Environment International 147 (2021) 106321

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Prenatal methylmercury exposure and DNA methylation in seven-year-old children in the Seychelles Child Development Study

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ARTICLE INFO

Handling Editor: Marti Nadal

Keywords:
Methylmercury
MeHg
Epigenetic
DNA methylation
Neurodevelopment
Fish consumption
Early life

ABSTRACT

Background: Methylmercury (MeHg) is present in fish and is a neurotoxicant at sufficiently high levels. One potential mechanism of MeHg toxicity early in life is epigenetic dysregulation that may affect long-term neurodevelopment. Altered DNA methylation of nervous system-related genes has been associated with adult mental health outcomes.

Objective: To assess associations between prenatal MeHg exposure and DNA methylation (at the cytosine of CG dinucleotides, CpGs) in three nervous system-related genes, encoding brain-derived neurotropic factor (*BDNF*), glutamate receptor subunit NR2B (*GRIN2B*), and the glucocorticoid receptor (*NR3C1*), in children who were exposed to MeHg *in utero*.

Methods: We tested 406 seven-year-old Seychellois children participating in the Seychelles Child Development Study (Nutrition Cohort 2), who were prenatally exposed to MeHg from maternal fish consumption. Total mercury in maternal hair (prenatal MeHg exposure measure) collected during pregnancy was measured using atomic absorption spectroscopy. Methylation in DNA from the children's saliva was measured by pyrosequencing. To assess associations between prenatal MeHg exposure and CpG methylation at seven years of age, we used multivariable linear regression models adjusted for covariates.

Results: We identified associations with prenatal MeHg exposure for DNA methylation of one *GRIN2B* CpG and two *NR3C1* CpGs out of 12 total CpG sites. Higher prenatal MeHg was associated with higher methylation for each CpG site. For example, *NR3C1* CpG3 had an expected increase of 0.03-fold for each additional 1 ppm of prenatal MeHg (B = 0.030, 95% CI 0.001, 0.059; p = 0.047). Several CpG sites associated with MeHg are located in transcription factor binding sites and the observed methylation changes are predicted to lead to lower gene expression.

Conclusions: In a population of people who consume large amounts of fish, we showed that higher prenatal MeHg exposure was associated with differential DNA methylation at seven years of age at specific CpG sites that may influence neurodevelopment and mental health.

1. Background

MeHg is found in fish and is a developmental neurotoxicant at high exposures (Bakir et al. 1973; Harada, 1995). There is substantial uncertainty whether there are any neurodevelopmental effects due to MeHg exposure from consumption of fish with naturally acquired background levels of MeHg contamination (Barbone et al. 2019; Grandjean et al. 1997; Llop et al., 2012; Strain et al. 2015; van Wijngaarden et al. 2017; Vejrup et al. 2016), but genetics may influence individuals' susceptibility to MeHg (Llop et al. 2017; Julvez et al. 2019).

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https://doi.org/10.1016/j.envint.2020.106321
Received 1 September 2020; Received in revised form 6 November 2020; Accepted 2 December 2020
Available online 16 December 2020
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Potential mechanisms linking MeHg exposure to neurodevelopmental outcomes include oxidative stress (Farina & Aschner, 2019), mitochondrial dysfunction (Cambier et al. 2009; Xu et al. 2019), and alterations in the epigenetic regulation of genes (Culbreth and Aschner, 2019).

Epigenetic marks in the form of DNA methylation are propagated from one generation of cells to the next and thus exist as heritable memory in the cell. DNA methylation regulates temporal and spatial patterns of transcription in response to internal and external signals and plays a critical role in cell differentiation and tissue organisation during general and neurodevelopment (Bale, 2015, Cantone & Fisher, 2013). Exposure early in life to metals such as lead, arsenic, and cadmium, has been associated with differential DNA methylation patterns (Engström et al., 2015; Gliga et al. 2018; Kippler et al. 2013), and *in vivo* experiments have demonstrated that arsenic and cadmium interact with DNA methyltransferases (Bommarito et al. 2017; Comparative Toxicogenomics Database: www.ctdbase.org).

