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METHYLMERCURY INDUCED TOXICOGENOMIC RESPONSE IN C57 AND SWV MOUSE EMBRYOS UNDERGOING NEURAL TUBE CLOSURE

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Abstract

Methylmercury (MeHg) is a developmental neurotoxicant and teratogen and is hypothesized to perturb a wide range of biological processes, like other metals including arsenic (As) and cadmium (Cd). Common inbred mouse strains including C57 (sensitive) and SWV (resistant) display differences in sensitivity to metals such as As and Cd when exposed during neurulation. In this study, we investigated the impact of MeHg on neurulation, assessing for potential differences in sensitivity and associated toxicogenomic response in C57 and SWV mouse embryos. Parallel with morphological assessments of neural tube closure, we evaluated quantitative differences in MeHg-induced alterations in expression between strains at the gene level and within gene-enriched biological processes. Specifically, we observed differing sensitivities to MeHg-induced impacts on neural tube closure between C57 and SWV embryos in a time-dependent manner. These observations correlated with greater impact on the expression of genes associated with development and environmental stress-related pathways in the C57 compared to the SWV. Additional developmental parameters (e.g. mortality, growth effects) evaluated showed mixed significant effects across the two strains and did not support observations of differential sensitivity to MeHg. This study provides potential insights into MeHg-induced mechanisms of developmental toxicity, alterations associated with increased MeHg sensitivity and common biological processes affected by metals in embryos undergoing neurulation.

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1. INTRODUCTION

Mercury is a well-documented developmental neurotoxicant in humans. Mercury is naturally abundant in the earth's crust and is released into the environment via a wide range of human activity, including the release from coal-fired power plants. Mercury is bioconverted to methylmercury (MeHg) by microorganisms present in aquatic environments and is of public health concern due to bioaccumulation in fish and subsequent high consumption of contaminated fish in certain populations [1,2]. The single alkyl form, monomethylmercury represents the major form of MeHg (~99%) found in fish muscle tissue as compared to other methylated forms of mercury (i.e. dimethylmercury) [3,4]. Infamous cases of human exposure occurring in Japan (1950s) and Iraq (1973) have demonstrated MeHg's ability to impose neurological detriment and produce a wide array of teratogenic outcomes following exposure *in utero* [5,6]. In rodent studies, MeHg exposure *in utero* is associated with developmental delays, altered behavior, structural abnormalities and the neural tube defect (NTD) exencephaly [7–9]. Despite a lack of molecular studies investigating the effects of MeHg during neurulation, toxicological studies assessing MeHg impacts within other developmental time periods suggest that MeHg alters development by modulating cellular proliferation, altering structural proteins, producing cell death, disrupting the ubiquitin proteasome system and inducing oxidative stress and mitochondrial toxicity [10–15]. Studies comparing MeHg to other environmental metal contaminants (e.g. cadmium (Cd)) suggest that these other metals might also commonly impact development via these proposed mechanisms of toxicity [16]. The potential for adverse developmental consequences due to early gestational exposures to MeHg suggests a need for studies to address the unknown mechanistic relationship between MeHg and early stages of development.

The C57 and SWV mouse strains display differing sensitivities to various environmental teratogens when exposed *in utero* during early development [17–19]. Specifically, the C57 is more sensitive to metals such as Cd and arsenic (As) than the SWV during the period of neurulation [20–22]. This suggests that these strains might also show similar disparity in sensitivity to MeHg. Thus, in parallel with our previously published study [22] where we evaluated the toxicogenomic impact of Cd on neurulation, we compared C57 and SWV mouse embryos exposed to MeHg (i.p. 4mg/kg BW, GD8.0) assessing for effects on neural tube closure, fetal development and corresponding alterations in gene expression. In a time-dependent manner, we identified differential MeHg-induced impacts on gene expression and related GO biological processes between C57 and SWV in association with impacts on neural tube closure.

2. METHODS

2.1 Animals and Methylmercury Exposure

As previously described [22], C57BL/6J (C57) and SWV strains were maintained at the University of Washington in the Department of Environmental and Occupational Health Sciences. Colonies of C57 and SWV mice were originally established from Jackson laboratories and Dr. Phillip Mirkes laboratory (University of Washington, Department of Pediatrics), respectively. Under a 12h light/dark cycle, mice were housed in filter-covered transparent plastic cages in climate-controlled rooms. Water and food were available *ad libitum*. All experiments and animal care were completed in agreement with the University of Washington Animal Care Committee. Timed matings were achieved by placing individual male mice into cages containing two females in the evening. Copulatory plugs were identified in the early morning (8:00am $\pm$ 0.5h) the following day and designated as GD0. Pregnant dams were administered single doses via intraperitoneal injection (ip) on GD 8.0, 8:00am ($\pm$ 1h), with vehicle control (deionized water, 10ul/g) or monomethylmercury

hydroxide (4mg/kg/BW) (Alfa Aesar, Ward Hill, MA) (working concentration of 2mM). The MeHg concentration was chosen based on past studies suggesting embryonic developmental toxicity without causing maternal toxicity [9,23].

