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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.

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.

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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. 4 mg/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 12 h light/dark cycle, mice were housed in filter-covered transparent plastic cages in climate-controlled rooms. Water and food were available *ad libitium*. 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 (8:00 am ± 0.5 h) the following day and designated as GD0. Pregnant dams were administered single doses via intraperitoneal injection (i.p.) on GD8.0, 8:00 am (±1 h), with vehicle control (deionized water, 10 μl/g) or monomethylmercury hydroxide (4 mg/kg/BW) (Alfa Aesar, Ward Hill, MA) (working concentration of 2 mM). 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 24 h (8.5 and GD9.0) 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, 12 h; Thelier Stage (TS) ~13, GD9.0, 24 h, 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 500 μl of RTL Cell Lysis buffer (Qiagen, Valencia, CA) and homogenized with a 30G needle. RNA was purified using the RNAeasy 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 three independent litters of MeHg or vehicle-exposed embryos for 12 h assessments and four independent litters for 24 h assessments, with the exception of MeHg-exposed SWV embryos at 24 h ($n = 3$) (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 oligonuceotide 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 10 min. 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 2 h 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 14 h (Amersham Biosciences, Piscataway, NJ). cRNA was purified using the RNAeasy 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 20 min. Hybridization (10 μg cRNA) was completed for 18 h at 300 rpm and 37 °C. Array chips were washed with 0.75× TNT for 1 h at 46 °C and incubated with AlexaFlour 647-streptavidin (Molecular Probes, Eugene, OR) for 30 min. After 4 washes in 1× TNT and 2 washes in 0.05% Tween-20, slides were dried at 2000 rpm for 3 min and assessed for smears. Arrays were scanned on the Axon GenePix 4000 Scanner (Axon Instruments, Union City, CA) at 635 nm, PMT 600 V 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).

Model 1: $\mathrm{Log}_2[\mathrm{Exp}_n]\mathrm{C57} = B_0 + B_{\mathrm{Time}}x_1 + B_{\mathrm{MeHg}}x_2 + B_{\mathrm{MeHg\_Time}}x_1x_2$

Model 2: $\mathrm{Log}_2[\mathrm{Exp}_n]\mathrm{SWV} = B_0 + B_{\mathrm{Time}}x_1 + B_{\mathrm{MeHg}}x_2 + B_{\mathrm{MeHg\_Time}}x_1x_2$

Model 3: $\mathrm{Log}_2[\mathrm{Exp}_n]\mathrm{C57\_SWV} = B_0 + B_{\mathrm{Time}}x_1 + B_{\mathrm{MeHg}}x_2 + B_{\mathrm{Strain}}x_3 + B_{\mathrm{MeHg\_Time}}x_1x_2 + B_{\mathrm{Time\_Strain}}x_1x_3 + B_{\mathrm{MeHg\_Strain}}x_2x_3 + B_{\mathrm{MeHg\_Time\_Strain}}x_1x_2x_3$

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 h 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}, $p < 0.01$, Models 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 ($p < 0.01$, Models 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}, $p < 0.01$, Models 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 + 12 h, 24 h), as well as additional litters of embryos isolated GD8.0 + 6–24 h, 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 + 240 h (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 developmen-