Very little is known about the epigenetic effects of MeHg, and the available evidence comes mainly from *in vitro* and experimental *in vivo* studies (reviewed in Culbreth and Aschner, 2019). For example, Bose and co-workers reported a global decrease in DNA methylation in neural stem cells exposed to MeHg (Bose et al. 2012). In a few epidemiological studies, prenatal exposure to MeHg from fish consumption was associated with both global and site-specific changes in DNA methylation in children (Bakulski et al. 2015; Cardenas et al. 2017a; Cardenas et al. 2017b). Thus, it is possible that DNA methylation acts as a response mechanism to MeHg exposure or a long-term mediator of mercury-associated effects. Additionally, earlier epidemiological studies have not examined genetic sites associated specifically with neurodevelopment nor have they studied in populations outside the USA or with higher MeHg exposures.

Here, we examined the association between MeHg and DNA methylation using Nutrition Cohort 2 of the Seychelles Child Developmental Study (SCDS), the largest prospective study specifically designed to examine the effect of prenatal MeHg exposure from fish consumption on child development. The study population is from the Republic of Seychelles and includes 1522 mother–child pairs. The participants in the SCDS have mean Hg levels of 4.0 ppm in hair compared with 0.20 ppm in a population in the USA and 0.77 ppm in Italy (Strain et al. 2015; Miklavčič et al. 2013; McDowell et al. 2004). We analysed associations between prenatal MeHg exposure and DNA methylation in three nervous system related genes in a subpopulation of mother–child pairs from the SCDS.

We selected the *BDNF*, *GRIN2B*, and *NR3C1* genes because of their crucial roles in neurodevelopment and function, and their previously reported associations with MeHg exposure. Brain-derived neurotropic factor (BDNF) is a small secreted growth factor important for memory and learning (Arango-Lievano et al. 2019). In an experimental study in mice, changes in *Bdnf* expression and methylation were linked to perinatal MeHg exposure (Onishchenko et al. 2008). *GRIN2B* codes for the NR2B subunit of N-methyl-D-aspartate receptor (NMDAR), a glutamate receptor, and prenatal expression of *GRIN2B* plays an important role in brain development (Myers et al. 2019). The glutamate system is a well-established target of MeHg (Farina et al. 2011). *NR3C1* encodes the glucocorticoid receptor, which is a crucial regulator of stress responses (Kim & Iremonger, 2019). Glucocorticoid receptor function has been shown to be affected by MeHg in human cell and zebrafish models (Spulber et al. 2018). In humans, increased *NR3C1* methylation in the placenta has been associated with higher MeHg exposures (Appleton et al. 2017). For *BDNF* and *NRC31*, higher DNA methylation in regulatory regions has been associated with adult mental health outcomes (Zheleznyakova et al. 2016; Nöthling et al. 2019, Holmes et al. 2019). Here, we hypothesise that increased prenatal MeHg exposure (measured as MeHg in the mother's hair) is associated with differential DNA methylation of *BDNF*, *GRIN2B*, and *NR3C1*.

2. Materials and Methods

2.1. Study participants

The SCDS Nutrition Cohort 2 was designed to evaluate whether MeHg exposure from maternal fish consumption during pregnancy is associated with child neurodevelopmental outcomes and if this relationship is influenced by nutrition and genetics. Between 2008 and 2011, a total of 1522 mothers were recruited at their first antenatal visit at eight health centres across Mahé, the main island of the Seychelles (Strain et al. 2015). Inclusion criteria included being native Seychellois, being $\geq$ 16 years of age, having a singleton pregnancy, and having no obvious health concerns. At the seven-year examination 1,467 children were assessed, representing a follow-up success of over 95 percent. Biological sampling included maternal hair collected at delivery and saliva samples of the children at seven years. Blood samples were not collected from the children. For sampling of saliva, the children were not allowed to eat or drink for at least one hour before sampling to prevent food particles in the samples. Saliva samples were collected in 15-mL polystyrene tubes (Sarstedt, Nümbrecht, Germany) and stored at −80℃ then transported to Lund University, Sweden, where the DNA extractions were performed.