2.2 Embryo RNA Isolation

Pregnant dams were euthanized at GD8.0 + 12 and 24h (8.5 and GD 9.0) for gene expression assessments of MeHg exposed and control C57 and SWV embryos. The uterine horns were placed in cold CMF-EBSS (Gibco, Carlsbad, CA). Whole embryos were isolated from the uterus. All extraembryonic membranes, including the yolk sac were removed. Embryos were quickly examined for intended developmental stage of interest (GD8.5, 12h; Thelie Stage (TS) ~13, GD9.0, 24h, TS~14). Litters, which were identified to not be the correct stage due to missed pregnancies (>0.5 – 1.0 TS), were not used for this study. Pooled litters of embryos were kept separate, placed in liquid nitrogen and stored at –80°C. At a later date, litters were placed in 500ul of RTL Cell Lysis buffer (Qiagen, Valencia, CA) and homogenized with a 30G needle. RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) and assessed using the “6000” assay on the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

2.3 Gene Expression Analysis

Gene expression analysis was conducted using the Mouse Codelink Uniset I platform at the Fred Hutchinson Cancer Research Center Functional Genomic Laboratory (Seattle, WA). Specifically, in both strains, we assessed 3 independent litters of MeHg or vehicle exposed-embryos for 12h assessments and 4 independent litters for 24h assessments, with the exception of MeHg-exposed SWV embryos at 24h (n=3) (Supplemental Table 1). In total, we conducted gene expression analysis using 27 arrays. Using the manufacturer’s suggested protocol for the Codelink Mouse Uniset I 20K oligonucleotide array (GE Healthcare Life Sciences, Uppsala, Sweden), first and second strand cDNA synthesis was completed using 1 µg of total RNA. Samples were mixed with T7-(dT)24 primers and bacterial control mRNAs and denatured at 70°C for 10min. Superscript II RnaseH-reverse transcriptase, DTT, and dNTPs (Gibco, Carlsbad, CA) were added and samples were incubated at 42°C. Additional dNTPs, Rnase H and DNA polymerase were inserted into the mix and incubated for 2h at 16°C. Double-stranded cDNA was purified by QIAquick spin column (Qiagen, Valencia, CA). cRNA synthesis was completed by *in vitro* transcription (IVT) which consisted of mixing purified ds cDNA, ATP, GTP, CTP, UTP, biotin-11-UTP and the enzyme mixture and incubated at 37°C for 14h (Amersham Biosciences, Piscataway, NJ). cRNA was purified using the RNeasy kit (Qiagen, Valencia, CA). RNA quality was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cRNA was fragmented at 94°C for 20min. Hybridization (10ug cRNA) was completed for 18h at 300rpm and 37°C. Array chips were washed with 0.75X TNT for 1h at 46°C and incubated with AlexaFluor 647-streptavidin (Molecular Probes, Eugene, OR) for 30min. After 4 washes in 1X TNT and 2 washes in 0.05% Tween-20, slides were dried at 2000rpm for 3min and assessed for smears. Arrays were scanned on the Axon GenePix 4000 Scanner (Axon Instruments, Union City, CA) at 635nm, PMT 600V and 10 µM resolution.

Raw expression values were produced for all 20,290 probes using the Codelink Expression Analysis software v2.0 (GE Healthcare Life Sciences, Uppsala, Sweden). Raw intensities were normalized using the global median and transformed by log base 2 (Bioconductor (limma package), Seattle, WA). False discovery rates were calculated to control for multiple testing. In limited cases (<10%), more than one probe was present for a particular gene of interest. These probes were assessed separately. We refer to probes as “genes” in this manuscript to simplify the language used herein. We explored genes that were impacted by MeHg during this window of development in C57 and SWV embryos. We employed three

linear models using ANOVA ($p < 0.01$) to identify genes that were 1) differentially expressed based on the effect of time and MeHg treatment in the C57 (Model 1), 2) differentially expressed based on the effect of time and MeHg treatment in the SWV (Model 2), and 3) either commonly or differentially expressed based on the effect of time, MeHg treatment, and strain (Model 3) (Bioconductor, Seattle, WA).

$$\text{Log}_2[\text{Exp}_n]\text{C57} = B_0 + B_{\text{Time}}x_1 + B_{\text{MeHg}}x_2 + B_{\text{MeHg} \times \text{Time}}x_1x_2 \quad \text{Model 1}$$

$$\text{Log}_2[\text{Exp}_n]\text{SWV} = B_0 + B_{\text{Time}}x_1 + B_{\text{MeHg}}x_2 + B_{\text{MeHg} \times \text{Time}}x_1x_2 \quad \text{Model 2}$$

$$\text{Log}_2[\text{Exp}_n]\text{C57-SWV} = B_0 + B_{\text{Time}}x_1 + B_{\text{MeHg}}x_2 + B_{\text{Strain}}x_3 + B_{\text{MeHg} \times \text{Time}}x_1x_2 + B_{\text{Time} \times \text{Strain}}x_1x_3 + B_{\text{MeHg} \times \text{Strain}}x_2x_3 + B_{\text{MeHg} \times \text{Time} \times \text{Strain}}x_1x_2x_3 \quad \text{Model 3}$$

