GSH supplementation protects mixed cortical cultures containing neurons and glia from MeHg-associated cytotoxicity (Rush et al. 2012). Co-exposure with the organic anion transporter and MRP inhibitor MK571 increased cellular Hg levels and toxicity, suggesting that an MRP isoform that is sensitive to MK571 (MRP1, MRP2, MRP4, or MRP5) may play a role in Hg efflux (Rush et al. 2012, Lania-Pietrzak *et al.* 2005, Pratt *et al.* 2005, Reid *et al.* 2003, Yamazaki *et al.* 2005). Our studies show MeHg induces MRP-7 gene expression, consistent with vertebrate studies that show MRPs can be induced by inorganic Hg and MeHg (Toyama *et al.* 2007, Della Torre et al. 2012, Liu *et al.* 2003). We also show that acute and chronic MeHg exposure results in a large induction of *gst-38* gene expression (approximately 4- to 35-fold) suggesting that GST-38 responds immediately and during long-term exposure and may be involved in reducing cellular Hg levels through conjugating GSH to MeHg (or Hg) to facilitate MRP-7-associated cellular export.

This study also provides insight into the intracellular HSP stress response involved in MRP-7-associated inhibition of MeHg toxicity. Acute exposure of animals (with or without MRP-7 expression) to MeHg results in a dramatic increase in *hsp-16.1* gene expression suggesting that Golgi-associated Ca^{2+} regulation is particularly vulnerable to MeHg-associated toxicity. These results are consistent with vertebrate studies that show that cellular toxicity involves disruption of intracellular Ca^{2+} homeostasis (Atchison & Hare 1994). The induction of the ER-associated *hsp-4* and mitochondria-associated *hsp-6* in the *mrp-7* RNAi background when the intracellular Hg levels are high (ie, chronic exposure) suggests that MRP-7 expression limits MeHg-associated ER and mitochondria toxicity. These results are also consistent with vertebrate and our recent nematode studies that show that MeHg confers mitochondria deficits and ER stress (Rudgalvyte et al. 2013, Vanduyn et al. 2010).

It is intriguing to consider that MeHg-associated MRP expression or function could play a role in the development and/or progression of PD. At least three theoretical possibilities for the increased loss of DA neurons in PD have been proposed: 1) a genetic or *in utero* event or exposure results in a decrease in DA neurons at birth followed by a normal rate of neuronal loss due to aging; 2) an environmental insult sometime after birth decreases the number of DA neurons followed by normal aging-related rate of decline; 3) an unknown factor increases the rate of DA neuronal death or vulnerability during the lifespan (Langston 1990). Low chronic exposure to MeHg in humans results in neurological and behavioral effects that may not manifest for many years after exposure (Yorifuji *et al.* 2011) It is possible that MeHg exposure *in utero* or postnatally may result in DA neuronal loss and/or increase DA neuron vulnerability to environmental neurotoxicants. Our DA neurodegeneration studies are in line with the last two theoretical possibilities on DA neuronal loss in PD, as animals

that are exposed to low chronic levels of MeHg soon after hatching and into early adulthood show DA neuron degeneration.

A limited availability of GSH to interact physically or functionally with MRP may increase DA neuron vulnerability. PD patients often have a 40–50% reduction in GSH within the substantia nigra the neurons often show significant hallmarks of oxidative stress (Zeevalk *et al.* 2008). Chronic exposure to MeHg through food sources such as fish may also reduce GSH levels, as vertebrate studies have shown that the toxicant can inhibit intracellular GSH accumulation (Yee & Choi 1996, Franco *et al.* 2009, Amonpatumrat *et al.* 2008). If conjugation of MeHg or Hg to GSH is necessary for MRP to transport the toxicant out of the cell, a MeHg-associated reduction in GSH could inhibit Hg efflux through MRP exacerbating DA neurodegeneration in normal and (pre-clinical) PD patients.

Although there have been no published reports of MRPs associated with the development of PD, polymorphisms in another class of membrane transporters involved in cellular efflux of toxicants, MDR1/ABCB1, have been associated with the disease. In a study of Chinese PD patients, three SNPs in MDR1 were significantly associated with the risk for PD in males over age 60 (Lee et al. 2004). Also individuals who had a history of pesticide exposure and had at least one mutant isoform of MDR1 had a 5-fold increased risk of the disease compared to the non-exposed patients (Drozdzik *et al.* 2003). The correlation between an MDR1 polymorphism, pesticide exposure, and PD was also found to be significant in a study of French patients (Dutheil et al. 2010). These studies suggest that phase III proteins may play a role inhibiting DA neuron vulnerability in PD.

