Epigenetics and adverse health outcomes

Silenced by the past?

Lisette van der Knaap

Epigenetics and adverse health outcomes
Silenced by the past?

Lisette van der Knaap

ISBN: 978-94-6169-765-3

(Cover) layout and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands
Cover photo: iStock photo [51039654]
shutterstock_237482068

Epigenetics and Adverse Health Outcomes

Silenced by the past?

Epigenetica en nadelige gezondheidsuitkomsten

Uitgeschakeld door het verleden?

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

dinsdag 1 december 2015 om 11.30 uur

door

Lisette Jacoline van der Knaap
geboren te Hulst

Erasmus University Rotterdam

PROMOTIECOMMISSIE:

Promotoren	Prof.dr. F.C. Verhulst Prof.dr. A.J. Oldehinkel
Overige leden	Prof.dr. S.A. Kushner Prof.dr. R.P.M. Steegers – Theunissen Dr. B.T. Heijmans
Copromotoren	Dr. F.V.A. van Oort Dr. H. Riese

Paranimfen	Steven van der Knaap Johanna Schäfer

CONTENTS

Chapter 1

General introduction

GENERAL INTRODUCTION

Adverse life events are strong risk factors for adverse health outcomes, such as psychiatric problems, obesity and related cardiovascular disease and diabetes [1]. The pathways through which stressful events can promote the development of such divergent disorders in humans remain largely unknown. Often there is no convincing support for a specific genetic contribution to the development of these disorders. In recent years, a promising explanation has risen from the field of epigenetics (derived from the Greek "epi", which means "on top of"). Epigenetics refers to the modification of DNA or histone proteins physically associated with the DNA, resulting in functional or operational changes without changing the primary sequence composition. These changes in gene expression may result in phenotypical variations that could be expressed as adverse health outcomes. Epigenetic modifications can be actively remodeled by environmental signals, and may therefore be considered as a "candidate mechanism for the environmental 'programming' of gene expression" [2].

To date, the most well-known epigenetic modification is DNA methylation, an important regulator of gene expression through chemical modifications to the DNA. This modification involves the addition of a methyl group (CH_3) to a cytosine base to form 5-methylcytosine in a cytosine-phosphate-guanine (CpG) dinucleotide combination (methylation at non-CpG sites has also been known to occur, but predominantly in plants and embryonic stem cells). CpG dinucleotides are underrepresented in the genome, i.e., this combination of nucleotides occurs less frequently (about 20%) than would be expected based on chance. This CpG depletion occurs because methylated CpG sites throughout the genome (70-80% of CpG sites are methylated) are at risk to be spontaneously deaminated (removal of an NH_2 group), resulting in the conversion of 5-methylcytosine into the base thymine (resulting in a TpG dinucleotide) [3]. However, there are regions that contain a much higher frequency of CpG dinucleotides. These regions are known as CpG islands and can be found in the promoter regions (i.e. the start site) of genes. Whereas single CpG sites throughout the genome are mostly methylated, CpG sites in CpG islands are often unmethylated [4]. When these CpG sites in the CpG island become methylated, the gene is generally silenced as gene expression is reduced. This can be achieved by directly preventing transcriptional proteins to access the gene, or by recruiting methyl-CpG-binding domain proteins (MBDs). These proteins recruit other proteins that modify histones, which results in a structural change that makes the DNA inaccessible for other transcriptional proteins.

EPIGENETICS IN BEHAVIORAL RESEARCH – PRELIMINARY FINDINGS

Animal research has made a large contribution in understanding the processes of epigenetic regulation, particularly in a gene involved in the stress response, the glucocorticoid receptor gene (*Nr3c1*) [2, 5]. Studies in rats showed that variations in maternal care towards offspring could result in altered hypothalamic-pituitary-adrenal (HPA) axis responses and behavioral responses to stress, through differences in *Nr3c1* methylation levels. Glucocorticoid receptors regulate the release of glucocorticoids by providing negative feedback to the hypothalamus-pituitary-adrenals axis (HPA-axis). This inhibits the release of other hormones (corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH)) that stimulate the release of glucocorticoids from the adrenals. Low levels of maternal licking and grooming (LG) and arched back nursing (ABN) behavior towards the offspring in the first week of life were associated with higher *Nr3c1* promoter methylation levels in the hippocampus of rat pups. As a result, expression of the glucocorticoid receptors was decreased and feedback inhibition was reduced, resulting in elevated stress responses and more fearful behavior [2, 5-7]. Cross fostering of the pups resulted in a methylation and behavior pattern that matched the behavior of the foster mother. This indicates that epigenetic processes that regulate expression of the glucocorticoid receptor in the brain mediate the relationship between early life stress (reduced LG and ABN) and adult HPA-axis responses [5].

These findings in animal studies have been replicated in humans [8-13]. McGowan and colleagues (2009) reported more hippocampal DNA methylation in the *NR3C1* gene promoter in victims of suicide with a history of childhood abuse than in those without a history of childhood abuse and non-abused controls [8]. In studies using peripheral DNA, from blood or saliva, *NR3C1* methylation was found to be higher in individuals exposed to childhood abuse or neglect [10, 11] or other stressful life events (such as loss of a family member or parental depression) during childhood [11, 13]. These studies suggest that the same epigenetic mechanisms tested in animals are also present in humans and that the relationship between adverse life events and adverse health outcomes may be mediated by DNA methylation.

OTHER POTENTIAL PREDICTORS OF EPIGENETIC MODIFICATION

Although the association between early life adversity and DNA methylation has best been described for *NR3C1*, this association has also been investigated for the serotonin transporter gene (*SLC6A4*). The serotonin transporter protein is an important regulator of serotonergic neurotransmission through the reuptake of serotonin (5-hydroxtryptamine, 5HT) in brain synapses. Alterations in serotonergic neurotransmission and

genetic variation in this gene (e.g. 5HT-linked polymorphic region, *5HTTLPR*) have been associated with an increased risk for various psychiatric disorders [14-17]. Several studies have reported an association between childhood adversity and higher levels of *SLC6A4* methylation [18-24].

Notwithstanding the importance of these studies in humans, they were relatively small in size and vulnerable to confounding factors. The focus of these studies was predominantly on traumatic experiences (such as sexual abuse or neglect), and less on more common stressful life events (such as parental divorce or the loss of a family member) that may also affect DNA methylation. Additionally, most studies investigated adversity experienced in childhood, as the previously mentioned animal studies reported higher methylation levels only when exposed to reduced maternal care in the first week of life. In humans, however, adolescence is also an important period of development, often accompanied by an increased susceptibility to stress-related mental disorders [25, 26]. Yet, the impact of adversity in adolescence on *NR3C1* and *SLC6A4* methylation had not been investigated.

OUTCOMES OF EPIGENETIC MODIFICATION

In humans, many studies have shown robust associations of early adverse life events with a wide range of disorders in later life, such as psychiatric, cardiovascular and metabolic disorders, as well as for risk factors of these disorders, such as obesity [1, 27, 28]. Not only have robust associations been found for disorders, but also with increased responsiveness of the autonomic nervous system and the HPA-axis to stress [29-31]. However, not many studies have investigated the relationship between DNA methylation and these different outcomes.

For psychiatric problems such as anxiety and depression, only a few studies have investigated the association with *SLC6A4* or *NR3C1* methylation. These studies were cross-sectional or retrospective; prospective studies on this association are lacking. Similarly, research on the association between *NR3C1* methylation and HPA-axis regulation is scarce. The few studies that did investigate this association varied greatly in their measures of HPA-axis regulation and had a small sample size [9, 11, 32], and therefore require replication in a larger cohort.

The association between *SLC6A4* or *NR3C1* methylation and obesity has received even less attention. Although genetic variants of these genes have been associated with cardiovascular problems, metabolic disorders and obesity, the association between DNA methylation and obesity has only been reported once for *SLC6A4* [33] and has not been investigated for *NR3C1*.

Besides psychiatric, cardiovascular and metabolic disorders, exposure to adverse life events has also been linked with substance use [34, 35]. As DNA methylation by environmental factors is likely to affect multiple genes across the genome, it would be interesting to investigate whether substance use may be related to DNA methylation in a gene involved in dopamine regulation. Frequent substance use has been associated with altered dopamine levels in the brain, which is degraded by the enzyme catechol-O-methyltransferase (COMT). Variations in enzyme activity can influence vulnerability to substance use, and perhaps differences in COMT gene (*COMT*) expression through methylation may also affect the tendency for substance use. Again, little is known about the association between *COMT* methylation and substance use: only the associations between *COMT* methylation and tobacco smoking [36] and alcohol use [37] have been investigated in two studies with a small sample size. For cannabis use, this association has not been reported.

To investigate the relationship between DNA methylation in *NR3C1*, *SLC6A4* and *COMT* and the before-mentioned outcomes, large longitudinal studies are required that examine a wide range of exposures and outcomes, including long-term outcomes.

RESEARCH AIMS AND THESIS OUTLINE

In this thesis I have investigated whether DNA methylation may serve as an underlying biological mechanism that predisposes individuals with a history of adversity to adverse health outcomes. DNA methylation could be an eligible mediator of the association between adversity and adverse health outcomes (illustrated in **Figure 1**) when there is an association between adverse life events and DNA methylation, and between DNA methylation and adverse health outcomes. We therefore studied the associations of DNA methylation with both possible predictors and possible health outcomes.

THESIS OUTLINE

I first investigated whether stressful life events, during childhood and adolescence, are associated with higher CpG methylation in the genes encoding the glucocorticoid receptor (*NR3C1*, **Chapter 2**) and the serotonin transporter (*SLC6A4*, **Chapter 3**). I then investigated whether higher methylation levels of *NR3C1* and *SLC6A4* were associated with internalizing problems, i.e. anxiety and depression problems in **Chapter 4**. Next, I investigated whether *NR3C1* methylation was associated with HPA-axis dysregulation in **Chapter 5**. In **Chapter 6**, I investigated whether methylation of *SLC6A4* and *NR3C1* was associated with obesity, as obesity in adolescence is an important risk factor for

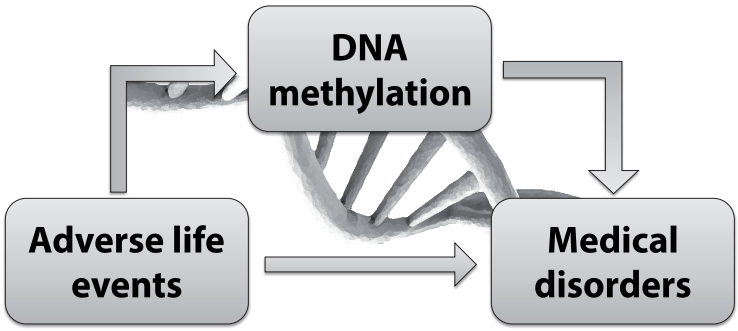


Figure 1. Proposed mediation model.

cardiovascular and metabolic disorders later in life. Next, to explore possible epigenetic associations with substance use, I investigated whether *COMT* methylation was associated with substance use in adolescents in **Chapter 7**. In **Chapter 8** I discuss the main findings of this thesis, and combine the information from **Chapters 2-4,** by investigating whether DNA methylation acted as a mediator in the association between SLEs and internalizing symptom scores. A summary of all findings is given in **Chapter 9**.

SETTING: THE TRAILS STUDY

This study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective population study in which Dutch adolescents are followed from childhood (10-12 years) into adulthood (25 years and older). Assessment waves have been conducted biennially or triennially, and five assessment waves have been completed thus far. A detailed description of sampling and methods can be found in de Huisman *et al* [38], Ormel *et al* [39], and Oldehinkel *et al* [40]. With its prospective design, its large representative population and the availability of a large number of measures on outcomes, life histories and biological samples, TRAILS offers good data for this project.

REFERENCES

1. McEwen BS. Protective and damaging effects of stress mediators. *New Engl J Med* 1998; **338**(3)**:** 171-179.
2. Meaney MJ. Epigenetics and the biological definition of gene x environment interactions. *Child Dev* 2010; **81**(1)**:** 41-79.
3. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J *et al.* Initial sequencing and analysis of the human genome. *Nature* 2001; **409**(6822)**:** 860-921.
4. Illingworth RS, Bird AP. CpG islands--'a rough guide'. *FEBS Lett* 2009; **583**(11)**:** 1713-1720.
5. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR *et al.* Epigenetic programming by maternal behavior. *Nat Neurosci* 2004; **7**(8)**:** 847-854.
6. Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A *et al.* Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* 1997; **277**(5332)**:** 1659-1662.
7. Meaney MJ, Diorio J, Francis D, Widdowson J, LaPlante P, Caldji C *et al.* Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. *Dev Neurosci* 1996; **18**(1-2)**:** 49-72.
8. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2009; **12**(3)**:** 342-348.
9. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (*NR3C1*) and infant cortisol stress responses. *Epigenetics* 2008; **3**(2)**:** 97-106.
10. Perroud N, Paoloni-Giacobino A, Prada P, Olie E, Salzmann A, Nicastro R *et al.* Increased methylation of glucocorticoid receptor gene (*NR3C1*) in adults with a history of childhood maltreatment: a link with the severity and type of trauma. *Transl Psychiatry* 2011; **1:** e59.
11. Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. *PLoS One* 2012; **7**(1)**:** e30148.
12. de Rooij SR, Costello PM, Veenendaal MV, Lillycrop KA, Gluckman PD, Hanson MA *et al.* Associations between DNA methylation of a glucocorticoid receptor promoter and acute stress responses in a large healthy adult population are largely explained by lifestyle and educational differences. *Psychoneuroendocrinology* 2012; **37**(6)**:** 782-788.
13. Essex MJ, Boyce WT, Hertzman C, Lam LL, Armstrong JM, Neumann SM *et al.* Epigenetic vestiges of early developmental adversity: childhood stress exposure and DNA methylation in adolescence. *Child Dev* 2013; **84**(1)**:** 58-75.
14. Malison RT, Price LH, Berman R, van Dyck CH, Pelton GH, Carpenter L *et al.* Reduced brain serotonin transporter availability in major depression as measured by [123I]-2 beta-carbomethoxy-3 beta-(4-iodophenyl)tropane and single photon emission computed tomography. *Biol Psychiatry* 1998; **44**(11)**:** 1090-1098.
15. Owens MJ, Nemeroff CB. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clin Chem* 1994; **40**(2)**:** 288-295.
16. Collier DA, Stober G, Li T, Heils A, Catalano M, Di Bella D *et al.* A novel functional polymorphism within the promoter of the serotonin transporter gene: possible role in susceptibility to affective disorders. *Mol Psychiatry* 1996; **1**(6)**:** 453-460.

17. Sen S, Burmeister M, Ghosh D. Meta-analysis of the association between a serotonin transporter promoter polymorphism (5-HTTLPR) and anxiety-related personality traits. *Am J Med Genet B Neuropsychiatr Genet* 2004; **127B**(1)**:** 85-89.
18. Alasaari JS, Lagus M, Ollila HM, Toivola A, Kivimaki M, Vahtera J *et al.* Environmental stress affects DNA methylation of a CpG rich promoter region of serotonin transporter gene in a nurse cohort. *PLoS One* 2012; **7**(9)**:** e45813.
19. Beach SR, Brody GH, Todorov AA, Gunter TD, Philibert RA. Methylation at *SLC6A4* is linked to family history of child abuse: an examination of the Iowa Adoptee sample. *Am J Med Genet B Neuropsychiatr Genet* 2010; **153B**(2)**:** 710-713.
20. Beach SR, Brody GH, Todorov AA, Gunter TD, Philibert RA. Methylation at 5HTT mediates the impact of child sex abuse on women's antisocial behavior: an examination of the Iowa adoptee sample. *Psychosom Med* 2011; **73**(1)**:** 83-87.
21. Kang HJ, Kim JM, Stewart R, Kim SY, Bae KY, Kim SW *et al.* Association of *SLC6A4* methylation with early adversity, characteristics and outcomes in depression. *Prog Neuropsychopharmacol Bol Psychiatry* 2013; **44:** 23-28.
22. Olsson CA, Foley DL, Parkinson-Bates M, Byrnes G, McKenzie M, Patton GC *et al.* Prospects for epigenetic research within cohort studies of psychological disorder: a pilot investigation of a peripheral cell marker of epigenetic risk for depression. *Biol Psychol* 2010; **83**(2)**:** 159-165.
23. Ouellet-Morin I, Wong CC, Danese A, Pariante CM, Papadopoulos AS, Mill J *et al.* Increased serotonin transporter gene (SERT) DNA methylation is associated with bullying victimization and blunted cortisol response to stress in childhood: a longitudinal study of discordant monozygotic twins. *Psychol Med* 2013; **43**(9)**:** 1813-1823.
24. Vijayendran M, Beach SR, Plume JM, Brody GH, Philibert RA. Effects of genotype and child abuse on DNA methylation and gene expression at the serotonin transporter. *Front Psychiatry* 2012; **3:** 55.
25. Veenema AH. Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: what can we learn from animal models? *Front Neuroendocrinol* 2009; **30**(4)**:** 497-518.
26. McGorry PD, Purcell R, Goldstone S, Amminger GP. Age of onset and timing of treatment for mental and substance use disorders: implications for preventive intervention strategies and models of care. *Curr Opin Psychiatry* 2011; **24**(4)**:** 301-306.
27. Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB. The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology* 2008; **33**(6)**:** 693-710.
28. Wegman HL, Stetler C. A meta-analytic review of the effects of childhood abuse on medical outcomes in adulthood. *Psychosom Med* 2009; **71**(8)**:** 805-812.
29. Heim C, Newport DJ, Heit S, Graham YP, Wilcox M, Bonsall R *et al.* Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *JAMA* 2000; **284**(5)**:** 592-597.
30. Van den Bergh BR, Van Calster B, Smits T, Van Huffel S, Lagae L. Antenatal maternal anxiety is related to HPA-axis dysregulation and self-reported depressive symptoms in adolescence: a prospective study on the fetal origins of depressed mood. *Neuropsychopharmacology* 2008; **33**(3)**:** 536-545.
31. Bosch NM, Riese H, Reijneveld SA, Bakker MP, Verhulst FC, Ormel J *et al.* Timing matters: long term effects of adversities from prenatal period up to adolescence on adolescents' cortisol stress response. The TRAILS study. *Psychoneuroendocrinology* 2012; **37**(9)**:** 1439-1447.

32. Edelman S, Shalev I, Uzefovsky F, Israel S, Knafo A, Kremer I *et al.* Epigenetic and genetic factors predict women's salivary cortisol following a threat to the social self. *PLoS One* 2012; **7**(11)**:** e48597.
33. Zhao J, Goldberg J, Vaccarino V. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *Int J Obes (Lond)* 2013; **37**(1)**:** 140-145.
34. Anda RF, Croft JB, Felitti VJ, Nordenberg D, Giles WH, Williamson DF *et al.* Adverse childhood experiences and smoking during adolescence and adulthood. *JAMA* 1999; **282**(17)**:** 1652-1658.
35. Dube SR, Anda RF, Felitti VJ, Edwards VJ, Croft JB. Adverse childhood experiences and personal alcohol abuse as an adult. *Addict Behav* 2002; **27**(5)**:** 713-725.
36. Xu Q, Ma JZ, Payne TJ, Li MD. Determination of Methylated CpG Sites in the Promoter Region of Catechol-O-Methyltransferase (*COMT*) and their Involvement in the Etiology of Tobacco Smoking. *Front Psychiatry* 2010; **1:** 16.
37. Abdolmaleky HM, Cheng KH, Faraone SV, Wilcox M, Glatt SJ, Gao F *et al.* Hypomethylation of *MB-COMT* promoter is a major risk factor for schizophrenia and bipolar disorder. *Hum Mol Genet* 2006; **15**(21)**:** 3132-3145.
38. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, Huizink AC *et al.* Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. *Int J Epidemiol* 2008; **37**(6)**:** 1227-1235.
39. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, Veenstra R *et al.* The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. *J Am Acad Child Adolesc Psychiatry* 2012; **51**(10)**:** 1020-1036.
40. Oldehinkel AJ, Rosmalen JG, Buitelaar JK, Hoek HW, Ormel J, Raven D *et al.* Cohort Profile Update: the TRacking Adolescents' Individual Lives Survey (TRAILS). *Int J Epidemiol* 2015; **44**(1)**:** 76-76n.

Chapter 2

Glucocorticoid receptor gene (*NR3C1*) methylation following stressful events between birth and adolescence. The TRAILS study

L.J. van der Knaap
H. Riese
J.J. Hudziak
M.M.P.J. Verbiest
F.C. Verhulst
A.J. Oldehinkel
F.V.A. van Oort

Translational Psychiatry, 2014, 4, e381, doi:10.1038/tp.2014.22.

