

Published in final edited form as:

Neurochem Res. 2013 August ; 38(8): 1543–1552. doi:10.1007/s11064-013-1054-8.

Early-life exposure to methylmercury in wildtype and *pdr-1/parkin* knockout *C. elegans*

Ebany J. Martinez-Finley^{1,2,3}, Sudipta Chakraborty^{3,4}, James C. Slaughter⁵, and Michael Aschner^{1,2,3}

¹Division of Pediatric Toxicology, Vanderbilt University Medical Center, Nashville, TN

²Center in Molecular Toxicology, VUMC, Nashville, TN

³Vanderbilt Brain Institute, VUMC, Nashville, TN

⁴Center for Molecular Neuroscience, VUMC, Nashville, TN

⁵Department of Biostatistics, VUMC, Nashville, TN

Abstract

We examined the impact of early-life exposure to methylmercury (MeHg) on *Caenorhabditis elegans* (*C. elegans*) *pdr-1* mutants, addressing gene-environment interactions. We tested the hypothesis that early-life exposure to MeHg and knockout (KO) of *pdr-1* (mammalian: *parkin*/PARK2) exacerbates MeHg toxicity and damage to the dopaminergic (DAergic) system. *pdr-1* KO worms showed increased lethality and decreased lifespan following MeHg exposure. Mercury (Hg) content, measured with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was increased in *pdr-1* KO worms compared to wildtype (N2) controls. ²⁷7' dichlorodihydrofluorescein diacetate (H2DCF-DA) assay revealed a significant increase in reactive oxygen species (ROS) in both strains following MeHg exposure; however, while N2 worms showed an increase in *skn-1* transcript levels following MeHg exposure, there was no difference in *skn-1* induction in *pdr-1* KO worms. Dopamine-dependent behavioral analysis revealed an effect of MeHg on N2 wildtype worms, but no effect on *pdr-1* KO worms. Taken together, these results suggest that *pdr-1* KO worms are more sensitive to MeHg than wildtype worms, but MeHg does not exacerbate behavioral changes related to the absence of *pdr-1*.

Keywords

Methylmercury; parkin; early-life exposure; dopamine; large amino acid transporter

INTRODUCTION

Aging, early-life toxicant exposure and genetic predisposition, all likely contribute to the etiology of neurodegenerative disease. Several studies support an association between fish consumption or occupational exposure to mercury (Hg) and increased prevalence of Parkinson's disease (PD) [1–4]. Challenges in determining the contributions of early-life

Contact Information:

Ebany J. Martinez-Finley, PhD, ebany.martinez.finley@vanderbilt.edu

Sudipta Chakraborty, sudipta.chakraborty@vanderbilt.edu

James C. Slaughter, james.c.slaughter@vanderbilt.edu

Michael Aschner, PhD, michael.aschner@vanderbilt.edu

Vanderbilt University Medical Center, Division of Pediatric Toxicology, 11425 MRB IV, 2215-B Garland Ave. Nashville, TN 37232-0414, Tel : 615-322-8024, Fax: 615-936-4080

exposures to etiologies of neurodegenerative diseases are the long aging period and presence of several confounds that can occur during the aging process. The nematode, *C. elegans*, is a unique model system, with a short lifecycle allowing temporal resolution in determining long-term effects of early-life exposures. Hg is a persistent environmental, bioaccumulative metal. Studies with methylmercury (MeHg) show that developmental exposure has long-term consequences. Pathophysiological effects may manifest years or even decades after exposure making it imperative to study the effects of early-life exposure in later life stages. We tested whether early-life exposure to MeHg, and genetics, are important factors in toxicity and neurodegeneration later in life.

Dopamine neurons are known to be sensitive to environmental neurotoxicants [5, 6] and high Hg levels have been detected in PD patients [1, 2]. Autosomal recessive juvenile Parkinson's disease (ARJP) has been linked to mutations in the human *parkin* (PARK2) gene [7, 8]. *C. elegans* contain an ortholog for *parkin* called *pdr-1*. *pdr-1* shares homology and conserved function to the human gene and is expressed in most neurons [9]. *C. elegans* hermaphrodites possess eight DAergic neurons, with a fully characterized genome that contains all genes responsible for DA biosynthesis, packaging and reuptake [10] and behavioral assays, including the basal slowing response, have been shown to be good indicators of DAergic function [11].

Several studies have highlighted the effects of MeHg on the dopaminergic (DAergic) system. While DAergic neurons are not the only subpopulation susceptible to MeHg, MeHg inhibits DA uptake, and systemic or intrastriatal MeHg administration increases rat striatal DA release [12, 13]. *In vitro* rat synaptosomes show age-dependent sensitivity to MeHg, characterized by higher DA release and lower DAT activity [4, 14]. Delayed effects on a number of DAergic parameters, including DA levels, turnover and uptake, occur in rat offspring at weaning following *in utero* MeHg exposure [15]. Transient effects on DA receptor number associated with behavioral dysfunctions are noted in rat pups exposed at late gestation to a single high-dose of MeHg [16].

MeHg also modifies kinase signaling pathways, like activation of c-jun N-terminal kinases [17]; shown to play an important role in the degeneration of DAergic neurons [16]. Oxidative stress and lipid peroxidation are shared mechanisms in mediating neuronal death in both MeHg and neurodegenerative diseases [1]. *SKN-1* (mammalian: Nrf2) is also posited to be an important mediator of DAergic loss due to its involvement in the stress response and its expression DAergic neurons [18].

MeHg entry into cells occurs through the large amino acid transporter (LAT1), and levels of LAT1 are highly expressed during the prenatal period due to its role in amino acid transport during neurodevelopment [19], making the developing brain particularly vulnerable to MeHg. The MeHg-cysteine complex closely mimics the structure of methionine, making it a substrate for LAT1 [20, 21]. *C. elegans* have nine genes [*aat-1* to *aat-9* (amino acid transporter catalytic chain)] that encode homologues of LAT1. Three of these genes (*aat-1*, *aat-2*, and *aat-3*) have a much higher degree of similarity to the mammalian homologs [22].

