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GENETIC POLYMORPHISMS AFFECTING SUSCEPTIBILITY TO MERCURY NEUROTOXICITY IN CHILDREN: SUMMARY FINDINGS FROM THE CASA PIA CHILDREN'S AMALGAM CLINICAL TRIAL

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Abstract

Mercury (Hg) is neurotoxic, and children may be particularly susceptible to this effect. A current major challenge is identification of children who may be uniquely susceptible to Hg toxicity because of genetic predisposition. We examined the possibility that common genetic variants that are known to affect neurologic functions or Hg handling in adults would modify the adverse neurobehavioral effects of Hg exposure in children. Three hundred thirty subjects who participated as children in the recently completed Casa Pia Clinical Trial of Dental Amalgams in Children were genotyped for 27 variants of 13 genes that are reported to affect neurologic functions and/or Hg disposition in adults. Urinary Hg concentrations, reflecting Hg exposure from any source, served as the Hg exposure index. Regression modeling strategies were employed to evaluate potential associations between allelic status for individual genes or combinations of genes, Hg exposure, and neurobehavioral test outcomes assessed at baseline and for 7 subsequent years during the clinical trial. Among boys, significant modification of Hg effects on neurobehavioral outcomes over a broad range of neurologic domains was observed with variant genotypes for 4 of 13 genes evaluated. Modification of Hg effects on a more limited number of neurobehavioral

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Conflict of Interest

The authors declare that there are no conflicts of interest.

outcomes was also observed for variants of another 8 genes. Cluster analyses suggested some genes interacting in common processes to affect Hg neurotoxicity. In contrast, significant modification of Hg effects on neurobehavioral functions among girls with the same genotypes was substantially more limited. These observations suggest increased susceptibility to the adverse neurobehavioral effects of Hg among children, particularly boys, with genetic variants that are relatively common to the general human population. These findings advance public health goals to identify factors underlying susceptibility to Hg toxicity and may contribute to strategies for preventing adverse health risks associated with Hg exposure.

Keywords

Mercury; Children; Neurobehavioral function; Neurotoxicity; Single nucleotide polymorphism; Genetic susceptibility; Allelic status

1. Introduction

Mercury (Hg), particularly in its elemental (Hg^0) and methylated (CH_3Hg^+) forms, poses a risk of neurotoxicity to humans (Clarkson, 2002). Hg^0 in the vapor form, once inhaled, readily crosses the blood brain barrier and preferentially distributes to those portions of the central nervous system (CNS) that affect personality and behavior, the toxic effects, hence, being largely neuropsychiatric in nature. Methylmercury (MeHg), in contrast, more preferentially distributes to those portions of the brain that control sensorimotor function, the principal effects being disturbances in coordination, equilibrium and motor control. Continuous exposure to MeHg, particularly *in utero*, may also lead to decrements in cognitive functions in offspring (Grandjean et al., 1997). Two large prospective studies initiated in the mid to late 1980s among residents of the Seychelle Islands and the Faroe Islands evaluated the potential developmental toxicity of MeHg among children exposed *in utero* from mothers' consumption of fish and/or pilot whale, respectively. No adverse neurobehavioral effects were observed among children in the Seychelle Child Development study (Myers et al., 1995), whereas mild deficits in learning, memory and attention, as well as in visuospatial and motor functions, were observed among Faroe Island children evaluated 7 years after birth when stratified on umbilical cord blood Hg levels (Grandjean et al., 1997). In the assessment of the potential neurobehavioral effects of Hg^0 in children, 2 randomized clinical trials (DeRouen et al., 2006; Bellinger et al., 2006) conducted between 1996-2006 compared the performance of children with and without Hg-containing dental amalgam fillings on an extensive battery of neurobehavioral tests over a period of 5 to 7 years following dental treatment beginning at a mean age of 10 years. Neither study found significant differences in neurobehavioral performance over the course of the trial when comparing children with and without dental amalgam fillings.

None of the aforementioned studies was designed to evaluate special sensitivities, such as genetic predisposition, that might affect Hg neurotoxicity in children. Such studies are relevant, however, in light of recent findings (Basu, et al., 2013; Echeverria et al., 2005, 2006, 2010; Goodrich et al., 2011; Gundacker et al., 2007, 2009; Heyer et al., 2004, 2008, 2009; Schläwicke Engstrom et al., 2008; Wang et al., 2012) identifying common variants of

numerous genes that modify the effects of Hg on neurologic functions and/or Hg handling in human subjects. Identification of genetic polymorphisms that affect Hg neurotoxicity is of particular importance with regard to assessment of Hg risks in children, who may be uniquely susceptible, compared with adults, because of their smaller body mass and more rapidly developing, hence more fragile, nervous and metabolic systems (Landrigan and Goldman, 2011).

The aim of the present study was to address this concern by identifying altered risks of Hg neurotoxicity potentially associated with common variants (single nucleotide polymorphisms [SNPs], insertions/deletions) in specific candidate genes that are postulated to independently confer increased susceptibility to neurological disorders and/or to modify Hg metabolism in human subjects. Specifically, we evaluated the extent to which performance on a wide variety of neurobehavioral tests was modified by Hg exposure in relation to genotype (wildtype or variant) for 27 variants in 13 different genes among subjects who participated as children and adolescents in a recently completed prospective, randomized dental amalgam clinical trial between ages 8 and 18 and for whom longitudinal (annual) neurobehavioral assessments and quantitative measures of Hg exposure over 7 years of follow-up were available. For these studies, we employed urinary Hg concentrations, representing Hg exposure *from any source* whether or not related to dental amalgam, as the Hg exposure measure. We evaluated the potential modifying effects of genetic variants on the dose-response effects of Hg exposure on neurobehavioral functions within a wide range of neurologic domains including Attention, Learning & Memory, Executive Function, Visual Spatial acuity, and Motor function. Additionally, because previous studies (Woods et al., 2007) suggested likely sex-related differences in Hg handling and susceptibility to Hg toxicity, we made these assessments independently in boys and girls.

2. Methods

2.1. The study population

The present study included 330 subjects who participated as children in the Casa Pia Clinical Trial of Dental Amalgams in Children (DeRouen et al., 2002; 2006) conducted between 1996 and 2006. Participants in the clinical trial included 279 boys and 228 girls, aged 8–12 years at baseline, who were students of the Casa Pia school system in Lisbon, Portugal. Founded more than 200 years ago to provide an education for orphaned and homeless children, the Casa Pia system now uses a combination of public and private support to provide a quality education for children from a much wider variety of backgrounds of which approximately 20% are boarding students and 80% are from middle- to upper-class socioeconomic families who pay tuition for their children to attend. Current enrollment of the 10 schools comprising the Casa Pia system exceeds 4,700 students in grades from 1 through 12. For the clinical trial, children were initially randomized to Hg amalgam (treatment) or composite resin (control) dental treatment groups. Subjects were evaluated at baseline and at 7 subsequent annual intervals following initial dental treatment using an extensive battery of neurobehavioral assessments (Martins et al., 2005). Follow-up data were obtained on a similar number of subjects in each treatment group. Baseline urinary

Hg concentrations were 1.5 ± 1.2 (0.1-7.7) and 1.4 ± 1.1 (0.0-8.6) $\mu\text{g/g}$ creatinine for amalgam and composite groups, respectively. These compare with a mean urinary Hg level of 0.358 (0.313-0.408) $\mu\text{g/creatinine}$ among a national representative sample of 12-19 year old U.S. children (Centers for Disease Control and Prevention, 2007). The possible sources of exposure accounting for this difference in urine Hg levels and public health implications have been discussed in detail (Woods et al., 2012, 2013). Mean urinary Hg concentrations by treatment group and by sex for each year of the clinical trial have been previously described (Woods et al., 2007). A detailed description of the study design and methods, including factors measured over the course of the study and how these factors were considered in constructing analytical models has been published (DeRouen et al., 2002).

2.2. Neurobehavioral tests

A comprehensive neurobehavioral test battery was used in these analyses, including measures from the Rey Auditory Verbal Learning Test (RAVLT), subtests from the Wide Range Assessment of Visual Motor Abilities (WRAVMA), the Wechsler Adult Intelligence Scale-III (WAIS-III), the Wechsler Memory Scale for Adults-III (WMS-III), Standard Reaction Time (SRT), Finger Tapping, Trailmaking A and B, and the Stroop word, color and word-color tests. The validity and rationale underlying the selection and use of these tests in the clinical trial as well as the baseline neuropsychological performance of all subjects have been described (Martins et al., 2005; Townes et al., 2008).

Table 1 lists the 23 neurobehavioral tests that were assessed and their test abbreviations referenced in subsequent tables. Tests are organized within the 5 behavioral domains (Attention, Learning & Memory, Executive Function, Visual Spatial acuity and Motor function) that were evaluated in the clinical trial (DeRouen et al., 2006). Arrows depict whether the test score increases or decreases in magnitude with improved performance. Diminished or adversely affected performance associated with Hg exposure or gene variant status is described as occurring in the direction of impaired performance, whereas enhanced or beneficially affected performance associated with either of these variables is described as occurring in the direction of improved performance. The Comprehensive Test Of Nonverbal Intelligence (CTONI) (Portuguese translation) was given to each child at the beginning of the clinical trial to obtain a measure of IQ at baseline. Social and cultural issues associated with measurements and interpretation of IQ test results among children in cross-cultural contexts have been previously described (Martins et al., 2005; Woods et al., 2013).

2.3. Genotyping assays

Genotyping was performed on DNA extracted from buccal cell samples that were obtained from study subjects following completion of the clinical trial (n=199) or from blood samples that were acquired at baseline for various clinical assessments (n=152). Genotyping was performed by the Functional Genomics and Proteomics Laboratory of the NIEHS Center for Ecogenetics and Environmental Health at the University of Washington using commercially available genotyping assays. Details of genotyping methods, including quality control procedures employed, are described in previous publications (Echeverria et al., 2010; Heyer et al., 2004; 2009; Woods et al., 2005).