ABSTRACT

Stress early in life is a known risk factor for the development of affective disorders later in life. Epigenetic mechanisms, such as DNA methylation, may have an important role in mediating that risk. Recent epigenetic research reported on the long-term relationship between traumatic stress in childhood and DNA methylation in adulthood. In this study, we examined the impact of various types of stress (perinatal stress, stressful life events (SLEs) and traumatic youth experiences) on methylation of the glucocorticoid receptor gene (*NR3C1*) in the blood of a population sample of 468 adolescents (50.4% female, mean age 16.1 years). Second, we determined whether stress at different ages was associated with higher *NR3C1* methylation. *NR3C1* methylation rates were higher after exposure to SLEs and after exposure to traumatic youth experiences. *NR3C1* methylation in adolescence was not higher after exposure to perinatal stress. Experience of SLEs in adolescence was associated with a higher *NR3C1* methylation, independently of childhood SLEs. We demonstrate that not only traumatic youth experiences but also (more common) SLEs are associated with higher *NR3C1* methylation. In addition, our findings underline the relevance of adolescent stress for epigenetic changes in the *NR3C1* gene.

INTRODUCTION

Severe maltreatment or neglect in childhood are known risk factors for development of affective disorders and have been associated with altered programming of the hypothalamic-pituitary-adrenal (HPA) axis [1-4]. Epigenetic modifications are thought to link early-life stress to later susceptibility to affective disorders, such as anxiety or depression, through interference with the development and functioning of the HPA axis early in life [5]. More recently, the notion that stress can have a direct effect on epigenetic modifications across the life span has been proposed, which in turn affects brain plasticity and may lead to anxiety [6]. The epigenetic process of DNA methylation involves the addition of methyl groups on cytosine-guanine dinucleotides (CpGs) in gene promoters and regulatory regions, which regulate gene transcription[7]. The presence of these methyl groups is associated with reduced gene expression by reducing access to the DNA. Methyl groups on CpGs in regulatory regions for transcription factors can directly interfere with the binding of transcription factors to their recognition elements. Methylated regions can also repress transcription indirectly by attracting methylated DNA-binding proteins, which can alter the chromatin formation, disabling access to DNA for transcription [8-12]. While much research focuses on the function of epigenetic modifications, less is known about how they are environmentally induced.

Whereas the genome is fixed, the epigenome is considered to be dynamic –that is, under influence of environmental factors [13, 14]. In rats, exposure to early-life stress, measured as reduced levels of maternal licking and grooming (LG) behavior towards their offspring, led to increased methylation in the glucocorticoid receptor (GR) gene (*Nr3c1*) of their offspring. The GRs regulate the release of glucocorticoids through a negative feedback mechanism in the HPA-axis. Increased methylation in offspring and reduced expression of GRs in the hippocampus by low maternal LG behavior resulted in a diminished feedback sensitivity of the HPA-axis [15]. Differences in methylation between offspring from high-LG and low-LG mothers persisted into adulthood, illustrating long lasting effects of early programming on the epigenome.

These animal findings were translated to humans by McGowan *et al* [16]. They reported increased levels of methylation and decreased levels of GR-expression in post-mortem hippocampal tissue of suicide completers who were abused during childhood, compared to non-abused suicide completers and non-abused controls [16]. Other studies using peripheral DNA, from blood of infants, adolescents or adults, have shown increased levels of *NR3C1* methylation in response to perinatal stress [17-19] and abuse or neglect during childhood [20, 21]. Most studies thus far reported on DNA methylation in adults after enduring stress or traumatic events such as abuse or neglect [16, 20-22]. Fewer studies have investigated whether *NR3C1* methylation in humans can be induced by other,

more common, stressful life events (SLEs – for example, parental divorce, loss of a family member) as well [21, 23, 24].

Besides types of stress, the role of timing of stress on methylation is understudied. Humans are subjected to a high variety of stressors throughout life. The perinatal period and childhood years are regarded as sensitive periods for the developing brain in which the organism could be particularly susceptible to epigenetic modifications that influence HPA-axis development [25]. However, some brain regions keep developing at least until early adulthood [26], hence there is a possibility that epigenetic modifications are not restricted to childhood [27-29]. While there is preliminary evidence for epigenetic modification of *NR3C1* due to stressors in childhood [16, 20, 21], the adolescent period, in spite of its obvious importance as a period of increased susceptibility to stress-related mental disorders [30, 31], has had no examination of the impact of stress on *NR3C1* methylation independent of stress experienced in childhood [6].

We studied the effects of stress on *NR3C1* methylation in a large prospective population study of adolescents in two ways. First, we examined the impact of various types of stress (for example, perinatal stress, SLEs and traumatic youth experiences) on *NR3C1* methylation. Second, we determined whether stress at different ages was associated with higher *NR3C1* methylation. Based on previous findings [15-22, 32] we hypothesized that perinatal stress, many SLEs, and traumatic youth experiences would relate to higher *NR3C1* methylation in adolescence. We further expected that SLEs experienced in childhood would, independently of later adolescent stress, relate to increased *NR3C1* methylation in adolescence.

MATERIALS & METHODS

Sample selection

Data from the TRAILS (TRacking Adolescents' Individual Lives Survey) study were used. TRAILS is a prospective population study of Dutch adolescents (N = 2230) who are being followed from pre-adolescence into adulthood. Assessment waves are conducted biennially or triennially, and four assessment waves have been completed so far. Written consent was obtained from each subject and their parents at every assessment wave. The present study involves data collected during the first assessment wave (T1, 2001–2002, mean age 11.1 years, s.d. = 0.55), second (T2, 2003–2004, mean age 13.6 years, s.d. = 0.53), third (T3, 2005–2007, mean age 16.3 years, s.d. = 0.71) and fourth (T4, 2008–2010, mean age 19.1 years, s.d. = 0.60) assessment waves. At T3, 715 TRAILS subjects (focus sample) participated in more extensive experimental data collection. Adolescents with an increased risk of mental health problems had a greater chance of being selected for the experimental session. Increased risk was defined as having at least

one of the following risk factors: child temperament (high frustration and fearfulness, low effortful control), parental psychopathology (depression, anxiety, addiction, psychoses or antisocial behavior) and environmental risk (living in a single-parent family), all measured at T1. Although high-risk adolescents were slightly oversampled (66% of the focus sample, the remaining 34% of the focus sample were selected at random from the low-risk TRAILS participants), the sample included the total range of mental health problems present in a community population of adolescents. The study was approved by the Dutch Central Medical Ethics Committee and all subjects received compensation for their participation. A detailed description of sampling and methods can be found in Huisman *et al* [33] and Ormel *et al* [34]. DNA had been isolated from blood for 654 of these subjects. Initial selection for methylation analyses ($N = 475$) was obtained by excluding subjects with non-Dutch ethnicity ($N = 58$), unknown or insufficient DNA concentration ($N = 116$), and randomly excluding one of each sibling pair ($N = 5$). Following drop-out after DNA methylation analyses, 468 subjects were eligible for analysis. Our subsample subjects did not differ significantly ($P>0.05$) from the TRAILS focus sample with regard to sex, socioeconomic status (T1), age (T3), internalizing problems (T3) and externalizing problems (T3).

Stress measures

Perinatal stress was operationalized as the sum of maternal psychological problems during pregnancy or the three months after delivery, preterm delivery (≤33 weeks), low birth weight (≤2500 g), hospitalization of mother or child within 1 month after delivery, and maternal alcohol use or smoking during pregnancy. For birth weight and gestational age we used records of the Preventive Child Healthcare services[35]. The other stressors were measured in a detailed interview with the parents at T1.

SLEs experienced between ages 0 and 15 years were assessed for the age categories of 0–5, 6–11, 12–13 and 14–15 years, as described by Bosch *et al* [4]. Information on SLEs in *early childhood (0-5 years) and middle childhood (6-11 years)* was collected during a detailed interview with the parents at T1, and included the number of times the child had experienced parental divorce, hospitalization, the death of a family member or friend, out-of-home placement, parental addiction or parental mental health problems [4]. The total number of SLEs experienced in *early adolescence (12-13 years)* was assessed with a self-report questionnaire at T2 [4]. The 25 SLEs included illness or injury of the participant, a family member or a friend; parental divorce; death of family member or friend; changes in family composition; parental unemployment; conflicts with family or friends; and being bullied. SLEs in *middle adolescence (14-15 years)* were assessed at T3 in an Event History Calendar Interview [36] with the adolescent. The list of possible events consisted of conflicts, physical or sexual intimidation, victim of bullying/gossiping, loss or lack of friends, psychological/addiction problems of family or friends, out-of-home

placement, running away from home, death/illness of family member, hospitalization of participant, and parental divorce. On the basis of the above-mentioned event measures, we calculated a measure of total SLEs experienced between ages 0 and 15 years by standardizing the sum score of the number of events for each age category, and summing the standardized scores. This procedure was chosen to account for differences in the number of possible SLEs by age group. The resulting total sum score (SLEs 0-15 years) was standardized for analyses.

Traumatic youth experiences. At T4, information on sexual, physical and other traumatic experiences before the age of 16 years was obtained with a 16-item self-report questionnaire. To determine sexual abuse, the participants were asked if an adult family member, friend of the family or stranger had ever, before the participant was 16, showed his/her genitals or masturbated in front of them; had sexually assaulted them; had forced them to touch him/her in a sexual manner; had attempted to have intercourse or had actually had intercourse with them. To determine physical abuse, participants were asked if a parent or caretaker had ever, before the participant was 16, hit them with a belt, brush, stick or other hard object; had hit them with a fist or kicked them very hard; had shaken or pinched them; had beaten them up (that is, hit them in succession) or had threatened them with a knife or other weapon. To determine other trauma, participants were asked if, before the age of 16, they had been involved in a life-threatening accident; had witnessed severe injury or death; had been a victim of physical violence or assault; had been threatened with a weapon, had been held captive or abducted or had been involved in a fire, flood or other (natural) disaster. Answers were coded into a single exposure or multiple exposures to traumatic youth experiences. We had no data on the timing of traumatic life stress. We cross-tabbed the three SLE profiles with traumatic experiences (Supplementary Table 1) and found that traumatic experiences were not strongly overrepresented in the childhood SLE group or the adolescence SLE group.

Amplicon selection

Three amplicons (genomic regions) within the *NR3C1* cytosine-guanine dinucleotide (CpG) island in the promoter-region were selected for analyses (Figure 1). CpG-island position was determined using criteria from UCSC genome browser – that is, a genomic region with a CpG content of 50% or greater, length greater than 200bp, and a ratio greater than 0.6 of observed number of CG dinucleotides to the expected number on the basis of the number of Gs and Cs in the segment (UCSC human Feb. 2009 assembly GRCh37/hg19, (http://genome.ucsc.edu). Two primer sets were designed using the software EpiDesigner by Sequenom (www.epidesigner.com), covering the edges of the *NR3C1* CpG-island. Additionally, we analyzed a region of the CpG-island using the primer set previously used by McGowan *et al* [16]. This genomic region encompasses exon

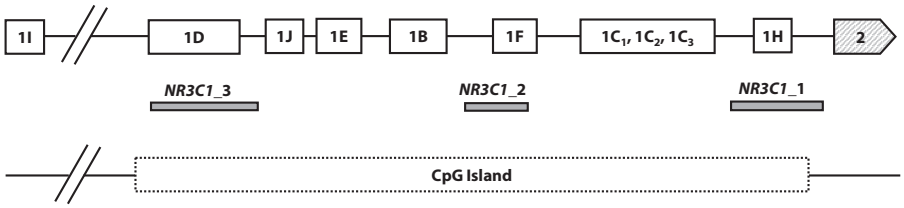


Figure 1. Schematic representation of the glucocorticoid receptor gene (*NR3C1*). Amplicons (*NR3C1*_1, *NR3C1*_2 and *NR3C1*_3, shown in grey) are shown in relation to the *NR3C1* CpG-island (chr5:142782072-142785071, dotted box) and untranslated first exons (line boxes) upstream of exon 2 (striped box). Image based on Labonte *et al* [37] and Turner *et al* [38].

1_F which corresponds to the rat exon 1_7. Sequence information and primer properties can be found in Supplementary Figure 1 and Supplementary Table 2.

DNA methylation

Analysis. DNA was extracted from whole-blood samples using a manual salting-out procedure as described by Miller *et al* [39]. DNA-methylation rates were analyzed using bisulfite-treated DNA, PCR, reverse transcription, base-specific cleavage of *in vitro* transcribed RNA product, and mass spectrometry (Sequenom EpiTYPER, San Diego, CA, USA). Bisulfite conversion of DNA was performed using EZ-96 DNA Methylation Kit (Shallow; Zymo Research, CA, USA). Bisulfite treatment was performed according to manufacturers' protocol. It must be noted that bisulfite conversion does not differentiate between the different types of cytosine methylation (e.g. hydroxymethylation). PCR, reverse transcription, cleavage and mass spectrometry were performed in triplicate according to EpiTYPER protocol. Amplification conditions can be found in Supplementary Table 3. The mass signal patterns generated are translated to quantitative methylation rates for different CpG units by the MassARRAY EpiTYPER analyzer software from Sequenom. (v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA). Fragments with CpG dinucleotides are referred to as CpG units. One CpG unit can contain one or more CpG dinucleotide.

Data cleaning. All samples were analyzed in triplicate, and samples with a s.d. of ≥10% between replicates were removed for analysis. CpG units with ≥25% missing values were not included in the analyses. For each CpG unit, methylation scores of the triplicates were averaged. We accounted for mass change in CpG units by single nucleotide length polymorphisms (only when minor allele frequency >5%) by removing CpG units from analyses containing the single nucleotide length polymorphism and removing units with overlapping mass caused by single nucleotide length polymorphisms in non-CpG units. Amplicon 1 consisted of 11 eligible CpG units, amplicon 2 contained 10 and amplicon 3 contained nine eligible CpG units.

Statistical analyses

Main analyses. Linear regression analyses were conducted to examine the effects of type and timing of stress on *NR3C1* methylation in adolescence. Amplicons were analyzed separately [40]. For each CpG unit, methylation was mean-centered (resulting in mean methylation of 0, with original s.d.), to account for high methylation in a small number of CpG units. As individuals could have drop-out in one or more CpG units, an average methylation score for each amplicon was calculated by taking the average of the mean-centered methylation scores of the CpG units within an amplicon. Separate models were run for perinatal stress, SLEs (0-15 years), the three categories of traumatic youth experiences (sexual, physical and other trauma). Smoking, gender and age at T3 were considered as potential confounders, but including them in the models appeared not to change the regression coefficients of stress variables with more than 10%. Therefore, these confounders were not included in the final models. To test our hypothesis on the timing of stress and *NR3C1* methylation, we ran a linear regression model with the two SLEs variables (0-11 and 12-15 years).

As a *post-hoc* robustness check, we repeated analyses on type and timing of stress on methylation of amplicon 2 on an additional random sample of 454 TRAILS subjects, who were not part of the T3 focus sample. Information on the other two amplicons was not available for this sample. Amplicon 2 was chosen *a priori* for its correspondence with the study by McGowan *et al* [16] and the known presence of the nerve-growth-factor-inducible-protein-A (NGFI-A)-binding site.

Exploratory analyses. In addition to the analyses of the three above-mentioned amplicons, we performed exploratory linear regression analyses of type and timing of stress on mean-centered methylation scores of three individual CpG units, selected based on their relatively high methylation rates and s.d.'s (Supplementary Table 4).

The tests were conducted using SPSS (IBM SPSS, v.20.0. Armonk, NY: IBM Corp), whereas the latent profile analysis (LPA) was performed in Mplus 5 (Los Angeles, CA: Muthén & Muthén).

RESULTS

Approximately half of the sample was female (50.4%) and the mean age of the adolescents was 16.1 years at the time of the DNA collection, with a range from 14 to 18 years. Methylation was not correlated with age. Table 1 presents the descriptive statistics of perinatal stress, SLEs and traumatic youth experiences (uncentered, unstandardized).

Table 1. Descriptives of perinatal stress, SLEs and traumatic youth experiences.

	median (min-max)	N (%)
Perinatal stress	1 (0-6)	
SLEs (0-15yrs)		
0-15yrs	6 (0-23)	
0-5yrs	1 (0-10)	
6-11yrs	1 (0-8)	
12-13yrs	2 (0-12)	
14-15yrs	1 (0-11)	
Traumatic Youth Experiences (0-16yrs)		
Sexual abuse		
None		403 (84.8%)
Single exposure		22 (4.6%)
Repeated exposure		12 (2.5%)
Physical abuse		
None		253 (53.2%)
Single exposure		170 (35.8%)
Repeated exposure		14 (2.9%)
Other trauma		
None		319 (67.2%)
Single exposure		90 (18.9%)
Repeated exposure		28 (5.9%)

Abbreviation: SLE, stressful life event.

Life stress and methylation

The results of the linear regression models with stress variables as predictors of *NR3C1* methylation are presented in Table 2. Exposure to SLEs 0–15 years and to traumatic youth experiences significantly predicted higher methylation rates in amplicon 1. In amplicon 2, only single exposure to sexual abuse predicted higher methylation rates (B=0.44, P<0.001). For amplicon 3, repeated exposure to other traumatic youth experiences was associated with lower methylation rates (B=-0.26, P<0.01).

Secondly, we analyzed timing of SLEs. We had no specific hypotheses for early versus late adolescence or for early versus late childhood stress [4]. As these variables are correlated more strongly within childhood and adolescence age groups than between (see Supplementary Table 5), we considered creating a childhood SLE variable and an adolescence SLE variable. We used latent LPA to explore profiles of SLE in our sample. We found three distinct profiles (see Supplementary Table 6 and Supplementary Figure 2): a 'low stress group' at all ages, a 'high childhood stress group (0-5 years and 6-11

Table 2. Life stress and *NR3C1* methylation scores by amplicons.

	***NR3C1*_1**			***NR3C1*_2**			***NR3C1*_3**		
	B	**s.e.**	**P**	**B**	**s.e.**	**P**	**B**	**s.e.**	**P**
Perinatal stress	0.03	0.02	.14	-0.01	0.02	.78	0.01	0.02	.53
Traumatic youth experiences[a]									
Sexual abuse									
single exposure	**0.37**	**0.09**	**<.0001**	**0.44**	**0.12**	**<.001**	-0.08	0.10	.40
repeated exposure	**0.50**	**0.12**	**<.0001**	0.13	0.17	.45	-0.26	0.13	.05
Physical abuse									
single exposure	0.01	0.04	.73	0.02	0.06	.68	-0.02	0.05	.66
repeated exposure	**0.49**	**0.12**	**<.0001**	-0.09	0.15	.56	-0.18	0.12	.15
Other trauma									
single exposure	0.09	0.05	.06	-0.04	0.07	.52	-0.10	0.06	.08
repeated exposure	**0.53**	**0.08**	**<.0001**	0.07	0.11	.56	**-0.26**	**0.09**	**<.01**
SLEs[b]									
Total (0-15 years)	**0.05**	**0.02**	**.02**	0.03	0.03	.25	-0.03	0.02	.20
Childhood (0-11 years)	0.01	0.02	.57	0.00	0.03	.97	-0.03	0.02	.22
Adolescence (12-15 years)	**0.05**	**0.02**	**<.01**	0.04	0.03	.13	-0.01	0.02	.62

Abbreviations: B, regression coefficient; SLEs, stressful life events. Linear regression of early-life stress and multivariate regression analyses of SLEs in two age categories on *NR3C1* methylation scores. Bold numbers indicate significant results (P<0.05). [a] No exposure is the reference category. [b] Z-scores.

years)' and a 'high adolescent stress group (12-13 years and 14-15 years)'. Sample size was too small to use the three profiles as predictor of *NR3C1* methylation; however, the LPA analysis provided the support to distinguish between SLEs in childhood (0-11 years) and SLEs in adolescence (12-15 years). The variables were constructed by summing standardized scores for each age period. The SLE scores (0-11 years and 12-15 years) were standardized before analyses.

Experience of SLEs in adolescence was associated with a higher methylation score independently of childhood SLEs in amplicon 1 (B=0.05, p<0.01), but not in amplicons 2 and 3 (Table 2).

Exploratory analyses

Exploratory analyses on CpG unit-specific methylation (Table 3) showed higher methylation rates with more exposure to SLEs 0-15 years and to traumatic youth experiences in all three CpG units. Perinatal stress was not related to CpG unit-specific methylation. Additionally, the CpG unit-specific analyses show that SLEs in adolescence predicted

Table 3. Life stress and *NR3C1* methylation scores of the three highest methylated CpG units.