For this study, we identified samples from the first 450 children examined at age seven years. We excluded mother–child pairs for one of each twin pair or if a maternal hair sample was unavailable for measurement of Hg, leaving 406 eligible mother–child pairs. The study was conducted according to guidelines laid down in the Declaration of Helsinki and all study procedures involving participants were reviewed and approved by the Seychelles Ethics Board, the Research Subjects Review Board at the University of Rochester, and the Regional Ethics Committee at Lund University, Sweden.

2.2. Measurements of biomarkers of methylmercury exposure

We measured total Hg in maternal hair as a proxy for foetal exposure to MeHg; >80% of total Hg in hair is MeHg (Cernichiari et al. 1995a; National Research Council, 2000). Maternal hair was collected at delivery and the longest available segment reflecting the gestational period (assuming a growth rate of 1.1 cm/month) was identified for analysis. Total Hg content in the hair was measured as previously described (Cernichiari et al. 1995b) and reported in parts per million (ppm). A previous study reported the estimated number of fish meals consumed by cohort mothers during pregnancy (8.52 ± 4.56 fish meals per week; Strain et al. 2015); therefore, the hair mercury measure was presumed to recapitulate average steady-state exposure from habitual fish consumption during gestation.

2.3. DNA methylation

For the DNA extraction from saliva, Omega Bio-Tek E.Z.N.A. kit (Omega Bio-tek, Norcross, GA, USA) was used following the manufacturer's instructions. After extraction, the DNA was stored at −20℃ for further analyses. The methylation analysis was performed by investigators blinded to the biomarkers of Hg exposure; for this purpose, the DNA samples were coded and randomised in 96-well plates. Bisulfite treatment was performed on 112–375 ng DNA using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions and the treated DNA was stored at −20℃ until further analysis.

The analysed genes and CG dinucleotide (CpG) sites are depicted in Fig. 1. Two of the methylation assays (for *GRIN2B* and *NR3C1*) were adapted from previously published papers (Alavian-Ghavanini et al. 2018; Efstathopoulos et al. 2018). A 169-bp fragment of *GRIN2B* (containing 4 CpG sites) located in an intergenic predicted promoter region, a 162-bp fragment of *NR3C1* (5 CpG sites) located in exon 1F, and a 218-bp fragment of *BDNF* (5 CpG sites) located in intron 3, were amplified