2.4. Human subjects considerations

All parents or guardians of children who participated in the clinical trial gave written consent, and all children provided signed assent, for the treatments and assessments made during the course of the trial, including collection of blood samples. Written consent was also obtained from all participants who provided buccal cell samples for genotyping subsequent to completion of the clinical trial. The study protocols for both the clinical trial and the present genotyping study were approved by the institutional review boards at the University of Lisbon and the University of Washington.

2.5. Urinary Hg analysis

A urine sample was collected from each child at baseline of the clinical trial and at each subsequent annual visit to the University of Lisbon School of Dental Medicine for dental, neurologic, and neurobehavioral evaluations. Strictly maintained sterile conditions and handling procedures precluded contamination of urine samples by Hg or any other substance. Analysis of total mercury (Hg) was performed by continuous flow, cold vapor spectrofluorometry, as previously described (Pingree et al., 2001). Urinary creatinine concentrations were measured using a standard colorimetric procedure (Sigma #555-A; Sigma-Aldrich, St. Louis, MO, USA). Urinary Hg concentrations (HgU) were calculated as micrograms per gram creatinine ($\mu\text{g/g}$ creatinine).

2.6. Assessment of Hg exposure

In the present genotyping study, we modified the clinical trial approach in 2 essential ways. First, rather than using the assignment to Hg amalgam or composite resin treatment groups, we employed urinary Hg concentrations (HgU) measured annually for all participants to calculate *acute* and *chronic* measures of Hg exposure. This allowed us to capture the effects of *all Hg exposure*, whether related to dental amalgam, diet, or other environmental sources. This decision was based on the fact that assignment to Hg amalgam or composite resin treatment group accounted for, at most, 17% of the variation in HgU among boys ($r^2=0.171$) and 15% among girls ($r^2=0.154$), both occurring in year 2 of the clinical trial, and indicating considerable background Hg exposure unrelated to dental amalgam (Woods et al., 2013). Moreover, recent studies (Sherman et al., 2013) demonstrate that urine may contain inorganic Hg derived from demethylation of ingested methylmercury from fish, a major constituent of the Portuguese diet. Secondly, we evaluated the potential modifying effects of specific genetic variants on the dose-response effects of Hg exposure, as represented by urinary Hg levels, on neurobehavioral performance. Because previous studies (Woods et al. 2007) suggested possible sex-related differences in Hg handling and susceptibility to Hg toxicity, we made these assessments separately in boys and girls.

2.7. Study design

We examined whether allelic status of specific genes affected the dose-response relationship between urinary Hg concentration and tests of neurobehavioral functions among children who had been evaluated annually from baseline through 7 years of follow-up of the clinical trial. The wide range in ages of subjects at the beginning of the trial (8-12 years), the duration of the study (which included passage through puberty for most subjects), and the

associated change in specific tests administered to subjects based upon their age group during the course of the trial (e.g., child versus adult versions of some tests) introduced significant complexity into the interpretation of repeated measures analyses for this study. We, therefore, evaluated the *acute* effects of Hg exposure on performance using the concurrent urinary Hg concentrations (HgU) and neurobehavioral test performance measures at the end of the 2nd year of follow-up (Year 2), where mean HgU reached a peak among both boys and girls in the clinical trial cohort and where cumulative effects of Hg would be minimal. Similarly, we estimated the *chronic* effects of Hg exposure by examining the relationship between a cumulative measure of HgU over the entire study period and performance outcomes during the final study year (Year 7).

The measure of *acute* Hg exposure was calculated as the natural log of HgU adjusted by 1 ($\ln[\text{HgU}+1]$). The natural log best accommodates how exposures are distributed biologically, whereas adding 1 minimizes the influence of very small changes in HgU at very low levels (which could have otherwise dominated the analyses). The measure of *chronic* Hg exposure was evaluated using cumulative HgU calculated as the natural log of the sum of HgU from baseline and each year of follow-up also adjusted by 1 ($\ln[(\text{HgU}) + 1]$). A small number of subjects (28) who were followed for the full 7 years of the clinical trial had missed one or more intermediate annual evaluations. Subjects missing 3 or more evaluations ($n=2$, both female) were eliminated from the *chronic* exposure analyses. Those missing 1 ($n=22$, 9 female) or 2 ($n=4$, 3 female) annual evaluations were included in the *chronic* Hg exposure analyses with their mean HgU concentrations substituted for the missing years. *Chronic* Hg exposure analyses conducted with and without these subjects did not significantly differ. Of note, the *acute* and *chronic* measures of Hg exposure were found to have a Pearson correlation of 0.36, indicating that they share only 13% common variance (r^2) and, hence, are not highly correlated.

2.8. Statistical analyses

The effect of specific gene variants on the dose-response association between Hg exposure and neurobehavioral performance was the principal focus of this study. Thus, our analytical protocol focused on gene-Hg interactions (genexHg), independently evaluating the impact of allelic status of any gene individually and in combination on performance of each behavioral test. In all cases, boys and girls were evaluated independently, as were the effects of *acute* and *chronic* Hg exposures. Statistical analyses were performed using SPSS Version 19 (IBM®SPSS®, Chicago, IL, USA).

Initial analyses were conducted using a full interaction model consisting of Hg exposure (either *acute* or *chronic* as defined above), dichotomous allelic status for each gene (WT or not WT [HetMut or Mut]), and their interaction terms. Covariates in this model included age at assessment, race, and non-verbal IQ (determined at baseline). These covariates were selected because of their potential to bias neurobehavioral test performance in relation to Hg exposure (Echeverria et al. 1995) or other stressors (Krieg et al. 2001) and because data pertinent to these specific variables were available from the clinical trial from which subjects in the present study were acquired (Martins et al. 2005; Townes et al. 2003). Restricting our analyses to these 3 covariates, which are not highly correlated, minimized

the possibility of having an over-determined model. Other factors potentially affecting behavioral test performance in relation to Hg exposure, including home environment, parent's socioeconomic status, or medical histories, were comparable among essentially all subjects (DeRouen et al., 2002) and were, therefore, not included as covariates. All behavioral tests with significant interaction terms in the above model were re-evaluated for dose-response associations between Hg exposure and test performance independently for subjects with WT or not WT allelic status for each SNP. This final model, which included Hg exposure, age at assessment, race and non-verbal IQ at baseline, provided analyses of Hg dose-response relationships for behavioral performance within each genotypic group unencumbered by interaction terms, and where the differences in dose-response by allelic status (interactions) could be directly compared. Further details pertinent to these analyses have been described (Woods et al., 2013).

Additional analyses were conducted to evaluate the possibility of interaction between combinations of genes that might potentially modify Hg neurotoxicity via shared mechanisms on the same or related neurologic processes. These analyses were performed using multi-regression techniques as described above but included dummy variables for potentially modifying gene alleles as additional covariates in the regression models. Clustering of effects was assessed by determination of significant dose-response associations between Hg exposure and test performance with or without additional alleles in the analysis.

Prior to fitting the regression models, we examined the assumptions of the model by scrutinizing the distributions and variances of all neurobehavioral tests and $\ln(\text{HgU})$. Most distributions had no significant deviation from normality or inflated variance. After fitting each model, we examined the standardized residuals for statistical outliers. In the rare event that an outlier was found, it was removed and the model was refit.

For all variants, we evaluated the effects of genotype on test outcomes independent of Hg exposure. Few associations were observed, and most were of only borderline significance in terms of suggesting independent effects of gene status on neurobehavioral performance. Findings pertinent to variants of CPOX are published (Woods et al., 2012).

2.9. Selection of candidate genes

Using a search of the PubMed database and the current literature, we identified 13 candidate genes that, as variant forms, are reported to be associated with various neurobehavioral/neuropsychiatric disorders and/or changes in Hg handling in adults and that, therefore, might be expected to modify the effects of Hg on neurobehavioral performance among children. We limited the selection of candidate genes to those whose variants were considered *a priori* to modify the effects of Hg on as broad a range of neurobehavioral and Hg toxicokinetic functions as possible, with minimal overlap or redundancy. These included variants of 9 genes (CPOX, COMT, TDO2, GRIN2A, GRIN2B, SLC6A4, KIBRA, APOE) that are reported to modify performance in adults on at least 1 of the 5 neurologic domains evaluated, whereas those affecting Hg handling included variants of 4 genes (MT1M, MT2A, GSTT1, SEPP1) that are reported to modify Hg toxicokinetics and/or tissue distribution via possibly different mechanisms or processes. When minor allelic frequencies

(MAF) of any gene were less than 20%, power calculations were performed to ensure that statistical significance could be attained given the numbers of boys and girls genotyped as wildtype (WT) and variant (Het, Mut, deletion, insertion/deletion), respectively. These genes and their variants, along with the distribution of their wildtype and variant forms and their minor allelic frequencies, are presented in Table 2. Conventional abbreviations for gene names are employed. A synopsis of pathways or processes affected by each gene, along with their neurologic relevance, is presented in Table 3.

3. Results

3.1. Genetic Variants that modify the effects of Hg on a Broad Range of Neurobehavioral functions in Children

Of the 13 genes evaluated, 4 (CPOX, MT1M, MT2A, COMT) were found to have variant forms that significantly modified the effects of Hg exposure on neurobehavioral performance outcomes within 3 or more neurological domains in children. Significant findings for these as well as for other genes evaluated are summarized in Table 4 (Boys) and Table 5 (Girls).

