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DNA methylation profiles at birth and child ADHD symptoms

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ABSTRACT

Attention deficit/hyperactivity disorder (ADHD) is a common and highly heritable psychiatric disorder. In addition, early life environmental factors contribute to the occurrence of ADHD. Recently, DNA methylation has emerged as a mechanism potentially mediating genetic and environmental effects.

Here, we investigated whether newborn DNA methylation patterns of selected candidate genes involved in psychiatric disorders or fetal growth are associated with ADHD symptoms in childhood. Participants were 426 children from a large population based cohort of Dutch national origin. Behavioral data were obtained at age 6 years with the Child Behavior Checklist. For the current study, 11 regions at 7 different genes were selected. DNA methylation levels of cord blood DNA were measured for the 11 regions combined and for each region separately. We examined the association between DNA methylation levels at different regions and ADHD symptoms with linear mixed models.

DNA methylation levels were negatively associated with ADHD symptom score in the overall analysis of all 11 regions. This association was largely explained by associations of DRD4 and 5-HTT regions. Other candidate genes showed no association between DNA methylation levels and ADHD symptom score. Associations between DNA methylation levels and ADHD symptom score were attenuated by co-occurring Oppositional defiant disorder and total symptoms.

Lower DNA methylation levels of the 7 genes assessed at birth, were associated with more ADHD symptoms of the child at 6 years of age. Further studies are needed to confirm our results and to investigate the possible underlying mechanism.

1. Introduction

Attention deficit/hyperactivity disorder (ADHD) is a neuro-developmental disorder characterised by delayed brain maturation (Shaw et al., 2007 Dec 4). However, the exact underlying mechanism of ADHD is poorly understood. Twin studies have provided evidence that ADHD has a genetic basis and heritability is estimated to be around 0.76, ranging from around 60 to over 0.95 (Faraone et al., 2005 Jun 1). Despite the high heritability, few definite risk genes have been identified (Faraone and Mick, 2010 Mar). The numerous candidate gene studies and several genome wide association scans have largely been without consistent findings (Zhou et al., 2008 Dec 5; Romanos et al., 2008 May). The complex phenotype, the very polygenic genotype or gene–environment

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interactions may explain this inconsistency between behavioral and molecular genetic studies (Maher and 2008 Nov 6).

Recent studies have demonstrated a possible role of DNA methylation in schizophrenia, depression and suicidal behavior (Petronis, 2004; Mill et al., 2008; Autry and Monteggia, 2009). Epigenetics may represent a mechanism explaining the occurrence of disease, in particular as a postulated mechanism linking environmental risk factors to biological mechanisms (Plazas-Mayorca and Vrana, 2011 Jan 7). Early onset, heritable neurodevelopmental disorders are candidates for epigenetic research as neurons are generated early during development. Epidemiological studies have associated prenatal exposure to adverse environmental factors like smoking (Langley et al., 2005 Dec), toxins (Braun et al., 2011 Nov) or maternal stress (O'Connor et al., 2002 Jun) with the risk of ADHD. These observed environmental risk factors exert an effect mainly during the prenatal or perinatal period, suggesting that timing of exposure is important for the susceptibility of the developing brain. Low birth weight, preceded by intrauterine growth restriction, can be seen as an indicator of a poor prenatal environment. Low birth weight also elevates the risk of adverse behavioral outcomes and is a known risk factor for ADHD (Heinonen et al., 2010).

Against this background, we investigated whether methylation patterns of neuronal genes observable at birth are associated with ADHD symptoms at age 6 years. As ADHD is observed more often in children born small-for-gestational age, we examined whether prenatal factors influence the occurrence of ADHD by alterations in DNA methylation.

DNA methylation patterns were assessed in cord blood to examine the association with ADHD prospectively. Although confounding cannot be excluded this design reduces the chance of reverse causation as it dissects whether differences in DNA methylation are a cause or a consequence of the disorder. However, unmeasured prenatal confounding factors cannot be ruled out.

Because of inaccessibility of brain tissue in living subjects, in the current study we examined DNA derived from leukocytes. It is hypothesized that environmental factors impacting the epigenetic marks in the fetal brain also induce peripheral epigenetic alterations (Talens et al., 2010 Sep). Based on the literature we selected a few genes, of which variations are assumed to affect nervous system development and metabolism (glucocorticoid receptor (NR3C1), methylenetetrahydrofolate-reductase (MTHFR)), presynaptic genes (Dopamine Receptor D4 (DRD4) and serotonin transporter protein (5-HTT)) or fetal growth (insulin-like growth factor 2 (IGF2DMR), H19, potassium channel protein (KCNQ1OT1)).