The studies described herein were designed to establish novel and translational information on mechanisms associated with DAergic neurodegeneration, especially after early life exposure to MeHg and loss of the activity of parkin. Owing to parkin's expression in neurons and MeHg's involvement in DA neurodegeneration we tested the hypothesis that early-life exposure to MeHg and knockdown of *pdr-1* exacerbates MeHg toxicity, and loss of DAergic function later in life.

MATERIALS AND METHODS

Note: Methylmercury is toxic and all mercurials were handled as potentially highly toxic compounds and disposed of properly.

1.1 *C. elegans* maintenance

C. elegans strains were handled and maintained at 20°C as previously described [23]. Worms were grown on plates containing nematode growth medium (NGM) or 8P seeded with either *Escherichia coli* strain OP50 or NA22, respectively, as previously described [23]. The hermaphroditic wildtype N2 Britol strain was used as a control for all experiments. The VC1024 (*pdr-1* (gk448) III) strain was used for *pdr-1* knockout (KO) experiments. VC1024 was backcrossed four times. All strains were obtained from the *Caenorhabditis* Genetics Center, Minneapolis, MN.

1.2 MeHgCl treatments

To obtain a synchronous population prior to treatment, worms were treated with an alkaline bleach solution. Methylmercuric chloride (CH₃HgCl; Sigma-Aldrich) treatments (0–50 μM) were performed for 30 minutes using synchronized L1 worms to determine appropriate dosing. Five thousand (lifespan, lethality, behavior and broodsize), 10,000 (DCF assay), 20,000 (RNA), 50,000 (MeHgCl analyses) or 150,000 (dopamine) nematodes were treated with 0, 10 or 20 μM MeHgCl. After treatment, worms were washed three times with M9 buffer (KH₂PO₄; Na₂HPO₄; and NaCl) and either plated on seeded NGM plates or collected for immediate analysis. A sample size of six (n = 6) represents the total number of independent worm preparations; each independent experiment was carried out with 5,000–150,000 worms (see above).

1.3 Lethality

Following treatment and washing, worms were plated on seeded 60 mm NGM plates and allowed to grow for 24 hours. Worms were then counted and scored using a grid system. Nematodes on 4 of the 64 grids were counted and the number of worms per grid was averaged and multiplied by 64, and results expressed as percent control.

1.4 Lifespan and brood size

For determination of lifespan, 40 nematodes from each dose group were picked to a fresh NGM plate 24 hours following treatment. The worms were counted every day and scored as live or dead. Live *C. elegans* were picked to new plates every day during the egg-laying period of their lifecycle and once egg-laying ceased they were picked every other day until no live *C. elegans* remained.

For broodsize, four worms were placed individually on four NGM plates per treatment concentration 24 hours after MeHgCl treatment. Every 24 hours, the worm was transferred to a new NGM plate until no new progeny were generated in a 24 hour period. The progeny on each plate were counted and all plates from one hermaphrodite were added together and expressed as total progeny.

1.5 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) for Mercury content

The protocol used for ICP-MS was carried out as previously described [23]. Briefly, worms were treated with MeHgCl, followed by three washes. Samples were then allowed to air dry until a dehydrated pellet was obtained. Next, the dried sample was transferred to preweighed Teflon jars and reweighed. Concentrated acids HNO₃, HCl, H₂SO₄ were added, and the samples were placed in heat block at 100°C under the hood overnight. The following day

digested samples were reweighed and placed in preweighed metal-free 15mL centrifuge tubes. They were then analyzed by ICP-MS, which has a Hg limit-of-detection in the parts per trillion range.

1.6 RNA isolation

Following treatments, synchronized L1 stage worms were washed 3 times with M9 and frozen at -80°C until RNA purification. Worm pellets were resuspended in Trizol (200 μL /100 μL pellet); the protein and other impurities were separated from nucleic acids using chloroform. The pellet was then washed with 70% EtOH; DNA was digested using RNase free DNase kit (Ambion). RNA concentrations were determined (OD 260nm) using a ND-1000 spectrophotometer (NanoDrop®). Purified mRNA was stored at -80°C .

1.7 cDNA synthesis

Reverse transcription reactions were performed in 20 μL following the manufacturer's protocol (Applied Biosystems). A 10 μL reaction mixture containing, 10X RT buffer, 25X dNTP mix (100mM), 10X random primers, Multiscribe reverse transcriptase and sterile nuclease-free water was added to 10 μL of RNA (75ng). The contents were mixed gently and incubated at 25°C for 10 minutes, 37°C for 120 minutes followed by inactivation by heating at 85°C for 5 minutes. Synthesized cDNA was stored at -20°C until used. The suitability of the cDNA for PCR was determined using 2 μL of cDNA added to a 40 μL PCR. A control reaction containing 2 μl of purified mRNA was run without reverse transcription to test for presence of DNA contamination in the purified mRNA samples. The PCR was analyzed on a 1.5% (w/v) agarose gel and cDNA was stored at -20°C .

1.8 Primers

Oligonucleotide sequences for pdr-1: 5' GGACGGAGAAGCATGATTTG3' and 5' GCACATGACTGCGAGGACTA3'; aat-1: 5' TATTCCGGTGCTCGTGAAGGACAA3' and 5' GCTGTTCCGATTGCAAGCCAGTAA3'; aat-2: 5' ACTTCCTTCTGCTACATCGGGCAT3' and 5' CGCCGGATGCATAATTGCTGTTCT3'; aat-3: 5' ACTGGATTGCTATTGGAGGAGCCA3' and 5' ACAAGAAGCACGCACCCAATGAAG3'; skn-1: 5' AGTGTGCGCGTTCCAGATTTCC3' and 5' GTCGACGAATCTTGCGAATCA3'; gapdh (housekeeping): 5' CAATGCTTCCTGCACCACTA3' and 5' CTCCAGAGCTTTCCTGATGG3'. The specificity of each primer pair was confirmed by the identification of a single PCR product of predicted size on 1.5% (w/v) agarose gels. Oligonucleotide sequences were designed based on published literature.

1.9 Semi-quantitative determination of transcript levels by Real-time PCR

Real-time PCR was conducted in a Gene Amp 7300 sequence detection system (Applied Biosystems) using a 96-well plate (MicroAmp™ Fast). The relative quantification was determined with the $2^{-\Delta\Delta\text{Ct}}$ method [24]. GAPDH was used as an internal control.