3.1.1 CPOX—Of the 27 variants evaluated, a SNP in exon 4 of the gene encoding the heme pathway enzyme, coproporphyrinogen oxidase (CPOX4, rs1131857), conveyed the broadest range of effects in the present study, modifying performance on multiple neurobehavioral tests within all 5 neurological domains (Attention, Learning & Memory, Executive Function, Visual Spatial acuity and Motor function) in response to *chronic* Hg exposure among boys. Whereas no dose-response measures among boys genotyped as CPOX WT were significant, 15 of 23 dose-response associations between Hg and neurobehavioral test performance among boys genotyped as CPOX4 Het or Mut were significant, and all were in the direction of impaired performance with increasing Hg exposure (Table 4). A similar dichotomy of effects was observed between girls genotyped as CPOX WT or CPOX4 Het or Mut, although with substantially fewer significant findings, and limited largely to tests of Learning and Memory in response to *acute* Hg exposure (Table 5). Scatter plots depicting the associations between specific tests of neurobehavioral performance and *chronic* Hg exposure among boys genotyped as WT or Het/Mut for CPOX rs1131857 are shown in Figures 1A and 1B. A second CPOX variant (CPOX5, rs1729995), which encodes a synonymous mutation in the CPOX enzyme (E330E) was also evaluated in terms of mediating effect modification of Hg exposure on neurobehavioral test performance in children. None was observed for either boys or girls genotyped as CPOX5 in response to either *acute* or *chronic* Hg exposure. These findings have been described in detail (Woods et al., 2012) and are the first to demonstrate genetic susceptibility to the adverse neurobehavioral effects of Hg exposure in children.

3.1.2 MT1M and MT2A—The Metallothioneins (MT) are a multigene family of proteins that are involved in Hg distribution and excretion (Aschner et al., 2006). Two MT isoforms, MT1 and MT2, in particular, are implicated in the dispersal and storage of Hg in the CNS (West et al., 2008). Studies in humans have suggested that SNPs of these MT isoforms, MT1M (rs2270837) and MT2A (rs10636), affect Hg body burden, and therefore potentially

modify the adverse effects of Hg on neurobehavioral functions (Gundacker et al., 2007; Wang et al., 2012). Testing this hypothesis in children, we observed numerous significant adverse effects, particularly in relation to *chronic* Hg exposure, among boys genotyped as Het/Mut for either MT1M or MT2A, occurring principally within the domains of Visual Spatial acuity and Learning & Memory, but also on tests of Attention and Motor function (Table 4). A scatter plot depicting the association between performance on the ravl1 tr1-5 test of Learning & Memory and *chronic* Hg exposure among boys genotyped as WT or Het/Mut for MT1M rs2270837 is shown in Figure 1C. Additionally, we found significant potentiation of the adverse effects of *chronic* Hg exposure on tests of Attention, Visual Spatial acuity, Learning & Memory, and Motor Function among boys carrying Het/Mut variants of both MT1M and MT2A genes (Woods et al., 2013). In contrast, we found no significant modification of Hg effects on any tests of neurobehavioral function among boys genotyped as WT for either gene. Among girls, we found significant modification of Hg effects on neurobehavioral function only for the ravl1 tr8 test of Learning and Memory ($p < 0.01$) in relation to *chronic* Hg exposure among those genotyped as Het/Mut for MT1M rs70837 (Table 5). These results support a role for metallothioneins in protection from the adverse neurobehavioral effects of Hg in children and demonstrate increased susceptibility to those adverse effects among boys carrying Het or Mut variants of these genes. Detailed findings are published (Woods et al., 2013).

3.1.3 COMT—Catechol-*O*-methyltransferase (COMT) is a ubiquitously expressed enzyme that maintains neurologic functions by regulating the availability of key neurotransmitters such as dopamine (Weinshilboum et al., 1999). Polymorphisms in the COMT gene that modify the activity of the COMT enzyme are known to underlie a number of neuropsychiatric disorders as well as process-specific functions that accompany neurobehavioral tasks (Barnett et al., 2009; Nackley et al., 2009). We examined the hypothesis that 4 genetic variants of COMT (rs4680, rs4633, rs4818 and rs6269), individually or in combination, would modify the adverse neurobehavioral effects of Hg exposure in children. Analyses demonstrated that 3 of these 4 COMT SNPs (rs4680, rs4633 and rs6269) modified the effects of Hg exposure on a wide range of neurobehavioral functions within the domains of Attention and Learning & Memory, among boys. We found comparable effects among boys having a COMT haplotype that included the Mut form of rs4680 but not among those whose haplotype did not include rs4680 Mut. Therefore, we attribute the adverse Hg effects observed to rs4680, a variant form of COMT common to approximately 40% of subjects in the general population. Scatter plots depicting the associations between specific tests of neurobehavioral performance and *acute* Hg exposure among boys genotyped as WT or Mut for COMT rs4680 are shown in Figures 1D and 1E.

In additional studies, we evaluated the potential contribution of CPOX4, MT1M and MT2A to Hg effects on neurobehavioral functions among subjects genotyped as COMT WT or Mut, to test the possibility that clusters of SNPs of different genes affecting the same test outcomes would explain more of the effects associated with COMT variants alone. Significantly less impaired performance was observed in each case among both boys and girls when CPOX or MT variants were included in the models, suggesting that these variants do not act in concert to modify the effects of Hg on neurobehavioral functions among

subjects genotyped as COMT Mut. Details of these findings are published (Woods et al., 2014).

3.2. Genetic Variants that modify the effects of Hg on a Limited Range of Neurobehavioral functions in Children

Among the 13 genes evaluated (Table 2), 8 were found to have variant forms that significantly modified the effects of Hg exposure on neurobehavioral test performance within a limited number of neurologic domains in children. We note that, for all genetic variants evaluated, the numbers of significant findings may reflect the limited population size when stratified by gender and may be more fully substantiated when assessed in a larger population of Hg-exposed subjects.

3.2.1 TDO2—Tryptophan 2,3-dioxygenase (TDO2) is the first and rate-limiting enzyme in the catabolism of tryptophan, the precursor of serotonin (5-HT). TDO2 is a hemoprotein whose synthesis and activity are dependent on heme availability (Ren and Correia, 2000). Thus, heme deficiency impairs TDO2 activity and tryptophan degradation, leading to increased tryptophan availability and, consequently, increased brain 5-HT levels. Elevated 5-HT in the CNS may underlie neurobehavioral disorders associated with porphyria (Litman and Correia, 1983) and autism (Nabi et al., 2004) as well as with increased anxiety-related behaviors (Kanai et al., 2009). We evaluated the possibility that genetic variants of TDO2 that presumably compromise TDO2 activity and increase 5-HT levels would exacerbate the adverse effects of Hg on neurobehavioral functions in children. Among the 7 TDO2 SNPs we evaluated (Table 2), only rs3755907 (TDO2-907) substantially modified the adverse effects of Hg exposure among children in this study. Significant effects among boys were seen principally on tests of Motor function including pegs dominant ($p < 0.05$), pegs non-dominant ($p < 0.04$) and finger tapping dominant ($p < 0.04$) relative to *acute* Hg exposure. Significant effects among boys were also observed on the Stroop word test of Attention ($p < 0.05$) and the SRT test of Visual-Spatial acuity ($p < 0.005$) in relation to *chronic* Hg exposure (Table 4). In contrast, among girls, tests of Executive Function and Learning & Memory were modified in relation to *chronic* Hg exposure, however, in the direction of improved performance (Table 5). SNP rs3755907 (TDO2-907) is in linkage disequilibrium with rs3755908, rs3755910 and rs3775085 (Comings et al., 1996), but we found no modification of Hg effects among subjects who were HetMut for these SNPs. It is possible, therefore, that effects observed in relation to TDO2-907 are the result of chance observation. Additionally, we evaluated the effects of Hg on neurobehavioral functions among boys genotyped as HetMut for both TDO2-907 + CPOX4 (rs1131857), hypothesizing that the activity of the enzyme encoded by TDO2-907 would be further compromised by heme deficiency associated with CPOX4 + Hg (Li and Woods, 2009). We found no substantial differences in test performance among such subjects than observed among boys genotyped as CPOX4 and Hg alone.

3.2.2 GRIN2A and GRIN2B—GRIN2A and GRIN2B (glutamate receptor, ionotropic, N-methyl D-aspartate 2A and 2B) encode subunits of N-methyl-D-aspartate (NMDA) glutamatergic receptors, which mediate excitatory neurotransmission in the CNS. The NMDA channel has been shown to be involved in long-term potentiation, an activity-

dependent increase in the efficiency of synaptic transmission thought to underlie Attention as well as Learning & Memory domains that are detrimentally affected by Hg (Adams et al., 2004; Baraldi et al., 2002; Endeley et al., 2010). We assessed the possibility that variants of these genes would increase susceptibility to the adverse effects of Hg exposure in children, particularly on tests within the domains of Attention and Learning & Memory.

For **GRIN2A**, we found 4 significant genexHg interactions among boys genotyped as HetMut for GRIN2A rs727605, including 2 tests of Attention (Stroop Word [$p<0.03$] and Spatial Span [$p<0.05$]) as well as one test of Learning & Memory (ravlt tr1 [$p<0.04$]) (Table 4). Among boys genotyped as HetMut for GRIN2A rs1070503, we found 2 significant interactions including ravlt tr5 (Learning & Memory)($p<0.05$) and Finger Tapping-non dominant (Motor function) ($p<0.006$). We also found 4 significant genexHg interactions among boys who were HetMut for both GRIN2A SNPs. These include ravlt Sum 1-8 (Learning & Memory) ($p<0.04$), WCS #categories correct (Executive Function) ($p<0.009$), Finger Tapping-non-dominant (Motor function) ($p<0.004$), and WMS-III Spatial span (Attention) ($p<0.02$) (not shown). Additionally, since heme is postulated to regulate the expression of N-methyl-D-aspartate receptors in neurons (Chernova et al., 2006; Smith et al, 2012), we evaluated the effects of Hg on neurobehavioral functions among boys genotyped as HetMut for [GRIN2A rs727605 + CPOX rs1131857] or [GRIN2A rs100503 + CPOX rs1131857], hypothesizing that GRIN2A-associated glutamatergic processes that regulate cognition via NMDA receptors (Adams et al., 2004) would be further compromised by heme deficiency associated with CPOX rs1131857 (CPOX4) +Hg (Li and Woods, 2009). Consistent with this hypothesis, both [GRIN2A rs727605 + CPOX4] and [GRIN2A rs1070503 + CPOX4] were associated with impaired the Executive Function test WCS #categories correct, ($p<0.04$ and $p<0.05$, respectively) among boys genotyped as HetMut for both variants in relation to *acute* Hg exposure. These findings support the view that both GRIN2A and CPOX participate in the processing of glutamatergic receptors effecting development of executive function and that compromised processing associated with SNPs of either or both genes is further impaired by Hg exposure. No comparable interactive effects were observed among girls.