2. Methods and materials

2.1. Design and study population

This study was embedded in the Generation R Study, an ongoing population-based birth cohort from fetal life onwards. The Generation R Study, designed to identify early environmental and genetic determinants of growth, development and health, has been previously described in detail (Jaddoe et al., 2012). Assessments with physical examinations, biological samples and detailed questionnaires were performed.

Participants comprised a subset ($n = 540$) of the original Generation R cohort. To reduce the effect of population stratification, the current study was restricted to children of Dutch national origin, which was based on the country of birth of the parents and grandparents. Of the selected children, sufficient quantity and quality of DNA derived from cord blood was available. 448 children were randomly drawn from the cohort. The sampling strategy was to use stratification with oversampling of children diagnosed with ADHD ($n = 92$). This improved power of the analyses and achieve a more normal distribution of ADHD symptoms, which are highly right skewed in the general population as most individuals have relatively few ADHD symptoms and only a minority have high ADHD symptoms (Supplementary Figs. 1 and 2).

In addition, 100 children with the smallest birth weight for gestational age (<-1.75 SD) were selected to investigate the role of prenatal factors in the etiology of ADHD. However, we also ran the analyses without this sample. In 426 of all selected children, parents provided data on ADHD symptoms by filling out the Child Behavior Checklist.

The study has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam.

2.2. Measurements of DNA methylation and genotyping

It is widely recognized that presynaptic genes are the most important components in the etiology of ADHD (Gizer et al., 2009 Jul). Based on literature, genes were selected on the basis of their potential involvement in neurotransmitter systems and neurodevelopment (Supplemental Table 1). The number of DNA methylation regions ($n = 12$) assessed was limited by the total amount of DNA (500 ng) used. Hence, we chose to determine the CpG islands of the genes, and selected the middle region or outer regions per island or the region previously investigated by other groups: Two regions of the 5-HTT gene were selected from a publication by Philibert where the relationship with depression and alcohol dependency has been investigated (Philibert et al., 2008 Jul 5).

Previously, Wong et al. described epigenetic differences in twins in the region of the DRD4 gene and one of the regions in the 5-HTT gene (Wong et al., 2010 Aug 16).

We additionally selected regions of the IGF2DMR and H19 gene that are implicated in fetal growth. Talens et al. reported about the variation and stability of DNA methylation of the IGF2DMR and H19 region (Talens et al., 2010 Sep).

Epigenetic regulation of the NR3C1 has been associated with childhood abuse (McGowan et al., 2009 Mar). Primers of the other regions at the KCNQ1OT1, MTHFR and NR3C1 genes were designed using the online tool of EpiDesigner (epidesigner.com).

For convenience, we described the three regions at 5HTT and NR3C1 genes as region A, B or C (Supplemental Table 1).

The assessment of the selected region near the gene CCNL1/LEKR1, implicated in fetal growth, did not succeed due to technical issues.

Isolated genomic DNA (500 ng) from cord blood samples was treated with sodium bisulphite for 16 h using the EZ-96 DNA methylation kit (Shallow) (Zymo Research, Irvine, CA, USA). This was followed by PCR amplification, fragmentation after reverse transcription and analysis on a mass spectrometer (Sequenom, Inc, San Diego, USA). This generated mass spectra that were translated into quantitative DNA methylation levels of different CpG sites by MassARRAY EpiTYPER Analyzer software (v1.0, build 1.0.6.88 Sequenom, Inc, San Diego, USA).

Samples were randomly divided over bisulphite conversion and PCR amplification batches.

For each individual, the assays were amplified from the same bisulphite-treated DNA. All methylation measurements were done in triplicate from the same bisulphite-treated DNA.

Genotyping was performed for DRD4-48 base pair variable number tandem repeat (DRD4-48 bp VNTR) and a 44 bp insertion/deletion segment of the serotonin transporter gene 5-HTT (5-HTTLPR) as described previously (Luijk et al., 2011 Dec).

Frequency distributions conformed to the Hardy–Weinberg equilibrium.