1.10 DCF assay for oxidative stress

The formation of reactive oxygen species (ROS) was evaluated with 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA). Synchronized L1s were treated with MeHg as described earlier, and washed 4 additional times in M9 buffer. H2DCF-DA (1mM in DMSO) was then added to the tube with worms (in M9) for one hour in the dark. After 1h, the worms were washed three times with M9 to remove all H2DCF-DA content outside the worms. Worms were frozen and thawed twice and homogenized by sonication and then centrifuged. The supernatants were transferred to a 96-well plate and their fluorescence

levels (excitation: 485 nm; emission: 535 nm) detected with a FLEXstation III (Molecular Devices) pre-heated at 37°C. The fluorescence from each well was measured initially and at 1 hour. Values from final readings (one hour time point) minus initial readings were adjusted for background signal and reported as percent fluorescence relative to their respective control strain.

1.11 Basal slowing behavioral analysis

The behavioral analysis was adapted from a previously published protocol [11]. Well-fed L4 worms from the same treatment group were placed on two sets of 60×15mm plates: one with ~20μL bacteria spread in a ring with an inner diameter of ~1cm and an outer diameter of ~3.5cm and one without bacteria (both incubated at 37°C overnight and cooled to room temperature prior to assay). Bacterial mechanosensation induces the dopamine-mediated slowing of locomotion in the presence of food (bacteria), and can be measured by counting the number of body bends per 20-second interval. Locomotor rates were then compared between the well-fed worms placed on plates of food versus those placed on plates without food and is referred to as the change (Δ) in body bends/20 seconds. A lower value represents less slowing on food, indicating deficits in DAergic function. The *cat-2* strain is tyrosine hydroxylase deficient and therefore defective in bacterial mechanosensation, making it a positive control [11].

1.12 Statistical analysis

Statistics were carried out and graphs were generated with GraphPad Prism. Briefly, we used a sigmoidal dose-response model with a top constraint at 100% to draw the curves and determine the LD₅₀. The Kaplan-Meier method was used to estimate survival curves, and curves were compared using the log rank test or Wald test of coefficients from a Cox proportional hazards regression. Statistical analysis of significance was carried out by one-way ANOVA for brood size, and change in DA-mediated locomotor activity, and two-way ANOVA for ROS content, transporter levels, *skn-1* and MeHg content, followed by *post-hoc* Bonferroni or student's t-test when $p < 0.05$. In all figures, error bars are \pm SEM.

RESULTS

2.1 MeHg-induced lethality in N2 and *pdr-1*KO worms (Figure 1)

Assessment of lethality following a 30 minute exposure revealed a leftward-shift in the curve for *pdr-1* mutants compared to N2 wildtype worms. LD₅₀ for N2 = $24 \pm 0.059 \mu\text{M}$; LD₅₀ for *pdr-1*KOs = $17 \mu\text{M} \pm 0.051$. These data suggest that *pdr-1* KO worms are more sensitive to MeHg than N2 wildtypes. The doses used in this study, 10 and 20 μM , were chosen based on the LD₅₀ values for each strain.

2.2 Lifespan and brood size (Figures 2 and 3)

Log-rank test revealed that MeHg had a significant effect on lifespan in *pdr-1*KO (** $p < 0.05$), worms which was not seen in wildtype worms ($X^2(2, N = 120) = 0.9708$, $p = 0.6154$, n.s.) (fig. 2A/B). Knockout of *pdr-1* alone had an effect on lifespan, which was exacerbated by MeHg (fig. 2) ($X^2(2, N = 120) = 11.99$, ** $p = .0025$). Overall, there was a Dose effect ($p < 0.001$) and a Strain effect ($p = 0.003$). The broodsize analysis revealed a significant effect of Dose ($F(2,30) = 13.99$, *** $p < 0.0001$) and Strain ($F(1,30) = 20.37$, *** $p < 0.0001$) but not a Dose x Strain interaction ($F(2, 30) = 1.741$, $p > 0.05$, n.s.) (fig. 3). *Post-hoc* analysis revealed a significant difference between N2 0 μM and N2 20 μM (** $p = 0.0055$), *pdr-1*KO 0 μM and *pdr-1*KO 20 μM (## $p = 0.0099$) and between 20 μM N2 and 20 μM *pdr-1*KO (^^^ $p = 0.0009$).

2.3 Mercury content (Figure 4)

Hg accumulation was higher in *pdr-1KO* worms compared to wildtype worms at the 20 μM dose, but failed to reach significance at the 10 μM dose. Overall, there was a significant effect of Dose ($F(2,20)=15.83$, $***p<0.0001$). Although there was a difference in *pdr-1KO* worms compared to wildtypes, the Strain effect failed to reach statistical significance ($F(1,20)=3.912$, $p=0.06$, n.s.), as did the Dose x Strain interaction ($F(2, 20) = 2.51$, $p>0.05$, n.s.). There was a significant difference between *pdr-1KO* 0 μM and *pdr-1KO* 20 μM ($*p=0.016$), and the difference between the 20 μM N2 wildtype and 20 μM *pdr-1KO* failed to reach statistical significance ($p=0.06$).

2.4 *aat-1/aat-2/aat-3* expression (Figure 5)

Expression of these putative MeHg transporters was measured to determine if higher intraworm Hg concentration in *pdr-1KO*s was due to increased transporter expression. ***aat-1* expression** There was a significant effect of Dose ($F(2,45) = 4.30$, $*p < 0.05$) but no significant effect of Strain ($F(1,45) = 1.51$, n.s.), nor was there a Dose x Strain interaction ($F(2,45) = 0.07$, n.s.). *Post-hoc* analysis revealed a significant difference between 0 μM wildtype worms and 10 μM ($*p<0.05$) and 20 μM ($**p<0.005$) wildtype worms, and there was a trend toward an increase in expression in *pdr-1KO*s. ***aat-2* expression** There was no significant effect on any of the parameters measured; Dose ($F(2,32) = 0.50$, n.s.), Strain ($F(1,32) = 2.42$, n.s.), or Dose x Strain interaction ($F(2,32) = 0.36$, n.s.). *Post-hoc* analysis revealed a significant difference between 0 μM wildtype worms and 10 μM and 20 μM wildtype worms ($*p<0.05$), and a significant difference between wildtype 0 μM and *pdr-1KO* 0 μM ($**p<0.005$). ***aat-3* expression** There was no significant effect on any of the parameters measured; Dose ($F(2,31) = 0.5466$, n.s.), Strain ($F(1,31) = 1.504$, n.s.), or Dose x Strain interaction ($F(2,31) = 0.1420$, n.s.). *Post-hoc* analysis revealed significant differences between 0 μM wildtype and *pdr-1KO* 0 μM ($*p<0.05$).