In summary our findings show that a putative MRP, MRP-7, inhibits MeHg-associated DA neurodegeneration. We also show that the transporter plays a role in efflux of Hg from the cell and is expressed in the DA neurons. These studies indicate that MRP expression or function may significantly affect DA neuron vulnerability to both endogenous and exogenous xenobiotics, and suggest a novel therapeutic target to inhibit PD-associated pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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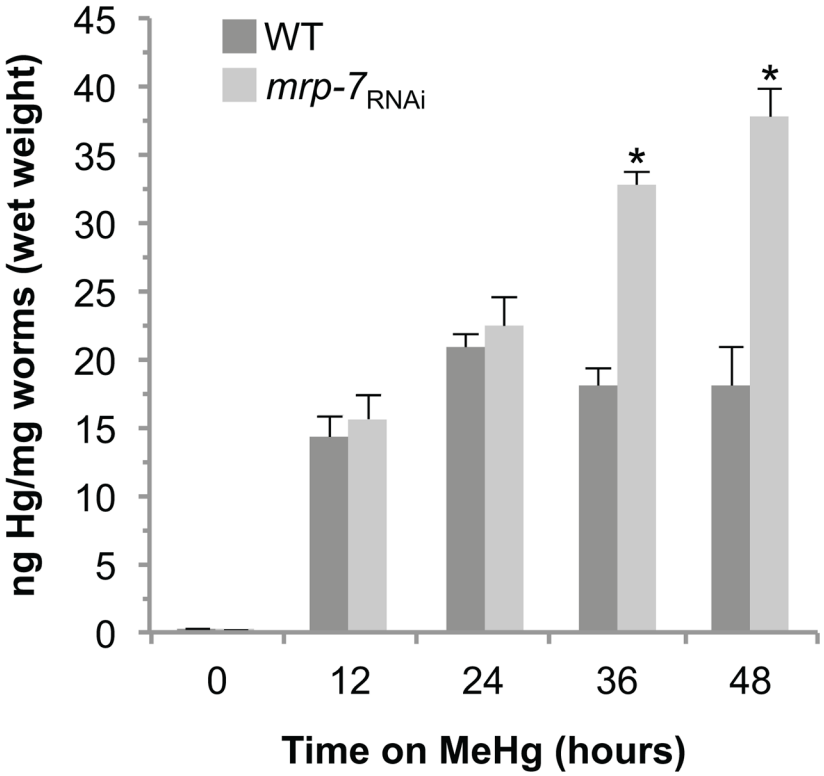

Figure 1.
Sequence alignment of human MRP1 with *C. elegans* MRP-7. ClustalW was used to align human MRP1 (accession NP_004987.2) with *C. elegans* MRP-7 (accession NP_507812.3). Identical residues are highlighted in dark gray and similar residues are highlighted in light gray. Thick black lines indicate the transmembrane domains of MRP1 and the conserved Walker A, Walker B and ABC signature sequences are boxed.