All genes identified to be significantly altered by MeHg (Models 1–3, $p < 0.01$) were plotted via cross scatter plot to display the magnitude, directionality and significance (symbol indicates model significance) of effect induced by MeHg exposure in C57 and SWV strains 12 and 24 hours post-injection (h). Less than 1% of genes identified to be significantly altered by MeHg were represented by more than one unique probe. Gene Ontology (GO) terms were classified by biological process, molecular function and cellular component [24]. To explore enrichment of GO terms, we completed GO analysis using MappFinder within genes identified to be significantly altered with MeHg exposure (ANOVA, B_{MeHg} , $p < 0.01$, Model 1–3). Enriched GO terms were identified using set criteria consisting of permutation value ($p < 0.02$), Z-score ($Z > 2.0$) and the number of genes altered within each GO category (≥ 3 genes). Using the GO-application GO-Quant [25], we quantified the extent of MeHg-induced impact within each enriched GO biological process for both C57 and SWV embryos by calculating the absolute average magnitude in change (fold ratio) associated with MeHg in all significant genes ($p < 0.01$, Model 1–3) at each respective timepoint. Significantly overrepresented terms with a minimum of 6 genes altered within each specific GO biological process are presented. Between C57 and SWV embryos, we investigated the MeHg response within genes related to system development (GO:48731) and response to stress (GO:6950) biological processes by creating cross-scatter plots for genes identified to be significantly altered with MeHg exposure (B_{MeHg} $p < 0.01$, Model 1–3). These two categories were chosen due to significant enrichment in MeHg exposed embryos, differences between C57 and SWV in the magnitude of effect within genes of these two categories, and previous studies suggesting these pathways to be disrupted by MeHg. The GO term “system development” is used to refer to genes related to many developmental processes, including organ, CNS, neural tube, eye, and heart development. The majority of genes related to the GO term “response to stress” are associated with classical environmental stress-related effects, including induction of apoptosis, response to DNA damage stimulus and negative regulation of cellular proliferation. Results from this study were compared with previously published analyses examining the toxicogenomic response associated with Cd exposure in C57 embryos undergoing neurulation [22] to identify common toxicogenomic responses associated with MeHg and Cd. We accomplished this using GO-Quant [25] by examining quantitative changes (absolute average fold change) in genes significantly altered by MeHg or Cd within common enriched GO biological processes (same criteria as above).

2.4 Reproductive and Developmental Assessments

Embryos used for gene expression studies (GD8.0 + 12h, 24h), as well as additional litters of embryos isolated GD8.0 + 6–24h, were examined under a microscope to assess closure of the anterior neural tube. Specifically, we evaluated for MeHg's impact on fusion of the neuroectodermal layers within the mid/hind brain region of the neural tube. Observations were made within this region of the neural tube due to correspondence with the development of exencephaly [26] and similarities in developmental progress between strains during this time period of neurulation [22,27]. At the timepoints assessed, we did not observe significant differences between control C57 and SWV embryos in terms of neural tube closure. Total implantations were also recorded at this time.

Separate litters were assessed on GD8.0 + 240h (GD18) to assess for gross reproductive and developmental effects (Supplemental Table 2). Pregnant mice were euthanized via inhalation of isofluroane and cervical dislocation. Fetuses were removed from each dam and sacrificed by overexposure to isofluorane. The total number of fetuses, implantation sites and resorptions (RR) were evaluated. Growth measurements including body weight (BW), head diameter (HD) and crown-rump length (CR) were also evaluated. Fetuses were assessed for the external malformation exencephaly, which is recognized as incomplete closure of the cranial region. On a litter basis, mean averages were calculated for all reproductive and developmental effects. Student t-tests were conducted to calculate for significant differences between MeHg exposed and control fetuses (one-sided, $p < 0.05$).

Due to mixed strain MeHg specific impacts on evaluated developmentally-related endpoints, we used logistic regression to assess multiple endpoints across time simultaneously to assess for differences in sensitivity. We assessed MeHg impacts on the prevalence in each litter of neural tube closure and exencephaly in C57 and SWV embryos across time, combining assessments from GD8.0 + 6–24h (GD8.25–9.0) with GD8.0 + 240h (GD18.0). Parallel comparisons with previously published Cd exposure data were used to evaluate strain sensitivity due to previously established strain differences in sensitivity with Cd [20,22]. Differences between the variables MeHg, Time, and Strain as well as the interaction of these variables were determined based upon whether they significantly reduced the deviance in the logistic regression analysis ($p < 0.05$). The prevalence of the endpoint for each litter was used in the analysis and there was significant extra-binomial variation between the litters. To account for this extra-binomial variation between litters we included a dispersion parameter in the logistic regression [28]. A similar analysis on the prevalence of resorptions in these litters was conducted.

3. RESULTS

3.1 Effect of MeHg on Neural Tube Closure

In Figure 1, we demonstrate the effect of MeHg on neural tube closure and the formation of exencephaly in C57 and SWV embryos across time in relation to previous assessments made with Cd [22]. MeHg significantly delayed closure of the neural tube in both C57 and SWV embryos ($p < 0.05$, B_{MeHg}). Additionally, we observed C57 and SWV embryos to be significantly differentially impacted by MeHg ($p < 0.05$, $B_{\text{MeHg_Strain}}$). Specifically, at GD8.0 + 12h, MeHg exposed C57 embryos displayed a 25% increase in open neural tubes compared to control whereas in the SWV no differences were observed between MeHg exposed and control ($< 1\%$). Whereas at GD8.0 + 24h, MeHg-exposed C57 and SWV embryos displayed similarities in increases in open neural tubes above controls (15%, C57; 22% SWV). At GD8.0 + 240h (or GD18), exencephaly was not observed in either C57 or SWV fetuses. Measurements of delayed neural tube closure with MeHg exposure were not as robust as assessments made with Cd exposure (4mg/kg, GD8.0) associated with induced

exencephaly at GD8.0 + 240h. In a strain-dependent manner, MeHg disrupted viability and fetal development in C57 and SWV fetuses (t-test $p < 0.05$) (Supplemental Table 2). Differences in sensitivity were not observed between strains when these endpoints were evaluated simultaneously (not shown) ($p > 0.05$, $B_{\text{MeHg_Strain}}$).