2.3. Assessment of behavioral problems

We measured behavioral problems of the children at six years of age by using the Child Behavior Checklist (CBCL/1,5-5). The CBCL is a parent report questionnaire that contains 99 problem items rated on a 3-point scale: 0 (not true), 1 (somewhat or sometimes true) and 2 (very true or often true). The 6-item DSM-oriented scale for ADHD was selected for our analyses since its items were chosen to closely map onto DSM-IV criteria for ADHD. The items included "Can't sit still, restless, or hyperactive," "Can't concentrate, can't pay attention for long" and "Quickly shifts from one activity to another". By summing scores of these selected items, a DSM-Oriented Scale of Attention-Deficit/Hyperactivity can be computed that has been used for continuous analyses. Higher scores represent higher severity. Good reliability and validity have been reported for the CBCL (Achenbach and Rescorla, 2000). The CBCL in general have shown to be a robust predictor of clinically-diagnosed ADHD (Hudziak et al., 2004 Oct).

For sample selection and dichotomous analyses the Diagnostic Interview Schedule for Children–Parent version (DISC-P) was used. The DISC-P is a structured interview with the parents, assessed during home visits, that define diagnoses according to the criteria specified by the Diagnostic and Statistical Manual of Mental Disorders (4th ed. DSM-IV, APA, 1994). The reliability and validity of the DISC-P have been reported to be acceptable (Shaffer et al., 2000 Jan).

The DISC was only assessed in a selected risk and control group of the Generation R study group. For this reason, data from DISC was available in 116 children (mainly cases) of the current study population. To maximize the power for analyses we used CBCL scores as they were present in 426 children. Median CBCL ADHD symptom score in children that fulfilled criteria of ADHD according to DISC-P was 8.0 (95% CI–7.2; 8.4), and 2.61 (95% CI–2.5; 3.0) in children that did not fulfill these criteria (Supplemental Fig. 3).

2.4. Covariates

Possible determinants of DNA methylation and ADHD were derived from the literature (Steegers-Theunissen et al., 2009; McGuinness et al., 2012 Feb; Adkins et al., 2011 Aug; Schroeder et al, 2011 Dec).

Information on maternal age, education, parity, prenatal smoking, alcohol use, folic acid supplement use and child national origin was obtained by questionnaires during pregnancy. Educational level of the mother was assessed by the highest completed education and reclassified into three categories: no or primary, secundary or higher education. Maternal prenatal smoking and alcohol use were classified as 'no use', 'use until pregnancy was confirmed' and 'continued use during pregnancy'. Height and weight were measured without shoes and heavy clothing; body mass index was calculated from weight and height (kg/m^2). At 20 weeks pregnancy, we measured maternal psychological problems using the Brief Symptom Inventory (De Beurs, 2004). Child gender, birth weight, Apgar score one minute after birth, and the mode of delivery were derived from medical records completed by midwives and gynecologists. To define gestational age at birth, we used fetal biometry measured at the first prenatal visit. In addition of selection of children of Dutch national origin, child genetic ancestry was determined by principle component analyses of genome wide association data, as described previously (Jaddoe et al., 2010 Nov).

2.4.1. Statistical analysis

First, we explored whether individual genetic variations may underlie variation in DNA methylation levels. The 5-HTT and DRD4 gene are characterized by a variable nucleotide repeat that have been linked to ADHD in childhood (Gizer et al., 2009 Jul; Faraone et al., 2001 Jul). We examined the association between genetic variants DRD4-48 bp VNTR and 5-HTTLPR and DNA methylation levels in the measured regions in the DRD4 and 5-HTT gene. In the current population, we tested whether these variations are associated with ADHD symptoms at age 6 years using linear regression models.

Primarily, we investigated the association between DNA methylation and ADHD symptom score. To further explore our results we repeated the analyses using a dichotomous outcome defined by a cut-off of 2 on the ADHD symptom scale of the CBCL. We also examined the association between DNA methylation levels and ADHD diagnosis based on DISC-P interview.

In children with ADHD the risk for comorbid psychiatric disorders is high. We tested specificity for the association between DNA methylation on ADHD symptoms by adding symptom scores of the other CBCL syndrome scales and total symptom scores to the model.

Additionally, to test whether results depended on the sampling strategy with an oversampling of children born small-for-gestational age, all analyses were repeated excluding this sample.

DNA methylation levels were treated as continuous variables. To approximate normal distribution, variables of DNA methylation levels were transformed by the square-root. For the analyses, the triplicate measurements and DNA methylation levels of the separate CpG units within a region were treated as clustered variables and not as a mean.

For an initial overall analysis of methylation and ADHD symptom score, DNA methylation levels of all CpG units in the 11 regions together were combined in one analysis. We present the complete data from all regions selected and chose a hierarchical approach to reduce the risk of type I error rather than to report selectively. Next to specific effects of DNA methylation, general effects on the level of DNA methylation have been described. Besides, the specific regions are correlated; a combined analysis increases the power and helps detect small effects of DNA methylation. Consequently, we have to be very careful to interpret specific effects on DNA methylation as the focus was on the overall effect.