2.5 Reactive oxygen species (ROS) (Figure 6)

To study MeHg-induced stress response, we measured the production of ROS. Using the DCF assay we show a significant increase in ROS in both strains 1 hour after 10 μM MeHg treatment (fig. 6). The effect of Dose was significant ($F(2,14) = 5.029$, $*p<0.05$), but the effect of Strain ($F(1,14) = 0.1179$, n.s.) and the Dose x Strain interaction ($F(2,14) = 0.03061$, n.s.), were not significant. Sodium nitroprusside (NaNP) was used as a positive control and MeHg in the absence of worms was used as a negative control. NaNP was significantly different from 0 μM wildtype and 0 μM *pdr-1KO* ($*p<0.05$; $\#p<0.05$).

2.6 MeHg increases *skn-1* expression in wildtype but not *pdr-1KO* worms (Figure 7)

Whole extracts were used to probe for *skn-1* transcriptional activity following 30 minute exposure to MeHg. Using qRT-PCR, we show that wildtype worms significantly upregulate *skn-1* in response to MeHg (fig. 7), but *pdr-1KO*s do not. Differences in the average threshold cycle (ΔCt) were determined and normalized to the expression of GAPDH. The Strain effect was significant ($F(1,50)=12.71$, $***p<0.001$), as was the effect of Dose ($F(2,50)=5.73$, $**p<0.01$). There was no Dose x Strain interaction ($F(2,50)=1.46$, n.s.). These data suggest that wildtype worms mounted an antioxidant response, whereas *pdr-1KO* worms failed to do so in response to MeHg.

2.7 Basal slowing behavioral analysis (Figure 8)

We measured DA-mediated behavior at 72 hours post exposure (adult stage) to determine if there was a decrease in functional DA. For each strain, locomotion rates in the absence and presence of bacteria (supplemental fig. 1) were calculated, and results are presented as Δ body bends/20 seconds (fig. 8). Asterisks indicate values significantly different from the

slowing seen in the wildtype untreated N2 worms. Higher values indicate functioning DAergic neurons, while lower values indicate dysfunction. When the change in locomotor activity (Δ body bends/20 seconds) was compared by one way ANOVA, it was statistically significant ($F(8,26)=22.60$, $***p<0.0001$). *Post-hoc* analysis showed all doses/strains were significantly different from 0 μ M wildtype ($**p<0.05$, $***p<0.0005$). There was an effect of MeHg on wildtype worms but not on *pdr-1*KO worms. *cat-2* unexposed worms were significantly different from all MeHg-treated wildtype and *pdr-1*KO worms irrespective of dose ($^{\wedge}p<0.05$).

DISCUSSION

Due to the known effects of MeHg on the DAergic system [4, 12, 13, 16] and the increased risk for damage to the DAergic system with *parkin* mutations [7, 8], we tested the hypothesis that loss of *pdr-1* exacerbates MeHg toxicity, with significant decreases in DAergic function following early-life exposure to MeHg. Because *parkin* is implicated in ARJP, the juvenile form of PD, our exposures were carried out in early-life stages. Mammals are susceptible to MeHg during the perinatal stage, including gestation and early postnatal periods [25].

Lethality was used as a short-term determinant of toxicity, revealing a leftward-shift in the curve for *pdr-1*KO worms compared to wildtype worms. The shift is indicative of increased sensitivity of *pdr-1*KOs to MeHg (fig. 1). This increased sensitivity to MeHg in *pdr-1*KOs has not been previously described, but is not surprising considering that environmental contaminants, including metals, have been implicated in the pathogenesis of PD [5, 6, 23].

Survival assays revealed decreased lifespan in *pdr-1*KO worms compared to wildtype worms, which was exacerbated by exposure to MeHg (figs. 2A/B). The lack of a MeHg effect in wildtype worms is consistent with an earlier report [23], and the decrease in lifespan in *pdr-1*KO worms compared to wildtype worms is corroborated by studies in *Drosophila* that show significant decreases in lifespan in flies lacking *parkin* compared to control flies [26]. To our knowledge this is the first report on the effect of MeHg on *pdr-1*KO lifespan. Broodsize was decreased in both strains at the 20 μ M dose of MeHg (fig. 3), consistent with the literature from MeHg-exposed wildtypes [18, 23]. While this is the first report of the effect of early-life MeHg on *parkin*, studies in *parkin* mutant mice, show no differences in viability or reproduction compared to controls [27], analogous to our data.

Intraworm Hg burden was higher in *pdr-1*KO worms compared to wildtypes (fig. 4), suggesting that they either take up more Hg or fail to extrude Hg. To date, there are no studies implicating *parkin* in regulating LAT1 expression; however, a role for *parkin* in ubiquitination and degradation of the divalent metal transporter 1 (DMT1) *in vitro* [28] and *in vivo* (unpublished observation) has been demonstrated. Accordingly, *parkin* may have the ability to modulate the *aat*'s, thereby increasing susceptibility to MeHg toxicity.