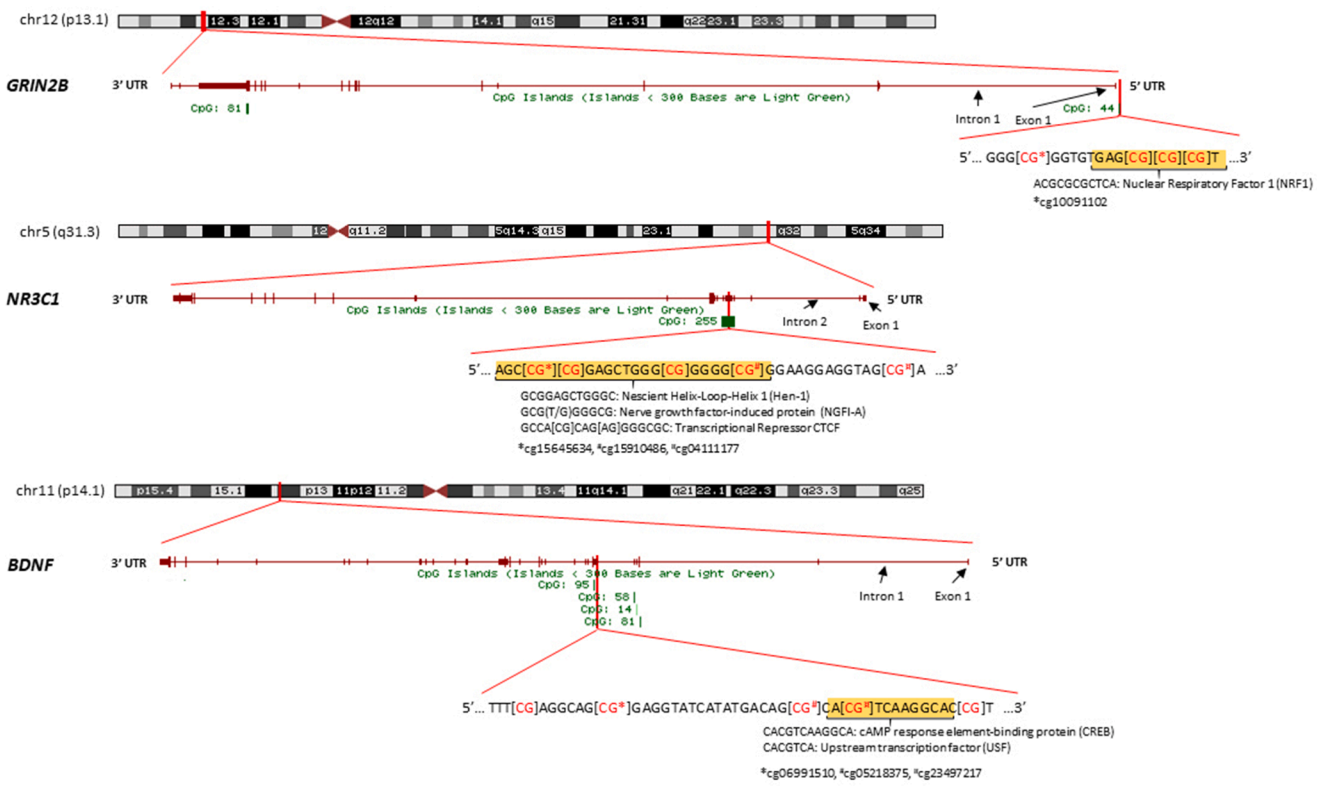

Fig. 1. The locus of each gene along with close-ups of the analysed CpG sites are shown. The CpG sites are named according to their position. Stars and hashtags indicate the ID for CpG sites on the Illumina 450 K BeadChip array. Sequences highlighted in yellow show transcription factor binding sites predicted using the ConSite web-based tool. A transcription factor binding sites for NRF1 was identified in Alavian-Ghavanini et al. (2018). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, Massachusetts) using 1 μL (*GRIN2B*) or 2 μL (*NR3C1* and *BDNF*) of bisulfite-treated DNA. The assay conditions used for PCR and pyrosequencing of the three genes are summarised in Supplementary Table 1. PyroMark Q96 ID (Qiagen, Hilden, Germany) was used for sequencing the amplified PCR products following the manufacturer's protocol, and the percentage of DNA methylation was calculated with PyroMark Q96 Software 2.5.8 (Qiagen). Samples that failed during the pyrosequencing run were removed from the analysis. Percentage of DNA methylation was transformed to M−values for each CpG site, $M_i = \log_2\left(\frac{p_i}{1-p_i}\right)$ (Du et al 2010).

2.4. Prediction of transcription factor binding sites

Transcription factor binding sites (TFBS) for *GRIN2B*, *NR3C1*, and *BDNF* were predicted using the ConSite web-based tool (Sandelin et al., 2004), which predicts TFBS by identifying significantly similar regions in alignments of homologous sequences. In addition, TFBS were identified with the University of California-Santa Cruz Genome Browser (http://genome.ucsc.edu/) using the human GRCh37/hg19 assembly released in 2009 and the TFBS conserved and uniform TFBS tracks. The TFBS conserved track predicts TFBS conserved in human, mouse, and rat, whereas the uniform TFBS track predicts TFBS based on ChIP-seq experiments performed by the ENCODE project (Landt et al. 2012).

2.5. Statistical analysis

Pearson correlations were computed between the DNA methylation levels at each CpG site and maternal hair concentrations. In primary models, linear regression analyses investigated associations between M−values of DNA methylation and prenatal MeHg exposure (maternal mercury in hair). Twelve models (unadjusted and adjusted) were used to examine associations between the methylation for each CpG in the child and Hg concentrations in maternal hair. In previous studies of non-Seychelles cohorts, we found sex-specific associations of DNA methylation with metals other than Hg (arsenic and cadmium) (Broberg et al. 2014; Kippler et al. 2013). To see if Hg also showed a sex-specific association with DNA methylation (albeit in a different population), we therefore performed exploratory analyses examining whether the Hg concentration relationships are distinct for boys and girls. To this end, additional regression models were fitted to test for an interaction between child sex and maternal hair Hg.