	***NR3C1*_1CpGU12**			***NR3C1*_2CpGU13**			***NR3C1*_2CpGU14**		
	B	**s.e.**	**P**	**B**	**s.e.**	**P**	**B**	**s.e.**	**P**
Perinatal stress	0.05	0.11	.64	0.05	0.20	.81	-0.05	0.09	.58
Traumatic youth experiences[a]									
Sexual abuse									
single exposure	**1.95**	**0.52**	**<.001**	**5.06**	**0.95**	**<.0001**	**0.91**	**0.43**	**.04**
repeated exposure	**3.07**	**0.70**	**<.0001**	**4.21**	**1.29**	**<.01**	0.50	0.59	.40
Physical abuse									
single exposure	0.07	0.24	.78	0.34	0.45	0.46	0.15	0.20	.46
repeated exposure	**2.79**	**0.67**	**<.0001**	**2.68**	**1.20**	**.03**	-0.34	0.54	.52
Other trauma									
single exposure	0.36	0.28	0.20	-0.02	0.53	.98	0.07	0.24	.78
repeated exposure	**3.32**	**0.46**	**<.0001**	**3.86**	**0.83**	**<.0001**	0.22	0.39	.57
SLEs[b]									
Total (0-15yrs)	**0.38**	**0.12**	**<.01**	**0.45**	**0.21**	**.03**	**0.24**	**0.09**	**.01**
Childhood (0-11yrs)	0.10	0.12	.41	-0.05	0.21	.82	**0.21**	**0.09**	**.02**
Adolescence (12-15yrs)	**0.41**	**0.12**	**<.001**	**0.68**	**0.21**	**<.01**	0.10	0.09	.26

Abbreviations: B, regression coefficient; CpG, cytosine–guanine dinucleotide; SLE, stressful life events. Linear regression of early life stress and multivariate regression analyses of SLEs in two age categories on *NR3C1* methylation scores. Bold numbers indicate significant results (P<0.05). [a] No exposure is the reference category. [b] Z-scores.

higher *NR3C1* methylation in *NR3C1*_1CpGU12 and *NR3C1*_2CpGU13, whereas childhood SLEs predicted higher *NR3C1* methylation in *NR3C1*_2CpGU14.

In the *post hoc* robustness check (Supplementary Table 7), we repeated the analyses for amplicon 2 exclusively. The significant association with single exposure to sexual abuse in the original sample failed to replicate in the additional sample.

DISCUSSION

In this study, experience of multiple SLEs and exposure to traumatic experiences between birth and adolescence were associated with higher *NR3C1* methylation rates in adolescents. In contrast with our initial expectation, we found that not perinatal or childhood stress, but rather SLEs in adolescence were associated with higher *NR3C1* methylation. To the best of our knowledge no other comparable studies have focused on the timing of stressful events when investigating DNA methylation. Our results on traumatic

youth experiences are consistent with prior studies [16, 20-22], with higher methylation rates in individuals who have experienced traumatic youth experiences.

The absence of a significant relationship between *NR3C1* methylation and perinatal stress in our sample appears to contrast with previous human studies that showed higher *NR3C1* methylation after exposure to perinatal stress [17-19]. *NR3C1* methylation in newborns was positively associated with depressed maternal mood in the third trimester of pregnancy [17]. In addition, war stress during pregnancy affected newborn *NR3C1*-methylation rates [19]. As these studies involve newborns, no statement could be made on the effect of stressful experiences on *NR3C1* methylation later in life. Radtke *et al* [18] and colleagues reported higher *NR3C1* methylation in 25 adolescents with maternal exposure to intimate partner violence during pregnancy. However, they did not consider the possible influences of more recent stressful experiences on *NR3C1* methylation of the adolescents.

Our timing analysis on SLEs indicates that *NR3C1* methylation was independently associated with SLEs in adolescence. Although this may not be very surprising given that adolescence is a significant neurodevelopmental stage, this is the first study to show that adolescent stress actually co-occurs with higher *NR3C1* methylation. Our study did not support the notion of a sensitive period to SLEs in childhood for *NR3C1* methylation in adolescents, despite suggestive findings in animal studies [15, 32, 41] and a study on long-term effects of adversities on cortisol stress response in our TRAILS participants [4]. However, in line with our results, a study in rodents did not report alterations in *NR3C1* methylation following early life stress [42], and others reported changes in *NR3C1* methylation following chronic and acute stress in adult rats [43]. Possibly, some epigenetic modifications by stress exposure may be short-term effects, which may allow for a more adaptive stress regulation. This may also explain the discrepancy between our study and those investigating methylation in newborns following maternal stress. Recent evidence on active removal of methyl groups [44, 45] gives rise to the possibility of dynamic regulation and reversibility of DNA methylation. Reversal of *NR3C1* methylation in adult rats was proven possible through pharmacological manipulation [46], but it is currently unknown if active demethylation can be triggered by environmental factors, - for example, positive events following a stressful early life.

We expected to find associations with stress in amplicon 2 in particular. This amplicon is identical to the one studied by McGowan *et al* [16], and covers the exon 1_F (analog to first exon 1_7 in rats [47]) promoter containing the transcription factor NGFI-A binding site. In rats and humans, DNA methylation inhibited binding of NGFI-A to its binding site, causing a reduction in transcriptional activity [16, 48]. However, most of the associations with stress measures were in amplicon 1, at the edge of the CpG-island (Figure 1). It is likely that the environment exerts its influence on other alternative first exon promoters spanning the CpG-island as well, since methylation is highly variable between individuals in these

promoters [49]. Further, the presence of other CpG-rich transcription factor binding sites may also play a role [49]. Recently, childhood abuse was related to methylation of other first exon promoters than the exon 1_F promoter in human post-mortem brain tissue [37].

The exploratory analyses on individual CpG units with relatively high methylation scores showed more pronounced relations between stress and methylation than the main analyses. Additionally, these analyses also showed higher methylation in a single CpG unit after childhood stress exposure. Differences between CpG units emphasize the need to understand which regions have a regulatory function and may be more responsive to environmental stimuli. In our *post hoc* robustness check on amplicon 2 the significant association with sexual abuse in the original sample failed to replicate. This may be due to the fewer individuals that have experienced a single exposure to sexual abuse in the replication sample or the initial association may have been due to chance.

It has to be noted that our assessment of SLEs differed between time periods because we aimed to measure age-appropriate SLEs for the different developmental stages of childhood and adolescence, and used different informants to optimize the reliability of event recall. As a consequence, some SLEs that were assessed in adolescence were not measured in childhood, including conflicts with family and friends, being bullied, sexual intimidation and loss or lack of friends. These added events in adolescence may have contributed to the difference in methylation rates we found for childhood and adolescence SLEs. As children lack the capacity to remember early childhood experiences, childhood SLEs were based on parent-reports. SLEs in adolescence were based on self-report, because the parents may no longer be aware of all aspects of their children's lives as they grow towards independence. Considering the nature of our study design and our desire to include as many relevant SLEs as possible, we could not fully overcome these dissimilarities but we do acknowledge that they warrant caution when interpreting the results. It is possible, for instance, that self-reported SLEs more closely reflect the actual stress levels experienced than parent-reported events. Furthermore, some SLEs that were assessed only in adolescence may be more chronic than the SLEs measured in childhood (e.g. sexual intimidation or lack/loss of friends) and thus have a more lasting influence on stress levels. Hence, although our findings suggest that SLEs in adolescence are independently associated with *NR3C1* methylation, we cannot completely exclude the possibility that this can be ascribed to specific SLEs measured in adolescence but not in childhood, or to informant differences.

Our study has several strengths: The TRAILS study provides data on *NR3C1* methylation in a large population based sample. Furthermore, we had a detailed account of SLEs between birth and adolescence. This study is the first to explore the effect of SLEs during childhood and adolescence separately. A limitation of the study was that blood was collected at T3 only, preventing analyses of changes in methylation. For this reason we could not establish any causal links between SLEs or trauma and methylation. Also, our

sample overrepresents adolescents with an increased risk of mental health problems. However, the use of sampling weights to reproduce the distribution in the total TRAILS sample [50] did not affect our results. In addition, unlike for the SLEs measure, no timing data were available for traumatic youth experiences. Another limitation is that our robustness check was only possible for amplicon 2.

Together, our findings add to the existing literature by showing that both SLEs and traumatic stress affect *NR3C1* methylation in adolescents. In addition, it is the first study to show epigenetic effects of stress experienced in adolescence.

REFERENCES

1. Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB. The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology* 2008; **33**(6)**:** 693-710.
2. Heim C, Newport DJ, Wagner D, Wilcox MM, Miller AH, Nemeroff CB. The role of early adverse experience and adulthood stress in the prediction of neuroendocrine stress reactivity in women: a multiple regression analysis. *Depress Anxiety* 2002; **15**(3)**:** 117-125.
3. Holsboer F. The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* 2000; **23**(5)**:** 477-501.
4. Bosch NM, Riese H, Reijneveld SA, Bakker MP, Verhulst FC, Ormel J *et al.* Timing matters: long term effects of adversities from prenatal period up to adolescence on adolescents' cortisol stress response. The TRAILS study. *Psychoneuroendocrinology* 2012; **37**(9)**:** 1439-1447.
5. Xiong F, Zhang L. Role of the hypothalamic-pituitary-adrenal axis in developmental programming of health and disease. *Front Neuroendocrinol* 2013; **34**(1)**:** 27-46.
6. Hunter RG, McEwen BS. Stress and anxiety across the lifespan: structural plasticity and epigenetic regulation. *Epigenomics* 2013; **5**(2)**:** 177-194.
7. Razin A, Cedar H. DNA methylation and gene expression. *Microbiol Rev* 1991; **55**(3)**:** 451-458.
8. Razin A. CpG methylation, chromatin structure and gene silencing-a three-way connection. *EMBO J* 1998; **17**(17)**:** 4905-4908.
9. Watt F, Molloy PL. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. *Genes Dev* 1988; **2**(9)**:** 1136-1143.
10. Bird AP, Wolffe AP. Methylation-induced repression--belts, braces, and chromatin. *Cell* 1999; **99**(5)**:** 451-454.
11. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* 2006; **31**(2)**:** 89-97.
12. Szyf M. The dynamic epigenome and its implications in toxicology. *Toxicol Sci* 2007; **100**(1)**:** 7-23.
13. Faulk C, Dolinoy DC. Timing is everything: the when and how of environmentally induced changes in the epigenome of animals. *Epigenetics* 2011; **6**(7)**:** 791-797.
14. Szyf M. The early life environment and the epigenome. *Biochim Biophys Acta* 2009; **1790**(9)**:** 878-885.
15. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR *et al.* Epigenetic programming by maternal behavior. *Nat Neurosci* 2004; **7**(8)**:** 847-854.
16. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2009; **12**(3)**:** 342-348.
17. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (*NR3C1*) and infant cortisol stress responses. *Epigenetics* 2008; **3**(2)**:** 97-106.
18. Radtke KM, Ruf M, Gunter HM, Dohrmann K, Schauer M, Meyer A *et al.* Transgenerational impact of intimate partner violence on methylation in the promoter of the glucocorticoid receptor. *Transl Psychiatry* 2011; **1:** e21.
19. Mulligan CJ, D'Errico NC, Stees J, Hughes DA. Methylation changes at *NR3C1* in newborns associate with maternal prenatal stress exposure and newborn birth weight. *Epigenetics* 2012; **7**(8)**:** 853-857.

20. Perroud N, Paoloni-Giacobino A, Prada P, Olie E, Salzmann A, Nicastro R *et al.* Increased methylation of glucocorticoid receptor gene (*NR3C1*) in adults with a history of childhood maltreatment: a link with the severity and type of trauma. *Transl Psychiatry* 2011; **1:** e59.
21. Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. *PLoS One* 2012; **7**(1)**:** e30148.
22. Perroud N, Dayer A, Piguet C, Nallet A, Favre S, Malafosse A *et al.* Childhood maltreatment and methylation of the glucocorticoid receptor gene *NR3C1* in bipolar disorder. *Br J Psychiatry* 2013**:** e-pub ahead of print 6 June 2013; doi: 2010.1192/bjp.bp.2112.120055.
23. de Rooij SR, Costello PM, Veenendaal MV, Lillycrop KA, Gluckman PD, Hanson MA *et al.* Associations between DNA methylation of a glucocorticoid receptor promoter and acute stress responses in a large healthy adult population are largely explained by lifestyle and educational differences. *Psychoneuroendocrinology* 2012; **37**(6)**:** 782-788.
24. Essex MJ, Boyce WT, Hertzman C, Lam LL, Armstrong JM, Neumann SM *et al.* Epigenetic vestiges of early developmental adversity: childhood stress exposure and DNA methylation in adolescence. *Child Dev* 2013; **84**(1)**:** 58-75.
25. Murgatroyd C, Spengler D. Epigenetics of early child development. *Front Psychiatry* 2011; **2:** 16.
26. Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* 2009; **10**(6)**:** 434-445.
27. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML *et al.* Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* 2005; **102**(30)**:** 10604-10609.
28. Talens RP, Christensen K, Putter H, Willemsen G, Christiansen L, Kremer D *et al.* Epigenetic variation during the adult lifespan: cross-sectional and longitudinal data on monozygotic twin pairs. *Aging Cell* 2012; **11**(4)**:** 694-703.
29. Wong CC, Caspi A, Williams B, Craig IW, Houts R, Ambler A *et al.* A longitudinal study of epigenetic variation in twins. *Epigenetics* 2010; **5**(6)**:** 516-526.
30. Veenema AH. Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: what can we learn from animal models? *Front Neuroendocrinol* 2009; **30**(4)**:** 497-518.
31. McGorry PD, Purcell R, Goldstone S, Amminger GP. Age of onset and timing of treatment for mental and substance use disorders: implications for preventive intervention strategies and models of care. *Curr Opin Psychiatry* 2011; **24**(4)**:** 301-306.
32. Murgatroyd C, Patchev AV, Wu Y, Micale V, Bockmuhl Y, Fischer D *et al.* Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nat Neurosci* 2009; **12**(12)**:** 1559-1566.
33. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, Huizink AC *et al.* Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. *Int J Epidemiol* 2008; **37**(6)**:** 1227-1235.
34. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, Veenstra R *et al.* The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. *J Am Acad Child Adolesc Psychiatry* 2012; **51**(10)**:** 1020-1036.
35. Reijneveld SA, Brugman E, Verhulst FC, Verloove-Vanhorick SP. Identification and management of psychosocial problems among toddlers in Dutch preventive child health care. *Arch Pediatr Adolesc Med* 2004; **158**(8)**:** 811-817.
36. Caspi A, Moffitt TE, Thornton A, Freedman D, Amell JW, Harrington H *et al.* The life history calendar: A research and clinical assessment method for collecting retrospective event-history data. *Int J Methods Psychiatr Res* 1996; **6**(2)**:** 101-114.

37. Labonte B, Yerko V, Gross J, Mechawar N, Meaney MJ, Szyf M *et al.* Differential glucocorticoid receptor exon 1(B), 1(C), and 1(H) expression and methylation in suicide completers with a history of childhood abuse. *Biol Psychiatry* 2012; **72**(1)**:** 41-48.
38. Turner JD, Alt SR, Cao L, Vernocchi S, Trifonova S, Battello N *et al.* Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. *Biochem Pharmacol* 2010; **80**(12)**:** 1860-1868.
39. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3)**:** 1215.
40. Warnecke PM, Stirzaker C, Melki JR, Millar DS, Paul CL, Clark SJ. Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. *Nucleic Acids Res* 1997; **25**(21)**:** 4422-4426.
41. Murgatroyd C, Wu Y, Bockmuhl Y, Spengler D. Genes learn from stress: How infantile trauma programs us for depression. *Epigenetics* 2010; **5**(3).
42. Daniels WM, Fairbairn LR, van Tilburg G, McEvoy CR, Zigmond MJ, Russell VA *et al.* Maternal separation alters nerve growth factor and corticosterone levels but not the DNA methylation status of the exon 1(7) glucocorticoid receptor promoter region. *Metab Brain Dis* 2009; **24**(4)**:** 615-627.
43. Witzmann SR, Turner JD, Meriaux SB, Meijer OC, Muller CP. Epigenetic regulation of the glucocorticoid receptor promoter 1(7) in adult rats. *Epigenetics* 2012; **7**(11)**:** 1290-1301.
44. Branco MR, Ficz G, Reik W. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. *Nat Rev Genet* 2012; **13**(1)**:** 7-13.
45. Bhutani N, Burns DM, Blau HM. DNA demethylation dynamics. *Cell* 2011; **146**(6)**:** 866-872.
46. Weaver IC, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ *et al.* Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. *J Neurosci* 2005; **25**(47)**:** 11045-11054.
47. Turner JD, Schote AB, Macedo JA, Pelascini LP, Muller CP. Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage? *Biochem Pharmacol* 2006; **72**(11)**:** 1529-1537.
48. Weaver IC, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S *et al.* The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. *J Neurosci* 2007; **27**(7)**:** 1756-1768.
49. Turner JD, Pelascini LP, Macedo JA, Muller CP. Highly individual methylation patterns of alternative glucocorticoid receptor promoters suggest individualized epigenetic regulatory mechanisms. *Nucleic Acids Res* 2008; **36**(22)**:** 7207-7218.
50. Oldehinkel AJ, Ormel J, Bosch NM, Bouma EM, Van Roon AM, Rosmalen JG *et al.* Stressed out? Associations between perceived and physiological stress responses in adolescents: the TRAILS study. *Psychophysiology* 2011; **48**(4)**:** 441-452.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. The number of traumatic events experienced for subjects in each of the LPA categories.

		LPA category SLEs		
		Childhood	**Adolescence**	**Overall low**
Sexual abuse	**no**	74 (18.0%)	36 (8.8%)	300 (73.2%)
	single	3 (13.6%)	6 (27.3%)	13 (59.1%)
	repeated	3 (25.0%)	2 (16.7%)	7 (58.3%)
Physical abuse	**no**	46 (18.0%)	21 (8.2%)	189 (73.8%)
	single	30 (17.3%)	19 (11.0%)	124 (71.7%)
	repeated	4 (26.7%)	4 (26.7%)	7 (46.7%)
Other trauma	**no**	54 (16.7%)	29 (9.0%)	241 (74.4%)
	single	19 (20.9%)	8 (8.8%)	64 (70.3%)
	repeated	7 (24.1%)	7 (24.1%)	15 (51.7%)

Note: Displayed percentages reflect the proportion of subjects who experienced no, single or repeated trauma over the three LPA categories. See method section for details on the 'other trauma' category.