The increase in ROS (fig. 6) in both strains is consistent with the literature on MeHg [1]. We, and others, have shown that MeHg triggers oxidative stress and lipid peroxidation in multiple experimental models of neurodegeneration [18, 29]. Additionally, mutations in *parkin* in the human teratocarcinoma (NT-2) and neuroblastoma (SK-N-MC) cell lines have been shown to increase levels of 8-hydroxyguanine, protein carbonyls and 3-nitrotyrosine, and to decrease levels of glutathione (GSH), in response to H_2O_2 , MPP+ or 4-hydroxy-2-trans-nonenal (HNE) [30], consistent with increased oxidative stress. Post-mortem PD patients show increased lipid peroxidation, protein oxidation, 3-nitrotyrosine formation, DNA oxidation and breaks, and a decrease in the activities of ROS-scavenging enzymes and glutathione peroxidase [31]. An additional study in a human DA neuroblastoma cell line (SH-SY5Y) demonstrated that overexpression of parkin decreased the level of ROS and

protein carbonyls and attenuated DA-induced activation of c-Jun N-terminal kinase (JNK) and caspase-3 [32]. We didn't observe a significant increase in ROS levels in *pdr-1*KOs compared to wildtypes in response to MeHg exposure; this doesn't exclude the possibility that the DAergic neurons are more affected as we are measuring whole worm levels. The lack of a significant increase in ROS at the 20 μ M dose could be due to the increased death of worms at this dose and therefore a decrease in active mitochondria at the time point measured. An alternative explanation could be that the species that are measured by the dye are not the primary ROS that are generated at the 20 μ M dose.

Given the increase in ROS in both strains, we measured *skn-1* expression to determine if the presence of ROS was activating a stress response. *skn-1* induction was dose-dependent in wildtype worms, but there was a lack of a significant response in *pdr-1*KO worms (fig. 7). This lack of response by *pdr-1*KOs is important and likely contributes to the reduction in lifespan after MeHg exposure (fig. 2). Extended longevity correlates with enhanced resistance against oxidative damage [33, 34]; therefore, failure to initiate an oxidative stress response would decrease longevity which is evident in our lifespan data. *skn-1* mutants are sensitive to oxidative stress and have shortened lifespans [35]. Evidence from induced pluripotent stem cells (iPSCs) derived from two PARK2 patients showed increases in NRF2 with decreases in GSH compared to their control lines [36]. We did not observe increased *skn-1* levels in *pdr-1*KOs compared to wildtypes as was shown in the Imaizumi study, which may be due to the fact that we are measuring whole worm levels. While we did not directly measure GSH levels, we would predict that levels of GSH would be decreased in our *pdr-1*KOs in response to MeHg as many of the glutathione s-transferases (*gsts*) are under the control of the SKN-1-activated antioxidant response element (ARE) promoter. GSH and several *gsts* were reported to increase following MeHg exposure in wildtype *C. elegans* [18, 37]. *skn-1* has been shown to affect lifespan [35] and may participate in *pdr-1*KO-induced shortened lifespan. The lack of antioxidant response in *pdr-1*KOs can be exacerbated by the propensity of MeHg to bind to GSH thereby attenuating the cellular redox status and leaving neurons more susceptible to damage by ROS [38].

Functioning DAergic neurons are required for food sensing (mechanosensation) [11]. The basal slowing response behavioral assay revealed a decrease in the Δ body bends/20 seconds in wildtype but not in *pdr-1*KO worms (fig. 8), suggesting a decrease in DA-dependent function by 72 hours in wildtype worms but not *pdr-1*KOs. The absence of a MeHg effect in *pdr-1*KO worms may be due to the decreased functioning of the DAergic neurons even at the 0 μ M control dose and the presence of a 'floor' effect, as the control dose in *pdr-1*KO worms was similar to the 20 μ M MeHg dose in wildtype worms. Studies in *parkin* mutant mice indicate deficits in DA function, including abnormal baseline motor activity lack of amphetamine-induced increased locomotor activity, reduced amphetamine-induced dopamine release, decreased levels of VMAT2 and DAT protein and reduced [³H]-DA uptake [27]. An additional rodent study showed decreased DA release and uptake in young *parkin* knockout animals (Oyama et al., 2010). Full characterization of the DAergic system in *pdr-1*KOs following early-life MeHg will be necessary to understand if the lack of MeHg effect is limited to DA-mediated behavior or extends to other measurements in the DAergic system. Future studies will assess structural integrity of the DAergic neurons as well as attempt to quantify DA directly, which has proven to be difficult in worms (unpublished observations).

In summary, the combination of early-life exposure to MeHg and *pdr-1*KO had significant effects on oxidative stress and aging. Our data suggest that early-life MeHg exposure is a risk factor for loss of DAergic function later in life in wildtype worms but the combination of *pdr-1*KO and early-life MeHg does not further exacerbate the already reduced DAergic function produced by *pdr-1*KO alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Environmental Health Sciences (NIEHS) [R01ES07331, T32 ES007028] and the National Institutes of Health Loan Repayment Program. We would like to acknowledge the *C. elegans* Reverse Genetics Core Facility at UBC, which is part of the International *C. elegans* Gene Knockout Consortium and the *Caenorhabditis* Genetic Center (CGC) for providing the strains used in this manuscript. The authors would also like to acknowledge contributions from Chris Muller.

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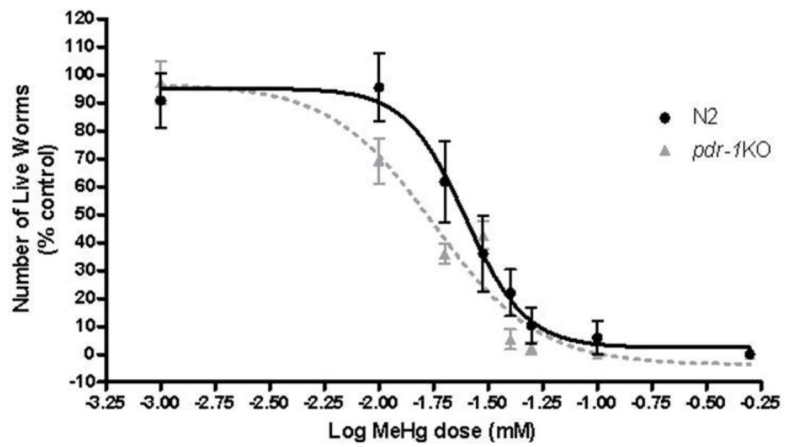
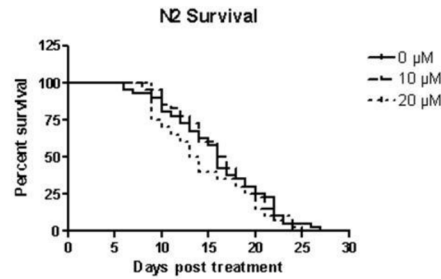
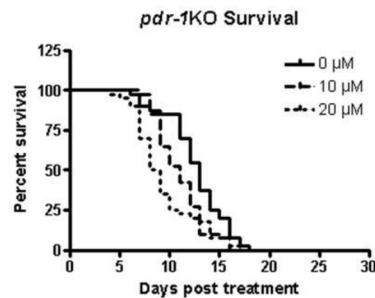


Figure 1. Dose-response curve of MeHg-induced lethality in N2 and *pdr-1KO* worms. *pdr-1KO* worms ($LD_{50} = 17 \mu\text{M}$, $n=10$) were more sensitive to MeHg than wildtype worms (LD_{50} for N2 = $25 \mu\text{M}$; $n=8$).