Covariates in the adjusted models were chosen because they have been associated with DNA methylation: maternal age at delivery, BMI at 20 months (as a surrogate for pre-pregnancy BMI), and Hollingshead Socioeconomic Status (SES), and child's sex, birth weight, and gestational age. Smoking and alcohol use during pregnancy were not included in the models due to their very low prevalence in our study population (Yeates et al. 2020). Model assumptions for the linear regression were verified through examination of the residuals for extreme values. The distribution of the residuals was checked for symmetry and each model showed symmetric distributions. All the statistical analyses were performed using R software (version 3.6.2; The R Foundation for Statistical Computing). A *p*-value of 0.05 (2-tailed) was chosen as the criterion for statistical significance in all analyses.

3. Results

3.1. Characteristics of the mothers and children

Descriptive statistics of characteristics of the mother–child cohort, including the biomarker of prenatal MeHg exposure (maternal Hg) and methylation of CpG sites in *GRIN2B*, *NR3C1*, and *BDNF* in the seven-

Table 1
Summary statistics for MeHg biomarkers and mother and child characteristics among pregnant women and their children, and DNA methylation in the children at seven years of age. (SD, standard deviation.)

Variables	N	Mean	SD	Min	Median	Max
Maternal hair Hg (ppm)	406	4.70	4.19	0.12	3.52	31.66
Maternal age (year)	406	26.56	5.92	16.27	25.65	44.84
Maternal BMI at 20 months (kg/m^2)	391	26.84	6.41	15.26	26.01	49.60
Hollingshead SES at 7 yrs	406	32.82	10.78	14	32	63
Gestational age at birth (week)	405	38.95	1.64	30	39	41
Weight at birth (kg)	406	3.16	0.52	1.10	3.19	5.20
GRIN2B CpG1 (%)	396	4.23	2.32	0	4.75	10.06
GRIN2B CpG2 (%)	396	4.76	2.03	0	5.07	9.05
GRIN2B CpG3 (%)	396	1.47	2.14	0	0	7.41
GRIN2B CpG4 (%)	396	2.27	2.50	0	0	8.40
NR3C1 CpG1 (%)	379	0.84	1.57	0	0	5.81
NR3C1 CpG2 (%)	378	0.22	1.00	0	0	10.67
NR3C1 CpG3 (%)	348	2.64	2.37	0	3.22	10
NR3C1 CpG4 (%)	348	0.54	1.72	0	0	21.43
NR3C1 CpG5 (%)	317	0.07	0.53	0	0	5.63
BDNF CpG1 (%)	390	0.47	1.09	0	0	13.76
BDNF CpG2 (%)	389	3.05	2.02	0	3.29	15.58
BDNF CpG3 (%)	382	2.54	2.21	0	3.04	15.88
BDNF CpG4 (%)	378	0.20	0.91	0	0	9.39
BDNF CpG5 (%)	377	0.64	1.27	0	0	7.65

year-old children are presented in Table 1. In our cohort, the mothers were on average 26.55 years old at childbirth and had an average pre-pregnancy BMI of 26.76. The children were 55% male and had a mean gestational age of 38.9 weeks and a mean birth weight of 3.14 kg. The mean concentration of Hg in maternal hair was 4.70 ppm, which was significantly higher than the concentration observed for other mothers in the SCDS Nutrition Cohort 2 who were not included in the present analysis (3.69 ppm, N = 979). DNA methylation in all three genes was generally low (median < 10%) in DNA from children's saliva.

3.2. Characteristics of the DNA methylation

Several transcription factor binding sites (TFBS) were identified as overlapping with the CpG sites analysed, particularly in *NR3C1* (Fig. 1 and Supplementary Table 2). In *NR3C1*, CpG sites 1–4 overlap with TFBS for nescient helix-loop-helix 1 (Hen-1), the transcriptional repressor CTCF, and nerve growth factor-inducible protein A (NGFI-A). The *BDNF* CpG4 (cg23497217) overlaps with TFBS for cAMP response element-binding protein (CREB) and upstream transcription factor (USF).