For **GRIN2B**, we found no significant genexHg interactions among boys genotyped as HetMut for rs1806201 for any tests of neurobehavioral function. However, among boys genotyped as HetMut for GRIN2B rs7301328, we observed significant genexHg interactions for ravlt tr8 (Learning & Memory)($p<0.07$), WSM-III visual reproductions delayed (Learning & Memory)($p<0.004$), and Finger Tapping-non dominant (Motor)($p<0.003$) (Table 4). These findings suggest modification of Hg effects by SNPs of GRIN2A and/or GRIN2B on tests of Attention and Learning & Memory as well as for Motor function among boys. Among girls (Table 5), we found significant effect modification only by GRIN2B rs7301328 on ravlt tr7 ($p<0.05$) in relation to *acute* Hg exposure, and ravlt tr8 ($p<0.03$) in relation to *chronic* Hg exposure, both tests of Learning & Memory. These findings are consistent with the postulated role of GRIN2A and GRIN2B in neuronal mechanisms underlying Attention and Learning & Memory and an adverse effect of Hg on these processes among subjects carrying variant forms of these genes.

3.2.3 BDNF—Brain-derived neurotrophic factor (BDNF) is a protein that regulates neuronal growth and differentiation in the peripheral and central nervous systems. A SNP (rs6265) at nucleotide 196 (G>A) in the gene encoding BDNF, resulting in a V66M substitution in the gene product, is associated with deficits in memory and motor skills and adverse mood as well as impairment of neurodevelopmental function in humans (Egan et al., 2003). Previous studies identified adults who were Het/Mut for rs6265 as having increased risk of neurobehavioral deficits associated with low-level occupational Hg exposure (Echeverria et al., 2005; Heyer et al., 2004). In the present study, we observed significant genexHg interactions among boys genotyped as Het/Mut for rs6265 for several tests of Learning & Memory, including ravlt tr7 ($p<0.01$), ravlt tr8 ($p<0.04$) and ravlt sum tr1-8 ($p<0.04$) in relation to *acute* Hg exposure (Table 4). We also observed a significant genexHg interaction among girls for ravlt tr1 ($p<0.008$) in relation to *acute* Hg exposure and for ravlt tr8 ($p<0.03$) in relation to *chronic* Hg exposure (Table 5). These findings suggest potential modification of Hg effects by BDNF rs6265 on tests of Learning and Memory among both boys and girls. A scatter plot depicting the association between performance on the ravlt tr7 test of Learning & Memory and *chronic* Hg exposure among boys genotyped as WT or Het/Mut for BDNF rs6265 is shown in Figure 1F.

3.2.4 GSTT1—GSTT1 encodes glutathione S-transferase theta-1, a member of a superfamily of enzymes that catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophilic and hydrophilic ligands including Hg (Goodrich et al., 2011). GSTT1 has a deletion polymorphism that results in impaired (or null) catalytic activity that may be associated with increased Hg body burden (Gundacker et al., 2007, 2009; Schläwiche Engström et al., 2008). We evaluated the effects of Hg exposure on neurobehavioral functions among children genotyped for the presence or absence of this deletion mutation. As shown in Table 4, we found significant interactions with *chronic* Hg exposure on tests of Attention (Stroop word [$p<0.004$]) and Learning & Memory (CMVT d-Prime [$p<0.05$]) and with *acute* Hg exposure for Pegs dominant (Motor) ($p<0.03$) among boys genotyped as having the GSTT1 deletion mutation. In addition, we observed a significant interaction with *chronic* Hg exposure for ravlt sum tr1-5 (Learning & Memory)($p<0.02$) among girls with the deletion mutant (Table 5). These findings suggest potential modification of Hg effects among both boys and girls with deletion mutations of GSTT1, consistent with increased Hg body burden observed among subjects with this variant (Gundacker et al., 2007). A scatter plot depicting the association between performance on the Stroop Word test of Attention and *chronic* Hg exposure among boys genotyped as WT (no deletion) or Het/Mut (deletion mutation) for GSTT1 is shown in Figure 1G.

3.2.5 SLC6A4—Solute Carrier 6A4, also known as 5-hydroxytryptamine (5-HT, serotonin) transporter (5-HTT), transports the neurotransmitter, serotonin, into presynaptic neurons and, hence, determines the magnitude and duration of serotonergic responses. A functional polymorphism in the promoter region of the 5-HTT gene known as the short (S) allele (5-HTTLPR-S) that decreases 5-HTT function has been reported to be associated with behavioral deficits, depressive symptoms, diagnosable depression, suicidal behavior and excessive shyness in children (Arbelle et al., 2003; Caspi et al., 2003; Lesch et al., 1996). Affective spectrum disorders including anxiety, depression and aggression-related

personality traits associated with this polymorphism are also reported (Collier et al., 1996). Since serotonergic neurotransmission influences many brain functions that may also be altered by Hg exposure, including cognition, attention, mood, social interaction and motor function, we hypothesized that a polymorphism that alters the functional expression of the 5-HTT gene might exacerbate Hg-induced neurobehavioral deficits in children. We evaluated the effects of Hg exposure among children genotyped as 5-HTTLPR-L (WT) versus those genotyped as LS or SS (variant). We observed significant variantxHg interactions for 2 tests of Attention, i.e., WAIS-III- digit span ($p < 0.01$) and Trails A ($p < 0.04$) and 2 tests of Learning & Memory (ravlt tr6 List B ($p < 0.05$) and ravlt sum 1-8 ($p < 0.003$) among boys with *chronic* Hg exposure (Table 4). No effect modification, however, was observed for either *acute* or *chronic* Hg exposure among girls genotyped with the variant form. These findings suggest the S allele of 5-HTTLPR may predispose boys to increased risk of adverse neurologic effects of Hg exposure. A scatter plot depicting the association between performance on the Digit Span test of Visual Spatial acuity and *chronic* Hg exposure among boys genotyped as WT or Het/Mut for the 5-HTTLPR deletion mutation is shown in Figure 1H.

3.2.6 KIBRA—The protein KIBRA (kidney and brain expressed protein), also known as WW domain-containing protein 1 (WWC1), is implicated in human cognition via an association of the intronic SNP rs17070145 with episodic memory performance in humans (Papassotiropoulos et al., 2006). This SNP is a common T>C exchange within the 9th intron of the KIBRA gene. CC carriers perform poorer on verbal and visual episodic memory tasks than CT and TT carriers (Schaper et al 2008). Neither the effect of this SNP on memory of healthy children nor its potential interaction with Hg exposure on memory or other neurobehavioral functions is known. We evaluated the effects of Hg exposure among children genotyped as CC (homozygous mutant) for KIBRA. Among boys, we observed significant genexHg interactions for ravlt tr7 List A ($p < 0.03$) and ravlt sum tr1-5 ($p < 0.01$) in the Learning & Memory domain, for Trails A (Attention) ($p < 0.02$), and for WAIS-III symbol search (Visual Spatial) ($p < 0.01$) for *chronic* Hg exposure (Table 4). No significant genexHg interactions were observed among girls. These findings suggest potential modification of Hg effects on tests of Learning & Memory as well as other domain functions among boys carrying the Mut variant of KIBRA rs17070145.

3.2.7 APOE—Apolipoprotein E (APOE) is an intracellular cholesterol and fatty acid transport protein that plays an important function in neuronal metabolism as well as in synaptogenesis during neurodevelopment (Buttini et al., 1999). Three common alleles of the APOE gene ($\epsilon 2$, $\epsilon 3$ and $\epsilon 4$) differ only on the basis of 1 or 2 amino acids on positions 112 and 158 (Wright et al., 2003). The APOE $\epsilon 4$ allele is associated in some studies with poor neural repair function and has been found to modify the adverse effects of prenatal Hg exposure on tests of neurobehavioral function among 2 year-old children (Ng et al., 2013). In the present study, we genotyped children for the principal alleles of the SNPs (rs429358, rs7412) encoding $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$ and evaluated the effects of Hg exposure on neurobehavioral functions among groups genotyped as $\epsilon 3$ ($\epsilon 3/\epsilon 3$) versus $\epsilon 4$ ($\epsilon 3/\epsilon 4 + \epsilon 4/\epsilon 4$). We found only 1 significant genexHg interaction among boys genotyped as $\epsilon 4$, specifically, ravlt sum tr1-8 (Learning & Memory) ($p < 0.02$) (Table 4). Among girls, we observed 2 significant genexHg

interactions, Stroop color word (Attention)($p < 0.04$) and Finger tapping dom (Motor) ($p < 0.005$), both in the direction of improved performance (Table 5). While these findings may be random in terms of suggesting an effect of APOE $\epsilon 4$ on the neurobehavioral effects of Hg in children, they are nonetheless consistent with those previously described by others (Ng et al., 2013) and may be more clearly substantiated if assessed in a larger population of Hg-exposed subjects.