(A)



(B)

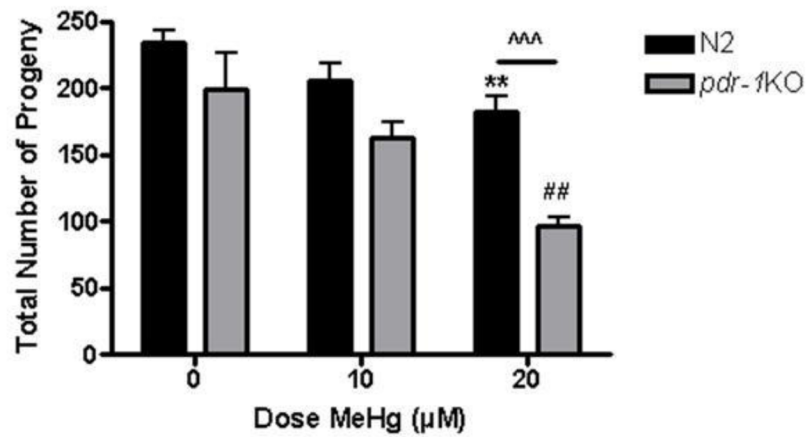


(C)

Strain	Median Survival (days) 0 μ M	Median Survival (days) 10 μ M	Median Survival (days) 20 μ M	P value
N2	16	16.5	13.5	p>0.05
<i>pdr-1</i> KO	13	11	8.5	**p<0.05

Figure 2.

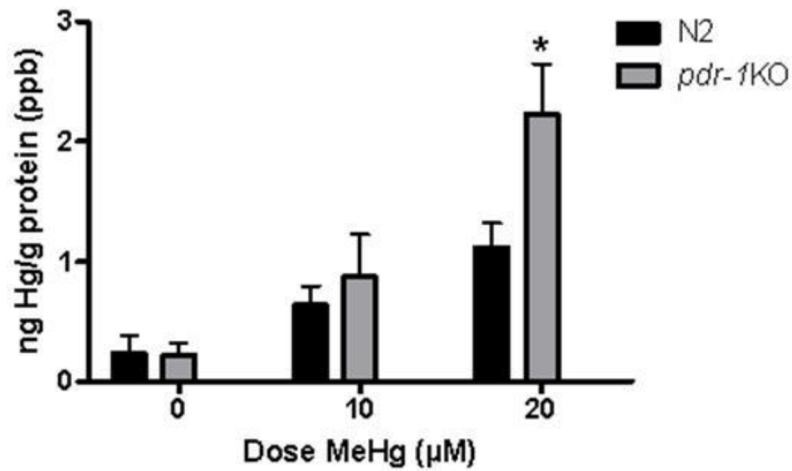
Lifespan is affected in *pdr-1*KO worms but not wildtype after MeHg exposure. (A) N2 wildtype worms do not show a decrease in lifespan following MeHg exposure (B) *pdr-1*KOs exhibit a reduced lifespan overall (compared to wildtype worms) and a further reduction in lifespan (**p<0.05) following 10 or 20 μ M MeHg exposure. Plotted values represent averages of three independent experiments, and the curves represent the best sigmoidal fit using log rank statistics.



Source of Variation	F value	P value
Dose	13.99	<0.0001
Strain	20.37	<0.0001
Dose x Strain	1.741	0.1927

Figure 3.

Brood size of wildtype and *pdr-1KO* nematodes following exposure to 10 or 20 μM MeHg. (A) Wildtype worms treated with 0, 10, or 20 μM MeHg (B) *pdr-1KO*s treated with 0, 10, or 20 μM MeHg. Total progeny was significantly decreased in both strains following 20 μM MeHg compared to 0 μM control. Two-way ANOVA revealed a significant Dose effect (<0.0001) and Strain effect (<0.0001) but not a Dose x Strain interaction ($p > 0.05$). *Post-hoc* analysis revealed a significant difference between N2 0 μM and N2 20 μM (** $p = 0.0055$), *pdr-1KO* 0 μM control and *pdr-1KO* 20 μM (## $p = 0.0099$) and between 20 μM N2 and 20 μM *pdr-1KO* (^^^ $p = 0.0009$).

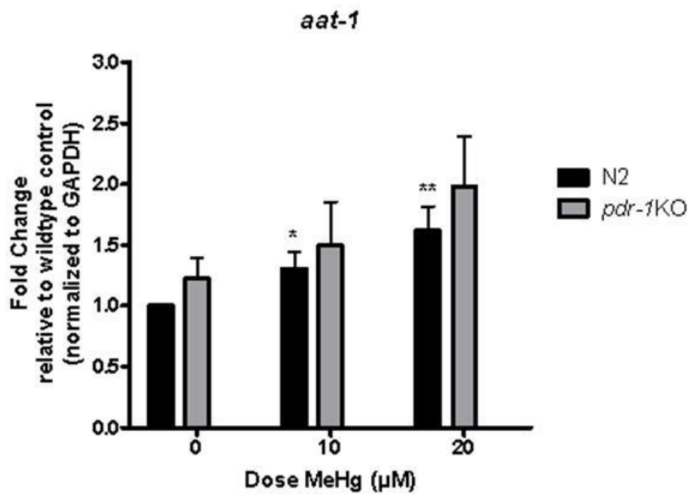


Source of Variation	F value	P value
Dose	15.83	0.0001
Strain	3.91	0.0619
Dose x Strain	2.51	0.1062

Figure 4.