As most CpG units within one region are correlated (except for 5HTT region A, MTHFR and NR3C1, all CpGs within regions assessed show correlations varying from 0.23 to 0.90 ($P < 0.001$)), the associations with DNA methylation level were calculated using Linear Mixed Models. Mixed models have the advantage to allow correlated random effects in individuals. Another advantage of this model is the ability to accommodate missing data points. Furthermore, using mixed models enables adjustment for relevant covariates on the raw data in the same model (Burton et al., 1998 Jun 15).

We also stratified the analysis on DNA methylation levels of DRD4 and 5-HTT regions by gender, and tested interactions between gender and ADHD symptoms.

To understand the effect of environmental factors on DNA methylation two models have been used. First, we only adjusted for bisulphite and PCR batch, child gender, genetic ancestry (principal components), gestational age at birth and age at assessment of CBCL. In the fully adjusted model, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use, child birth weight and Apgar score were added. This approach allowed us to evaluate the effect of exposures during pregnancy as possible explanatory factors behind any observational effect of methylation on ADHD symptoms (Supplemental Fig. 4). Analyses of the association of DNA

Table 1
Child and maternal characteristics in the study population.

	N=426
Child characteristics	
Gender, % boys	59.6
Birth order, % first born	68.9
Birth weight, g (sd)	3430 (580)
Gestational age, wk (sd)	40.2 (1.5)
Apgar score 1 min after birth (sd)	8.6 (1.3)
Mode of delivery, %	
Spontaneous vaginal	73.0
Instrumental vaginal	19.6
Caesarean section	7.4
Maternal characteristics	
Age (sd)	30.1 (4.7)
Body Mass Index, kg/m^2 (sd)	24.3 (4.0)
Educational level, %	
Primary	13.5
Secondary	55.3
High	31.1
Psychological symptoms, score, median (95% range)	0.15 (0.00–1.08)
Smoking during pregnancy, %	
Never	69.3
Until pregnancy was confirmed	8.8
Continued	21.9

Values represent means (SD) unless otherwise indicated.

methylation of any 5HTT or DRD4 regions with ADHD outcomes were adjusted for the respective 5HTT or DRD4 genotypes.

All analyses were performed using SPSS software, version 20.0 (IBM-SPSS, Chicago, IL, USA).

3. Results

Characteristics of the children and their mothers in the study population are presented in Table 1. The children were born after on average 40.2 (SD = 1.5) weeks of pregnancy. Mean birth weight was 3430 (SD = 580) grams. Mothers had a mean age of 30.1 (SD 4.7) years, 31.1% of the mothers was higher educated and 30.7% smoked during pregnancy.

Table 2 showed the association between adjacent DRD4-48 bp VNTR and 5-HTTLPR and DNA methylation levels. Children with one or two copies of the 7-repeat at the DRD4-48 bp VNTR have significantly lower methylation levels than children without a 7-repeat. Likewise, having a short allele of 5-HTTLPR was also associated with lower DNA methylation levels of the measured 5-HTT regions. In the current population the presence of a short allele of 5-HTTLPR was associated with an increase of ADHD symptoms (per copy of short allele: β-0.07 95% CI−0.09; −0.05, $P < 0.001$). DRD4-48 bp VNTR showed no association with ADHD symptom score (per copy of 7-repeat: β-0.01 95% CI−0.01; −0.04, $P = 0.30$).

Table 2
Associations between haploid genotype in candidate genes and DNA methylation.

	Crude analyses		Adjusted analyses	
	Beta (95%CI)	*P*-value	Beta (95%CI)	*P*-value
DRD4, number of 7-repeats (n = 500)	DRD4 region, % methylation			
0 (*n* = 326)	*ref*	*ref*	*ref*	*ref*
1 (*n* = 158)	−3.40 (−4.20;-2.60)	<0.001	−3.40 (−4.20;-2.60)	<0.001
2 (*n* = 16)	−7.10 (−9.20;-4.90)	<0.001	−6.70 (−8.80;-4.60)	<0.001
5-HTT, alleles-type (n = 512)	Combined 5-HTT regions, % methylation			
LL (*n* = 171)	*ref*	*ref*	*ref*	*ref*
SL (*n* = 239)	−0.23 (−0.32; 0.08)	0.25	−0.11 (−0.32; 0.09)	0.27
SS (*n* = 102)	−0.33 (−0.58;-0.08)	0.01	−0.33 (−0.58;-0.07)	0.01

Beta, 95% confidence intervals and *P*-values. Analyses are adjusted for bisulphite and PCR batch, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use and child gender, genetic ancestry (principle components), birth weight, Apgar score, gestational age at birth and age at assessment of CBCL.