The correlations between the individual CpG sites within each respective gene were at most 0.34 (*BDNF* CpG2 and CpG3; Pearson correlation coefficient), and were 0.27 between CpG sites in different genes (*NR3C1* CpG3 and *BDNF* CpG5).

3.3. Associations between prenatal Hg exposure and DNA methylation at seven years

Significant correlations between individual CpGs and maternal hair Hg were found for *NR3C1* CpG3 (r = 0.11, p = 0.048) and CpG5 (r = 0.16, p = 0.005) (Supplementary Table 3).

Of all the CpGs, *GRIN2B* CpG4 and *NR3C1* CpG3 and CpG5 showed significantly higher levels of DNA methylation with higher maternal hair Hg during pregnancy (Table 2). For example, *NR3C1* CpG3 had an expected increase in methylation of 0.03-fold for each additional 1 ppm of maternal hair Hg.

The interaction analysis showed that for *NR3C1* CpG5, there was a significant sex interaction for prenatal hair Hg (p = 0.027). Higher hair Hg concentrations were significantly associated with higher DNA methylation in boys (beta = 0.019, p = 0.001), but not in girls. For *BDNF* CpG5, a significant sex interaction was found for prenatal hair Hg (p = 0.009), where higher hair Hg was significantly associated with higher methylation in boys (beta = 0.034, p = 0.012) but not girls.

4. Discussion

Our study of 406 seven-year-old children in the SCDS Nutrition Cohort 2 showed that prenatal exposure to MeHg was associated with altered methylation of nervous system-related genes, in particular the glucocorticoid receptor *NR3C1*. As we discuss below, the methylation changes observed are predicted to result in lower gene expression, which in turn has been associated with adverse neurodevelopmental outcomes. Still, it is unclear to what extent the associations that we found represent toxic or adaptive responses following exposure to methylmercury, and if these epigenetic changes are predictive of neurodevelopment.

The NR3C1 receptor is a crucial factor in stress responses in the brain via its regulation of the hypothalamic–pituitaryadrenal axis (Kim & Iremonger 2019). We found that methylation at two out of the five CpG sites in *NR3C1* showed positive associations with prenatal Hg exposure. This result is in line with studies showing that increased *NR3C1* methylation in the placenta is associated with higher MeHg exposures (measured in toenails in 222 samples; Appleton et al. 2017). However, in the Appleton study, the authors did not differentiate between the individual CpG sites but used the average methylation for the entire gene. In

Table 2
Associations between *GRIN2B*, *NRC3C1*, and *BDNF* methylation (M−values) and Hg in maternal hair in unadjusted and adjusted linear regression models. Adjusted models are adjusted for maternal age and BMI, child sex, birth weight, gestational age, and family SES. Statistically significant associations (p < 0.05) are marked in bold.

Gene/CpG	Crude Models		Adjusted Models	
GRIN2B	N	Beta (SE), p value	N	Beta (SE), p value
CpG1	396	0.012 (0.013) p = 0.374	381	0.015 (0.013) p = 0.268
CpG2	396	0.013 (0.011) p = 0.259	381	0.014 (0.012) p = 0.216
CpG3	396	−0.005 (0.014) p = 0.738	381	−0.001 (0.015) p = 0.963
CpG4	396	0.029 (0.016) p = 0.061	381	**0.034 (0.016) p = 0.041**
NR3C1				
CpG1	379	−0.006 (0.11) p = 0.620	367	−0.012 (0.012) p = 0.321
CpG2	378	0.011 (0.006) p = 0.098	366	0.009 (0.007) p = 0.170
CpG3	348	**0.030 (0.015) p = 0.047**	336	**0.032 (0.016) p = 0.046**
CpG4	348	0.004 (0.010) p = 0.707	337	0.000 (0.010) p = 0.965
CpG5	317	**0.012 (0.004) p = 0.005**	307	**0.011 (0.005) p = 0.018**
BDNF				
CpG1	390	0.006 (0.008) p = 0.448	375	0.002 (0.008) p = 0.771
CpG2	389	0.014 (0.012) p = 0.232	375	0.014 (0.012) p = 0.236
CpG3	382	−0.009 (0.013) p = 0.521	367	−0.006 (0.014) p = 0.649
CpG4	378	−0.006 (0.006) p = 0.344	363	−0.007 (0.006) p = 0.245
CpG5	377	0.010 (0.010) p = 0.321	363	0.007 (0.010) p = 0.471