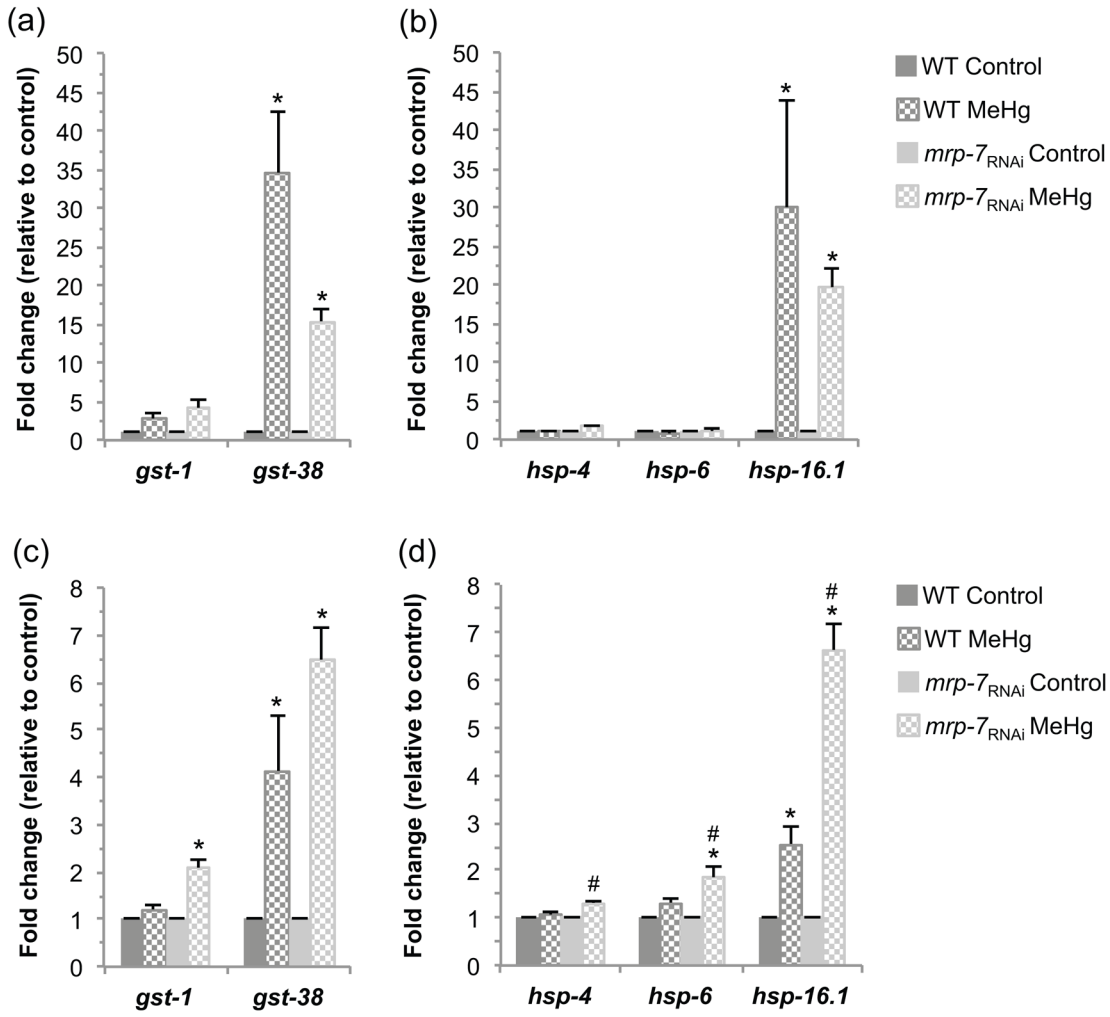

Figure 2.

MRP-7 inhibits MeHg-induced whole animal death. RNAi sensitive NL2099 worms were grown on RNAi bacteria to knock down *mrp-7* expression or empty vector for WT control and second generation L3 animals were exposed to the indicated concentrations of MeHg for 48 h. The number of live worms was counted and indicated as the percent of the total number of worms (> 50 per condition). The data is presented as mean ± SEM of 4 independent replicates. Data was analyzed by two-way ANOVA with Bonferroni post-tests. *mrp-7* RNAi is significantly different from WT with $p < 0.05$ (#) or $p < 0.001$ (*).