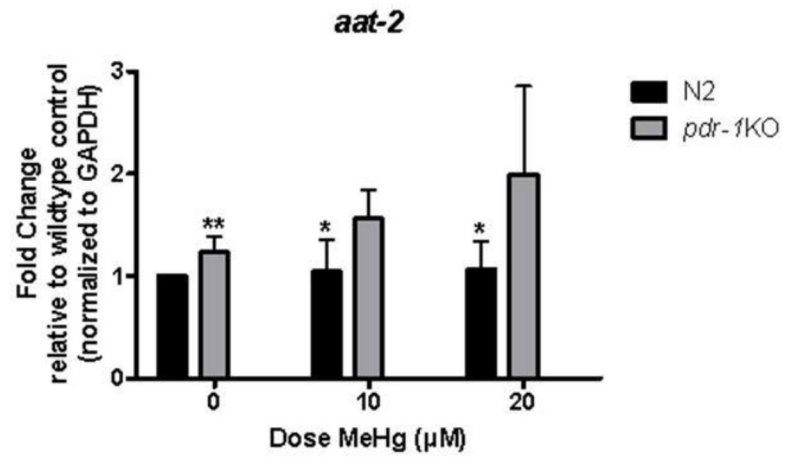
Hg content in wildtype and *pdr-1KO* worms. There was a significant effect of Dose and a trend toward a Strain effect. Hg content measured by ICP-MS. Two-way ANOVA revealed a statistically significant effect of Dose ($p < 0.0001$) but Strain ($p = 0.06$) and Dose x Strain interaction ($p > 0.05$) failed to reach significance. Post-hoc analysis revealed a significant difference between *pdr-1KO* 0 μM control and *pdr-1KO* 20 μM (* $p = 0.016$) and the difference between the 20 μM wildtype and 20 μM *pdr-1KO* failed to reach significance ($p = 0.06$). Results are presented as mean \pm SEM of 3–5 independent experiments.

(A)



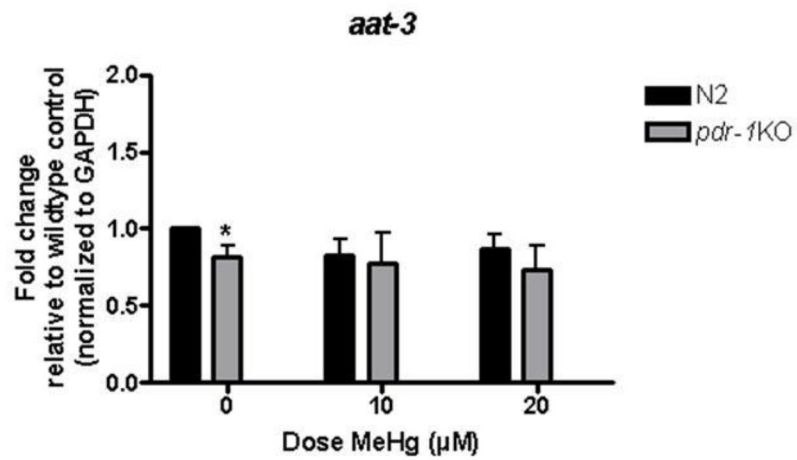
Source of Variation	F value	P value
Dose	4.30	0.0196
Strain	1.51	0.2253
Dose x Strain	0.07	0.9365

(B)



Source of Variation	F value	P value
Dose	0.50	0.6108
Strain	2.42	0.1299
Dose x Strain	0.36	0.7099

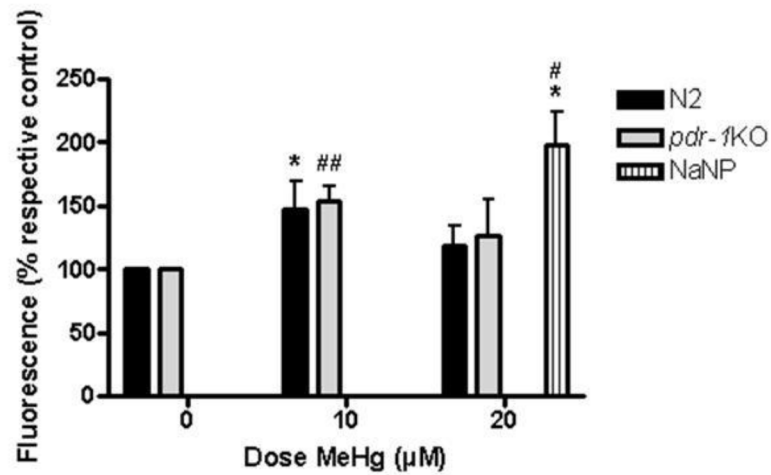
(C)



Source of Variation	F value	P value
Dose	0.5466	0.5844
Strain	1.504	0.2293
Dose x Strain	0.1420	0.8682

Figure 5.