3.2 MeHg-Induced Gene Expression Alterations in C57 and SWV Embryos

In Figure 2, we illustrate the effect of MeHg exposure on gene expression within C57 and SWV embryos over time (12 and 24h). The distribution of significantly altered genes identified for each of our three linear models (ANOVA) is shown with Venn diagrams in Figure 2A. Within the C57 (Model 1), we identified 399 (234 $\uparrow$, 165 $\downarrow$, average change across 12 and 24h) genes to be significantly impacted by MeHg exposure (B_{MeHg} , $p < 0.01$). Within these 399 genes, our Venn diagram identified 21 (13 + 8) genes showing significant time effects (B_{Time} , $p < 0.01$) and 102 (94 + 8) genes displaying significant MeHg-Time interaction effects ($B_{\text{MeHg_Time}}$, $p < 0.01$).

Likewise, in the SWV (Model 2), we identified 331 (81 $\uparrow$, 250 $\downarrow$, average change across 12 and 24h) genes significantly altered by MeHg exposure (B_{MeHg} , $p < 0.01$). We observed 78 genes showing both significant MeHg and Time (B_{Time} , $p < 0.01$) effects and 102 genes displaying significant MeHg and MeHg-Time interaction effects.

In Model 3, we used a 3-way ANOVA model to identify genes commonly or differentially expressed based on the effect of time, MeHg treatment and strain. As shown in our Venn diagram for Model 3, we identified 455 genes altered with MeHg treatment independent of strain (B_{MeHg} , $p < 0.01$). With this model we also observed 64 genes significantly impacted differentially between strains with MeHg ($B_{\text{MeHg_Strain}}$, $p < 0.01$) and 46 genes with a significant interaction between all three primary variables ($B_{\text{MeHg_Time_Strain}}$).

Cross-scatter plots of all genes identified to be significantly altered with MeHg (B_{MeHg} , $p < 0.01$, Model 1–3) are shown in Figure 2B and C. Fold change difference (log 2 ratio) in gene expression are displayed between MeHg treated and control C57 (x-axis) and SWV (y-axis) embryos using a log 2 scale ($1 = 2^1 \times$ fold change). We identified 884 genes across all models to be impacted by MeHg in the C57 and/or SWV. Genes in bold reflect gene expression alterations with large effects associated with MeHg exposure at each respective timepoint ($\sim 2 \times$ fold change in one of the two strains). These plots suggest several common and unique MeHg-induced alterations in gene expression comparing the two strains, in terms of magnitude in response and the directionality of effect.

We observed MeHg to commonly upregulate (or downregulate) expression of several genes in both strains represented by ($\square$) as well as only impact one of the two strains, C57 (Δ) or SWV ($\circ$). For example, following 12h exposure (Figure 2B, quadrant I), we observed *Eif2ak1* to be significantly upregulated in both C57 ($\sim \uparrow 3.0 \times$) and SWV ($\sim \uparrow 3.5 \times$) strains, whereas genes such as *Bbx* (Quadrant IV) and *Elavl4* (Quadrant III) were significantly altered in only the C57. In general we observed larger changes in the magnitude of response to MeHg 12h compared to 24h in genes that were significantly impacted by MeHg (B_{MeHg} , $p < 0.01$, Model 1–3).

3.3 Gene Ontology Analysis of MeHg-Induced Gene Expression Alterations

Gene Ontology (GO) analysis was completed to assess overrepresentation of GO categories within genes identified as significantly altered with MeHg (B_{MeHg} , Model 1–3) (not shown). In the C57, we identified enriched GO biological processes associated with classic environmental stress responses, including negative regulation of cell proliferation and positive regulation of apoptosis (Model 1, B_{MeHg} , $p < 0.01$). We also identified enriched GO biological processes such as cell development (e.g. cell motility, actin filament-based

process), developmental process (central nervous system development, eye development), biological regulation and metabolic related categories (e.g. biopolymer metabolism, RNA metabolism) (Model 1, B_{MeHg} $p < 0.01$). In the SWV, we observed common overrepresentation of biopolymer metabolism and unique enrichment in translation, cellular metabolism and RNA processing related-GO biological processes (Model 2, B_{MeHg} $p < 0.01$). GO analysis of genes commonly impacted between C57 and SWV (Model 3, B_{MeHg} $p < 0.01$) revealed enrichment in metabolic and oxidative stress related biological processes (not shown).

In Figure 3, we conducted quantitative analysis within genes of enriched GO biological processes identified in the C57 and/or SWV by calculating the absolute average change in intensity associated with MeHg in significantly altered genes within each GO category (Model 1–3, B_{MeHg} $p < 0.01$) across the two timepoints (12, 24h) assessed. We observed in the C57 at 12h compared to the SWV at 12h a greater impact in genes related to development processes, cellular development, biological regulation and response to stress categories, as well as represented subcategories. For example, we identified 72 genes to be significantly altered by MeHg and involved in system development in C57 and/or SWV embryos. The absolute average fold change associated with MeHg exposure equaled 0.79 in the C57 versus 0.52 in the SWV (log 2 scale), reflecting a greater impact on this subset of genes in the C57 compared to the SWV. In contrast, at 12h we observed larger changes in genes involved in RNA metabolic processes and related subcategories (e.g. RNA processing) in the SWV compared to the C57. At 24h smaller changes in all enriched categories (24h, 0.03 – 0.27) were evident in the range of log 2 ratios with MeHg in both strains compared to the 12h timepoint (0.30 – 1.04).