Next, we examined our primary hypothesis of an association between DNA methylation and ADHD symptom score measured at age 6 years (Table 3a). The table presents the models fully adjusted for bisulphite and PCR batch, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use, child gender, genetic ancestry (principle components), birth weight, Apgar score, gestational age at birth and age at assessment of CBCL. We found a negative relation between mean DNA methylation in the overall analysis of all regions measured. Children with higher methylation levels had less ADHD symptoms. This effect did not change meaningfully after correction for DRD4-48 bp VNTR and 5-HTTLPR indicating an independent effect of DNA methylation level ADHD symptom score (β-0.13, 95% CI−0.22; −0.04, $P = 0.01$).

In order to identify specific regions that contribute to the association between DNA methylation and ADHD symptoms in the overall analyses we subsequently explored specific regions. DNA methylation of specific regions that were negatively associated with ADHD symptom score were 5-HTT region B and DRD4 region. This direction is in line with the association between the ADHD risk variants and lower DNA methylation levels. Associations with KCNQ1OT1 (β-0.34 95% CI−0.63;−0.05, $P = 0.02$) were strongly attenuated and became non-significant after adjustment for maternal age, parity and educational level (Table 3a). To better understand the association between DNA methylation and ADHD symptoms, we repeated the analyses using a dichotomous variable of ADHD symptom score with a cut-off at 2 of the CBCL. The effects were consistent and remained significant after adjustment for potential confounders (Table 3b). We additionally tested whether any interaction between child 5HTTLPR genotype and DNA methylation predicted ADHD symptoms. There was no interaction effect between the 5HTTLPR variation and the respective DNA methylation levels (Methylation 5HTT regions × 5HTTLPR: β −8.87 95% CI−21.97; 4.64, $P = 0.20$) nor between the DRD4 VNTR 7 repeat and the respective DNA methylation levels on the level of ADHD problems (Methylation DRD4 region × DRD4 VNTR 7 repeat: β −2.03 95% CI−5.20; 1.14, $P = 0.21$).

To test whether the results depend on the comorbid psychiatric symptoms, symptom scores of other CBCL syndrome scales and total symptoms were added to the model. Associations between DNA methylation levels and ADHD symptom score were attenuated by oppositional defiant disorder (ODD) and total symptoms (overall regions: β-0.10 95% CI−0.22; 0.03, $P = 0.12$ and β-0.08 95% CI−0.21; 0.06, $P = 0.28$ respectively). In contrast, the association between child DNA methylation level and ADHD symptoms could not be explained by internalizing, affective disorder, anxiety or pervasive developmental disorder (PDD) problem scale (results not shown).

Analyses of the associations between mean DNA methylation in cord blood and child ADHD diagnosis based on DISC-P showed no associations (overall analyses β0.03 95% CI−0.17; 0.24, $P = 0.77$, other results not shown).

Next, we examined whether alterations in DNA methylation associated with ADHD symptoms, were influenced by prenatal environmental factors. To this aim, we also studied the associations without adjustment for indicators of maternal risks during pregnancy, including maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use, child birth weight and Apgar score. Results without adjustment for

Table 3
(a). DNA methylation and ADHD symptom score. (b). DNA methylation and ADHD dichotomous symptom score.

(a) $N = 426$	*Overall methylation*							
ADHD, symptom score (sqrt transformed)	All genes jointly analyzed, methylation %							
	β(95% CI)	*P*-value						
	−0.12 (−0.21;-0.02)	0.02						
Neurotransmitter systems								
ADHD, symptom score (sqrt transformed)	DRD4, methylation %		5-HTT region A, methylation %		5-HTT region B, methylation %		5-HTT region C, methylation %	
	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value
	−0.52 (−1.00;-0.05)	0.03	−0.00 (−0.15; 0.15)	1.00	−0.22 (−0.38;-0.06)	0.006	−0.09 (−0.26; 0.08)	0.30
Metabolism								
ADHD, symptom score (sqrt transformed)	NR3C1 region A, methylation %		NR3C1 region B, methylation %		NR3C1 region C, methylation %		MTHFR5, methylation %	
	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value
	−0.03 (−0.13; 0.06)	0.45	−0.08 (−0.20; 0.40)	0.18	0.03 (−0.08; 0.14)	0.56	0.05 (−0.04; 0.13)	0.28
Fetal growth								
ADHD, symptom score (sqrt transformed)	H19, methylation %		IGF2DMR, methylation %		KCNQ1OT1, methylation %			
	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value		
	−0.07 (−0.33; 0.20)	0.62	0.05 (−0.30; 0.41)	0.77	−0.29 (−0.61; 0.02)	0.07		