our study, CpG3 and CpG5 became more methylated with increasing MeHg exposure. CpG3 is located in a TFBS for Hen-1 and together with CpG4 is part of the binding site for the transcription factor NGFI-A. Weaver and co-workers (2007) elegantly showed *in vitro* that NGFI-A participates in epigenetic programming of glucocorticoid expression. They showed that increased methylation of CpG3 resulted in inhibition of NGF1-A binding and in turn lower *NR3C1* expression, whereas methylation of CpG4, which is at the end of the NGFI-A TFBS, did not have this effect on the interaction between NGFI-A and *NR3C1*. Lower levels of the glucocorticoid receptor would result in lower responsiveness to cortisol and other glucocorticoids, whose functions include decreasing inflammation and regulating stress responses (Rhen and Cidlowski 2005; Binder 2009). Therefore, changes in methylation that cause lower expression of *NR3C1* could result in dysregulation of the stress response.

GRIN2B encodes the NR2B subunit of N-methyl-D-aspartate receptors (NMDARs), which are receptors for the excitatory neurotransmitter glutamate and important for regulation of neural morphology, learning, and memory (Cull-Candy et al. 2001). For *GRIN2B*, we found higher DNA methylation at CpG4 with higher prenatal MeHg exposure. *GRIN2B* has, to our knowledge, not been studied in relation to MeHg before, but higher methylation in this region has been associated with prenatal bisphenol A exposure, albeit at CpG1, suggesting that this region is sensitive to prenatal chemical stressors (Alavian-Ghavanini et al. 2018). We have previously shown that in the rat hippocampus, higher DNA methylation in the homologous region correlates with lower gene expression (Alavian-Ghavanini et al. 2018). Furthermore, CpG4 is part of a conserved predicted binding site for nuclear respiratory factor 1 (Nrf1, (Alavian-Ghavanini et al. 2018), which regulates *Grin2b* expression (Priya et al. 2013) and is sensitive to DNA methylation (Choi et al. 2004; Domcke et al. 2015; Wang et al. 2017). Taken together, these observations suggest that higher methylation at CpG4 could lead to decreased *GRIN2B* expression. Little is known about the relations between hypermethylation of *GRIN2B* and neurodevelopmental outcomes, but genetic polymorphisms leading to decreased *GRIN2B* expression have consistently been found to be associated with neurodevelopmental diseases and disorders, such as attention deficit hyperactivity disorder, autism spectrum disorder, and schizophrenia (Dorval et al. 2007; Martucci et al. 2006; Guo et al. 2016).

Our findings also suggest that there are sex differences: for two CpG sites, boys showed higher DNA methylation with higher prenatal Hg concentrations whereas no associations were found in girls. There was a significant sex interaction for prenatal Hg and CpG5 in *NR3C1* as well as CpG5 in *BDNF*. Only *NR3C1* CpG5 was also associated with MeHg in the linear regression models. *BDNF* codes for neurotropin, which has a fundamental role in neural development, nerve cell survival, and synaptic plasticity (Pruunsild et al. 2007). Altered methylation of *BDNF* has been associated with developmental exposure to MeHg: decreased *BDNF* expression was associated with repressive epigenetic marks, including DNA hypermethylation, in mice exposed to MeHg during development (Onishchenko et al. 2008). Nevertheless, these interaction results should be interpreted cautiously, since this study had low power to detect interactions and the DNA methylation levels were low. Moreover, we have not found consistent evidence in the SCDS that boys or girls are more susceptible to Hg (Strain et al. 2015; Strain et al., accepted). Nevertheless, the observed sex differences are of interest because we and others have reported sex-specific epigenetic alterations associated with exposure to chemicals and metals (Broberg et al. 2014; Kippler et al. 2013; Kundakovic et al. 2015; Vilahur et al. 2015).