3.4 Impact of MeHg on System Development and Stress Response-related Gene Expression

As presented in Figure 4A, MeHg-induced gene expression alterations in C57 and SWV embryos in genes associated with the GO term “system development”. At 12h in quadrant I we observed common ($\square$) upregulation of *Eif2ak1*, *Crygc* and *Sema4d* and downregulation of *Mb* (quadrant III) (Model 3, $p < 0.01$) in both C57 and SWV embryos. In only the C57 (12h, Δ) we observed significant MeHg-induced upregulation of *Sall1*, *Foxf1a*, *Nte*, *Otx2* and *Bmp2* and downregulation of *Bcl11a* and *Neurod4* (Model 1, $p < 0.01$). At 24h (Figure 4B) no apparent significant changes were observed in system development-related gene expression in either strain.

In Figure 5A and B, MeHg-induced gene expression changes related to the GO term “response to stress” are presented for C57 and SWV embryos. In the C57 (Δ) we observed upregulation of *p53*, *Cdkn1a*, *Parp1*, *Trp53bp1* and *Gpx2* (Model 1, B_{MeHg} $p < 0.01$) (12h, quadrant I and IV of Figure 5A). Alterations in expression were more apparent at 12h with MeHg compared to 24h within this subset, with the exception of sustained upregulation of *Cdkn1a* in the C57 (quadrant I of Figure 5B).

3.5 GO Quant Analysis of GO categories altered by Cd and MeHg in the C57

In Figure 6, we present the impact of MeHg and Cd on gene expression within common enriched GO biological processes by comparing results from this study with previously published results from our lab assessing the effect of Cd on neurulation [22]. We identified seven categories to demonstrate significant enrichment with both metals in the C57. These categories include developmental process, anatomical structure development, system development, central nervous system development, brain development, negative regulation of cellular proliferation and response to UV. In general, we observed a similar response in terms of directionality (not shown) and magnitude with MeHg and Cd in significantly

altered genes ($p < 0.01$) associated with the aforementioned GO biological processes. For example, at 12h, MeHg and Cd induced alterations in central nervous development related genes (19 total) by ~ 0.7 (log 2 scale), while at 24h this response decreased (< 0.25) for both metals.

4. DISCUSSION

In this study we investigated the impact of MeHg exposure on neurulation in two mouse strains (C57 and SWV) previously shown to display differential sensitivity to the metals Cd and As. We determined MeHg effects on gene expression in parallel with assessments of neural tube closure to further explore the mechanism of MeHg-induced developmental toxicity and to evaluate responses associated with strain sensitivity.

MeHg's developmental toxic effects due to maternal exposure are well documented [5], yet early MeHg gestational exposure studies ($< \text{GD}10$) are limited. Early reports suggest MeHg (4mg/kg BW, single or multiple injection) induces malformations such as cleft palate but not exencephaly [23]. Higher doses (8mg/kg BW, 129/SvSl, i.p. single, GD9–13) result in increased incidence of exencephaly ($\sim 10\%$), high rates of resorptions ($\sim 73\%$) and maternal mortality [9]. In agreement with previous studies, we observed MeHg (4mg/kg BW) to alter growth (C57) and viability (SWV) (Supplemental Table 2) and also, we did not induce NTDs (exencephaly) in either C57 or SWV fetuses despite the suggestion of inhibition of neural tube closure in both C57 and SWV embryos (Figure 1). The use of a higher dose would have provided greater complexity due to maternal toxicity resulting in gross indirect maternal effects and the lack of embryos for microarray analysis due to a high resorption rate. We postulate that higher doses of MeHg compared to our study induce exencephaly and that early inhibition on neural tube closure (GD8.5, 9) is a positive indicator of the NTD exencephaly as observed in studies from our laboratory and others assessing Cd-induced NTDs [22,29].

Specific observations between C57 and SWV in this study correspond with earlier studies suggesting the C57 is more sensitive than the SWV when exposed to metals during neurulation [20–22]. Although differences observed in this study are more subtle compared to earlier studies that induced the full NTD phenotype with Cd and As, in this study, we observed significant differential effects on neural tube closure between the C57 and SWV (Figure 1). In particular, in a time dependent manner, we observed a greater impact on closure of the cranial region at GD8.0 + 12h in C57 compared to the SWV. In our previous Cd study [22], we observed a 30% increase in C57 embryos with an open neural tube at GD8.0 + 12h compared to SWV which showed a 5% *decrease* in open neural tubes comparing Cd exposed embryos to their respective control. These observations associated with 33% and 5% of Cd-exposed fetuses to develop exencephaly in the C57 and SWV, respectively, suggesting that differences at the 12h timepoint correlate with differential effects on neural tube development. On the other hand, unlike our previous study [22], all morphological parameters evaluated for Cd-toxicity (e.g. resorptions, growth effects) corresponded with the C57 being the more sensitive strain. Here, in this study, we observed significant effects on developmental growth (BW, CR, HD) in the C57 and not the SWV. In contrast, we observed increased resorptions in the SWV (at 240h) compared to the C57. Evaluation of these factors simultaneously suggest similar strain sensitivity to MeHg between C57 and SWV. Our observations reveal differences in sensitivity to MeHg between C57 and SWV strains in particular for neural tube development supported by morphological observations. They also highlight the complexity of identifying and interpreting strain sensitivity when assessing multiple parameters.

In parallel with morphological observations of greater effects on neural tube morphology in the C57 compared to the SWV at 12h, likewise, at 12h, we observed a greater impact in terms of overall magnitude in genes associated with GO terms, cellular development, development process and response to stress in the C57 compared to the SWV (Figure 3). These enriched GO biological processes have previously been identified to be perturbed by environmental stressors and, furthermore, linked with developmental toxicity.