(b) $N = 426$	*Overall methylation*							
ADHD, symptom score (>2) dichotomous	All genes jointly analyzed, methylation %							
	β(95% CI)	*P*-value						
	−0.25 (−0.43;-0.06)	0.009						
Neurotransmitter systems								
ADHD, symptom score (>2) dichotomous	DRD4, methylation %		5-HTT region A, methylation %		5-HTT region B, methylation %		5-HTT region C, methylation %	
	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value
	−1.04 (−2.03;-0.48)	0.04	−0.07 (−0.37; 0.22)	0.63	−0.35 (−0.66;-0.03)	0.03	−0.07 (−0.40; 0.26)	0.68
Metabolism								
ADHD, symptom score (>2) dichotomous	NR3C1 region A, methylation %		NR3C1 region B, methylation %		NR3C1 region C, methylation %		MTHFR5, methylation %	
	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value
	−0.11 (−0.29; 0.07)	0.22	−0.10 (−0.34; 0.15)	0.44	0.03 (−0.19; 0.25)	0.78	0.07 (−0.10; 0.24)	0.44
Fetal growth								
ADHD, symptom score (>2) dichotomous	H19, methylation %		IGF2DMR, methylation %		KCNQ1OT1, methylation %			
	β(95% CI)	*P*-value	β(95% CI)	*P*-value	β(95% CI)	*P*-value		
	−0.20 (−0.73; 0.33)	0.46	0.15 (−0.55; 0.85)	0.68	−0.50 (−1.11; 0.12)	0.11		

The beta represent the change in methylation % per squareroot (sqrt) transformed ADHD symptom score as analysed with linear mixed models.
Analyses are adjusted for bisulphite and PCR batch, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use and child gender, genetic ancestry (principle components), birth weight, Apgar score, gestational age at birth and age at assessment of CBCL. Analyses of DRD4 and 5-HTT regions are also adjusted for variable number tandem repeats.

indicators of maternal exposure were very similar, none of these covariates change the effect estimates substantially. This indicates that adverse prenatal environmental factors do not explain the association between child DNA methylation and ADHD symptoms. The only covariates related to both DNA methylation and ADHD symptoms were genetic ancestry (principle components) and gender.

When analyses were stratified by gender, the association between DNA methylation of all regions measured and ADHD symptom score was not meaningful different. There was no interaction effect of gender (*P*-value for interaction 0.56).

When analyses were repeated with exclusion of children born small-for-gestational age, the associations between DNA methylation level and ADHD symptoms were very similar, however, some associations were not significant as the group size was smaller (Supplemental Table 2).

4. Discussion

In this study we investigated the association of DNA methylation levels of neuronal and non-neuronal candidate genes with ADHD symptoms in childhood.

In an overall analysis across 11 regions, lower DNA methylation levels were associated with higher ADHD symptom scores. The results were independent of two selected DNA variants. However, we cannot rule out that other genetic variations in the vicinity of this region could directly influence DNA methylation measurements. The effect was largely explained by associations with DNA methylation levels in DRD4 and 5-HTT regions. However, this result should be interpreted with caution since our study had insufficient power to stringently correct for multiple testing in individual regions analyses.

DNA methylation was measured in cord blood collected directly after birth before any postnatal environmental influences exerted an effect. This created a unique opportunity to observe DNA methylation at baseline and investigate associations with ADHD in a prospective manner. However, since DNA methylation may be influenced by a variety of postnatal factors such as social environment, environmental toxins and drugs, this does not rule out environmental effects (Weaver et al., 2004 Aug; Rampon et al., 2000 Nov 7; Bollati et al., 2007 Feb 1; Cheng et al., 2008 Mar).

Several explanations may help to clarify the associations between lower levels of DNA methylation and more ADHD symptoms.

First of all, this association could be influenced by other confounding factors that might be in the causal pathway of environmental factors influencing the epigenome. We corrected our analysis for numerous potential confounding factors, however, there is little information from literature on what variables to control for.