***NR3C1*_1**

a|a|aggggcc|actt|ag|a|a|acctc|AGG**CGCG**[1]|a|att|a|ac|a|ac|a|a|A**CG**CT[2]|a|a|ag|agc|a|AGCCCTTG**CG**GGG**CG**GGGGTGG[3]|ag|a|ag|ag|a|a|a|a|a|AGTG**CG**[4]|aggtt|a|a|a|ag|ag|a|agt|A**CG**TCC[5]|ag|acctgttg|AGTTCTCTCTC**CG**[6]|ac|A**CG**CCC[7]|acttct|a|ac|ag|at|a|A**CG**C**CG**GCCC**CG**GC**CG**C[8]|agtctcc|a|AGTTG**CG**GGCTGTC[9]|AGCCCCC**CGCG**TGTGC[10]|accctc|A**CGCG**CCC**CG**C[11]|**ACG**CCCTCCTC[12]|a|agcc|AGG**CG**C**CG**C**CG**GGGCCTCCC**CG**G[13]|AGCC**CG**GGCT**CGCG**CT**CG**GG**CGCG**C**CG**GGGTGG**CG**TGC[14]|a|a|at|ATT**CG**GG**C G**[15]|agt|a|a|a|attc|ag|A**CGCG**GCTT[16]|AG**CG**TTC[17]|acc|A**CG**[18]|a|a|a|A**CG**GGTGT**CG**GG**CG**[19]|acccccttgg|aggg|a|a|agggg|ac|act|aggggg|ag|a|a|a|ag|aggcc|agg|attcc

***NR3C1*_2:**

tccctg|a|agcctcccc|ag|AGGG**CG**TGTC[1]|AGGC**CG**CC**CG**GCCC**CG**[2]|AG**CGCG**GC**CG**[3]|ag|A**CG**CTG**CG**GC[4]|AC**CG**TTTC**CG**TGC[5]|a|ACCC**CG**T[6]|AGCCCCTTT**CG**[7]|a|agtg|ac|ac|acttc|A**CG**C[8]|a|ACT**CG**GCC**CG**GC**CG**GC**CG**C**CG**C**CG**GGCC[9]|actc|A**CG**C[10]|agctc|AG C**CGCG**GG[11]|AGG**CG**CCC**CG**GCTCTTGTGGCC**CG**CC**CG**CTGTC[12]|ACC**CG**C[13]|aggggc|ACTGG**CG**G**CG**CTTGC**CG**CC[14]|a|aggggc|ag|AG**CG**[15]|AGCTCC**CG**[16]|agtgggtctgg|AGC**CGCG**G[17]|AGCTGG<u>G**CG**GGGG**CG**</u>GG[18]|a|agg|aggt|AG**CG**[19]|ag|a|a|a|ag|a|a|actgg|ag|a|a|act

***NR3C1*_3**

cccctc|agtcc|a|agggg|a|aggg|a|ACT**CG**TGGTC**CG**TCCTG[1]|ag|a|a|agg|ag|AGGGC**CG**TGGGG**CG**[2]|AGGGGTGCC**CG**TG**CG**GG[3]|a|AGCCCC**CG**CCCC[4]|agctcccttcccc|AGCT**CG**C**CGCG**T**CG**GG[5]|a|aggcttgg|A**CG**[6]|ATGC**CG**GG[7]|AC**CG**[8]|agcctcct|ACCTTGTG**CG**GC[9]|ac|ag|att|atg|atttttgtg|actctggg|a|a|aggttgtgctgtgtgggttt|agggtttgggg|aggt|a|ACTTTTG**CG**CCCCC[10]|ac|aggtg|ac|AT**CG** CTTGCC[11]|agctcctg|ac|A**CG**GG**CG**GGGGCTGC**CG**C[12]|agctcc|acct|a|ATCCTGCT**CG**GG**CG**CT**CG**GCC[13]|ac|agcc|actctctc|ACCTC C**CG**C**CGCG**CTC[14]|ag|actg|A**CG**G**CG**GCTCCCCCTGCTCTG[15]|ac|atcttg|a|ag

Supplementary Figure 1. CpG unit information for *NR3C1* amplicons 1, 2 and 3. Vertical lines represent position of splice sites on complementary RNA strand (not shown). CpG units are numbered and shown in uppercase. Methylation rates for gray CpG units could not be obtained or used for analyses. This could be due to equality in mass with another fragment or a mass outside the detection range of the mass spectrometer. Also, CpG units with >25% missing methylation values are left out of the analyses. Bold letters indicate CpG dinucleotides and the underlined sequence represents the human NGFI-A core recognition motif. Note: sequence shown is non-bisulfite treated. PCR product of *NR3C1*_2 is on the bisulfite treated antisense chain. PCR products of *NR3C1*_1 and *NR3C1*_3 are on the bisulfite treated sense chain. CpG units are numbered from 5′-3′.

Supplementary Table 2. Primer properties.

Name	Primer sequence[1]	Size (bp)	Position[2]	Ta (°C)	Units covered	CpGs covered
*NR3C1*_1	**F**: AAAGGGGTTATTTAGAAATTTTAGG **R**: AAAATCCTAACCTCTTTTCTCCCC	427	Chr5: 142782046-142782472	59	19 (11)	41 (22)
*NR3C1*_2	**F:** TTTTTGAAGTTTTTTTAGAGGG **R:** AATTTCTCCAATTTCTTTTCTC	322	Chr5: 142783585-142783906	63-54[3]	19 (10)	39 (19)
*NR3C1*_3	**F:** TTTTTAGTTTAAGGGGAAGGGAAT **R:** CTTCAAAATATCAAAACAAAAAAAACC	392	Chr5: 142784559-142784950	56	15 (9)	28 (20)

Note: [1]Primers are extended with tags according to Sequenom protocol (forward primers contained a 10 mer sequence tag (aggaagagag) to balance primer length, and reverse primers are equipped with a T7-promoter tag (cagtaatacgactcactataggg) and an 8 base pair insert to prevent abortive cycling (agaaggct) for initiation of *in vitro* transcription. [2]Position according to the UCSC human Feb. 2009 assembly (GRCh37/hg19). [3]Touchdown PCR. Ta: annealing temperature.

Supplementary Table 3. Amplification conditions

PCR conditions *NR3C1*_1 and *NR3C1*_3:			
1.	94°C	15 min	
2.	94°C	20 sec	45 cycles
	Annealing temp.	30 sec	
	72°C	1 min	
3.	72°C	3 min	
4.	4°C	5 min	
5.	15°C	Forever	

PCR conditions *NR3C1*_2:			
Touchdown: every cycle 1°C from 63°C to 54°C.			
1.	94°C	15 min	
2.	94°C	30 sec	10 cycles
	63-54°C.	30 sec	
	72°C	1 min	
3.	94°C	30 sec	35 cycles
	53°C.	30 sec	
	72°C	1 min	
4.	72°C	5 min	
5.	4°C	Forever	

Supplementary Table 4. Mean methylation, SD and range of individual CpG units within the three *NR3C1* amplicons.

		Methylation (%)			
		N	**mean**	**SD**	**Range**
*NR3C1*_1	CpGU3	441	4.62	1.65	0.00 - 8.50
	CpGU6	451	0.03	0.26	0.00 - 3.33
	CpGU8	453	1.58	0.75	0.00 - 4.50
	CpGU9	454	3.07	0.73	1.67 - 7.50
	CpGU10	454	2.51	1.16	0.50 - 6.67
	CpGU11	454	2.90	0.87	0.50 - 7.67
	CpGU12	453	9.89	2.45	2.00 - 16.00
	CpGU15	454	2.81	0.79	0.33 - 6.33
	CpGU16	454	0.96	0.63	0.00 - 6.33
	CpGU17	454	0.84	0.90	0.00 - 6.00
	CpGU19	454	0.21	0.33	0.00 - 2.00
*NR3C1*_2	CpGU2	450	1.35	0.57	0.00 - 4.00
	CpGU4	450	1.10	0.97	0.00 - 6.00
	CpGU5	450	2.04	0.84	0.00 - 5.67
	CpGU6	450	1.87	0.95	0.00 - 6.00
	CpGU7	449	2.32	1.27	0.00 - 8.33
	CpGU11	450	0.86	0.77	0.00 - 6.50
	CpGU13	395	12.23	4.20	4.00 - 27.00
	CpGU14	449	8.48	1.95	2.00 - 14.67
	CpGU17	449	0.83	1.19	0.00 - 9.33
	CpGU18	448	4.10	1.59	1.67 - 12.33
*NR3C1*_3	CpGU1	407	2.51	1.03	0.00 - 8.67
	CpGU2	406	3.64	1.58	1.00 - 11.00
	CpGU4	410	2.22	1.50	0.00 - 8.50
	CpGU5	404	4.62	1.91	1.00 - 11.00
	CpGU9	408	0.59	0.98	0.00 - 11.50
	CpGU10	412	0.37	0.67	0.00 - 5.00
	CpGU11	410	1.93	1.30	0.00 - 7.33
	CpGU12	404	2.10	1.36	0.00 - 10.00
	CpGU15	397	3.18	1.24	0.00 - 7.67

Note: *NR3C1*_1-3: amplicons within the *NR3C1* CpG island. CpGU: CpG unit, fragment of DNA containing one or more CpG dinucleotides. For CpG unit sequence information, see Supplementary Figure 1.

Supplementary Table 5. Correlations between stress measures.

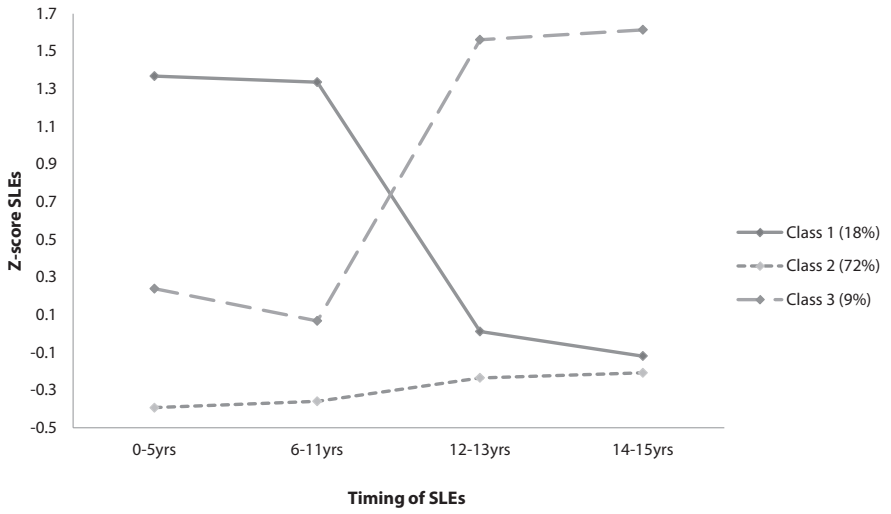
		SLEs 1				Trauma type		
		0-5	6-11	12-13	14-15	Sexual	Physical	Other
SLEs1	Perinatal stress	0.21**	0.13**	0.11*	0.08	-0.02	0.07	0.03
	0-5		0.49**	0.14**	0.09*	-0.01	0.05	0.07
	6-11			0.11*	0.03	0.13**	0.06	0.05
	12-13				0.34**	0.17**	0.13*	0.17**
	14-15					0.05	0.20**	0.13**
	0-15					0.12*	0.15**	0.14**

Note: Pearson or Spearman correlations used where appropriate. [1]Z-score, **p<.01, *p<.05.

Supplementary Table 6. Latent profile modeling of stress at different ages model fit statistics.

Classes	BIC	LMR-LRT 2LL	LMR-LRT p-value
1	5386.00	n.a.	n.a.
2	5211.91	198.47	0.02
3	5122.10	116.84	0.006
4	5078.80	71.78	0.33

Note: BIC = Bayesian Information Criterion; LMR-LRT = Lo-Mendell-Rubin likelihood ratio test; 2LL = 2 times the Loglikelihood Difference; n.a. = not applicable.



Supplementary Figure 2. Latent Profile Analyses.

Supplementary Table 7. Life stress and *NR3C1* methylation rates in amplicon 2 for the original and replication sample.

	Original sample				Replication sample			
	N	**B**	**SE**	**p**	**N**	**B**	**SE**	**p**
Perinatal stress		-0.01	0.02	.78		-0.04	0.02	.10
Traumatic youth experiences[2]								
Sexual abuse	*389*				*380*			
single exposure	*21*	**0.44**	**0.12**	**<.001**	*15*	-0.05	0.13	.67
repeated exposure	*11*	0.13	0.17	.45	*14*	-0.16	0.13	.22
Physical abuse	*247*				*265*			
single exposure	*160*	0.02	0.06	.68	*137*	0.01	0.05	.79
repeated exposure	*14*	-0.09	0.15	.56	*8*	0.08	0.17	.66
Other trauma	*308*				*319*			
single exposure	*85*	-0.04	0.07	.52	*75*	0.10	0.06	.10
repeated exposure	*28*	0.07	0.11	.56	*16*	0.11	0.12	.35
Stressful life events[1]								
Total (0-15 yrs)		0.03	0.03	.25		-0.01	0.02	.72
Childhood (0-11 yrs)		0.00	0.03	.97		0.01	0.02	.68
Adolescence (12-15 yrs)		0.04	0.03	.13		-0.03	0.02	.28

Note: Linear regression of early life stress and multivariate regression analyses of SLEs in two age categories on *NR3C1* methylation scores. Bold numbers indicate significant results. B=regression coefficient; SE=standard error. [1] Z-scores, [2] No exposure is the reference category

Chapter 3

Adverse life events and allele-specific methylation of the serotonin transporter gene (*SLC6A4*) in adolescents. The TRAILS study

L.J. van der Knaap
H. Riese
J.J. Hudziak
M.M.P.J. Verbiest
F.C. Verhulst
A.J. Oldehinkel
F.V.A. van Oort

Psychosomatic Medicine, 2015, 77(3), 246-255.

ABSTRACT

Objectives: Adverse life events increase vulnerability to affective disorders later in life, possibly mediated by methylation of the serotonin transporter gene (*SLC6A4*). We investigated the relationship of *SLC6A4* methylation with various types of adversity (perinatal adversity, traumatic youth experiences and stressful life events [SLEs]), as well as with the timing of SLEs (during childhood [0–11 years] or during adolescence [12–15 years]). In addition, we investigated whether different serotonin-transporter-linked polymorphic region genotypes were equally sensitive to SLE-related methylation.

Methods: In a population sample of 939 adolescents (mean age=16.2 years), we assessed *SLC6A4* methylation, *SLC6A4* functionality (serotonin-transporter-linked polymorphic region "long" and "short" alleles, and rs25531), and adverse life events.

Results: Only a higher number of SLEs was positively associated with higher *SLC6A4* methylation (B=0.11, p=.011). Adolescent SLEs were associated with higher *SLC6A4* methylation (B= 0.13, p= .004) independently of childhood SLEs (B=0.02, p=.57). L-allele homozygotes showed a greater impact of SLEs on methylation (B=0.37, p<.001) than did s-allele carriers (B=0.04, p=.66), resulting in higher levels of *SLC6A4* methylation for l-allele homozygotes among those experiencing high levels of SLEs.

Conclusions: Our findings demonstrate a higher level of *SLC6A4* methylation after SLEs in adolescents, with a more pronounced association for SLEs during adolescence than during childhood. Considering the allele-specific sensitivity of *SLC6A4* methylation to SLEs, this study may help clarify the role of *SLC6A4* in the development of affective disorders.

INTRODUCTION

Adverse life events increase vulnerability to developing affective disorders later in life. This process is presumably moderated by epigenetic modifications such as deoxyribonucleic acid (DNA) methylation, which are known to be influenced by environmental factors [1]. The addition of methyl groups occurs predominantly on cytosine-guanine dinucleotide combinations (CpGs); it affects transcriptional activity, generally reducing gene expression [2]. Previous studies have shown that stressful experiences can alter HPA-axis function by changing the epigenetic signature of stress-reactivity genes (e.g. the glucocorticoid receptor gene, *NR3C1*) [3-5], possibly increasing the risk of developing affective disorders [6]. Recently, Zhao and colleagues reported differences in methylation levels of the serotonin transporter (5HTT) gene (*SLC6A4*) in monozygotic twins discordant for depressive symptoms. These differences were attributed to unique environmental factors[7]. Alterations in serotonergic neurotransmission have been associated with altered neurodevelopment and the incidence of various psychiatric disorders [8, 9]. A genetic polymorphism in *SLC6A4* was also shown to interact with stressful life events (SLEs) in moderating the risk for depression [10]. Despite this, it is only recently that research on epigenetic modifications of genes involved in serotonergic neurotransmission following stress have attracted interest.

The serotonin transporter is an important regulator of serotonergic neurotransmission through the reuptake of serotonin (5-hydroxtryptamine, or 5HT) in brain synapses. Genetic variation of this gene, such as the 5HT-transporter-linked polymorphic region (*5HTTLPR*), has been associated, albeit not consistently[11], with an increased risk for psychopathology [12, 13]. *5HTTLPR* alleles are composed predominantly of short ("s") or long ("l") repeated elements and vary in their transcriptional activity, resulting in levels of *SLC6A4* mRNA that are lower in s-allele carriers than in l-allele carriers [14, 15]. The *5HTTLPR* polymorphism accounts for only part of the variation in mRNA expression [16-18]; higher levels of *SLC6A4* methylation have also been associated with reduced expression [18-20] and liability to depression [7, 20-22].

Few studies have investigated the effect of adverse life events on *SLC6A4* methylation in humans. Decreased *SLC6A4* methylation was observed in 82 infants exposed to second-trimester depressed maternal mood [23]. In other studies, childhood abuse [24-26], childhood adversities [21] and bullying victimization in childhood [27] were associated with higher methylation levels in children or adults. A recent study on stressful work environments in adulthood found lower *SLC6A4* methylation in a highly stressful environment [28]. In infant macaques, no effect of early life stress was found on *SLC6A4* methylation [19, 29]. However, sample sizes in these human and animal studies were relatively small.

Methylation levels have been shown to differ between *5HTTLPR* genotypes: a lower level of methylation has been reported for carriers of the l-allele [18-20]. These studies did

not investigate exposure to adverse life events, although it is important to know whether different genotypes are equally sensitive to be methylated after adverse life events [30]. Thus far, no interaction has been found between rearing type (nursery reared or mother reared) and genotype on *SLC6A4* methylation in macaques [19]. Similarly, in humans no interaction was found between work stress and *5HTTLPR* genotype on *SLC6A4* methylation in female healthcare professionals [28] and between sexual abuse and *5HTTLPR* genotype in four functional *SLC6A4* CpG residues in women [26]. Again, since the sample sizes in these three studies were small, investigation in a larger sample is necessary.

The objective of the current study was to investigate the effects of adverse life events between birth and adolescence on *SLC6A4* methylation in a large, representative, sample of adolescents. To investigate their relationship with *SLC6A4* methylation, we incorporated different types of adverse life events; perinatal adversity, stressful life events (SLEs) during childhood and adolescence, and traumatic youth experiences (TYEs). The effect of perinatal adversity on *SLC6A4* methylation in adolescence has never previously been examined. We are also the first to include SLEs experienced in both childhood and adolescence. This allowed us to analyze the independent associations of SLEs experienced during these two periods of life. Finally, we also examined allele-specific sensitivity for *SLC6A4* methylation in relation to SLEs.

METHODS AND MATERIALS

Sample selection

This study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective population study of Dutch adolescents, who have been followed from preadolescence into adulthood. Assessment waves are conducted biennially or triennially, and five assessment waves have been completed so far. Written consent was obtained from each subject and their parents at every assessment wave. The present study involves data collected during the first four assessment waves: T1, 2001-2002, N=2230, mean (standard deviation [SD]) age 11.1 (0.55) years; T2, 2003-2004, N=2149, mean (SD) age 13.6 (0.53) years; T3, 2005-2007, N=1816, mean (SD) age 16.3 (0.71) years; and T4, 2008-2010, N=1881, mean (SD) age 19.1 (0.60) years. The study was approved by the Dutch Central Medical Ethics Committee and subjects received compensation for their participation. A detailed description of sampling and methods can be found in Huisman *et al* [31] and Ormel *et al* [32]. The assessment at T3 included a blood draw (response N=1230). DNA was successfully isolated from blood for 1156 respondents. We excluded subjects with non-Dutch ethnicity (N=117). Next, samples with an insufficient DNA concentration were removed (N=79), and one of each sibling pair was randomly excluded (N=6). Few other samples could not be analyzed (N=15). In total, 939 subjects remained eligible for analysis. Adolescents who

had undergone venipuncture differed from adolescents who had not on sex (53.5% vs. 47.4% females), $X^2(1, 2230)=8.18$, p=.004), socioeconomic status (19.4% lowest, 49.3% middle and 31.4% highest class vs. 32.8% lowest, 49.9% middle and 17.3% highest, $X^2(2, 2188)=80.72$, p<.001) and age (16.2 years vs. 16.4 years, t(1817)=6.99, p<.001). The groups did not differ on measures of childhood and adolescence SLEs or perinatal adversity. At T4, responders differed from non-responders in regards to sex (54.7% vs. 37.8% females, p<.001) and age (mean age 19.0 vs. 19.4, p<.001).

Adverse life event measures

Perinatal adversity was operationalized as the sum of maternal psychological problems during pregnancy or the three months after delivery, preterm delivery (≤33 weeks), low birth weight (≤2500g), hospitalization of mother or child within one month after delivery, and maternal alcohol use or smoking during pregnancy. For birth weight and gestational age, we used the records of the Preventive Child Healthcare services [33]. The other stressors were measured in a detailed interview with the parents at T1.

SLEs experienced between ages 0 and 15 years were assessed for the age categories of 0-5, 6-11, 12-13 and 14-15 years, as described by Bosch *et al* [34]. Information on SLEs in *early childhood (0-5) and middle childhood (6-11)* was collected during a detailed interview with the parents at T1, and included the number of times the child had experienced parental divorce, hospitalization, the death of a family member or friend, out-of-home placement, parental addiction or parental mental health problems [34]. SLEs experienced in *early adolescence (12-13)* were assessed with a self-report questionnaire at T2 [34]. The 25 SLEs included illness or injury of the participant, a family member or a friend; psychopathology of family members; parental divorce; death of family member or friend; changes in family composition; parental unemployment; conflicts with family or friends; and bullying. SLEs in *middle adolescence (14-15)* were assessed at T3 in an Event History Calendar Interview[35] with the adolescent. The total number of events referred to the frequency of conflicts with family or friends, physical or sexual intimidation, bullying/gossiping, loss or lack of friends, mental health problems /addiction problems of family or friends, out-of-home placement, running away from home, death/sickness of a family member, hospitalization of participant, and parental divorce.