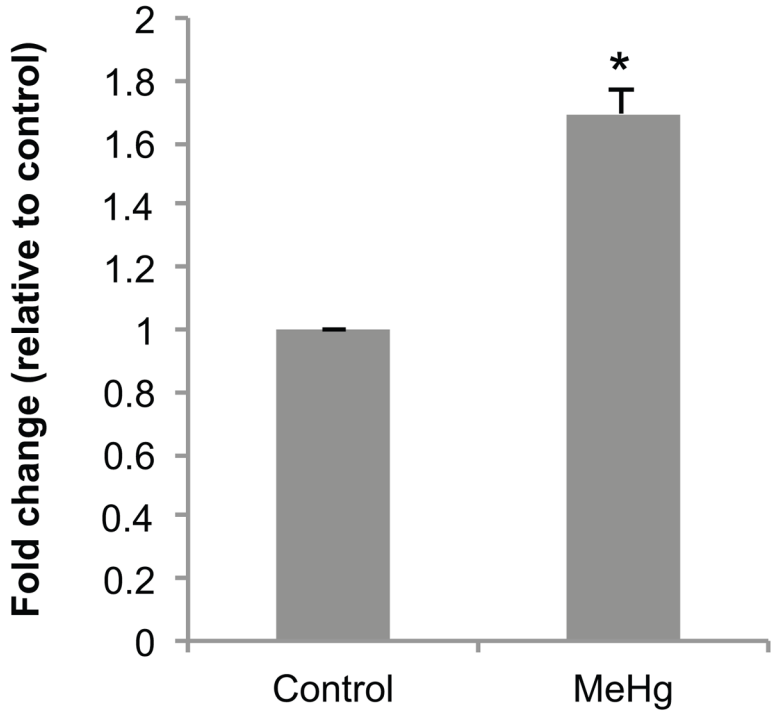

Figure 3.

MRP-7 inhibits the accumulation of MeHg. RNAi sensitive NL2099 worms were grown on RNAi bacteria knocking down *mrp-7* expression or empty vector for WT control and second generation adult animals were exposed to 1 μM MeHg for the indicated amount of time. Following exposure, worms were collected and the Hg content was measured by ICP-MS. Hg content was normalized to the wet weight of the worm samples and the data is presented as mean ± SEM of 4 independent replicates. Data was analyzed by two-way ANOVA with Bonferroni post-tests. *mrp-7* RNAi is significantly different from WT with $p < 0.001$ (*).

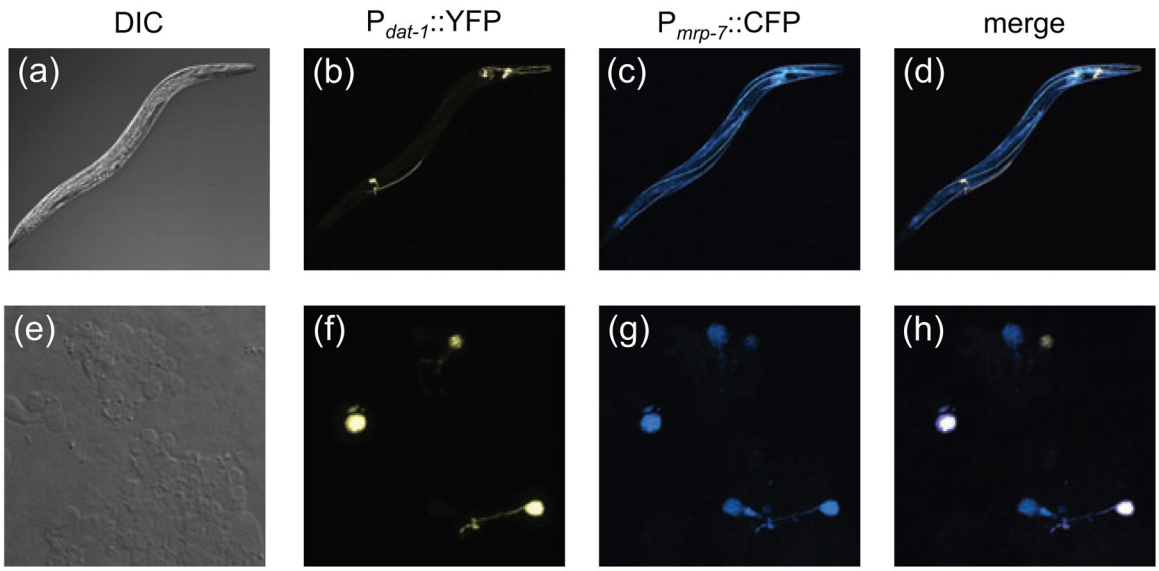

Figure 4.

GST and HSP mRNA expression are increased following MeHg exposure. Animals were exposed to 25 μM MeHg for 4 hours (a and b) or 400 nM MeHg for 4 days (b and d) and mRNA expression was measured by RT-PCR and analyzed using the ddCt method with normalization to GAPDH. dCt values for each gene were analyzed by one-way ANOVA followed by a Bonferroni post-test, * indicates a significant difference from the control treated group at $p < 0.001$ (a), $p < 0.01$ (b and d) or $p < 0.05$ (c), # indicates a significant difference between the WT and *mrp-7* RNAi MeHg treated groups at $p < 0.05$.