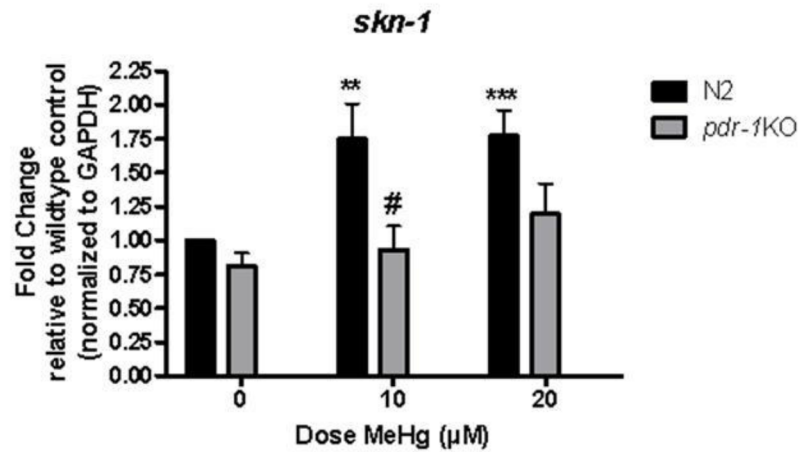
aat-1 and *aat-2* but not *aat-3* expression is higher in *pdr-1*KO worms than in wildtype worms. (A) *aat-1* expression in wildtype and *pdr-1*KO worms. Two-way ANOVA revealed a statistical significant Dose effect ($p=0.0196$) but not a Strain, or Dose x Strain interaction ($p>0.05$). *Post-hoc* analysis showed significance. **denotes significance from $0\mu\text{M}$ wildtype control ($*p<0.05$, $**p<0.005$). (B) *aat-2* expression in wildtype and *pdr-1*KO worms. Two-way ANOVA analysis showed no significant effect of any of the parameters measured ($p>0.05$). *Post-hoc* analysis showed significance. **denotes significance from $0\mu\text{M}$ wildtype control ($*p<0.05$, $**p<0.005$). (C) *aat-3* expression in wildtype and *pdr-1*KO worms. Two-way ANOVA revealed no significant Dose or Strain effects, or Dose x Strain interaction ($p>0.05$). *Post-hoc* analysis showed significance. **denotes significance from $0\mu\text{M}$ wildtype control ($*p<0.05$, $**p<0.005$). Results are presented as mean \pm SEM of 9–10 independent experiments.



Source of Variation	F value	P value
Dose	5.029	0.0226
Strain	0.118	0.7365
Dose x Strain	0.031	0.9699

Figure 6.

Reactive oxygen species (ROS) are elevated following MeHg treatment. Wildtype and *pdr-1KO* worms exhibit significantly higher ROS upon MeHg treatment. Two-way ANOVA showed a significant effect of Dose ($p < 0.05$), but the effect of Strain and the Dose x Strain interaction were not significant ($p > 0.05$). *denotes significance from $0\mu\text{M}$ wildtype ($*p < 0.05$). ##denotes significance from *pdr-1KO* $0\mu\text{M}$ (# $p < 0.05$; ## $p < 0.005$). NaNP=sodium nitroprusside ($500\mu\text{M}$) used as a positive control.



Source of Variation	F value	P value
Dose	5.73	0.0057
Strain	12.71	0.0008
Dose x Strain	1.46	0.2408

Figure 7.

skn-1 is induced upon exposure to 10 and 20 μM MeHg in wildtype worms but not *pdr-1*KOs. There was a significant Strain and Dose effect but not a Dose x Strain interaction. *Post-hoc* analysis revealed a significant difference between the 0 μM wildtype worms and the 10 μM wildtype (** $p=0.008$) and 20 μM wildtype strain (** $p=0.0008$). The difference between the 10 μM wildtype strain vs. the 10 μM *pdr-1*KO strain was also significant (# $p=0.02$). The difference between the 0 μM wildtype strain vs. the 0 μM *pdr-1*KO strain ($p=0.056$) and the 20 μM wildtype strain vs. the 20 μM *pdr-1*KO strain failed to reach significance ($p=0.06$).

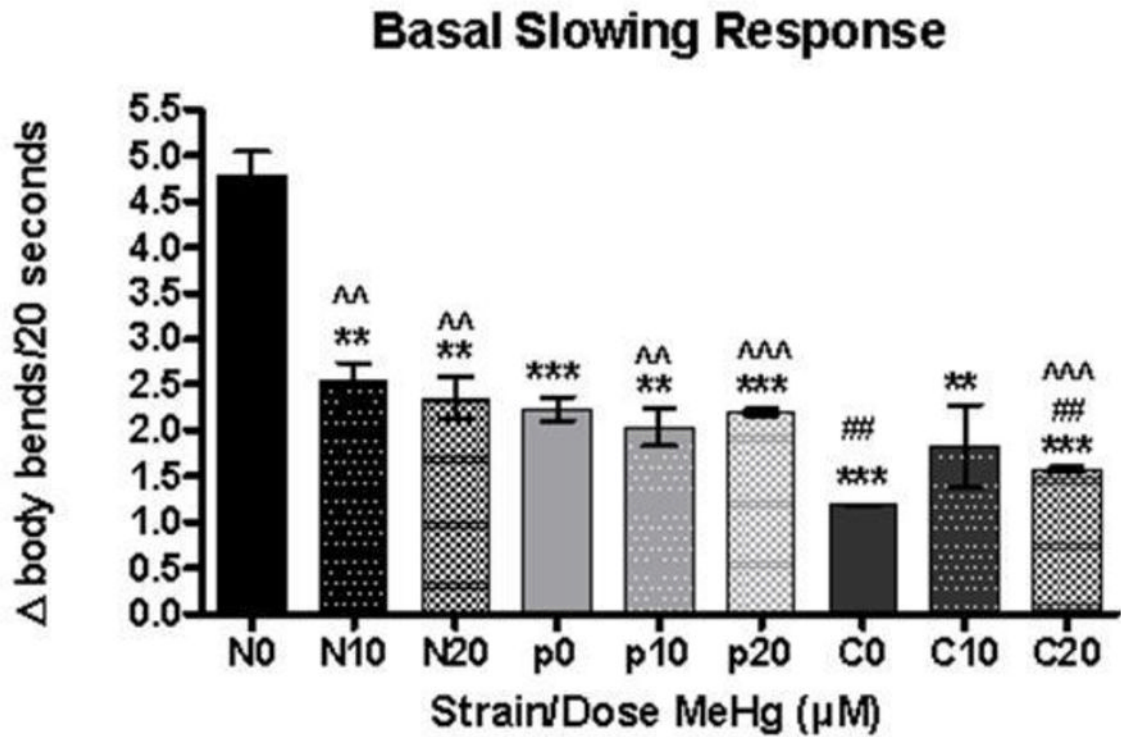


Figure 8.

MeHg has an effect of the Basal Slowing Response in wildtype but not *pdr-1*KO worms. Change in DA-mediated locomotor activity represented by ratio off food/on food. N=N2; p=*pdr-1*KO; C=*cat2* **denotes significance from 0μM wildtype control (**p<0.05, ***p<0.0005). ## denotes significance from 0μM *pdr-1*KO (##p<0.05). ^^denotes significance from 0μM *cat2*. Error bars represent the mean ± SEM of 25–40 individual worms.