Based on the above-mentioned event measures, we calculated a measure of total SLEs experienced between ages 0 and 15 years by standardizing the sum score of the number of events for each age category, and summing the standardized scores. This procedure was chosen to account for differences in the number of possible SLEs by age group. The resulting total sum score (SLEs 0-15) was standardized before analyses. The correlation coefficient between the 0-5 and 6-11 age categories was .48 (p<.001), and the correlation coefficient between the 12-13 and 14-15 age categories was .23 (p<.001). Other correlations between SLE categories were <.15 (.001<p<.050). We used one measure for child-

hood SLEs and one for SLEs in adolescence. These variables were constructed by summing standardized scores for the two variables of childhood SLEs and the two variables of SLEs in adolescence. The SLE scores (0-11 and 12-15) were standardized before analyses.

At T4, information on *TYEs*, i.e. sexual abuse, physical abuse and other traumatic youth experiences, was obtained with a 16-item self-report questionnaire designed for TRAILS (available upon request) and inspired by the Childhood Trauma Questionnaire [36]. To determine sexual abuse, the participants were asked if an adult family member, friend of the family or stranger had ever, before the age of 16, showed their genitals or masturbated in front of them; had sexually assaulted them; had forced them to touch them in a sexual manner; had attempted to have sexual contact or had actually had sexual contact with them. To determine physical abuse, participants were asked if a parent or caretaker had ever, before the age of 16, hit them with a belt, brush, stick or other hard object; had hit them with a fist or kicked them very hard; had shaken or pinched them; had beaten them up (i.e., hit them in succession) or had threatened them with a knife or other weapon. To determine 'other trauma', participants were asked if, before the age of 16, they had been involved in a life-threatening accident; had witnessed severe injury or death; had been a victim of physical violence or assault; had been threatened with a weapon, had been held captive or abducted; or had been involved in a fire, flood or other (natural) disaster. Answers were coded into a single exposure or multiple exposures to TYEs. We had no data on the timing of TYEs.

DNA methylation

Analysis. DNA was extracted from whole-blood samples using a manual salting-out procedure as described by Miller *et al* [37]. Using a primer set previously used by Philibert *et al* [20] (set B), we analyzed a genomic region that encompasses the CpG island surrounding exon 1 (Figure 1). We used forward primer GGTTATTTAGAGATTAGATTATGTGAGGGT and reverse primer CCTACAACAATAAACAAAAAAACCCC (Chr17:28562358–28562783, UCSC build (GRCh37/hg19), Feb. 2009, see Figure S1 for sequence details). Forward primers contained a 10 mer sequence tag (aggaagagag), and reverse primers are equipped with a T7-promoter tag (cagtaatacgactcactataggg) and an 8 base pair insert (agaaggct). DNA methylation levels were analyzed using the EpiTYPER method from Sequenom. Bisulfite conversion was followed by PCR amplification, reverse transcription, base-specific cleavage of in vitro transcribed RNA product, and mass spectrometry (Sequenom EpiTYPER, San Diego, CA, USA). Bisulfite conversion of DNA was performed using EZ-96 DNA Methylation Kit (Shallow; Zymo Research, CA, USA), according to manufacturers' protocol. PCR, reverse transcription, cleavage and mass spectrometry were performed in triplicate, according to EpiTYPER protocol. The mass signal patterns generated are translated to quantitative methylation levels for different CpG units by the MassARRAY EpiTYPER analyzer software from Sequenom (v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA).

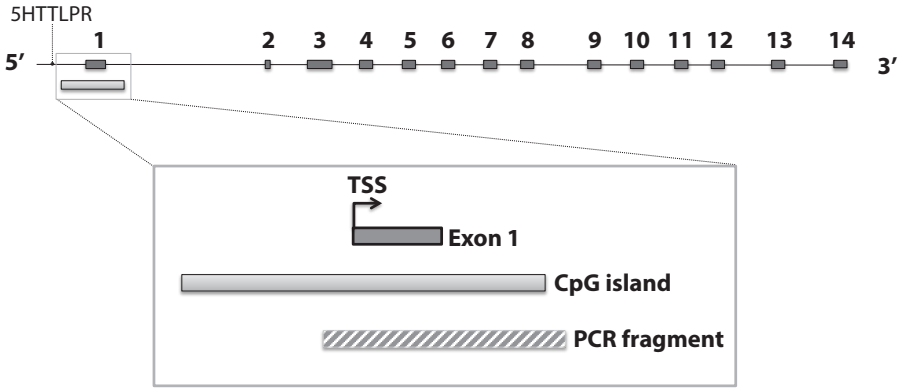


Figure 1. Schematic representation of the *SLC6A4* gene. Exons are represented by numbered black boxes. Approximate position of *5HTTLPR* is shown. The CpG island (Chr17:28562388-28563186) is represented by the gray box and is enlarged to show the positioning of the PCR fragment (Chr17:28562358-28562783) and their position relative to the TSS in exon 1 (based on Ensembl Transcript ID ENST00000394821). *5HTTLPR* is located outside the PCR fragment. *SLC6A4* = solute carrier family 6 (neurotransmitter transporter), member 4; *5HTTLPR* = serotonin-transporter–linked polymorphic region; CpG = cytosine-phosphateguanine; PCR = polymerase chain reaction; TSS = transcription start site.

Data cleaning. All samples were analyzed in triplicate, and samples with a standard deviation of ≥10% between replicates were removed for analysis. Fragments with CpG dinucleotides are referred to as CpG units. One CpG unit can contain one or more CpG dinucleotide. CpG units with a mass outside the range of the mass spectrometer, or with overlap in mass of another CpG unit, could not be analyzed (9 CpG units). We accounted for mass-change in CpG units by SNPs (only when the minor allele frequency >5%) by removing CpG units from analyses containing the SNP and from units with overlapping mass caused by SNPs in non-CpG units (1 CpG unit). For each CpG unit, methylation scores of the triplicates were averaged. CpG units with >25% missing values were removed (1CpG unit).

Genotyping

To determine the 5-HTT-linked-polymorphic-region genotype in the promoter region of *SLC6A4* we used sequence length analysis. The length assessment of the *5HTTLPR* alleles was measured by direct analysis on an automated capillary sequencer (ABI3730, Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). The call rate was 91.6%. The single nucleotide substitution (A>G) present in the *5HTTLPR* l-allele (rs25531) was genotyped using a custom made TaqMan assay (Applied Biosystems). Additional information is described in Nederhof *et al* [38]. The call rate for rs25531 was 96.5%. Concordance between DNA replicates showed an accuracy of 100%. Because the l_g-allele is considered functionally equivalent to the s-allele [39], it was recoded as an s-allele, and the l_a-allele was recoded as an l-allele. Genotype frequencies for *5HTTLPR* (L/L = 324, L/S = 455, S/S = 148) and rs25531 (A/A = 245, A/G = 473, G/G = 209) were in Hardy-Weinberg equilibrium

($\chi2$=0.31 [df = 1, p= 0.58] and $\chi2$= 0.45 [df = 1, p= 0.50], respectively). Allele frequencies were L = .59 and S = .41 for *5HTTLPR*, and A = .52 and G = .48 for rs25531. After recoding, new genotype frequencies, S/S (including S/Lg and Lg/ Lg) = 209, S/L (including Lg/ La)=473 and L/L (La/La only)=245 were still in Hardy-Weinberg equilibrium ($\chi2$=0.45, df = 1, p= 0.50), with allele frequencies for L=.52 and S=.48.

Covariates

We have explored the potential confounding of the analyses by age, daily smoking, oral contraceptive use, medication use, pubertal status and acute infection. Smoking habits, oral contraceptive use, pubertal status and medication use were measured in a self-report questionnaire. Pubertal development was measured using the pubertal development scale (PDS [40]) and was recoded into Tanner stages using the Shirtcliff method [41]. Only one of the respondents used fluoxetine (a selective serotonin re-uptake inhibitor), and removing this individual from the sample did not affect our results. As marker for acute infection we used high sensitive C-reactive protein (hsCRP). HsCRP was assessed in blood collected at the same blood draw as the blood for methylation analyses. The serum was transported to the laboratory for processing within four hours. On the same day, hsCRP was determined using a immunonephelometric method using a BN2, Siemens *Cardio*PhaseR hsCRP, with a lower detection limit of 0.175 mg/L. Intra-assay coefficients of variance ranged from 2.1 to 4.4, and inter-assay coefficients of variation ranged from 1.1 to 4.0.

Statistical analyses

Linear regression analyses were performed to examine the associations of type and timing of adverse life events with *SLC6A4* methylation in adolescence. Since randomly missing methylation values in higher methylated units (methylation values for individual units are presented in Table S1) may falsely lower the average methylation score, we opted to mean-center our methylation data to reduce bias in individual CpG unit methylation levels, while maintaining the individual variation in CpG units. We mean-centered the methylation score for each CpG unit, resulting in a mean methylation of 0, with original SD. An average *SLC6A4* methylation score was calculated by taking the average of the mean-centered methylation scores of the CpG units. Separate analyses were performed for perinatal adversity, SLEs (0-15) and the three categories of TYEs (sexual abuse, physical abuse, and other trauma). To study the relationship between childhood and adolescence SLEs and *SLC6A4* methylation, we performed a linear regression analysis with the two SLEs variables (0-11 and 12-15). Analyses were rerun with the putative confounders. Differences in methylation levels between genotypes were tested with ANOVA, and sex differences in methylation levels were tested with a T-test. We explored allele-specific methylation by adding *5HTTLPR* genotype (using s/s genotype as reference group) and an interaction term of genotype with SLE (0-15) to the

regression model with *SLC6A4* methylation as dependent variable. In case of a significant interaction term, analyses were stratified by genotype. All analyses were adjusted for sex. Statistical tests were performed in SPSS (IBM SPSS, v.21.0. Armonk, NY: IBM Corp). A p-value <.050 was considered statistically significant.

RESULTS

The sample consisted of 51.8% women and the average (SD) age of the adolescents was 16.2 (0.65) years at the time of DNA collection, with a range of 14-18 years. In Table 1 the descriptive statistics are given of the adverse life events in the total sample, and in the sample stratified by *5HTTLPR* genotype. In Figure 2 the centered *SLC6A4* methylation levels by genotype and sex are shown. *SLC6A4* methylation was higher in girls (N=486, mean [SD]=0.43% [1.27%]) than in boys (N=453, mean [SD]=-0.49% [1.27%]; t(937)=11.137, p<.001).

Table 1. Descriptives of adverse life event variables, by *5HTTLPR* genotype.

	Total	***5HTTLPR* genotype (N)**						
		s/s		**s/l**		**l/l**		
	N	**N**	**% or M(SD)**	**N**	**% or M(SD)**	**N**	**% or M(SD)**	**p**
***Perinatal adversity*[a]**	931	208	0.01(1.01)	467	-0.02 (0.97)	244	0.03 (1.08)	.81
***SLEs (0-15 years)*[a]**								
Total (0-15 years)	859	195	-0.03 (1.00)	432	0.04 (1.03)	222	-0.08 (0.92)	.29
Childhood (0-11 years)	939	209	-0.06(0.97)	473	0.01 (1.00)	245	0.03 (1.03)	.57
Adolescence (12-15 years)	859	195	0.03 (1.04)	432	0.05 (1.03)	222	-0.15 (0.84)	.045
***TYEs*[b]**								
Sexual abuse								
no exposure	795	184	93.4	388	91.3	213	93.4	
single exposure	38	7	3.6	20	4.7	10	4.4	.73
repeated exposure	28	6	3.0	17	4.0	5	2.2	
Physical abuse								
no exposure	529	133	67.5	258	60.7	134	58.8	
single exposure	310	62	31.5	156	36.7	85	37.3	.18
repeated exposure	23	2	1.0	11	2.6	9	3.9	
Other trauma								
no exposure	650	147	74.6	316	74.4	179	78.5	
single exposure	166	41	20.8	84	19.8	40	17.5	.69
repeated exposure	46	9	4.6	25	5.9	9	3.9	

5HTTLPR = serotonin-transporter–linked polymorphic region; s = short; l = long; M = mean; SD = standard deviation; SLEs = stressful life events; TYEs = traumatic youth experiences. The s-allele includes the s and lg alleles; the l-allele includes the l and la alleles. [a] z Score. [b] No abuse is the reference category.

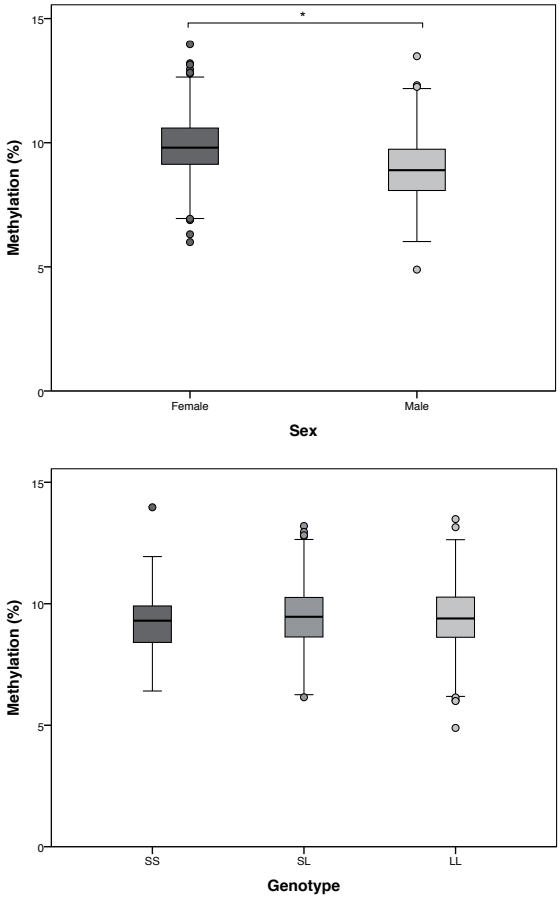


Figure 2. Boxplot of *SLC6A4* methylation scores (%) according to functional *5HTTLPR* genotype (i.e., including rs25531) and sex. Error bars represent the highest and lowest values that are not outliers. *SLC6A4* = solute carrier family 6 (neurotransmitter transporter), member 4; *5HTTLPR* = serotonin-transporter–linked polymorphic region. * $p < .001$.

Adverse life events and methylation

In Table 2 the results of the regression analyses with adverse life event variables as predictors for *SLC6A4* methylation are presented. Exposure to perinatal adversity or TYEs was not related to methylation. Exposure to SLEs (0-15) significantly predicted higher methylation levels. In the model including both SLEs during childhood and adolescence, exposure to SLEs in adolescence was related to higher methylation levels. No statistical significant interaction was found for adverse life event variables and sex on methylation.

Analyses with putative confounders showed little (<10%) change in the regression coefficients of adverse life event variables (Table S2-S5). In the analyses in the next sections these putative confounders were therefore not included.

SLEs and *5HTTLPR* genotype

Methylation levels did not differ between genotypes, F(2,924)=2.234, p=.11 (Figure 2). Adding *5HTTLPR* genotype in the regression model predicting methylation by SLEs (0-15) showed a significant interaction between genotype and SLEs (Table 3). This interaction did not differ as a function of sex (p-values >.050, see Table S6, for a graphical representation of this interaction, see Figure S2). The direction of the interaction was consistent over the majority of individual CpG units (Table S7), which suggest that the mean value of methylation is a good representation of the impact of SLEs on methylation across a larger region of the CpG island. The interaction was not significant when genotype was coded non-functionally, i.e., regardless of rs25531 genotype, (Table S8) but revealed a trend in the same direction. The interaction between genotype and perinatal adversity was not significant (p>.050). Due to the small number of participants who had experienced trauma, the interaction between TYEs and genotype could not be tested.

Table 2. Regression analyses of adverse life events and *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**p**	**95% CI of B**
***Perinatal adversity*[a]**	-0.07	0.04	.089	-0.15 - 0.01
***SLEs*[a]**				
Total (0-15 years)	**0.11**	**0.04**	**.011**	**0.03 - 0.20**
Childhood (0-11)	0.02	0.04	.57	-0.06 - 0.11
Adolescence (12-15)	**0.13**	**0.04**	**.004**	**0.04 - 0.22**
***TYEs*[b]**				
Sexual abuse				
single exposure	-0.13	0.21	.53	-0.54 - 0.28
repeated exposure	0.03	0.24	.91	-0.45 - 0.50
Physical abuse				
single exposure	-0.01	0.09	.94	-0.18 - 0.17
repeated exposure	-0.20	0.27	.46	-0.72 - 0.33
Other trauma				
single exposure	0.04	0.10	.69	-0.16 - 0.24
repeated exposure	0.08	0.15	.60	-0.21 – 0.36

SLC6A4 = solute carrier family 6 (neurotransmitter transporter), member 4; B = regression coefficient; SE = standard error; CI = confidence interval; SLEs = stressful life events; TYEs = traumatic youth experiences. Adjusted for sex. Bold numbers indicate significant (p < .05) results. All models were models with a single measure of adverse life events, except for one model in which childhood SLEs and adolescence SLEs were included together. [a] z Score. [b] No abuse is the reference category.

Table 3. Regression analyses of SLEs, genotype and the interaction between SLEs and *5HTTLPR* genotype, on *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**P**	**95% CI of B**
SLEs (0-15 years)[a]	0.03	0.09	.73	-0.15 - 0.21
s/l[b]	**0.26**	**0.11**	**.017**	**0.05 - 0.47**
l/l[b]	**0.30**	**0.12**	**.016**	**0.06 - 0.54**
SLEs (0-15 years)[a] X s/l[b]	0.01	0.11	.90	-0.20 - 0.23
SLEs (0-15 years)[a] X l/l[b]	**0.33**	**0.13**	**.010**	**0.08 - 0.59**

SLEs = stressful life events; *5HTTLPR* = serotonin-transporter–linked polymorphic region; *SLC6A4* = solute carrier family 6 (neurotransmitter transporter), member 4; B = regression coefficient; SE = standard error; CI = confidence interval; s = short; l = long; SNP = single nucleotide polymorphism. Adjusted for sex. Bold numbers indicate significant results. S-allele includes s and lg alleles; the l-allele includes the la allele. Table S8 shows the results when genotype was coded functionally while taking account of SNP rs25531. [a] z Score. [b] S/s is reference category.

Table 4. Regression analyses of SLEs and *SLC6A4* methylation stratified by *5HTTLPR* genotype.

	***SLC6A4* methylation**								
	s/s			**s/l**			**l/l**		
	B	**SE**	**p**	**B**	**SE**	**p**	**B**	**SE**	**p**
SLEs[a]									
Total (0-15 years)	0.04	0.09	.66	0.04	0.06	.49	**0.37**	**0.10**	**< .001**
Childhood (0-11)	-0.08	0.09	.34	0.00	0.06	.97	0.17	0.09	.066
Adolescence (12-15)	0.13	0.08	.11	0.06	0.06	.33	**0.36**	**0.11**	**.001**

SLEs = stressful life events; *SLC6A4* = solute carrier family 6 (neurotransmitter transporter), member 4; *5HTTLPR* = serotonin-transporter–linked polymorphic region; s = short; l = long; B = regression coefficient; SE = standard error. Adjusted for sex. Bold numbers indicate significant ($p < .05$) results. Total SLEs was entered in a model individually, whereas childhood SLEs and adolescence SLEs were included together in a model. [a] z Score.

Stratification by genotype (Table 4, Figure 3) showed that *SLC6A4* methylation was higher amongst l-allele homozygotes after exposure to more SLEs. S-allele carriers did not show a significant association between SLEs and methylation.