A few other epidemiological studies have investigated the epigenetic effects of MeHg on genes that are not directly involved in neurodevelopment. In an epigenome-wide association study of 141 children, Bakulski and co-workers (2015) found an association between low-level exposure to total Hg (median 1.4 μg/L) in cord blood and differential methylation of a region in Transcription Elongation Factor A (SII) N-Terminal and Central Domain Containing 2 (*TCEANC2*), a gene with unknown function, and they validated these results in an independent sample. Cardenas and co-workers (2017a) conducted a mother–child study in the USA, and reported that low-level prenatal MeHg exposure (maternal erythrocyte Hg = 3.8 ng/g) was associated with reduced methylation of a differentially methylated region in Paraoxonase 1 (*PON1*), a gene involved in Phase I biotransformation and fatty acid metabolism, in cord blood of boys. The reduced methylation persisted through childhood and was associated with one of the two neurodevelopmental outcomes studied. Thus, these studies, along with the results of the present study, suggest that DNA methylation may act as a long-term mediator of MeHg-associated effects.

The group of mother–child pairs selected for this study had slightly higher prenatal exposure to MeHg than the other mother–child pairs in the SDCS Nutrition Cohort 2. However, we do not consider this exposure to have resulted in any bias because we selected the first 450 children examined without any knowledge of their MeHg exposure levels. SDCS participants, however, have relatively high exposures to MeHg compared to other populations (e.g., in the USA and EU), and further studies are necessary to evaluate whether the associations with altered methylation that we observed are maintained at lower exposures.

One of the strengths of the study is that it is based on a large well-characterised cohort with exposure levels to MeHg several times higher than in Europe and in the US. Furthermore, we focused on DNA methylation in genes that have crucial roles in neurodevelopment and function. One limitation of our study is that we did not consider the effects of concurrent MeHg exposure or diet, which may also influence DNA methylation. The concurrent and post-natal environment, including the children's diets, might be expected to affect our measures, but if the prenatal effect is strong enough, it should override any post-natal effects, allowing the prenatal associations to be evident in children at seven years of age. Furthermore, we measured DNA methylation in saliva and not in the brain. Previous work has shown that the average level of DNA methylation correlates well between saliva and the brain ($r = 0.90$); however, for each CpG site and each gene, the correlation between brain and peripheral tissue methylation can vary widely (Braun et al., 2019). Using the website IMAGE-CpG, we interrogated the DNA methylation levels in the brain for the CpG sites used in this study (not all were present in the database) and found that they were low and in the same range as for saliva. Analysis of the brain–saliva correlation was not conclusive, however, probably due to the low number of individuals who were the basis for the comparisons ($N = 21$ or $N = 12$, depending on the analysis platform) and this issue needs further research. It should be noted that the effect estimates of the associations in this study were small and suggests the need for follow-up studies to examine whether the epigenetic associations found here are linked to cognitive and mental health later in life.

5. Conclusions

Epigenetic changes have potential for use as early and sensitive markers of exposure that can possibly predict later-life health outcomes (Greally and Jacobs 2013; Meehan et al. 2018; Marczylo et al. 2016). Future analysis is warranted to determine whether MeHg-related epigenetic changes in genes maintaining and regulating the nervous system are valid markers of effects on neurodevelopment and neurotoxicity. Specifically, we need to know whether these markers are valid in populations with a lower exposure to MeHg and whether the epigenetic alterations are linked to changes in the neurodevelopment of the exposed children.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We gratefully acknowledge the participation of all Seychellois citizens who took part in the study, the staff from the Child Development Centre, Seychelles for their assistance with data collection, and Dr. Karin Wahlberg for input to the manuscript.

Funding

This study was supported by the US National Institutes of Health (grants R01-ES010219 and P30-ES01247), Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), in kind support from the Government of Seychelles, Institute of Environmental Medicine, and Karolinska Institutet. The study sponsors had no role in the design, collection, analysis, or interpretation of data, in the writing of this article, or in the decision to submit the article for publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106321.

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