Second, genetic factors could underlie the association between DNA methylation and ADHD symptoms. We corrected for the child genetic variables in the selected regions. In this study we reported lower DNA methylation levels in subjects with the risk allele of the DRD4-48 bp VNTR or the 5-HTTLPR polymorphism. The association between low DNA methylation levels of DRD4 and 5-HTT regions was largely independent of the genetic variants DRD4-48 bp VNTR or 5-HTTLPR. In previous studies, the presence of the DRD4-48 bp VNTR was shown to affect mRNA expression *in vitro* (Schoots and Van Tol, 2003). However, we did not take into account other genetic variations than those two sites assessed, although several other polymorphisms exist in these regions.

Proper DNA methylation plays a critical role in embryonic development and cell differentiation. Methylation also plays a mediating role in gene expression (Suzuki and Bird, 2008 Jun). Although there is no simple relationship between DNA methylation and gene expression it is assumed that DNA methylation is associated with loss of gene expression (Reik and 2007 May 24). In reports of patients with psychiatric disorders, e.g. bipolar disorder, schizophrenia, anorexia nervosa and DNA methylation, patients were more likely to have hypomethylated (neuronal) candidate genes (Frieling et al., 2007; Abdolmaleky et al., 2006). This is in line with our study, where we report a negative association between DNA methylation levels and ADHD symptoms.

The selection of our candidate genes was based on previous psychiatric epigenetic and candidate gene studies. It is postulated that ADHD may be caused by an imbalance of neurotransmitters (Russell et al., 2000 Dec 20). Research has focused in particular on the dopaminergic system, since effective medication was reported to block the reuptake of dopamine by the dopamine transporter molecule (Krause et al., 2000 May 12). Early reports of animal models of ADHD resembling the human condition demonstrated increased DRD4 expression (Zhang et al., 2002 May; Zhang et al., 2001 Nov).

A study by Wang et al. suggests that peripheral DNA methylation of the serotonin transporter may be a marker of central serotonin transporter function (Wang et al., 2012). However, a meta-analyses of Gizer et al. indicated a modest but significant association between ADHD and the 5HTTLPR “long allele” (Gizer et al., 2009 Jul).

In mice, prenatal protein restriction leading to intrauterine growth retardation, significantly increased expressed dopamine related genes (Vucetic et al., 2010 Jun 30). However, in the current study the association between DNA methylation and ADHD symptoms was independent of birth weight. Possibly, although we corrected for many environmental factors, prenatal influences not reflected by changes in birth weight may underlie these behavioral symptoms.

In this study we did not observe any association between ADHD-associated prenatal exposures and child DNA methylation at birth. The observed effects are rather small, this makes it difficult to reveal a mediation by DNA methylation on the risk of ADHD symptoms. On the other hand, since no previous human study has succeed to link disease-associated exposures via DNA methylation to the occurrence of disease, it might be the case that both environmental exposure and DNA methylation have largely independent effects.

The association between DNA methylation patterns and ADHD diagnose based on DISC and CBCL ADHD symptom score show different results. An important explanation might be that using CBCL symptoms increases power as a symptom count was available in more children in the analyses and analysed continuously.

Second, there could be discrepancies in item specificity between the DISC and the CBCL instrument. Although the clinical utility of categorically defined ADHD is well established, there is also strong evidence that the liability to develop ADHD problems is continuous. Clustering of subjects in terms of subtypes, as DISC does, neglects variation in severity as children with moderately elevated symptom scores on several subtypes won’t meet DSM-IV criteria for ADHD while children with elevated symptom scores on one subtype will (Hudziak et al., 2004 Oct).

As ADHD is known to have high psychiatric comorbidity, alterations in DNA methylation might be the result of other disorders. Alterations in DNA methylation are reported to play a role in the aetiology of bipolar disorder, autism and obsessive compulsive disorder, among others (Seeman et al., 1993 Sep 30; Melnyk et al., 2012 Mar; Zill et al., 2012). In the current study, sensitivity analyses showed that ODD and total symptom scores attenuated the association between DNA methylation levels and ADHD symptom score. This suggests that the observed

associations may not be specific for ADHD. Most likely, this reflects our choice of candidate gene sites for the methylation analyses. All psychiatric genes have been implicated in several developmental disorders, and candidate genes specifically related to ADHD have not been identified.