Intricate signaling networks such as *Shh*, *Notch* and *Wnt* regulate cellular identity and behavior during neurulation. These networks regulate aspects of proliferation, differentiation and migration necessary for neural tube formation and subsequent CNS development. Researchers have postulated that neurulation is a time period of sensitivity underlying the development of NTDs as well as other complex diseases or adverse developmental outcomes such as autism [30] and other syndromes [31]. Disturbances to these networks might drive developmental abnormalities. In this study in the C57, MeHg induced alterations in several genes associated with development processes including organ, CNS, and eye development and also cell development (cell motility, cell migration), as shown in Figure 3. Many of these alterations work through these essential signaling pathways. For example we observed MeHg to alter gene expression of CNS differentiation factors *Otx2* and *Neurod4* (12h) (Figure 4A). *Otx2* defines the anterior rotation of the mid-hindbrain boundary by regulating *Shh* expression [32], while the transcription factor *Neurod4* facilitates proper neuronal cytoarchitecture along the midline via *Notch* signaling regulation [33]. Alterations in genes which predict cell fate and are responsible for development of the organism including the CNS might underlie observed MeHg-induced effects such as reduced brain size and neurobehavioral alterations witnessed in early gestational exposure studies (GD7–9, 6mg/kg BW) [34] as well as structural abnormalities, such as NTDs.

This study does not address how MeHg induces changes within CNS developmental networks. However, evidence suggests stress-response elements and signaling networks that regulate proliferation and differentiation are integrated [35]. Metals such as MeHg and Cd induce oxidative stress, either directly or indirectly, leading to reactive oxygen species (ROS) formation, lipid peroxidation and DNA damage with subsequent effects on cellular proliferation and cell viability. In this study, we observed MeHg (C57 only) to significantly induce expression of several genes known to be involved in stress response, including well-characterized genes involved in cell cycle arrest (*p53*, *Cdkn1a*) and DNA repair (*p53*, *Trp53bp1*, *Parp1*) (Figure 4B). *p53* is a key mediator in response to environmental stress, regulating cell cycle progression as well as apoptosis and DNA repair. MeHg induces the expression of *p53*, and the extent of MeHg-induced toxicity, including cell cycle arrest and cell death, is partially dependent on *p53* availability as observed in mouse embryonic fibroblasts and neuronal precursor cells [36,37]. MeHg induces expression of several downstream mediators of *p53* such as *Cdkn1a* and *Gadd45b* [13,38], which directly interact with elements promoting G1/S and G2/M cell cycle transitions [39,40]. Several factors modulate *p53* activity. For example *Parp1* and *Trp53bp1* induce the DNA repair response by promoting stability of the *p53* response through increased phosphorylation and inhibition of proteasomal degradation [41,42]. Our study suggests MeHg induces cellular stress, DNA damage and cell cycle arrest in C57 embryos corresponding with developmental toxicity. Furthermore, increased expression of these genes correlates with increased effects on neural tube closure in the C57 strain.

We observed similar impacts in genes involved in cell cycle and developmental related processes in comparisons with Cd and MeHg (Figure 6), which suggests that Cd and MeHg might elicit similar responses in inducing developmental toxicity. This study suggests potential shared mechanisms of toxicity by Cd and MeHg and indicates a need for studies assessing mixtures and their combined (synergistic or additive) impact on neurulation.

In summary, we report the first toxicogenomic study to investigate the effects of MeHg in C57 and SWV mouse embryos during the period of neurulation demonstrating MeHg to differentially impact neural tube closure in these two strains, as well as gene expression pathways representative of development and stress response pathways. In part, our results support previous observations of differences in sensitivity to metals between these two strains and identifies potential targets of interest for future mechanistic studies investigating the effects of metals on early gestational development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Anderson C, Heike K, Colquhoun D. Recommended fish intake is potentially dangerous due to high methylmercury content of certain fish. *Asia Pac J Clin Nutr* 2003;(12 Suppl):S67. [PubMed: 15023700]
2. Hightower JM, Moore D. Mercury levels in high-end consumers of fish. *Environ Health Perspect* 2003;111(4):604–608. [PubMed: 12676623]
3. Bloom N. On the chemical form of mercury in edible fish and marine invertebrate tissue. *Can. J. Fish Aquat. Sci* 1992;49:1010–1017.
4. Grieb TM, Bowie GL, Driscoll CT, Gloss SP, Schofield CL, Porcella DB. Factors affecting mercury accumulation in fish in the upper michigan peninsula. *Environmental Toxicology and Chemistry* 1990;9(7):919–930.
5. Myers GJ, Davidson PW, Shamlaye CF. A review of methylmercury and child development. *Neurotoxicology* 1998;19(2):313–328. [PubMed: 9553968]
6. Ratcliffe HE, Swanson GM, Fischer LJ. Human exposure to mercury: a critical assessment of the evidence of adverse health effects. *J Toxicol Environ Health* 1996;49(3):221–270. [PubMed: 8876653]
7. Burbacher TM, Rodier PM, Weiss B. Methylmercury developmental neurotoxicity: a comparison of effects in humans and animals. *Neurotoxicol Teratol* 1990;12(3):191–202. [PubMed: 2196419]
8. Gilbert SG, Grant-Webster KS. Neurobehavioral effects of developmental methylmercury exposure. *Environ Health Perspect* 1995;103 Suppl 6:135–142. [PubMed: 8549462]
9. Spyker JM, Smithberg M. Effects of methylmercury on prenatal development in mice. *Teratology* 1972;5(2):181–190. [PubMed: 4623447]
10. Castoldi AF, Barni S, Turin I, Gandini C, Manzo L. Early acute necrosis, delayed apoptosis and cytoskeletal breakdown in cultured cerebellar granule neurons exposed to methylmercury. *Journal of Neuroscience Research* 2000;59(6):775–787. [PubMed: 10700015]
11. Hwang GW, Furuchi T, Naganuma A. A ubiquitin-proteasome system is responsible for the protection of yeast and human cells against methylmercury. *FASEB J* 2002;16(7):709–711. [PubMed: 11978736]