3.2.8 SEPP1—SEPP1 encodes a selenoprotein (Selenoprotein P) containing multiple selenocysteine residues that confer antioxidant and metal binding functions (Goodrich et al., 2011). Several variants encoding different isoforms have been found for this gene, although no studies describing their effects on human behavior or performance have been reported. However, SEPP1 knock-out mice have low brain Se concentrations and develop progressive neurological dysfunction characterized by impaired movement and coordination (Nakayama et al., 2006). We evaluated the effects of Hg exposure on neurobehavioral functions among children genotyped as HetMut for the relatively common 3'UTR rs7579 SEPP1 variant. We found no significant interactions in relation to either *acute* or *chronic* Hg exposure.

4. Discussion

4.1. Genetic variation in the assessment of Hg risk

This study identifies significant adverse effects of low-level Hg exposure (such as that acquired from diet or dental amalgams) on a wide range of neurobehavioral functions among children who carry genetic variants that are relatively common within the general human population. These findings have potentially important implications for assessment, prediction and management of risks of neurobehavioral dysfunction associated with Hg exposure in children and adults as well. Foremost, the minor allelic frequencies (MAFs) for most of the individual SNPs evaluated in the present study cohort ranged from between 20% and 40%, accounting for the relatively high prevalence of adverse effects among subjects in response to Hg exposure observed. Inasmuch as the present study population was largely of European (Caucasian) ancestry, the findings of susceptibility to Hg toxicity observed here should be predictably similar among all populations of comparable descent. Moreover, although substantial differences in allelic frequencies of individual SNPs are known to occur across populations of different lineage, e.g., European, African, Asian, it is notable that common combinations of alleles (haplotypes) are largely shared across different populations (Gabriel et al., 2002), suggesting that the likelihood of genetic susceptibility to Hg toxicity observed here will be broadly relevant across other population groups. We note in this context that the present findings demonstrate genetic modification of neurobehavioral test outcomes in relation to Hg exposure but not specifically by Hg alone. Such findings point to the importance of genetic variation both as an etiologic factor underlying susceptibility to Hg toxicity as well as for assessment, prediction and management of Hg risk.

4.2. Children are more susceptible than adults to genetic modification of Hg neurotoxicity

The findings of the present study support and substantiate the well-established view that children have heightened susceptibility to the adverse effects of environmental chemical exposures compared with adults, particularly, with regard to Hg exposure. Here, children

who were genotyped with common variants of genes that have been previously shown to elicit no or only additive effects with Hg on neurobehavioral functions in adults, e.g., BDNF, CPOX, COMT, 5-HTTLPR (Echeverria et al., 2005, 2006, 2010; Heyer et al., 2004, 2008, 2009), displayed numerous significant interactive effects on comparable tests of neurobehavioral function at similar levels of Hg exposure. Since adverse neurobehavioral effects were found only among children having gene modifications, genexHg interactions are assumed to underlie these effects. In this regard, Hg directly affects dopaminergic (Eddins et al., 2008), serotonergic (Tsai et al., 1995) and other neurologic processes (Castoldi et al., 2008; Pieper et al., 2014), and these effects are likely to be exaggerated against enzyme or protein products of genetic variants that mediate or facilitate developing neurologic pathways. Of note, Hg²⁺ directly impairs the enzymatic activity of CPOX4 (Li and Woods, 2009) and also likely inhibits preferentially the S-containing met forms of the COMT val158met enzyme and the BDNF val66met protein product, both of which play critical roles in neurologic development (Barnett et al., 2009; Burkhalter et al., 2003). Moreover, genetic variants affecting Hg toxicokinetics, e.g., MT1M, MT2A, GSTT1 (Wang et al., 2012), might predispose children to higher concentrations of Hg in critical brain regions than would occur in adults with comparable exposures. Such effects directed against components of the developing central nervous system could underlie the preferential susceptibility of children and adolescents with these genotypes to the adverse neurologic effects of Hg exposure. Additionally, from a genomic perspective, pre-pubertal Hg exposure might act epigenetically to alter the timing of normal developmental regulation of gene expression and/or gene products in such a way as to maximize the impact of Hg interactions in younger children. Of note, epigenetic mechanisms have been reported to underlie the timing of toxic effects of a number of environmental chemicals, including Hg (Baccarelli and Bollati, 2009), and these alterations are particularly manifest in early development (Fleming et al., 2008). Epigenetic factors affecting the timing of gene expression might also provide a plausible mechanistic explanation for genexHg interactions whose effects were expressed differentially as a consequence of *acute*, e.g., COMT, BDNF, GRIN2A, GRIN2B, versus *chronic*, e.g., CPOX4, MT2A, GSTT1, 5-HTTLPR, KIBRA, Hg exposure among children in this investigation. Further studies are required to identify and elaborate the mechanisms by which the toxic effects of Hg are preferentially expressed in relation to genotype and timing of Hg exposure in children versus adults.

4.3. Boys are more susceptible to genetic modification of Hg neurotoxicity than girls

In all cases in which we observed significant genetic modification of Hg effects on neurobehavioral functions, such effects were found to be substantially more prominent among boys than girls in terms of both numbers of neurobehavioral domains as well as tests within such domains affected. Moreover, few adverse effects of Hg by sex were observed in the absence of genetic variation. These findings support the view that genetic predisposition is a significant factor in defining sex differences in susceptibility to the adverse neurobehavioral effects of Hg, particularly in children. Factors underlying these sex-specific effects may include differences in Hg toxicokinetics that predispose boys to greater Hg retention and tissue accumulation than girls. Notably, significant differences in Hg excretion between boys and girls were observed in the clinical trial from which subjects in the present study were drawn (Woods et al, 2007), suggesting that boys may retain and accumulate

significantly higher levels of Hg than girls over the course of comparable Hg exposure. Findings from animal studies (Hultman and Nielsen, 2001) demonstrating significantly higher renal Hg concentrations and whole body Hg retention in male compared with female mice following prolonged HgCl₂ exposure in the drinking water support this view. Genetic and hormonal differences affecting brain development, morphology and function between boys and girls might also underlie the sex-differences observed with respect to effects of genotype on Hg-related neurobehavioral functions. In this respect, estrogen regulation of COMT transcription (Xie et al., 1999) corresponds to significant sex differences in dopamine levels in the prefrontal cortex and other tissues (Harrison and Tunbridge, 2008), potentially contributing to sex differences in response to Hg exposure. Similarly, sex hormone regulation of BDNF signaling mediates sexually dimorphic axonal growth, resulting in generation of sex-specific neural circuits (Liu et al., 2012), also potentially underlying differences in response of boys and girls to Hg exposure (Spulber et al., 2010). Moreover, as noted above, epigenetic factors that differentially affect the timing, duration and magnitude of gene expression are also likely to contribute to the sex differences in susceptibility to Hg neurotoxicity observed (Jirtle and Skinner, 2007; Basu et al., 2013; Goodrich et al., 2013). In this regard, significant differences in DNA methylation patterns, methyl transferases, methyl binding proteins, and co-repressor proteins are currently recognized as contributing to the establishment and maintenance of sex differences in the brain and behavior (McCarthy et al., 2009), providing a plausible mechanistic basis for sex differences in response to Hg observed, both with respect to the numbers of genes and related neurologic processes affected as well as in the overall scope and direction of responses between boys and girls. Consistent with this view, the present findings are not unique to Hg exposure. Many classes of drugs and chemicals including other metals, pesticides, halogenated hydrocarbons and constituents of cigarette smoke are reported to differentially affect neurologic functions in males and females, particularly children (Woods et al., 2012). The finding of sex-specific responses to Hg exposure observed here in relation to common genetic variants highlights the importance of considering such differences in the development of strategies aimed as risk reduction from adverse Hg effects, particularly among children.

4.4. Implications for future research

The present studies focused exclusively on a candidate gene approach toward defining susceptibility to Hg neurotoxicity in children, based on *a priori* knowledge of genes whose variants are known or suspected to modify Hg handling and/or to increase the risk of neurologic disorders in humans. Although most of the variants investigated adversely modified the effects of Hg exposure on neurologic functions in children, the relatively small population size may have precluded detection of other significant genexHg effects associated with some variants as well as identification of potential effects associated with combinations of the variants evaluated. Replication of the present study in other, larger, cohorts is therefore essential both to confirm the present findings as well as to identify additional variants contributing to Hg toxicity. Beyond the present study design, investigations aimed at defining common pathways through which these and other variants might act to modulate the adverse effects of Hg could substantially advance our understanding of Hg toxicity. In particular, studies utilizing genome-wide genotyping and

network modeling techniques (e.g., Schadt et al., 2009), could allow more comprehensive genetic characterization of variants that potentially influence Hg toxicity and elucidate a broader set of pathways and/or biological subnetworks that underlie health risk susceptibilities from Hg exposure. A pathway-based approach to Hg risk assessment could also enhance abilities to make connections between genes and pathways underlying Hg neurotoxicity with those associated with other neurologic health risks, such as attention deficit or autism spectrum disorders. Additionally, investigation of epigenetic factors underlying global expression of genes through which Hg neurotoxicity is predominantly mediated may allow better characterization of timing, magnitude, direction and gender-specificity of these effects. It is already clear from these and other studies cited herein that the genes being identified expose relevant biology. Ultimately, elucidation of genome-wide pathways underlying Hg neurotoxicity will improve the power for prediction both of Hg-mediated adverse health risks as well as better inform the extent to which Hg toxicity can be mitigated by prevention of Hg exposure.

4.5. Conclusions

Numerous common genetic polymorphisms affect susceptibility to the adverse neurobehavioral effects of Hg in children. The genexHg interactions identified in this study contribute to public health goals to define factors underlying susceptibility to Hg toxicity and suggest risk assessment and intervention strategies for preventing adverse health effects associated with Hg exposure, irrespective of source, within the general population. The present findings also establish a scientific framework for additional candidate gene studies in other Hg-exposed populations as well as more comprehensive genome-wide, network-based studies to define multiple genetic variants and associated biochemical pathways underlying susceptibility to Hg neurotoxicity, particularly in children.