At the same time, the association between lower DNA methylation and more ADHD symptoms was independent of internalizing symptoms. This finding is in line with the observations from Kendler et al., that internalizing and externalizing disorders originate partly from different genetic mechanisms (Kendler et al., 2011 Jan; Kendler et al., 2003 Sep), whereas symptoms of ADHD and aggression are not only frequently co-morbid reflecting a more substantially shared genetic vulnerability (Hamshere et al., 2013 Aug 1). However, we have to be careful to translate findings in genetic studies to epigenetics and can only speculate about the parallels between underlying genetic and epigenetic structures in psychiatric disorders.

Epigenetics provide a possible mechanism for gene–environment interactions that have been observed in ADHD (Thapar et al., 2007 Jan). Several models postulating a complex interplay between the genome, epigenome and environmental factors have been proposed. Some specific tandem repeats are associated with alterations in epigenetic patterns (Bell et al., 2010). Other possibilities are an increased susceptibility by genetic variations for environmental factors to alter DNA methylation or, vice versa, an increased susceptibility for gene–environment interactions in the presence of altered DNA methylation. In the current study, we describe that DRD4-48 bp VNTR and 5-HTTLPR were associated with lower DNA methylation levels of the adjacent regions. The alterations in DNA methylation could be the result of interaction effects instead of direct genetic effects. However, testing interaction effects was not the core aim of the current study as we lacked hypotheses and, besides, sufficient power.

Several limitations of our study should be mentioned. First, in the current study attention problems were primarily assessed by means of a structured questionnaire and attention problems were considered as a continuous trait instead of a dichotomy. This has been shown to adequately represent attention problems on the population level and provides more power in the analyses. Further, the CBCL-ADHD symptom scale converges with the results of clinical interviews covering the DSM-IV-criteria (Biederman et al., 1993 Oct).

Second, our focus on selected regions in the genome made it unable to extrapolate our findings to other genomic regions. We performed an overall analysis of all regions assessed under the assumption that the variation of methylation has a uniform direction of effect. Separate analyses of the specific regions showed certain heterogeneity of effects across the different genomic regions, however, the direction of methylation effects at birth on ADHD symptoms was consistent.

Third, the DNA in the current study was isolated from leukocytes and not from brain tissue. The question rises how alterations in DNA methylation profiles in cord blood reflect DNA methylation profiles in other tissue of the child. This is a major problem in human studies. One post-mortem study analyzed an average of approximately 1500 regions, from 12 different tissues from different brain parts and other organs obtained from adult individuals found that only 34 CpGs were differently methylated among neural and non-neuronal tissues (Ghosh et al., 2010 Aug 16). Another study analyzing DNA methylation variation across brain and blood found that, although between-tissue variation exceed between-individual variation, inter-individual variation was reflected across brain and blood. This implies that peripheral tissues may be of use for epidemiological studies (Davies et al., 2012). Talens et al., reported for four of the eight investigated regions strong correlations between DNA methylation profiles in blood and buccal cells, tissues from different germ layers (Talens et al., 2010 Sep). However, no studies have compared fetal brain and peripheral tissues.

Finally, our study relied on genomic DNA extracted from whole blood. Epigenetic differences across samples could be derived from different cellular leukocyte populations, although cellular heterogeneity is considered to have limited impact (Talens et al., 2010 Sep).

To conclude, consistent with the epigenetic hypothesis of ADHD, a number of regions were found in which lower methylation levels measured at birth were associated with more ADHD symptoms in childhood. However, we cannot distinguish whether genetic, non-genetic intragenerational transmission, or unknown environmental factors underlie this association. Moreover, the observed association with ADHD symptoms was partly explained by co-occurring ODD and total symptoms. Further studies are needed to confirm our results and to investigate the possible underlying mechanism.

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Contributors

All authors participated in study design and editing and reviewed and approved the final version of the report. NHM collected, analyzed, and interpreted data and drafted the report. RST and HT formulated the original hypothesis, obtained funding and supervised the first author in drafting the report. RST and HT designed the study; HT supervised the data analysis and interpretation of data. BTH was involved in interpretation of data and contributed to drafts. PHC supervised statistical analyses. MIB-B was involved in data collection and data analysis. JR supervised data collection and input data. AU, LS and MPPJV set up and supervised DNA methylation data acquisition and laboratory analyses. AH, VJJ, FCV and EAPS initiated and designed the study, were responsible for the infrastructure in which the study is conducted and contributed to the original data collection.

Conflict of interest

Frank Verhulst is publisher of the Dutch translations of ASEBA from which he receives remuneration. All other authors report no financial relationships with commercial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpsychires.2013.10.017.

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