12. Kaur P, Aschner M, Syversen T. Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. *Neurotoxicology* 2006;27(4):492–500. [PubMed: 16513172]
13. Ou YC, Thompson SA, Ponce RA, Schroeder J, Kavanagh TJ, Faustman EM. Induction of the cell cycle regulatory gene p21 (Waf1, Cip1) following methylmercury exposure in vitro and in vivo. *Toxicol Appl Pharmacol* 1999;157(3):203–212. [PubMed: 10373404]
14. Ponce RA, Kavanagh TJ, Mottet NK, Whittaker SG, Faustman EM. Effects of methyl mercury on the cell cycle of primary rat CNS cells in vitro. *Toxicol Appl Pharmacol* 1994;127(1):83–90. [PubMed: 8048057]
15. O'Hara MF, Charlap JH, Craig RC, Knudsen TB. Mitochondrial transduction of ocular teratogenesis during methylmercury exposure. *Teratology* 2002;65(3):131–144. [PubMed: 11877777]
16. Yu X, Hong S, Moreira EG, Faustman EM. Improving in vitro Sertoli cell/gonocyte co-culture model for assessing male reproductive toxicity: Lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates. *Toxicol Appl Pharmacol* 2009;239(3):325–336. [PubMed: 19560483]
17. Finnell RH, Moon SP, Abbott LC, Golden JA, Chernoff GF. Strain differences in heat-induced neural tube defects in mice. *Teratology* 1986;33(2):247–252. [PubMed: 3738820]
18. Finnell RH, Shields HE, Taylor SM, Chernoff GF. Strain differences in phenobarbital-induced teratogenesis in mice. *Teratology* 1987;35(2):177–185. [PubMed: 3603402]
19. Naruse I, Collins MD, Scott WJ Jr. Strain differences in the teratogenicity induced by sodium valproate in cultured mouse embryos. *Teratology* 1988;38(1):87–96. [PubMed: 3140405]
20. Hovland DN Jr, Machado AF, Scott WJ Jr, Collins MD. Differential sensitivity of the SWV and C57BL/6 mouse strains to the teratogenic action of single administrations of cadmium given throughout the period of anterior neuropore closure. *Teratology* 1999;60(1):13–21. [PubMed: 10413334]
21. Machado AF, Hovland DN Jr, Pilafas S, Collins MD. Teratogenic response to arsenite during neurulation: relative sensitivities of C57BL/6J and SWV/Fnn mice and impact of the splotch allele. *Toxicol Sci* 1999;51(1):98–107. [PubMed: 10496681]
22. Robinson JF, Yu X, Hong S, Griffith WC, Beyer R, Kim E, et al. Cadmium-induced differential toxicogenomic response in resistant and sensitive mouse strains undergoing neurulation. *Toxicol Sci* 2009;107(1):206–219. [PubMed: 18974090]
23. Su MQ, Okita GT. Embryocidal and teratogenic effects of methylmercury in mice. *Toxicol Appl Pharmacol* 1976;38(1):207–216. [PubMed: 982469]
24. Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR. MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biol* 2003;4(1):R7. [PubMed: 12540299]
25. Yu X, Griffith WC, Hanspers K, Dillman JF 3rd, Ong H, Vredevogd MA, et al. A system-based approach to interpret dose- and time-dependent microarray data: quantitative integration of gene ontology analysis for risk assessment. *Toxicol Sci* 2006;92(2):560–577. [PubMed: 16601082]
26. Van Allen MI, Kalousek DK, Chernoff GF, Juriloff D, Harris M, McGillivray BC, et al. Evidence for multi-site closure of the neural tube in humans. *Am J Med Genet* 1993;47(5):723–743. [PubMed: 8267004]
27. Kuczuk MH, Scott WJ Jr. Potentiation of acetazolamide induced ectrodactyly in SWV and C57BL/6J mice by cadmium sulfate. *Teratology* 1984;29(3):427–435. [PubMed: 6463905]
28. McCullagh, P.; Nelder, JA. Generalized linear models. 2nd ed.. London: Chapman and Hall; 1989. p. 261xiii
29. Fernandez EL, Gustafson AL, Andersson M, Hellman B, Dencker L. Cadmium-induced changes in apoptotic gene expression levels and DNA damage in mouse embryos are blocked by zinc. *Toxicol Sci* 2003;76(1):162–170. [PubMed: 12915712]
30. Rodier PM, Ingram JL, Tisdale B, Nelson S, Romano J. Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. *J Comp Neurol* 1996;370(2):247–261. [PubMed: 8808733]