DISCUSSION

In the current study, having experienced SLEs was associated with higher *SLC6A4* methylation in adolescents. This association was more prominent for SLEs experienced in adolescence than SLEs experienced in childhood. We found that the relationship between SLEs and *SLC6A4* methylation was influenced by *5HTTLPR* genotype: methyla-

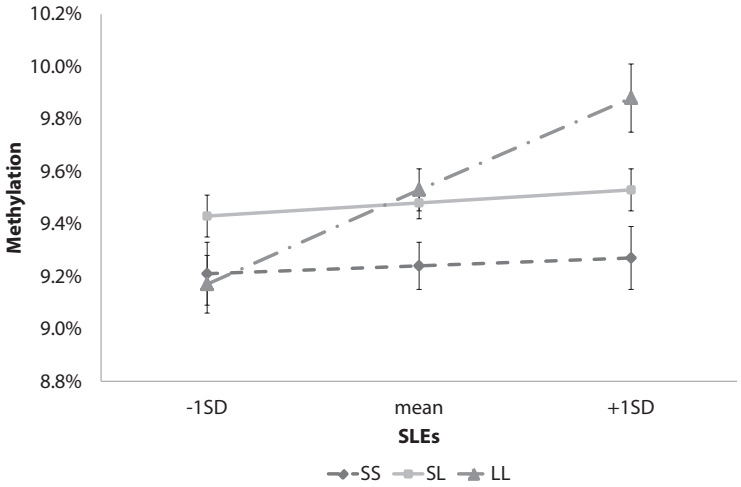


Figure 3. Interaction of *5HTTLPR* genotype and score for stressful life events (SLEs, 0-15 years) on mean-centered *SLC6A4* methylation scores (%). Error bars represent standard errors. *5HTTLPR* = serotonin-transporter–linked polymorphic region; SLEs = stressful life events; *SLC6A4* = solute carrier family 6 (neurotransmitter transporter), member 4; SD = standard deviation.

tion levels were higher in adolescents who were homozygous for the l-allele and experienced more SLEs, but SLEs were not associated with methylation for s-allele carriers. Like Philibert and colleagues [20], we found that sex affected *SLC6A4* methylation, with higher methylation levels in females than in males. The relationship between SLEs (0-15) and methylation did not differ between males and females.

Our finding that perinatal adversity was not associated with *SLC6A4* methylation contrasts with the study by Devlin and colleagues [23], who reported higher levels of *SLC6A4* methylation in cord blood after exposure to depressed maternal mood during pregnancy. Possibly, stress related methylation may have a relatively low temporal stability and may be a reflection of exposure to recent adverse life events. As our methylation data originated from blood collected in adolescence, methylation marks from the perinatal period may have been gradually lost over time, perhaps through active demethylation mechanisms [42]. This may also explain the lack of an association with perinatal stress and the difference in regression coefficients for childhood SLEs and adolescence SLEs. Since the assessment of SLE measures differed between time periods, it needs to be acknowledged that differences in measurements of SLEs in childhood and adolescence may also be reflected in our analyses. However, the advantage of this operationalization was that the SLE measures more accurately reflected the SLEs that were appropriate for the different developmental stages of childhood and adolescence.

Also, no association was found between TYEs and *SLC6A4* methylation. While this finding was unexpected and in contrast with other reports on higher methylation following childhood abuse [21, 24, 25], these studies had relatively small sample sizes (N=108-192), sometimes with specific study populations, and were thus in need of replication. More specific, Kang and colleagues [21] studied patients diagnosed with Major Depressive Disorder (MDD), and reported an association for family history of depression with methylation of *SLC6A4*. They suggest that this finding may be ascribed to a stressful childhood environment. It is possible that this association was affected by SLEs, which may explain the disparity between our study and theirs. Also, it cannot be ruled out that MDD itself may have affected methylation. In an earlier study in our (TRAILS) population, TYEs were associated with higher methylation of *NR3C1* [43], suggesting that methylation by TYEs may be dependent on the gene investigated. In addition, because rates of reported TYEs, particularly repeated sexual abuse and physical abuse of the sort that might plausibly influence methylation, were quite low, it is possible that the current investigation was not well powered to examine these effects.

Although carriers of the *5HTTLPR* l-allele have previously been associated with lower *SLC6A4* methylation [18-20, 28], we found no differences in methylation levels between genotypes. However, when taking the number of SLEs experienced into account, we found that l-allele homozygotes with few SLEs had lower methylation levels than s-allele carriers. It should be noted that, to the extent that there was a differential susceptibility effect in the current study, it was in the direction of the l-allele rather than the s-allele, considered to be the susceptibility allele [44]. In addition, the difference between the l-allele homozygotes and s-allele carriers was most pronounced at high levels of SLEs, suggesting that the effect might be best characterized as a vulnerability effect.

The genotype-SLE interaction on *SLC6A4* methylation seemed to contrast with the work of Caspi and colleagues [10], who showed that individuals with two l-alleles were not at risk for depression, regardless of how many SLEs they experienced. Replication of this finding, however, has not always been successful [45, 46]. More recently, Mueller *et al* [47] showed that only the experience of SLEs in the first five years of life showed an interaction with *5HTTLPR* in predicting depression in young adults. In TRAILS, there was no support for an interaction between *5HTTLPR* genotype and childhood adversities on depression [38]. To shed light on this paradox, it may be necessary to incorporate methylation patterns into these interactions: a highly methylated l-allele is considered to have a poor transcriptional activity, and methylation might nullify the protective function of the l-allele, resulting in a functionality equivalent to the s-allele. This suggestion is supported by a study that showed more unresolved loss or trauma in highly methylated l-allele carriers and low methylated s-allele homozygotes [48]. More research is needed to gain understanding of the relation between genetic and epigenetic variation and its

possible implication in clinical practice. A manuscript investigating the relation between methylation and *5HTTLPR* genotype on internalizing problems is in preparation.

Our study has several strengths. *SLC6A4* genotype and methylation data was obtained from a large population-based sample. The TRAILS study also provides a detailed account of SLEs between birth and adolescence, and is the first study to explore the effect of SLEs during childhood and adolescence on *SLC6A4* methylation separately. Several limitations should also be acknowledged. Because blood was collected only at the third assessment wave, changes in methylation by SLEs could not be analyzed, and no causal links could be established. Sample size was large, but not large enough to study genotype-specific methylation by TYEs. Another limitation is the lack of a replication sample, but obtaining a suitable replication sample of adolescents with comparable measures of adverse life events and population characteristics is very challenging. An important limitation concerns the use of peripheral tissue (blood) as a proxy of the target tissue (the brain), which is often inaccessible in living humans. However, correlations between methylation levels in the brain and blood have been found to be high [49]. Recently, preliminary evidence was provided that *SLC6A4* methylation in peripheral blood leukocytes reflects DNA methylation of a specific part of the brain involved in emotion regulation, i.e., the amygdala [50], suggesting that blood may be a valid biomarker for brain methylation. However, since we were unable to account for the cellular heterogeneity of the blood cells, potentially influenced by stress exposure, we could not rule out that the associations may be in part a reflection of differences in cellular composition. Finally, we analyzed one region of *SLC6A4,* located in the promoter and thus probably relevant for regulation of transcription, yet we suggest further research to include more regions of *SLC6A4*.

In conclusion, we showed that adverse life events affect *SLC6A4* methylation in adolescents, and that this association was *5HTTLPR* genotype dependent. Allele-specific methylation may be a mechanism through which a stressful environment can lead to vulnerability to affective disorders. Our findings are new, the first to be reported in a young sample of this size. They are important to the field, but we acknowledge that replication is warranted, also in light of generalization: our final sample individuals with low SES scores were underrepresented, and investigation in ethnicities other than white is warranted. The study can be regarded as a starting point for new ways to explore the complex relations between *SLC6A4*, stressful experiences, and the risk of developing affective disorders.

REFERENCES

1. Faulk C, Dolinoy DC. Timing is everything: the when and how of environmentally induced changes in the epigenome of animals. *Epigenetics* 2011; **6**(7)**:** 791-797.
2. Razin A, Cedar H. DNA methylation and gene expression. *Microbiol Rev* 1991; **55**(3)**:** 451-458.
3. de Rooij SR, Costello PM, Veenendaal MV, Lillycrop KA, Gluckman PD, Hanson MA *et al.* Associations between DNA methylation of a glucocorticoid receptor promoter and acute stress responses in a large healthy adult population are largely explained by lifestyle and educational differences. *Psychoneuroendocrinology* 2012; **37**(6)**:** 782-788.
4. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics* 2008; **3**(2)**:** 97-106.
5. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR *et al.* Epigenetic programming by maternal behavior. *Nat Neurosci* 2004; **7**(8)**:** 847-854.
6. Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB. The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology* 2008; **33**(6)**:** 693-710.
7. Zhao J, Goldberg J, Bremner JD, Vaccarino V. Association between promoter methylation of serotonin transporter gene and depressive symptoms: a monozygotic twin study. *Psychosom Med* 2013; **75**(6)**:** 523-529.
8. Malison RT, Price LH, Berman R, van Dyck CH, Pelton GH, Carpenter L *et al.* Reduced brain serotonin transporter availability in major depression as measured by [123I]-2 beta-carbomethoxy-3 beta-(4-iodophenyl)tropane and single photon emission computed tomography. *Biol Psychiatry* 1998; **44**(11)**:** 1090-1098.
9. Owens MJ, Nemeroff CB. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clin Chem* 1994; **40**(2)**:** 288-295.
10. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 2003; **301**(5631)**:** 386-389.
11. de Geus EJ, Middeldorp CM. Serotonin transporter gene: will epigenetics prove less depressing than genetics? *Psychosom Med* 2013; **75**(6)**:** 520-522.
12. Collier DA, Stober G, Li T, Heils A, Catalano M, Di Bella D *et al.* A novel functional polymorphism within the promoter of the serotonin transporter gene: possible role in susceptibility to affective disorders. *Mol Psychiatry* 1996; **1**(6)**:** 453-460.
13. Sen S, Burmeister M, Ghosh D. Meta-analysis of the association between a serotonin transporter promoter polymorphism (5-HTTLPR) and anxiety-related personality traits. *Am J Med Genet B Neuropsychiatr Genet* 2004; **127B**(1)**:** 85-89.
14. Heils A, Teufel A, Petri S, Stober G, Riederer P, Bengel D *et al.* Allelic variation of human serotonin transporter gene expression. *J Neurochem* 1996; **66**(6)**:** 2621-2624.
15. Lesch KP, Balling U, Gross J, Strauss K, Wolozin BL, Murphy DL *et al.* Organization of the human serotonin transporter gene. *J Neural Transm Gen Sect* 1994; **95**(2)**:** 157-162.
16. Bradley SL, Dodelzon K, Sandhu HK, Philibert RA. Relationship of serotonin transporter gene polymorphisms and haplotypes to mRNA transcription. *Am J Med Genet B Neuropsychiatr Genet* 2005; **136B**(1)**:** 58-61.
17. Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S *et al.* Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 1996; **274**(5292)**:** 1527-1531.

18. Philibert R, Madan A, Andersen A, Cadoret R, Packer H, Sandhu H. Serotonin transporter mRNA levels are associated with the methylation of an upstream CpG island. *Am J Med Genet B Neuropsychiatr Genet* 2007; **144B**(1)**:** 101-105.
19. Kinnally EL, Capitanio JP, Leibel R, Deng L, LeDuc C, Haghighi F *et al.* Epigenetic regulation of serotonin transporter expression and behavior in infant rhesus macaques. *Genes Brain Behav* 2010; **9**(6)**:** 575-582.
20. Philibert RA, Sandhu H, Hollenbeck N, Gunter T, Adams W, Madan A. The relationship of 5HTT (SLC6A4) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B**(5)**:** 543-549.
21. Kang HJ, Kim JM, Stewart R, Kim SY, Bae KY, Kim SW *et al.* Association of SLC6A4 methylation with early adversity, characteristics and outcomes in depression. *Prog Neuropsychopharmacol Bol Psychiatry* 2013; **44:** 23-28.
22. Olsson CA, Foley DL, Parkinson-Bates M, Byrnes G, McKenzie M, Patton GC *et al.* Prospects for epigenetic research within cohort studies of psychological disorder: a pilot investigation of a peripheral cell marker of epigenetic risk for depression. *Biol Psychol* 2010; **83**(2)**:** 159-165.
23. Devlin AM, Brain U, Austin J, Oberlander TF. Prenatal exposure to maternal depressed mood and the MTHFR C677T variant affect SLC6A4 methylation in infants at birth. *PLoS One* 2010; **5**(8)**:** e12201.
24. Beach SR, Brody GH, Todorov AA, Gunter TD, Philibert RA. Methylation at SLC6A4 is linked to family history of child abuse: an examination of the Iowa Adoptee sample. *Am J Med Genet B Neuropsychiatr Genet* 2010; **153B**(2)**:** 710-713.
25. Beach SR, Brody GH, Todorov AA, Gunter TD, Philibert RA. Methylation at 5HTT mediates the impact of child sex abuse on women's antisocial behavior: an examination of the Iowa adoptee sample. *Psychosom Med* 2011; **73**(1)**:** 83-87.
26. Vijayendran M, Beach SR, Plume JM, Brody GH, Philibert RA. Effects of genotype and child abuse on DNA methylation and gene expression at the serotonin transporter. *Front Psychiatry* 2012; **3:** 55.
27. Ouellet-Morin I, Wong CC, Danese A, Pariante CM, Papadopoulos AS, Mill J *et al.* Increased serotonin transporter gene (SERT) DNA methylation is associated with bullying victimization and blunted cortisol response to stress in childhood: a longitudinal study of discordant monozygotic twins. *Psychol Med* 2013; **43**(9)**:** 1813-1823.
28. Alasaari JS, Lagus M, Ollila HM, Toivola A, Kivimaki M, Vahtera J *et al.* Environmental stress affects DNA methylation of a CpG rich promoter region of serotonin transporter gene in a nurse cohort. *PLoS One* 2012; **7**(9)**:** e45813.
29. Kinnally EL, Feinberg C, Kim D, Ferguson K, Leibel R, Coplan JD *et al.* DNA methylation as a risk factor in the effects of early life stress. *Brain Behav Immun* 2011; **25**(8)**:** 1548-1553.
30. Schalkwyk LC, Meaburn EL, Smith R, Dempster EL, Jeffries AR, Davies MN *et al.* Allelic skewing of DNA methylation is widespread across the genome. *Am J Hum Genet* 2010; **86**(2)**:** 196-212.
31. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, Huizink AC *et al.* Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. *Int J Epidemiol* 2008; **37**(6)**:** 1227-1235.
32. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, Veenstra R *et al.* The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. *J Am Acad Child Adolesc Psychiatry* 2012; **51**(10)**:** 1020-1036.

33. Reijneveld SA, Brugman E, Verhulst FC, Verloove-Vanhorick SP. Identification and management of psychosocial problems among toddlers in Dutch preventive child health care. *Arch Pediatr Adolesc Med* 2004; **158**(8)**:** 811-817.
34. Bosch NM, Riese H, Reijneveld SA, Bakker MP, Verhulst FC, Ormel J *et al.* Timing matters: long term effects of adversities from prenatal period up to adolescence on adolescents' cortisol stress response. The TRAILS study. *Psychoneuroendocrinology* 2012; **37**(9)**:** 1439-1447.
35. Caspi A, Moffitt TE, Thornton A, Freedman D, Amell JW, Harrington H *et al.* The life history calendar: A research and clinical assessment method for collecting retrospective event-history data. *Int J Methods Psychiatr Res* 1996; **6**(2)**:** 101-114.
36. Bernstein DP, Fink L. *Childhood Trauma Questionnaire: A retrospective self-report manual*. The Psychological Corporation San Antonio, TX, 1998.
37. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3)**:** 1215.
38. Nederhof E, Bouma EM, Oldehinkel AJ, Ormel J. Interaction between childhood adversity, brain-derived neurotrophic factor val/met and serotonin transporter promoter polymorphism on depression: the TRAILS study. *Biol Psychiatry* 2010; **68**(2)**:** 209-212.
39. Hu XZ, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD *et al.* Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *Am J Hum Genet* 2006; **78**(5)**:** 815-826.
40. Williams JM, Dunlop LC. Pubertal timing and self-reported delinquency among male adolescents. *J Adolesc* 1999; **22**(1)**:** 157-171.
41. Shirtcliff EA, Dahl RE, Pollak SD. Pubertal development: correspondence between hormonal and physical development. *Child Dev* 2009; **80**(2)**:** 327-337.
42. Bhutani N, Burns DM, Blau HM. DNA demethylation dynamics. *Cell* 2011; **146**(6)**:** 866-872.
43. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ *et al.* Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry* 2014; **4:** e381.
44. Belsky J, Pluess M. Beyond diathesis stress: differential susceptibility to environmental influences. *Psychol Bull* 2009; **135**(6)**:** 885-908.
45. Munafo MR, Durrant C, Lewis G, Flint J. Gene X environment interactions at the serotonin transporter locus. *Biol Psychiatry* 2009; **65**(3)**:** 211-219.
46. Risch N, Herrell R, Lehner T, Liang KY, Eaves L, Hoh J *et al.* Interaction between the serotonin transporter gene (5-HTTLPR), stressful life events, and risk of depression: a meta-analysis. *JAMA* 2009; **301**(23)**:** 2462-2471.
47. Mueller A, Armbruster D, Moser DA, Canli T, Lesch KP, Brocke B *et al.* Interaction of serotonin transporter gene-linked polymorphic region and stressful life events predicts cortisol stress response. *Neuropsychopharmacology* 2011; **36**(7)**:** 1332-1339.
48. van IJzendoorn MH, Caspers K, Bakermans-Kranenburg MJ, Beach SR, Philibert R. Methylation matters: interaction between methylation density and serotonin transporter genotype predicts unresolved loss or trauma. *Biol Psychiatry* 2010; **68**(5)**:** 405-407.
49. Tylee DS, Kawaguchi DM, Glatt SJ. On the outside, looking in: A review and evaluation of the comparability of blood and brain "-omes". *Am J Med Genet B Neuropsychiatr Genet* 2013; **162**(7)**:** 595-603.
50. Riese H, van den Heuvel ER, Snieder H, den Dunnen WF, Plosch T, Kema IP *et al.* Association Between Methylation of the SLC6A4 Promoter Region in Peripheral Blood Leukocytes and Methylation in Amygdala Tissue. *Psychosom Med* 2014.

SUPPLEMENTARY INFORMATION

DNA fragment details

ggct|atct|ag|ag|atc|ag|acc|atgtg|AGGGCC**CGCG**GGT[1]|ac|a|a|at|AC**G**GC**CGCG**C**CG**G**CG**CCCCTC**CG**[TSS]C[2]|ac|agcc|AG**CG**C**CG**C**CG**GGTGCCT**CG**[3]|AGGG**CGCG**[4]|aggcc|AGCCC**CG**CCTGCCC[5]|AGCC**CG**GG[6]|acc|AGCCTCCC**CGCG**C[7]|agcctggc|AGGTGGGTC**CG**CTTTTCCTCTC**CG**CCT**CG**[8]|a|accc|A**CG**TTTCTTTCC[9]|ag|ACCTTCTTCCC**CG**CCT**CG**GGG[10]|aggggg|at|ag|a|AC**CG**CTG**CG**CCCC[11]|AC**CG**CCCTG**CG**[12]|agg|AGG**CG**[13]|agg|aggtgc|ATG**CG**CCCC[14]|AG**CG**GTGGG**CG**C**CG**G[15]|ATCCTGCCCCTG**CG**CCCTCC[16]|A**CG**CTC[17]|agc|a|ag|agcc|ag|agctg|a|agctg|AC**CG**GCC[18]|ag|agtggg|ag|A**CG**[19]|agg|a|A**CG**TGG[20]|AGTGCT**CG**[21]|a|AGTGGG**CG**GG**CG***T*[22]|*aggggg*ctcctttgtct|attgttgc|agg

Figure S1. Sequence of DNA fragment used for methylation analysis, positioned on the reverse strand. Forward and backward primers are underlined. Numbers represent CpG units and vertical lines represent splice sites. CpG units in grey could not be analyzed for methylation rates. Bases in italics are outside of CpG island (according to UCSC build GRCh37/hg19). TSS marks transcription start site and grey marked area covers exon 1 (based on Ensembl Transcript ID ENST00000394821).

Descriptives CpG unit methylation

Table S1. CpG unit methylation; range, mean and SD.

	N	Minimum	Maximum	M	SD
CpGU1	938	1.0%	8.0%	2.8%	0.9%
CpGU3	936	2.0%	12.0%	5.8%	1.6%
CpGU4	920	0.0%	19.0%	6.0%	2.5%
CpGU6	934	0.0%	10.0%	1.4%	1.6%
CpGU9	935	2.0%	14.0%	7.6%	1.9%
CpGU10	774	0.0%	10.0%	2.4%	1.5%
CpGU12	898	5.0%	27.0%	15.4%	3.3%
CpGU14	892	1.0%	23.0%	9.5%	3.2%
CpGU15	930	3.0%	20.0%	8.6%	2.3%
CpGU16	843	5.0%	31.0%	15.9%	3.8%
CpGU22	859	8.0%	47.0%	28.0%	5.3%

Note: M=mean, SD=standard deviation.