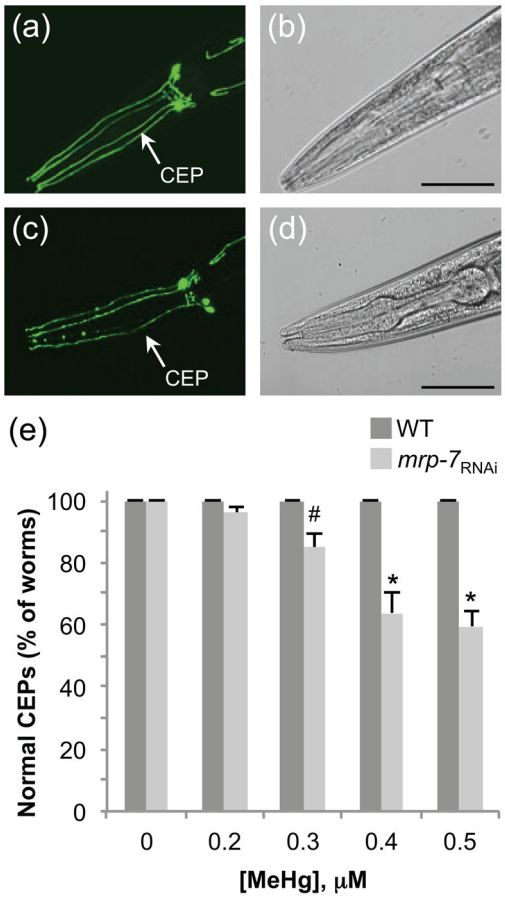

Figure 5.

mrp-7 mRNA levels are increased following chronic MeHg exposure. NL2099 L1 stage worms were grown on 400 nM MeHg for 4 days and *mrp-7* mRNA expression was measured by RT-PCR and analyzed using the ddCt method with normalization to GAPDH. dCt values for control and MeHg groups were compared using a student's t-test. * indicates a significant difference, $p < 0.05$.

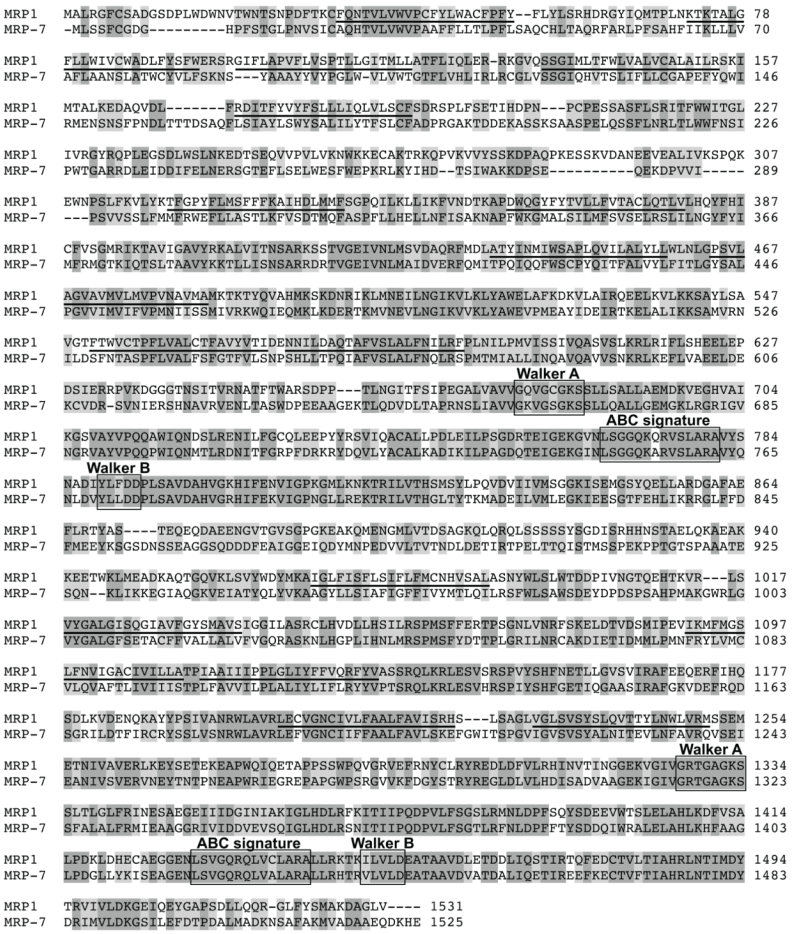

Figure 6.