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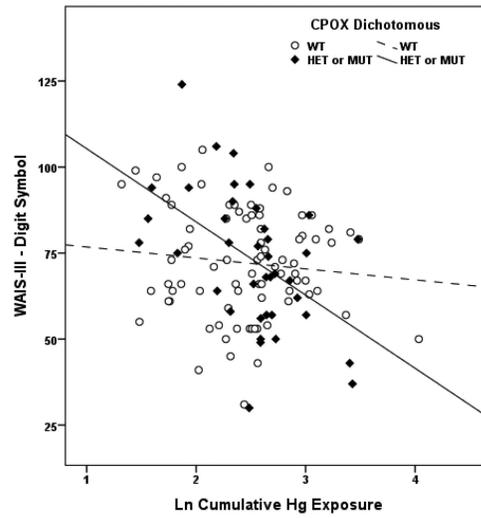
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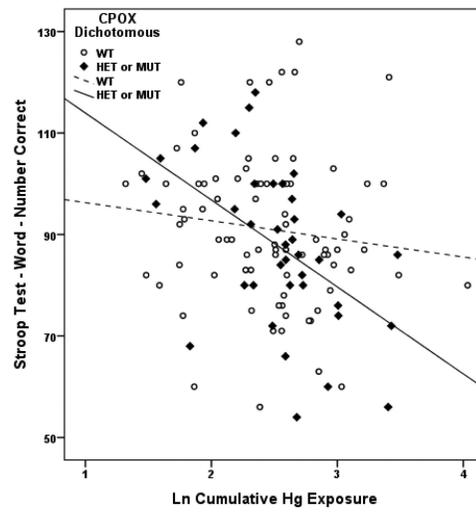
Highlights

- Genotype determines the effects of Hg on neurobehavioral functions in children
- Boys are more susceptible to genetic modification of Hg neurotoxicity than girls
- Multiple common variants underlie the wide prevalence of Hg neurotoxicity
- Genes identified expose relevant biology underlying susceptibility to Hg toxicity

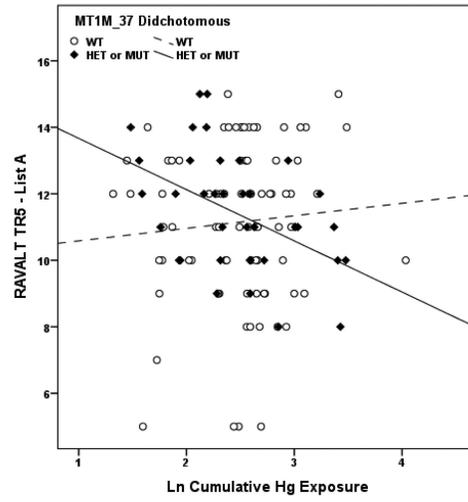
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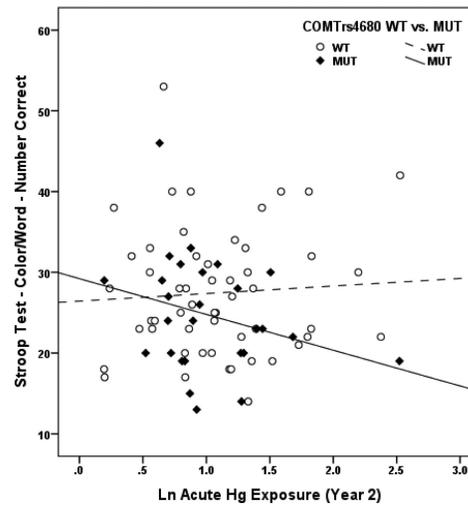
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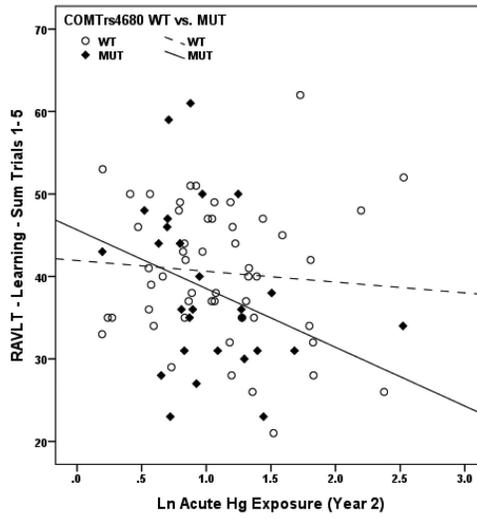
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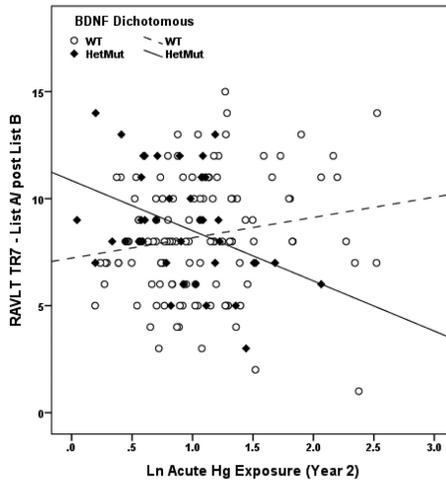
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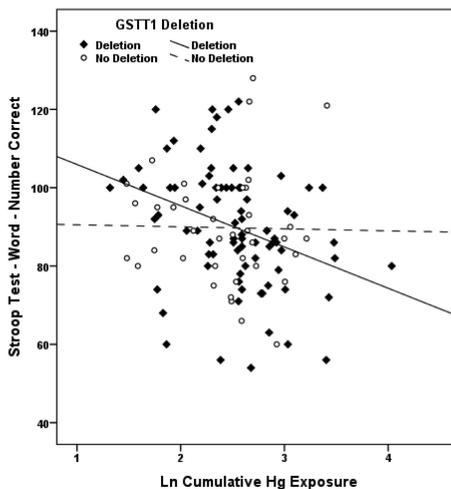
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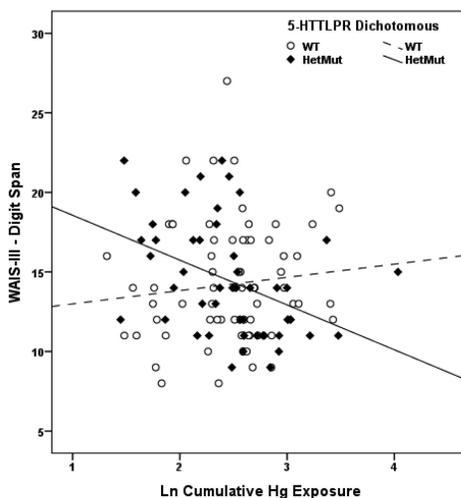


Figure 1.

Figures 1A-1H present scatter plots and simple linear regression fit lines of neurobehavioral test scores by acute or cumulative (chronic) Hg exposure plotted to distinguish boys genotyped as wildtype (WT) from those genotyped as variant with respect to the gene identified.

Figure 1A. Association between performance on the WAIS-III Digit Symbol test of Visual Spatial acuity and cumulative (chronic) Hg exposure among boys genotyped as WT (dashed line, open symbols) or Het/Mut (solid line, closed symbols) for coproporphyrinogen oxidase rs1131857 (CPOX4). Linear regression r^2 s are 0.015 and 0.259, respectively, indicating that Hg exposure accounted for 1.5% of performance variance among boys with WT status but for nearly 26% of performance variance among boys with Het or Mut gene status.

Figure 1B. Association between performance on the Stroop Word test of Attention and cumulative (chronic) Hg exposure among boys genotyped as WT (dashed line, open symbols) or Het/Mut (solid line, closed symbols) for coproporphyrinogen oxidase rs1131857 (CPOX4). Linear regression r^2 s are 0.011 and 0.251, respectively, indicating that Hg exposure accounted for 1.1% of performance variance among boys with WT status but for 25.1% of performance variance among boys with Het or Mut gene status.

Figure 1C. Association between performance on the Rey Auditory Verbal Learning Test (RAVLT) Trial5 List A test of Learning & Memory and cumulative (chronic) Hg exposure among boys genotyped as WT (dashed line, open symbols) or Het/Mut (solid line, closed symbols) for metallothionein MT1M rs2270837. Linear regression r^2 s are 0.006 and 0.210, respectively, indicating that Hg exposure accounted for 0.6% of performance variance among boys with WT status but for 21% of performance variance among boys with Het or Mut gene status.

Figure 1D. Association between performance on the Stroop Color/Word test of Attention and acute Hg exposure among boys genotyped as WT (dashed line, open symbols) or Mut (solid line, closed symbols) for catechol *O*-methyltransferase (COMT) rs4680. Linear regression r^2 s are 0.004 and 0.180, respectively, indicating that Hg exposure accounted for 0.4% of performance variance among boys with WT status but for 18% of performance variance among boys with Mut gene status.

Figure 1E. Association between performance on the Rey Auditory Verbal Learning Test (RAVLT) sum of Trials 1-5 test of Learning & Memory and acute Hg exposure among boys genotyped as WT (dashed line, open symbols) or Mut (solid line, closed symbols) for catechol *O*-methyltransferase (COMT) rs4680. Linear regression r^2 s are 0.007 and 0.150, respectively, indicating that Hg exposure accounted for 0.7% of performance variance among boys with WT status but for 15% of performance variance among boys with Mut gene status.

Figure 1F. Association between performance on the Rey Auditory Verbal Learning Test (RAVLT) Trial7 List A/Post B test of Learning & Memory and acute Hg exposure among boys genotyped as WT (dashed line, open symbols) or Het/Mut (solid line, closed symbols) for brain-derived neurotrophic factor (BDNF) rs6265. Linear regression r^2 s are 0.031 and 0.156, respectively, indicating that Hg exposure accounted for 3.1% of performance variance among boys with WT status but for 15.6% of performance variance among boys with Het or Mut gene status.