Analyses with confounders/covariates

Table S2a. Regression analyses of adverse life events and *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**p**	**95% CI of B**
***Perinatal adversity*[1]**	-0.07	0.04	0.118	-0.15 - 0.02
***SLEs*[1]**				
Total (0-15 years)	**0.11**	**0.04**	**0.012**	**0.03 - 0.20**
Childhood (0-11)	0.04	0.04	0.414	-0.05 - 0.12
Adolescence (12-15)	**0.12**	**0.05**	**0.009**	**0.03 - 0.21**
***TYEs*[2]**				
Sexual abuse				
single exposure	-0.18	0.22	0.405	-0.61 - 0.24
repeated exposure	-0.01	0.26	0.963	-0.52 - 0.49
Physical abuse				
single exposure	-0.02	0.09	0.823	-0.20- 0.16
repeated exposure	-0.21	0.27	0.429	-0.74 - 0.31
Other trauma				
single exposure	0.03	0.11	0.759	-0.19 - 0.26
repeated exposure	-0.17	0.20	0.392	-0.57 - 0.22

Covariates: Sex and use of oral contraceptives. **Note:** SLEs= Stressful life events; TYEs= Traumatic Youth experiences. [1]Z-score, [2]No abuse is the reference group.

Table S2b. Regression analyses of SLEs, genotype and the interaction between SLEs and *5HTTLPR* genotype, on *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**P**	**95% CI of B**
SLEs (0-15 years)[2]	0.04	0.09	0.656	-0.14 - 0.22
s/l[1]	**0.24**	**0.11**	**0.029**	**0.02 - 0.46**
l/l[1]	**0.27**	**0.13**	**0.032**	**0.02 - 0.52**
SLEs (0-15 years)[2] X s/l[1]	0.00	0.11	0.985	-0.22 - 0.22
SLEs (0-15 years)[2] X l/l[1]	**0.32**	**0.13**	**0.016**	**0.06 - 0.58**

Covariates: Sex and use of oral contraceptives. **Note:** SLEs= Stressful life events; [1] s/s is the reference group, [2]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Table S2c. Regression analyses of SLEs and *SLC6A4* methylation stratified by *5HTTLPR* genotype.

	***SLC6A4* methylation**								
	s/s			**s/l**			**l/l**		
	B	**SE**	**p**	**B**	**SE**	**p**	**B**	**SE**	**p**
***SLEs*[1]**									
Total (0-15 years)	0.05	0.09	0.591	0.04	0.06	0.515	**0.34**	**0.10**	**0.001**
Childhood (0-11)	-0.07	0.09	0.457	0.00	0.06	0.946	0.17	0.09	0.064
Adolescence (12-15)	0.13	0.09	0.134	0.05	0.06	0.414	**0.33**	**0.12**	**0.005**

Covariates: Sex and use of oral contraceptives. **Note:** SLEs= Stressful life events. [1]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Table S3a. Regression analyses of adverse life events and *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**p**	**95% CI of B**
***Perinatal adversity*[1]**	-0.07	0.04	0.085	-0.15 - 0.01
***SLEs*[1]**				
Total (0-15 years)	**0.11**	**0.04**	**0.009**	**0.03 - 0.20**
Childhood (0-11)	0.03	0.04	0.550	-0.06 - 0.11
Adolescence (12-15)	**0.13**	**0.04**	**0.003**	**0.04 - 0.22**
***TYEs*[2]**				
Sexual abuse				
single exposure	-0.13	0.21	0.525	-0.55 - 0.28
repeated exposure	0.02	0.24	0.938	-0.46 - 0.50
Physical abuse				
single exposure	0.00	0.09	0.983	-0.18 - 0.17
repeated exposure	-0.19	0.27	0.474	-0.72 - 0.33
Other trauma				
single exposure	0.05	0.11	0.683	-0.17 - 0.26
repeated exposure	-0.11	0.19	0.580	-0.48 - 0.27

Covariates: Sex and CRP (C-reactive protein, mg/l). **Note:** SLEs= Stressful life events; TYEs= Traumatic Youth experiences. [1]Z-score, [2]No abuse is the reference group.

Table S3b. Regression analyses of SLEs, genotype and the interaction between SLEs and *5HTTLPR* genotype, on *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**P**	**95% CI of B**
SLEs (0-15 years)[2]	0.03	0.09	0.748	-0.15 - 0.21
s/l[1]	**0.25**	**0.11**	**0.020**	**0.04 - 0.47**
l/l[1]	**0.30**	**0.12**	**0.017**	**0.05 - 0.54**
SLEs (0-15 years)[2] X s/l[1]	0.02	0.11	0.866	-0.19 - 0.23
SLEs (0-15 years)[2] X l/l[1]	**0.34**	**0.13**	**0.009**	**0.08 - 0.59**

Covariates: Sex and CRP (C-reactive protein, mg/l). **Note:** SLEs= Stressful life events; [1] s/s is the reference group, [2]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Table S3c. Regression analyses of SLEs and *SLC6A4* methylation stratified by *5HTTLPR* genotype.

	***SLC6A4* methylation**								
	s/s			**s/l**			**l/l**		
	B	**SE**	**p**	**B**	**SE**	**p**	**B**	**SE**	**p**
***SLEs*[1]**									
Total (0-15 years)	0.04	0.09	0.663	0.05	0.06	0.421	**0.37**	**0.10**	**<.001**
Childhood (0-11)	-0.08	0.09	0.346	0.00	0.06	0.998	0.17	0.09	0.065
Adolescence (12-15)	0.13	0.08	0.115	0.06	0.06	0.277	**0.37**	**0.11**	**0.001**

Covariates: Sex and CRP (C-reactive protein, mg/l). **Note:** SLEs= Stressful life events. [1]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Table S4a. Regression analyses of adverse life events and *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**p**	**95% CI of B**
***Perinatal adversity*[1]**	-0.07	0.04	0.103	-0.15 - 0.01
***SLEs*[1]**				
Total (0-15 years)	**0.12**	**0.04**	**0.009**	**0.03 - 0.20**
Childhood (0-11)	0.03	0.04	0.493	-0.06 - 0.12
Adolescence (12-15)	**0.13**	**0.05**	**0.005**	**0.04 - 0.22**
***TYEs*[2]**				
Sexual abuse				
single exposure	-0.12	0.21	0.555	-0.54 - 0.29
repeated exposure	0.09	0.25	0.727	-0.40 - 0.57
Physical abuse				
single exposure	0.02	0.09	0.865	-0.16 - 0.20
repeated exposure	-0.18	0.27	0.504	-0.71 - 0.35

Table S4a. Regression analyses of adverse life events and *SLC6A4* methylation. (continued)

	***SLC6A4* methylation**			
	B	**SE**	**p**	**95% CI of B**
Other trauma				
single exposure	0.06	0.11	0.610	-0.16 - 0.28
repeated exposure	-0.11	0.20	0.564	-0.50 - 0.27

Covariates: Sex and pubertal development. **Note:** SLEs= Stressful life events; TYEs= Traumatic Youth experiences. [1]Z-score, [2]No abuse is the reference group.

Table S4b. Regression analyses of SLEs, genotype and the interaction between SLEs and *5HTTLPR* genotype, on *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**P**	**95% CI of B**
SLEs (0-15 years)[2]	0.05	0.09	0.598	-0.13 - 0.23
s/l[1]	**0.27**	**0.11**	**0.013**	**0.06 - 0.49**
l/l[1]	**0.30**	**0.13**	**0.018**	**0.05 - 0.54**
SLEs (0-15 years)[2] X s/l[1]	0.00	0.11	0.993	-0.22 - 0.21
SLEs (0-15 years)[2] X l/l[1]	**0.30**	**0.13**	**0.021**	**0.05 - 0.56**

Covariates: Sex and pubertal development. **Note:** SLEs= Stressful life events; [1] s/s is the reference group, [2]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Table S4c. Regression analyses of SLEs and *SLC6A4* methylation stratified by *5HTTLPR* genotype.

	***SLC6A4* methylation**								
	s/s			**s/l**			**l/l**		
	B	**SE**	**p**	**B**	**SE**	**p**	**B**	**SE**	**p**
***SLEs*[1]**									
Total (0-15 years)	0.05	0.09	0.534	0.04	0.06	0.488	**0.36**	**0.10**	**<.001**
Childhood (0-11)	-0.07	0.09	0.452	0.00	0.06	0.980	0.17	0.09	0.069
Adolescence (12-15)	0.14	0.09	0.106	0.06	0.06	0.337	**0.35**	**0.11**	**0.002**

Covariates: Sex and pubertal development. **Note:** SLEs= Stressful life events. [1]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Table S5a. Regression analyses of adverse life events and *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**p**	**95% CI of B**
***Perinatal adversity*[1]**	-0.07	0.04	0.119	-0.15 - 0.02
***SLEs*[1]**				
Total (0-15 years)	**0.12**	**0.05**	**0.010**	**0.03 - 0.21**
Childhood (0-11)	0.04	0.04	0.335	-0.04 - 0.13
Adolescence (12-15)	**0.12**	**0.05**	**0.011**	**0.03 - 0.21**
***TYEs*[2]**				
Sexual abuse				
single exposure	-0.17	0.22	0.423	-0.60 - 0.25
repeated exposure	0.03	0.26	0.917	-0.49 - 0.54
Physical abuse				
single exposure	0.01	0.09	0.914	-0.17 - 0.19
repeated exposure	-0.19	0.27	0.475	-0.72 - 0.34
Other trauma				
single exposure	0.04	0.11	0.719	-0.18 - 0.27
repeated exposure	-0.18	0.20	0.382	-0.57 - 0.22

Covariates: Sex, age, use of oral contraceptives, CRP (C-reactive protein, mg/l) and pubertal development.
Note: SLEs= Stressful life events; TYEs= Traumatic Youth experiences. [1]Z-score, [2]No abuse is the reference group.

Table S5b. Regression analyses of SLEs, genotype and the interaction between SLEs and *5HTTLPR* genotype, on *SLC6A4* methylation.

	***SLC6A4* methylation**			
	B	**SE**	**P**	**95% CI of B**
SLEs (0-15 years)[2]	0.05	0.09	0.590	-0.13 - 0.24
s/l[1]	**0.26**	**0.11**	**0.021**	**0.04 - 0.48**
l/l[1]	**0.26**	**0.13**	**0.039**	**0.01 - 0.52**
SLEs (0-15 years)[2] X s/l[1]	0.00	0.11	0.985	-0.22 - 0.22
SLEs (0-15 years)[2] X l/l[1]	**0.30**	**0.13**	**0.026**	**0.04 - 0.56**

Covariates: Sex, age, use of oral contraceptives, CRP (C-reactive protein, mg/l) and pubertal development.
Note: SLEs= Stressful life events; [1] s/s is the reference group, [2]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Table S5c. Regression analyses of SLEs and *SLC6A4* methylation stratified by *5HTTLPR* genotype.

	***SLC6A4* methylation**								
	s/s			**s/l**			**l/l**		
	B	**SE**	**p**	**B**	**SE**	**p**	**B**	**SE**	**p**
***SLEs*[1]**									
Total (0-15 years)	0.06	0.09	0.493	0.05	0.06	0.432	**0.33**	**0.10**	**0.001**
Childhood (0-11)	-0.05	0.09	0.607	0.01	0.06	0.877	0.18	0.09	0.052
Adolescence (12-15)	0.12	0.09	0.148	0.06	0.06	0.365	**0.30**	**0.12**	**0.011**

Covariates: Sex, age, use of oral contraceptives, CRP (C-reactive protein, mg/l) and pubertal development.
Note: SLEs= Stressful life events. [1]Z-score. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles.

Three-way interaction of sex, *5HTTLPR* genotype and SLEs on methylation.

Table S6. Three-way interaction of sex, *5HTTLPR* genotype and SLEs on methylation.

	B	**SE**	**p**
sex[1]	**-0.68**	**0.18**	**<.001**
s/l[2]	**0.39**	**0.15**	**.008**
l/l[2]	**0.40**	**0.17**	**.023**
SLEs (0-15 years)[3]	0.04	0.11	.715
sex[1] * s/l[2]	-0.30	0.22	.176
sex[1] * l/l[2]	-0.22	0.25	.376
sex[1] * SLEs (0-15 years)[3]	-0.01	0.19	.979
s/l[2] * SLEs (0-15 years)[3]	0.03	0.13	.804
l/l[2] * SLEs (0-15 years)[3]	0.23	0.18	.207
sex[1] * s/l[2] * SLEs (0-15 years)[3]	-0.08	0.23	.741
sex[1] * l/l[2] * SLEs (0-15 years)[3]	0.16	0.27	.563

Note: [1]Female is the reference category; [2]s/s is the reference category; [3]Z-score. SLEs= Stressful life events. Bold numbers indicate significant results. S-allele includes s and l_g alleles, the l-allele includes the l_a allele.

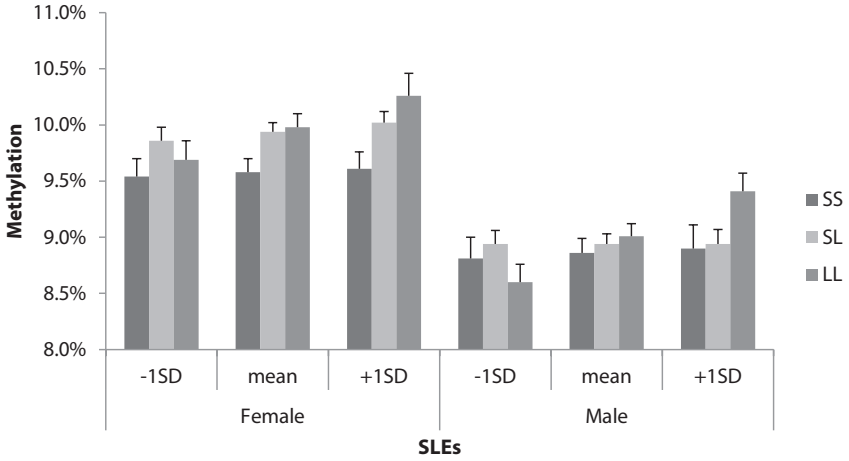


Figure S2. Graphical representation of the interaction between stressful live events (SLEs) and different genotypes on methylation levels in males and females.

CpG-unit specific interaction

Table S7. Regression analyses of SLEs, genotype and the interaction between SLEs and *5HTTLPR* genotype, on *SLC6A4* methylation per CpG unit.

Methylation		***SLEs (0-15 years)*^{2}**	**s/l^1**	**l/l^1**	***SLEs (0-15 years)*^{2} X s/l^1**	***SLEs (0-15 years)*^{2} X l/l^1**
	B	0.07	0.12	0.10	-0.04	0.06
CpGU1	**SE**	0.06	0.08	0.09	0.08	0.09
	P	0.241	0.102	0.243	0.612	0.475
	B	-0.02	0.24	0.19	0.15	0.31
CpGU3	**SE**	0.11	0.13	0.15	0.13	0.16
	P	0.844	0.078	0.214	0.269	0.054
	B	0.06	**0.46**	**0.51**	-0.17	0.16
CpGU4	**SE**	0.18	**0.22**	**0.25**	0.22	0.26
	P	0.732	**0.034**	**0.042**	0.422	0.545
	B	0.02	0.11	0.24	-0.07	0.05
CpGU6	**SE**	0.12	0.14	0.16	0.14	0.17
	P	0.851	0.439	0.132	0.612	0.746
	B	**0.38**	0.07	-0.17	-0.04	-0.07
CpGU9	**SE**	**0.13**	0.16	0.18	0.16	0.19
	P	**0.005**	0.665	0.353	0.822	0.723
	B	0.13	0.18	0.16	-0.02	-0.10
CpGU10	**SE**	0.12	0.15	0.17	0.14	0.17
	P	0.272	0.218	0.329	0.895	0.555
	B	0.03	-0.02	-0.24	0.17	0.22
CpGU12	**SE**	0.24	0.29	0.33	0.28	0.34
	P	0.907	0.948	0.469	0.549	0.513
	B	-0.08	0.49	**0.67**	0.30	0.42
CpGU14	**SE**	0.23	0.27	**0.31**	0.27	0.33
	P	0.727	0.072	**0.033**	0.270	0.196
	B	-0.02	0.29	0.39	0.00	0.21
CpGU15	**SE**	0.16	0.19	0.22	0.19	0.23
	P	0.886	0.132	0.081	0.980	0.358
	B	0.00	0.11	0.70	-0.01	**1.10**
CpGU16	**SE**	0.26	0.33	0.37	0.32	**0.38**
	P	0.991	0.747	0.060	0.969	**0.004**
	B	-0.22	0.67	0.71	-0.05	**1.28**
CpGU22	**SE**	0.33	0.41	0.47	0.40	**0.49**
	P	0.503	0.100	0.129	0.905	**0.008**

Note: Adjusted for sex. [1]s/s is reference category, [2]Z-score. Bold numbers indicate significant results. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles. SLEs= Stressful life events; CpGU= CpG unit.

Interaction without SNP recoding

Table S8. Regression analyses of SLEs, genotype and the interaction between SLEs and genotype, on *SLC6A4* methylation, regardless of rs25531.

	***SLC6A4* methylation**			
	B	**SE**	**p**	**95% CI of B**
SLEs (0-15 years)[2]	-0.01	0.11	.947	-0.22 - 0.21
s/l[1]	0.14	0.13	.302	-0.12 - 0.39
l/l[1]	0.10	0.14	.458	-0.17 - 0.37
SLEs (0-15 years)[2] X s/l[1]	0.14	0.13	.274	-0.11 - 0.39
SLEs (0-15 years)[2] X l/l[1]	0.24	0.14	.078	-0.03 - 0.51

Note: Adjusted for sex. [1]s/s is reference category, [2]Z-score. Bold numbers indicate significant results. The s-allele includes only the s-allele, the l-allele includes the l_g and l_a alleles. SLEs= Stressful life events.

Chapter 4

Methylation of *NR3C1* and *SLC6A4* and internalizing problems. The TRAILS study

L.J. van der Knaap
F.V.A. van Oort
F.C. Verhulst
A.J. Oldehinkel
H. Riese

Journal of Affective Disorders, 2015, 180, 97-103.

ABSTRACT

Background: The relationship between early adverse life events and later internalizing problems could be mediated by DNA methylation. Adversity has been associated with higher methylation levels in the glucocorticoid receptor gene (*NR3C1*) and the serotonin transporter gene (*SLC6A4*) in adolescents. We investigated cross-sectional and prospective associations of *NR3C1* and *SLC6A4* methylation with adolescents' clinical diagnoses of internalizing disorders and internalizing symptom scores.

Methods: In a population sample (mean age=16.2) we measured DNA methylation in three regions of *NR3C1* (*NR3C1*_1, N=454; *NR3C1*_2, N=904; *NR3C1*_3, N=412) and one region of *SLC6A4* (N=939) at baseline. Internalizing problems were operationalized as clinical DSM-IV diagnoses, assessed at 3 year follow-up with a diagnostic interview, and internalizing symptom scores, assessed with Self-Report questionnaires at baseline and follow-up.

Results: Only *NR3C1*_1 methylation was positively associated with risk of lifetime internalizing disorders, and with symptom scores at follow-up. However, after accounting for baseline symptom scores there was only a tendency for association with internalizing symptom scores at follow-up. There was no association between *SLC6A4* methylation and risk of lifetime internalizing disorders. *SLC6A4* methylation and internalizing symptom scores showed a tendency for association, also after accounting for baseline symptom scores.

Limitations: There was no repeated measure of DNA methylation to study causality between methylation and internalizing problems. Gene expression data were not available.

Conclusions: Although the role of gene methylation in the development of internalizing problems remains unclear, our findings suggest that gene methylation, particularly of *NR3C1*, may be involved in the development of internalizing problems in adolescence.

INTRODUCTION

Despite consistent reports of associations between adverse life events and anxiety and depression later in life [1, 2], not all individuals exposed to adversity develop internalizing problems. Heritability estimates ranging between 27% and 50% (e.g., [3, 4, 5]) suggest a genetic predisposition for the development of these disorders. Yet, GWAS studies have not shown convincing support for specific loci that contribute to the development of internalizing problems. In recent years, the focus of research on adverse life events and psychopathology has shifted from the interplay between genetic variation and the environment towards epigenetic variation and the environment. Epigenetic modifications such as DNA methylation, have become popular targets for investigation and evidence of the influence of the environment on DNA methylation is steadily growing [6-8]. DNA methylation involves the addition of a methyl group to a cytosine-phosphate-guanine (CpG) combination and generally inhibits gene expression [9-13] when located in the promoter region. Reduced expression of genes that regulate physiological processes that underlie normal behavior may contribute to the development of internalizing disorders [9].

Two genes of interest in the study of adverse life events and internalizing disorders are the serotonin transporter gene (*SLC6A4*) and the glucocorticoid receptor gene (*NR3C1*). Depression has consistently been linked to alterations in stress reactivity [14], and the glucocorticoid receptor (GR) is an important regulator of the stress response system by providing negative feedback in the HPA-axis. Higher *NR3C1* methylation levels results in an altered stress response [12, 15], which may underlie the development of internalizing problems [16].