31. Duru S, Ceylan S, Guvenç BH. Segmental costovertebral malformations: association with neural tube defects. Report of 3 cases and review of the literature. *Pediatr Neurosurg* 1999;30(5):272–277. [PubMed: 10461076]
32. Puelles E, Annino A, Tuorto F, Usiello A, Acampora D, Czerny T, et al. Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* 2004;131(9):2037–2048. [PubMed: 15105370]
33. Ohsawa R, Ohtsuka T, Kageyama R. Mash1 and Math3 are required for development of branchiomotor neurons and maintenance of neural progenitors. *J Neurosci* 2005;25(25):5857–5865. [PubMed: 15976074]
34. Dore FY, Goulet S, Gallagher A, Harvey PO, Cantin JF, D'Aigle T, et al. Neurobehavioral changes in mice treated with methylmercury at two different stages of fetal development. *Neurotoxicol Teratol* 2001;23(5):463–472. [PubMed: 11711249]
35. Hansen JM. Oxidative stress as a mechanism of teratogenesis. *Birth Defects Res C Embryo Today* 2006;78(4):293–307. [PubMed: 17315243]
36. Gribble, EJ. Cell cycle inhibition as a mode of abnormal development: the role of cell cycle checkpoint proteins and cyclin-dependent kinase inhibitors in neurodevelopmental toxicant defense, in Dept. of Environmental and Occupational Health Sciences. Seattle: University of Washington; 2005. Unpublished
37. Gribble EJ, Hong SW, Faustman EM. The magnitude of methylmercury-induced cytotoxicity and cell cycle arrest is p53-dependent. *Birth Defects Res A Clin Mol Teratol* 2005;73(1):29–38. [PubMed: 15641097]
38. Ou YC, Thompson SA, Kirchner SC, Kavanagh TJ, Faustman EM. Induction of growth arrest and DNA damage-inducible genes Gadd45 and Gadd153 in primary rodent embryonic cells following exposure to methylmercury. *Toxicol Appl Pharmacol* 1997;147(1):31–38. [PubMed: 9356304]
39. Bartek J, Lukas J. Pathways governing G1/S transition and their response to DNA damage. *FEBS Lett* 2001;490(3):117–122. [PubMed: 11223026]
40. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. *Oncogene* 2001;20(15):1803–1815. [PubMed: 11313928]
41. Haince JF, Kozlov S, Dawson VL, Dawson TM, Hendzel MJ, Lavin MF, et al. Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents. *J Biol Chem* 2007;282(22):16441–16453. [PubMed: 17428792]
42. Ward IM, Difilippantonio S, Minn K, Mueller MD, Molina JR, Yu X, et al. 53BP1 cooperates with p53 and functions as a haploinsufficient tumor suppressor in mice. *Mol Cell Biol* 2005;25(22):10079–10086. [PubMed: 16260621]

Figure 1. The impact of MeHg on closure of the cranial region of the neural tube in C57 and SWV embryos

Embryos isolated GD8.0 + 6, 12, 24h were examined for closure of the anterior neural tube within the hind/brain region. These results were combined with external malformation assessments made on GD8.0 + 240h (GD18), evaluating closure of the cranial region (exencephaly). Data points are based on litter averages ($n \geq 3$ for each group). We observed significant effects on neural tube closure in MeHg-exposed C57 and SWV embryos (B_{MeHg} , $p < 0.05$) and significant differential impacts by MeHg on neural tube closure between strains ($B_{\text{MeHg_Strain}}$, $p < 0.05$).

Figure 2. ANOVA distributions of MeHg-induced gene expression alterations in C57 and SWV embryos

(A) Venn diagrams display the distribution of genes identified to be significantly altered due to selected effects within each one of the three models. (B) Scatter plots display the magnitude of change (MeHg/Con) between MeHg and control in differentially expressed genes in C57 and SWV embryos (12h (A) and 24h (B) post-injection). Genes were identified as statistically significant due to a significant *B_{MeHg}* term ($p < 0.01$) in one of our three ANOVA models assessing for genes that are commonly or uniquely altered in the two strains. Genes identified by one of our three ANOVA models are displayed as (A) *B_{MeHg}* $p < 0.01$, in both strains (light gray boxes), (B) *B_{MeHg}* ($p < 0.01$), C57BL/6J only (dark gray triangles) and (C) *B_{MeHg}* ($p < 0.01$), SWV only (black circles).

Figure 3. Quantitative analysis of GO biological processes impacted by MeHg in C57 and SWV embryos

The color-coded diagram represents the absolute mean fold change (MeHg/control) of genes identified to be significantly altered with MeHg in any of the two strains (Models 1–3) within overrepresented GO subsets identified in the C57 and/or SWV. All selected GO subsets were identified as significantly impacted by MeHg in either C57 (*) or SWV ([^]) using MAPPFinder criteria ($Z > 2.0$, $p < 0.02$, number changed within each GOID > 5). The number of genes changed ($p < 0.01$) in either strain is in parentheses next to each GO categorical name. Descriptions on the right represent general description of GO term grouping.

Figure 4. MeHg-induced gene expression alterations associated with development

Cross scatter plots display the magnitude in fold change (MeHg/Con) associated with both C57 (x-axis) and SWV (y-axis) embryos 12h (A) and 24h (B) post injection (GD8.0) for genes that were identified as significantly altered in one of the three models and linked with the GO biological process, System Development (GO:48731).

Figure 5. MeHg-induced gene expression alterations associated with stress response

Cross scatter plot display the magnitude in fold change (MeHg/Con) associated with both C57 (x-axis) and SWV (y-axis) embryos 12h (A) and 24h (B) post injection (GD8.0) for genes that were identified as significantly altered in one of the three models and linked with the specific GO biological process, Response to Stress (GO:6950).

Figure 6. Quantitative analysis of commonly enriched GO biological processes affected by MeHg and Cd

The bar chart represents the absolute mean fold change (treatment/control) of genes identified to be significantly altered with MeHg or Cd [22] within commonly enriched GO biological process. All selected GO subsets were identified as significantly enriched by both MeHg and Cd in the C57 using our cutoff criteria of significance ($Z > 2.0$, $p < 0.02$, number changed within each GOID > 3). The total number of genes altered by MeHg and/or Cd ($p < 0.01$) in the C57 strain are displayed to the right of each GO category in parentheses.