Figure 1G. Association between performance on the Stroop Word test of Learning & Memory and cumulative (chronic) Hg exposure among boys genotyped as no deletion (dashed line, open symbols) or deletion mutation (solid line, closed symbols) for glutathione-S-transferase theta-1 (GSTT1). Linear regression r^2 s are 0.0003 and 0.114, respectively, indicating that Hg exposure accounted for essentially 0% of performance variance among boys without the deletion mutation but for 11.4% of performance variance among boys with the deletion polymorphism.

Figure 1H. Association between performance on the WAIS III Digit Span test of Visual Spatial acuity and cumulative (chronic) Hg exposure among boys genotyped as WT (LL) (dashed line, open symbols) or deletion (LS or SS) (solid line, closed symbols) for solute carrier 6A4 (SLC6A4), also known as 5-HTTLPR. Linear regression r^2 s are 0.011 and 0.175, respectively, indicating that Hg exposure accounted for 1.1% of performance variance

among boys without the deletion mutation but for 17.5% of performance variance among boys with one (LS) or two (SS) alleles for the deletion polymorphism.

Table 1

Neurobehavioral tests assessed with mean scores for Year 7 (final year of clinical trial)

Test / Domain	Test Abbreviation	Measure ^a	Boys Mean (SD)	Girls Mean (SD)
Attention (6 tests)				
Stroop Test – Color	strpcol	# correct ↑	66.16 (11.97)	69.25 (10.39)
Word	strpwd	# correct ↑	89.93 (15.16)	91.54 (15.19)
Color-Word	Strpc/wd	# correct ↑	41.74 (9.76)	43.93 (8.73)
WAIS-III – Digit Span	digspn	# correct ↑	14.30 (3.67)	14.14 (2.76)
WAIS- III – Spatial Span	spatspn	# correct ↑	15.83 (3.03)	15.61 (3.12)
Adult Trailmaking A	TrailsA	Time (sec) ↓	26.43 (10.63)	30.25 (11.42)
Visual-Spatial (3 tests)				
Standard Reaction Time	SRT	Time (sec) ↓	0.74 (0.15)	0.77 (0.13)
WAIS III – Digit Symbol	digitsym	# correct ↑	72.02 (16.48)	76.58 (13.85)
Symbol Search	symsea	# correct ↑	32.99 (8.82)	34.59 (8.01)
Executive Functioning (2 tests)				
Wisconsin Card Sort – Categories Completed	Card Sort-Cat	# categories ↑	3.05 (1.38)	3.09 (1.47)
Adult Trailmaking B	TrailsB	Time (sec) ↓	65.97 (26.94)	63.10 (23.80)
Learning & Memory (8 tests)				
RAVLT Trial 1 – List A	ravlt tr1	# correct ↑	5.62 (1.49)	6.12 (1.86)
Trial 5 – List A (fifth repetition)	ravlt tr5	# correct ↑	11.23 (2.20)	11.55 (2.23)
Trial 6 – List B	ravlt tr6	# correct ↑	4.73 (1.38)	5.28 (1.56)
Trial 7 – List A/Post B	ravlt tr7	# correct ↑	9.85 (2.57)	10.23 (2.48)
Trial 8 – List A after 20'	ravlt tr8	# correct ↑	9.29 (2.73)	10.08 (2.76)
WMS-III – Visual Reproductions – Immediate	VisRep-Imm	# correct ↑	34.69 (4.66)	36.62 (2.97)
-Delayed	VisRep-Del	# correct ↑	32.01 (6.98)	34.93 (4.02)
CVMT d-Prime	CVMT	Score ↑	1.50 (.94)	1.61 (.87)
Motor (4 tests)				
WRAPMA –Pegs – Dominant Hand	pegdom	# Pegs ↑	47.35 (8.51)	49.92 (6.28)
Non Dominant Hand	pegndom	# Pegs ↑	44.37 (7.59)	45.03 (6.10)
Finger Tapping – Dominant Hand	ftapdom	# Taps ↑	52.66 (5.53)	48.55 (5.83)
Non Dominant Hand	ftapndom	# Taps ↑	46.54 (5.79)	42.53 (5.78)

^a Arrows show direction of improved performance.

Table 2

Summary of genes and gene variants investigated

Gene#	Gene	Chromosome	SNP	Variation	N	WT	HET	MUT	MAF%
1	CPOX	3	rs1131857	A>C	328	224	91	13	24
			rs1729995	G>A	329	190	117	22	24
2	MT1M	16	rs2270837	G>A	330	204	114	12	27
			rs2270836	C>T	330	134	126	70	33
3	MT2A	16	rs10626	G>C	330	200	106	24	24
4	COMT	22	rs4680	G>A	330	115	162	53	39
			rs4633	C>T	330	114	165	51	39
			rs4818	C>G	330	120	156	54	32
			rs6269	A>G	330	83	172	75	37
5	TDO2	4	rs13434811	G>T	330	269	56	5	11
			rs2271537	G>T	330	117	147	66	48
			rs2292537	A>G	330	254	71	5	11
			rs3755907	G>A	330	242	78	10	18
			rs3755908	T>C	330	242	78	10	18
			rs3755910	C>A	330	312	18	0	3
6	GRIN2A	16	rs727605	C>T	330	134	150	46	26
			rs1070503	C>T	330	232	89	9	25
			rs1806201	G>A	330	206	106	18	31
7	GRIN2B	12	rs7301328	G>C	330	114	157	59	40
			rs6265	G>A	330	238	80	12	23
8	BDNF	11	rs6265	G>A	330	238	80	12	23
9	GSTT1	22	gene deletion		330	246 ^a		84	25
10	SLC6A4	17	5-HTTLPR ins/del		330	194 LL	91 LS	45 SS	15
11	KIBRA	5	rs17070145	C>T	330	126	144	38	46
12	APOE	19	rs429358	T>C	330	237	85	8	15
			rs7412	C>T	330	287	38	5	7
13	SEPP1	5	rs7579	C>T	330	223	92	15	27

WT:Homozygous major variant; HET:Heterozygous; MUT:Homozygous minor variant; MAF: Minor Allelic Frequency

^aData for GSTT1 are no deletion (WT) vs deletion (Mut).

Table 3

Synopsis of gene functions and neurologic relevance.

Gene(s)	Variant(s)	Pathway/Process	Neurologic Relevance
CPOX	rs1131857	Heme biosynthesis	Neuronal receptor processing Energy (ATP) Production Modulation of neuronal gene expression
MTIM	rs2270837	Hg dispersal/storage	Protection & maintenance of neurologic processes
MT2A	rs10636	Hg dispersal/storage	Protection & maintenance of neurologic processes
COMT	rs4680	Catecholamine deactivation	Maintenance of cognitive & executive functions
TDO2	rs3755907	Tryptophan catabolism	Serotonin (5-HT) availability & signaling
GRIN2A	rs727605 rs1070503	N-methyl-D-aspartate receptor processing	Glutamatergic (excitatory) neurotransmission
GRIN2B	rs1806201 rs7301328	N-methyl-D-aspartate receptor processing	Glutamatergic (excitatory) neurotransmission
BDNF	rs6265	Neuronal growth and differentiation	Memory and motor functions
GSTT1	Deletion	Hg-GSH conjugation	Facilitation of Hg excretion
SLC6A4 (5-HTTLPR)	Insertion/deletion	Serotonin uptake	Maintenance of mood, cognitive & motor functions
KIBRA	rs17070145	Synaptic plasticity	Verbal & visual episodic memory
APOE	rs429358 rs7412	Cholesterol & fatty acid transport; neuronal repair	Maintenance of neuronal and cognitive integrity
SEPP1	rs7579	Hg binding	Protection & maintenance of neurologic processes

Table 4

Individual Gene SNPxHg Results Among BOYS. Data depict significant ($p < 0.05$) dose-response effects for Acute and Chronic Hg exposure on performance on specific neurobehavioral tests among boys genotyped as variant for the gene indicated. No significant dose-response measures among boys genotyped as WT for any gene were observed (not shown).

Gene	SNP (rs#)	Test	Domain	Acute Hg Exposure			Chronic Hg Exposure		
				Beta	SE	p value	Beta	SE	p value
CPOX	1131857			WT = 114, Het = 45, Mut = 1			WT = 82, Het = 38, Mut = 1		
		strpwd	Attn				-18.1	4.89	0.001
		strpcol	Attn	-8.4	2.8	0.005	-12.55	4.05	0.004
		strpc/wd	Attn	-3.5	1.68	0.04			
		digspn	Attn				-3.57	1.12	0.003
		spatspn	Attn				-2.45	0.97	0.02
		trailsA*	Attn				11.25	3.83	0.006
		SRT*	VisSpa				0.15	0.05	0.007
		digitsym	VisSpa				-20.7	6.08	0.002
		trailsB*	ExFn				22.26	7.03	0.003
		ravlt tr5	L&M				-1.78	0.8	0.03
		ravlt tr7	L&M				-2.24	0.82	0.01
		ravlt tr8	L&M				-2.14	0.83	0.01
		pegdom	Motor				-7.16	2.9	0.02
		pegndom	Motor				-5.34	2.7	0.06
ftapdom	Motor	-3.25	1.53	0.04	-3.46	1.65	0.04		
ftapndom	Motor				-5.83	1.66	0.001		
MT1M	2270837			WT = 106, Het = 49, Mut = 3			WT = 82, Het = 35, Mut = 3		
		ravlt tr5	L&M	-1.22	0.58	0.04	-1.56	0.53	0.006
		ravlt tr8	L&M	-1.69	0.67	0.02	-2.11	0.74	0.007
		ftapdom	Motor	-4.75	1.53	0.003			
MT2A	10636			WT = 96, Het = 49, Mut = 13			WT = 74, Het = 36, Mut = 10		
		strpcol	Attn	-6.88	2.62	0.01			
		digitsym	VisSpa				-12.9	4.77	0.01
		symsea	VisSpa				-8.21	2.62	0.003
		ravlt tr5	L&M				-1.96	0.86	0.03
		ravlt tr8	L&M				-6.49	2.72	0.007
COMT	4680			WT = 53, Het = 79, Mut = 27			WT = 40, Het = 57, Mut = 23		
		strpwd	Attn	-12.43	6.21	0.06			
		strpcol	Attn	-9.32	4.09	0.03			
		strpc/wd	Attn	-7.92	2.76	0.009			
		digspn	Attn	-2.11	0.93	0.03			
		spatspn	Attn				-5.05	1.95	0.02