MRP-7 is expressed in *C. elegans* DA neurons. Confocal imaging was used to visualize the expression of YFP and CFP in RJ1089 [P_{dat-1}::YFP; P_{mrp-7}::CFP] young adult nematodes (a–d) and primary cultures (e–f). DA neurons were identified by the presence of YFP (b and f). MRP-7 expression (c and g) was detected in the DA neurons as well as other cells (d and h), but not all cells (a and e).

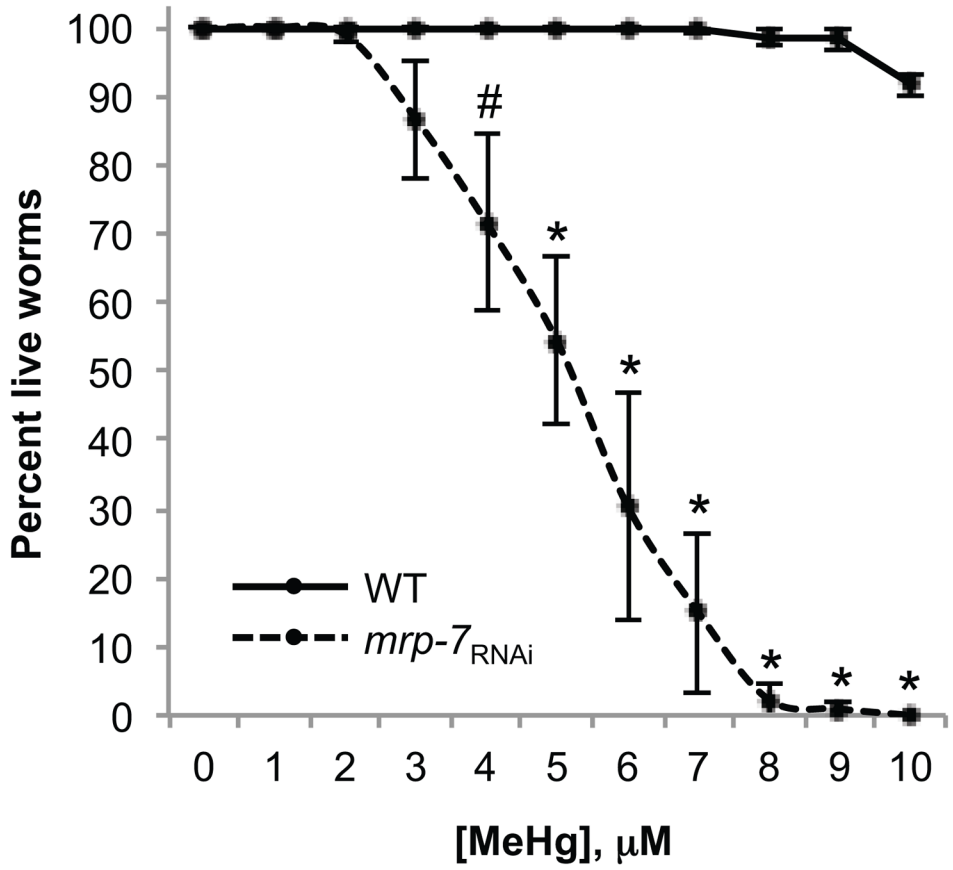

Figure 7.

MRP-7 inhibits MeHg-associated DA neuron degeneration. RNAi sensitive RJ928 L1 stage worms were grown on plates containing MeHg and seeded with RNAi bacteria targeting *mrp-7* or empty vector. After 4 days, the DA neurons were visualized using a fluorescent microscope and scored for degeneration. Worms are considered normal if all 4 CEP processes are intact from the cell body to the tip of the nose. MeHg (0.5 μM) does not affect the DA neurons in WT animals (a) however exposure to 0.5 μM MeHg causes degeneration of the DA neurons in the absence of *mrp-7* (c). The DIC images (b, d) illustrate that there are no gross morphological differences between WT and *mrp-7* RNAi, and the animals are of similar size (scale bar is 50 μM). Quantification of degeneration is presented as the percent of worms with normal CEPs (e). The data is presented as mean ± SEM of 5 independent replicates. Data was analyzed by two-way ANOVA with Bonferroni post-tests. *mrp-7* RNAi is significantly different from WT with $p < 0.01$ (#) or $p < 0.001$ (*).