Gene	SNP (rs#)	Test	Domain	Acute Hg Exposure			Chronic Hg Exposure		
				Beta	SE	p value	Beta	SE	p value
		ravlt tr5	L&M	-2.66	1.08	0.02			
		ravlt tr6	L&M	-1.68	0.69	0.02			
		ravlt tr7	L&M	-2.83	1.19	0.03			
		ravlt tr8	L&M	-3.70	1.48	0.02			
		ravlt 1-5	L&M	-12.21	4.26	0.01			
		digitsym	VisSpa				-32.46	11.28	0.01
		symsea	VisSpa				-16.49	5.52	0.008
COMT	4633			WT = 50, Het = 84, Mut = 25			WT = 38, Het = 61, Mut = 21		
		strpcol	Attn	-7.60	3.70	0.05	-13.79	6.23	0.04
		strpc/wd	Attn	-7.98	2.73	0.01			
		digitspn	Attn	-2.04	0.98	0.05			
		spatspn	Attn				-4.58	1.84	0.02
		ravlt tr5	L&M	-2.7	1.06	0.02			
		ravlt tr6	L&M	-1.65	0.69	0.02			
		ravlt tr7	L&M	-2.8	1.16	0.03			
		ravlt tr8	L&M	-3.69	1.44	0.02			
		ravlt 1-5	L&M	-12.38	4.2	0.008			
		digitsym	VisSpa				-35.71	10.87	0.005
		symsea	VisSpa				-16.19	5.53	0.01
COMT	6269			WT = 42, Het = 83, Mut = 34			WT = 34, Het = 60, Mut = 26		
		strpcol	Attn	-5.31	2.59	0.05			
		digitspn	Attn	-1.72	0.79	0.04			
		digitsym	VisSpa				-17.92	6.8	0.01
		symsea	VisSpa				-8.48	3.83	0.04
		ravlt tr5	L&M	-2	0.78	0.01			
		ravlt tr7	L&M	-1.96	0.94	0.04			
		ravlt tr8	L&M	-2.63	1.04	0.02			
		ravlt 1-5	L&M	-8.41	3	0.008			
TDO2	3755907			WT = 119, Het = 38, Mut = 3			WT = 88, Het = 29, Mut = 3		
		strpwd	Attn				-14.66	7.12	0.05
		SRT ^a	VisSpa				0.16	0.06	0.005
		pegsdom	Mot	-3.72	1.8	0.05	-9.36	3.9	0.02
		pegndom	Mot	-3.59	1.97	0.04			
		ftapdom	Mot	-2.83	1.35	0.04			
GRIN2A	727605			WT = 57, Het = 77, Mut = 24			WT = 42, Het = 61, Mut = 17		
		strpwd	Attn	-6.28	2.78	0.03			
		spatspn	Attn				-1.54	0.64	0.02
		ravlt tr1	L&M	-0.71	0.34	0.04			

Gene	SNP (rs#)	Test	Domain	Acute Hg Exposure			Chronic Hg Exposure		
				Beta	SE	p value	Beta	SE	p value
GRIN2A	1070503			WT = 114 , Het = 40, Mut = 4			WT = 82, Het = 35, Mut = 3		
		ravlt tr5	L&M	-1.13	0.65	0.05			
		ftapndom	Mot	-4.03	1.38	0.006			
GRIN2B	7301328			WT = 54, Het = 80, Mut = 24			WT = 41, Het = 60 , Mut = 19		
		ravlt tr8	L&M	-1.07	0.58	0.07			
		VisRep-Del	L&M				-4.55	1.54	0.004
		ftapndom	Motor	-3.21	1.06	0.003	-3.65	1.26	0.005
BDNF	6265			WT = 118, Het = 35, Mut = 5			WT = 88, Het = 29, Mut = 3		
		ravlt tr7	L&M	-2.11	0.82	0.01			
		ravlt tr8	L&M	-1.99	0.89	0.03			
		ravlt 1-8	L&M	-0.71	0.34	0.04			
GSTT1	Deletion			No Deletion = 110, Deletion = 48			No Deletion = 81, Deletion = 39		
		strpwd	Attn				-9.76	3.29	0.004
		CMVT	L&M				-0.42	0.22	0.05
		pegsdom	Motor	-2.26	1.02	0.03			
SLC6A4	Ins/del			WT = 103, Het = 41, Mut = 14			WT = 70, Het = 38, Mut = 12		
(5-HTTLPR)		digitspn	Attn				-2.32	0.86	0.01
		TrailsA ^a	Attn				5.72	2.76	0.04
		ravlt 1-8	L&M				-7.09	2.22	0.003
		ravlt 6	L&M				-0.68	0.33	0.05
KIBRA	17070145			WT = 65, Het = 75, Mut = 18			WT = 48, Het = 60, Mut = 12		
		TrailsA ^a	Attn				6.42	2.71	0.02
		symsea	VisSpa				-6.34	2.42	0.01
		ravlt 1-5	L&M				-7.22	2.68	0.01
APOE	ε4			ε3(ε3/ε3) = 112, ε4(ε3/ε4 + ε4/ε4) = 44					
		ravlt 1-8	L&M				-9.15	3.71	0.02

^aTrailmaking A and SRT increase in magnitude (time) with impaired performance. All other tests decrease in magnitude (score) with impaired performance. Beta is the standardized value.

Table 5

Individual Gene SNPxHg Results Among GIRLS. Data depict significant ($p < 0.05$) dose-response effects for Acute and Chronic Hg exposure on performance on specific neurobehavioral tests among girls genotyped as variant for the gene indicated. No significant dose-response measures among girls genotyped as WT for any gene were observed (not shown).

Gene	SNP (rs#)	Test	Domain	Acute Hg Exposure			Chronic Hg Exposure		
				Beta	SE	p value	Beta	SE	p value
CPOX	1131857			WT = 97, Het = 42, Mut = 12			WT = 73, Het = 34, Mut = 11		
		ravlt tr5	L&M	-1.34	0.47	0.006			
		ravlt tr8	L&M	-1.69	0.55	0.003			
		ravlt 1-8	L&M	-4.06	1.64	0.02			
		trailsB*	ExFn				8.88	3.33	0.008
MT1M	2270837			WT = 86, Het = 57, Mut = 8			WT = 68, Het = 44, Mut = 7		
		ravlt tr8	L&M				-1.74	0.65	0.01
COMT	4680			WT = 56, Het = 74, Mut = 22			WT = 45, Het = 58, Mut = 16		
		trailsA*	Attn	7.49	1.51	0.0001			
		trailsB*	ExFn	6.52	2.81	0.03			
		ravlt tr8	L&M				-2.29	1.06	0.05
COMT	4633			WT = 58, Het = 72, Mut = 22			WT = 46, Het = 57, Mut = 16		
		trailsA*	Attn	6.18	2.00	0.007			
		trailsB*	ExFn	6.52	2.81	0.03			
COMT	4818			WT = 55, Het = 70, Mut = 27			WT = 43, Het = 54, Mut = 22		
		strpcol	Attn	8.39	3.16	0.01			
		strpwd	Attn	11.90	4.68	0.02			
		strpcolwd	Attn	4.84	1.85	0.02			
COMT	6269			WT = 35, Het = 81, Mut = 36			WT = 28, Het = 61, Mut = 30		
		ravlt tr6	L&M	0.81	0.37	0.04			
TDO2	3755907			WT = 108, Het = 37, Mut = 6			WT = 89, Het = 25, Mut = 5		
		Card Sort-Cat	ExFn				6.63	3.27	0.05
		Visrep-Imm	L&M				2.36	1.00	0.03
GRIN2B	7301328			WT = 51, Het = 68, Mut = 32			WT = 40, Het = 55, Mut = 24		
		ravlt tr7	L&M	-0.70	0.35	0.05			
		ravlt tr8	L&M				-0.92	0.51	0.08
		pegsdom	Motor	2.67	0.76	0.001			
BDNF	6265			WT = 107, Het = 37, Mut = 7			WT = 89, Het = 28, Mut = 5		
		ravlt tr1	L&M	-7.19	2.25	0.008			
		ravlt tr8	L&M				-1.80	0.77	0.03
GSTT1	deletion			No Deletion = 119, Deletion = 32			No Deletion = 93, Deletion = 26		

Gene	SNP (rs#)	Test	Domain	Acute Hg Exposure			Chronic Hg Exposure		
				Beta	SE	p value	Beta	SE	p value
		Trails A	Attn	6.80	3.20	0.04			
		pegsndom	Motor	-3.94	1.87	0.04			
APOE	$\epsilon 4$			$\epsilon 3(\epsilon 3/\epsilon 3) = 104, \epsilon 4(\epsilon 3/\epsilon 4 + (\epsilon 4/\epsilon 4) = 41$					
		strpcol	Attn				<i>8.14</i>	<i>3.18</i>	<i>0.04</i>
		ftapdom	Motor				<i>7.13</i>	<i>2.46</i>	<i>0.005</i>

* *Trailmaking A and B increase in magnitude (time) with impaired performance. All other tests decrease in magnitude (score) with impaired performance. Values in italics indicate results that significantly improved with Hg exposure (in the unexpected direction). Beta is the standardized value.*