Similarly, alterations in serotonergic transmission have been associated with anxious or depressed mood [17, 18]. Serotonin levels are regulated by serotonin transporter (5HTT) availability and can be altered by selective serotonin reuptake inhibitors (SSRIs), a frequently prescribed medication for both mood and anxiety disorders.

Only a few studies have investigated the relationship between *NR3C1* or *SLC6A4* methylation and internalizing problems. For *NR3C1,* one study on *NR3C1* methylation and depression showed uniformly low methylation in both patients with major depressive disorder (MDD) and controls [19], whereas a more recent study showed lower methylation levels in patients with MDD than controls [10], both in the 1F promoter region. Methylation of *SLC6A4* has been positively associated with depressive symptoms in monozygotic twin pairs [13] and patients with MDD [20], and a positive trend was found for methylation with lifetime history of MDD [11] (for a graphical representation of the genomic regions studied, see Supplementary Figures S1 (*SLC6A4*) and S2 (*NR3C1*)). The link between *NR3C1* and *SLC6A4* methylation and anxiety has not been investigated thus far. Yet this link seems plausible: for some anxiety disorders an association with hyperactivity of the

HPA axis has been reported [21], which suggests that *NR3C1* methylation may be related to anxiety. Also, the effectiveness of SSRIs on the treatment of anxiety disorders makes *SLC6A4* a plausible candidate for the involvement in anxiety disorders.

Whereas the existing literature focused on concurrent associations, we aimed to study whether *NR3C1* and *SLC6A4* methylation levels are associated with internalizing problems, both concurrently and prospectively. The few studies on the relationship between methylation and internalizing problems up till now have shown mixed findings, possibly due to their small sample sizes (12< N <192). To overcome this limitation, we used prospective data of a large sample of 945 adolescents, in whom internalizing disorders often have a first onset [22]. Given that adverse life events have often been associated with higher methylation levels and more internalizing problems, we hypothesized that higher levels of methylation are associated with higher internalizing symptom scores and a higher risk of developing any internalizing disorder. In two studies, a polymorphism in the *SLC6A4* gene, the 5HTT-linked polymorphic region (*5HTTLPR*), has been shown to modify methylation levels when associated with stressful life events [23] or unresolved loss or trauma [24]. We have therefore included this polymorphism as a possible moderator of the association between *SLC6A4* methylation levels and internalizing symptom scores.

METHODS

Sample selection

This study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective population study in which Dutch adolescents are followed from preadolescence into adulthood. Assessment waves are conducted biennially or triennially, and five assessment waves have been completed so far. Written consent was obtained from each subject and their parents at every assessment wave. The present study involves data collected during the third (T3, 2005-2007, N=1816, mean age 16.3, SD 0.71) and fourth (T4, 2008-2010, N=1881, mean age 19.1, SD 0.60) assessment waves. The study was approved by the Dutch Central Medical Ethics Committee and subjects received compensation for their participation. A detailed description of sampling and methods can be found in Huisman *et al* [25] and Ormel *et al* [26].

The T3 assessment involved a blood draw, from which DNA was successfully extracted in 1156 T3 participants. Selection for methylation analyses was based on the availability of blood, Dutch ethnicity, and sufficient DNA concentration. We randomly excluded one of each sibling pairs and removed DNA samples which were not suitable for analyses. Together, this resulted in a total of 945 individuals (48.3% males) suitable for methylation analyses. Assessment waves T3 and T4 will henceforth be referred to as baseline and follow-up.

DNA methylation analyses

DNA selection and methylation analyses. DNA was extracted from whole-blood samples using a manual salting-out procedure as described by Miller *et al* [27]. We used primer sets previously used by McGowan *et al* [28] for *NR3C1* and Philibert *et al* [11] (set B) for *SLC6A4* , which encompass regions of the CpG island (i.e., a region with a higher frequency of CpG sites than expected) in the promoter regions of both genes. Two additional *NR3C1* primer sets were designed (with EpiDesigner by Sequenom) and analyzed in a subsample (N=475) of the individuals initially selected for methylation analyses, to increase the coverage of the CpG island (described in detail in van der Knaap *et al* [8]). For *NR3C1*, DNA regions were numbered (*NR3C1*_1 to 3) according to their position in the CpG island. More details and graphical representations of these specific DNA regions can be found in van der Knaap *et al* [8] for *NR3C1*, and van der Knaap *et al* [23] for *SLC6A4*. Forward primers contained a 10 mer sequence tag (aggaagagag), and reverse primers are equipped with a T7-promoter tag (cagtaatacgactcactataggg) and an 8 base pair insert (agaaggct). DNA methylation levels were analyzed using the EpiTYPER method from Sequenom. Bisulfite conversion was followed by PCR amplification, reverse transcription and base-specific cleavage. Fragments were analyzed on a mass spectrometer (Sequenom EpiTYPER, San Diego, CA, USA). Bisulfite conversion of DNA was performed using EZ-96 DNA Methylation Kit (Shallow) (Zymo Research, CA, USA), according to manufacturers' protocol. PCR, reverse transcription, cleavage and mass spectrometry were performed in triplicate, according to EpiTYPER protocol. The mass signal patterns generated were translated to quantitative methylation levels for different CpG-units by the MassARRAY EpiTYPER analyzer software from Sequenom (v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA). Fragments with CpG dinucleotides are referred to as CpG units. One CpG unit can contain one or more CpG dinucleotides. CpG units with a mass outside the range of the mass spectrometer or overlap in mass of another CpG unit could not be analyzed.

Data cleaning procedures. All samples were analyzed in triplicate, and methylation levels of the triplicates were averaged for each CpG unit [8]. Samples with a standard deviation of ≥10% between replicates and CpG units with >25% missing values were removed (*NR3C1*_1: CpGU4; *NR3C1*_3: CpGU13; *SLC6A4*: CpGU17). We accounted for mass-change in CpG units by SNPs (only when minor allele frequency >5%) by removing CpG units containing SNPs (*SLC6A4*: CpGU18) equal in mass to non- or other CpG units containing SNPs (none in our sample). In total, 11 CpG units remained eligible for *NR3C1*_1, 10 for *NR3C1*_2, 9 for *NR3C1*_3, and 11 for *SLC6A4*. Overall, we obtained methylation levels of *NR3C1*_1 for 454 individuals, of *NR3C1*_2 for 904 individuals, of *NR3C1*_3 for 412 individuals, and of *SLC6A4* for 939 individuals.

5HTTLPR and rs25531 genotyping

Sequence length analysis was used to determine the 5-HTT-linked-polymorphic-region genotype in the promoter region of *SLC6A4*. The length assessment of the *5HTTLPR* alleles was measured by direct analysis on an automated capillary sequencer (ABI3730, Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). The call rate was 91.6%. The single nucleotide substitution (A>G) present in the *5HTTLPR* l-allele (rs25531) was genotyped using a custom made TaqMan assay (Applied Biosystems). Additional information is described in Nederhof *et al* [29]. The call rate for rs25531 was 96.5%. Concordance between DNA replicates showed an accuracy of 100%. Because the l_g-allele is considered functionally equivalent to the s-allele [30], it was recoded as an s-allele, and the l_a-allele was recoded as an l-allele. The genotype frequencies for *5HTTLPR* (s/s: N=209, s/l: N=473, l/l: N=245) were in Hardy-Weinberg equilibrium (χ^2=0.45, df = 1, p=.50).

Internalizing problems

Symptom scores: Internalizing symptom scores were assessed dimensionally at baseline with the Internalizing Problems scale from the Youth Self-Report (YSR [31], 31 items; α= .89), and at follow-up with the Adult Self-Report (ASR [32], 39 items; α= .93) [33]. Specific problems scales from the YSR and ASR included DSM-IV Affective Problems scale (YSR: 13 items, α=.78; ASR: 14 items, α= .84) and DSM-IV Anxiety Problems scale (YSR: 6 items; α= .65; ASR: 7 items, α= .75). Mean item scores were used. All items referred to the past six months.

Clinical DSM-IV diagnoses: Internalizing disorders were assessed at follow-up using the Composite International Diagnostic Interview (CIDI; Version 3.0 [34]). The CIDI is a structured diagnostic interview that yields lifetime and current diagnoses according to criteria of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM–IV; American Psychiatric Association, 2000). Of all participants at follow-up, 84.2% (N=1584) agreed to have the diagnostic interview. In addition to lifetime prevalence, the CIDI generates the age of first onset, the age at which the last episode started, and the age at which the last episode ended.

We constructed a binary score indicating the presence or absence of lifetime diagnosis of any internalizing disorder, subdivided into Depression and Anxiety. Depression included Major Depressive Disorder and Dysthymia; Anxiety included Adult Separation Anxiety, Agoraphobia, Generalized Anxiety Disorder, Panic Disorder, Obsessive Compulsive Disorder, Separation Anxiety Disorder, and Social Phobia. We did not exclude individuals with comorbid anxiety and depressive disorders, because comorbid anxiety and depressive disorder are highly common and this may result in non-representative groups.

Statistical analyses

Missing data on internalizing symptom scores and CIDI diagnoses were imputed. Using the Multiple Imputations procedure in SPSS (version 21) twenty datasets were generated and subsequently used to generate pooled estimates from analyses. DNA methylation data was mean-centered, a method previously used in van der Knaap *et al* [8], and averaged over all CpG units within each DNA region.

Descriptive statistics were calculated and associations among internalizing problems and methylation values were examined with the use of Spearman correlations. The association between methylation levels and internalizing disorders was analyzed with a series of binary logistic regression models, each with the methylation level at a specific DNA region as predictor and the lifetime prevalence of an internalizing disorder as outcome variable. Associations between methylation levels and internalizing symptom scores, and the interaction between methylation and *5HTTLPR* in predicting internalizing symptom scores, were explored with linear regression analyses. In case of a significant interaction we will stratify the analyses by genotype. Methylation level at a specific DNA region was specified as predictor and internalizing symptom scores at follow-up was specified as outcome variable. In addition, we fitted a model that included internalizing symptom scores at baseline as additional predictor, to test whether methylation levels would predict a change in symptom scores over time. Each model contained age at baseline and sex as covariates, since age and sex may affect both methylation patterns [35] and the prevalence of internalizing problems [36]. To explore whether the association between DNA methylation and internalizing problems differs between sexes, we also tested interactions between methylation and sex in the models, and stratified the analyses by sex in case of a significant interaction effect.

RESULTS

Descriptive statistics of internalizing problems and methylation values are presented in Table 1 and Table 2. Bivariate associations between internalizing problems and DNA methylation are presented in Table 3. On average, slightly fewer internalizing problems were reported at follow-up than at baseline, but this does not necessarily imply a lack of emergence of new symptoms. The correlation between internalizing symptom scores at baseline and at follow-up is not very high (0.58), which indicates that a large proportion of individuals experience either a loss of symptoms or an emergence of new symptoms. We did not find an interaction between methylation and *5HTTLPR* genotype in predicting internalizing symptom scores prior to imputation (all ps>.05, see Supplementary Table S1), suggesting that *5HTTLPR* does not moderate the association between *SLC6A4* methylation levels and internalizing problems. None of the interactions with sex was

Table 1. Descriptive statistics of internalizing problems

	N	yes (%)
CIDI		
Lifetime internalizing disorder	945	277 (29.3%)
Lifetime anxiety disorder	945	193 (20.5%)
Lifetime depression disorder	945	149 (15.8%)
	N	**mean (sd)**
YSR (Assessment wave 3)		
Age	945	16.2 (0.7)
Internalizing symptom scores	945	0.3 (0.2)
ASR (Assessment wave 4)		
Age	945	19.0 (0.6)
Internalizing symptom scores	945	0.2 (0.2)

ASR=adult self-report; YSR=Youth self-report; CIDI=Composite international diagnostic interview. ASR and YSR scores are based on mean symptom scores for internalizing, anxiety and affective/depressive problems (N=945).

statistically significant, except for the interaction with *NR3C1*_3 methylation when adjusted for baseline symptom scores (see Supplementary Table S2). For boys, low *NR3C1*_3 methylation was associated with an increase in internalizing symptom scores, whereas no effect was found in girls (see Supplementary Table S2a).

NR3C1 methylation at DNA region 1 was associated with an increased risk for lifetime internalizing disorders (Table 4). An absolute increase of 1% methylation (percentage point) in this region indicated an almost 3-fold higher odd of suffering from an internalizing disorder at age 19. No associations were found between methylation in other DNA regions of *NR3C1* and lifetime internalizing disorders, or between *SLC6A4* methylation and lifetime internalizing disorders.

Higher methylation levels of *NR3C1* in DNA region 1 were also associated with higher internalizing symptom scores at follow-up (Table 5; for a graphical representation see Supplementary Figure S3). This association was not found in the other DNA regions of *NR3C1*. When accounting for internalizing symptom scores at baseline, the association became non-significant, indicating no prediction of symptom score change over time. Methylation levels of *SLC6A4* were not significantly associated with internalizing symptom scores at follow-up, but showed a tendency for association in the same direction, also when accounting for internalizing symptom scores at baseline.

Post hoc analyses

To explore whether the associations were driven by either depression or anxiety problems, we performed post hoc analyses using either depression or anxiety disorders, or symptom scores as outcome variable. Firstly, for anxiety and depression disorders, *NR3C1*

Table 2. Range, mean and SD of individual CpG units within the three *NR3C1* regions.

	N	Min	Max	Mean	SD
***NR3C1*_1**					
CpGU3	441	0.0%	8.5%	4.6%	1.6%
CpGU6	451	0.0%	3.3%	0.0%	0.3%
CpGU8	453	0.0%	4.5%	1.6%	0.7%
CpGU9	454	1.7%	7.5%	3.1%	0.7%
CpGU10	454	0.5%	6.7%	2.5%	1.2%
CpGU11	454	0.5%	7.7%	2.9%	0.9%
CpGU12	453	2.0%	16.0%	9.9%	2.4%
CpGU15	454	0.3%	6.3%	2.8%	0.8%
CpGU16	454	0.0%	6.3%	1.0%	0.6%
CpGU17	454	0.0%	6.0%	0.8%	0.9%
CpGU19	454	0.0%	2.0%	0.2%	0.3%
***NR3C1*_2**					
CpGU2	904	0.0%	4.0%	1.3%	0.5%
CpGU4	903	0.0%	7.0%	1.3%	1.0%
CpGU5	904	0.0%	5.7%	1.7%	1.0%
CpGU6	904	0.0%	7.0%	1.5%	1.1%
CpGU7	903	0.0%	8.3%	2.5%	1.3%
CpGU11	904	0.0%	6.5%	0.7%	0.7%
CpGU13	819	1.0%	27.5%	10.1%	4.8%
CpGU14	903	1.5%	14.7%	7.5%	2.1%
CpGU17	902	0.0%	9.3%	0.7%	1.0%
CpGU18	900	1.0%	12.3%	4.3%	1.6%
***NR3C1*_3**					
CpGU1	407	0.0%	8.7%	2.5%	1.0%
CpGU2	406	1.0%	11.0%	3.6%	1.6%
CpGU4	410	0.0%	8.5%	2.2%	1.5%
CpGU5	404	1.0%	11.0%	4.6%	1.9%
CpGU9	408	0.0%	11.5%	0.6%	1.0%
CpGU10	412	0.0%	5.0%	0.4%	0.7%
CpGU11	410	0.0%	7.3%	1.9%	1.3%
CpGU12	404	0.0%	10.0%	2.1%	1.4%
CpGU15	397	0.0%	7.7%	3.2%	1.2%
SLC6A4					
CpGU1	938	0.5%	8.3%	2.8%	0.9%
CpGU3	936	2.3%	12.0%	5.8%	1.6%
CpGU4	920	0.0%	19.0%	6.0%	2.5%
CpGU6	934	0.0%	10.3%	1.4%	1.6%
CpGU9	935	2.0%	14.0%	7.6%	1.9%
CpGU10	774	0.0%	9.5%	2.4%	1.5%
CpGU12	898	5.0%	27.3%	15.4%	3.3%
CpGU14	892	0.7%	22.7%	9.5%	3.2%
CpGU15	930	2.7%	20.0%	8.6%	2.3%
CpGU16	843	5.0%	31.0%	15.9%	3.8%
CpGU22	859	7.7%	47.0%	28.0%	5.3%

A CpG unit (CpGU) is a fragment of DNA containing one or more CpG dinucleotides. *NR3C1*=Glucocorticoid receptor gene. *SLC6A4*=Serotonin transporter gene

Table 3. Bivariate associations between internalizing problems and *NR3C1* and *SLC6A4* methylation.

			Internalizing symptom scores						Clinical diagnoses			DNA methylation		
			Baseline			**Follow-up**						***NR3C1***		
			INT	**DEP**	**ANX**	**INT**	**DEP**	**ANX**	**INT**	**DEP**	**ANX**	***NR3C1*_1**	***NR3C1*_2**	***NR3C1*_3**
Internalizing symptom scores	**baseline**	**DEP**	,80**											
		ANX	,77**	,55**										
	follow-up	**INT**	,58**	,52**	,41**									
		DEP	,52**	,53**	,37**	,86**								
		ANX	,54**	,43**	,48**	,79**	,68**							
clinical diagnoses		**INT**	,38**	,33**	,30**	,42**	,39**	,35**						
		DEP	,34**	,34**	,28**	,37**	,39**	,30**	,62**					
		ANX	,31**	,24**	,24**	,32**	,29**	,30**	,72**	,25**				
DNA methylation	***NR3C1***	***NR3C1*_1**	,12*	,12**	,10*	,15**	,14**	,14**	,21**	,21**	,17**			
		***NR3C1*_2**	,02	,00	,00	-,04	-,05	-,02	-,01	-,00	,02	,08		
		***NR3C1*_3**	-,08	-,13**	-,04	-,07	-,08	-,09	-,07	-,04	-,06	-,22**	,01	
	SLC6A4	***SLC6A4***	,15**	,12**	,11**	,11**	,15**	,10**	,11**	,08*	,11**	-,05	,16**	,13**

Numbers shown are Spearman correlation coefficients. INT=internalizing problems, DEP=depression, ANX=anxiety. *NR3C1*= Glucocorticoid receptor gene, *SLC6A4*=Serotonin transporter gene. See method section for further details.**p<0.01. *p<0.05.

methylation levels at *NR3C1*_1 were positively associated with the risk of a depressive (OR=3.71, 95%CI=2.00–6.89, p<.001) and an anxiety (OR=2.41, 95%CI=1.35–4.31, p=.003) disorder. For *SLC6A4*, higher levels of methylation were associated with a higher odds for anxiety (OR=1.16, 95%CI=1.02–1.33, p=.026), but not depression (OR=1.04, 95%CI=0.90–1.21, p=.613). Secondly, for symptom scores, *NR3C1* methylation levels in *NR3C1*_1 were positively associated with depressive symptom scores at follow-up (B=0.13, SE=0.03, p<.001), but became non-significant when accounting for depressive symptom scores at baseline (B=0.05, SE=0.03, p=.119). *SLC6A4* methylation levels were also positively associated with depressive symptom scores at follow-up (B=0.02, SE=0.01, p=.011), and this association remained significant when accounting for depressive symptom scores at baseline (B=0.02, SE=0.01, p=.015). For anxiety symptom scores, we only found an association between *NR3C1* methylation in *NR3C1*_1 and higher anxiety symptom scores at follow up (B=0.13, SE=0.04, p<.001), which remained significant after accounting for anxiety symptom scores at baseline (B=0.07, SE=0.03, p=.044). No associations were found between *SLC6A4* methylation levels and anxiety symptom scores (B=0.01, SE=0.01, p=.250).

Table 4. Associations between methylation levels of *NR3C1* and *SLC6A4* and lifetime internalizing disorders.

Methylation	**Lifetime internalizing disorder**			
	OR	**95% CI of OR**	**p**	**N**
*NR3C1*_1	**2.91**	**1.70 - 4.98**	**<.001**	454
*NR3C1*_2	0.97	0.76 - 1.24	.809	904
*NR3C1*_3	0.65	0.38 - 1.11	.113	412
Methylation	**Lifetime internalizing disorder**			
	OR	**95% CI of OR**	**p**	**N**
SLC6A4	1.08	0.96 - 1.22	.193	939

Results were derived from binary logistic regression analyses; bold numbers indicate significant results. All models were adjusted for sex and age. *NR3C1*=Glucocorticoid receptor gene; *SLC6A4*=Serotonin transporter gene.

DISCUSSION

We found that *NR3C1* methylation at age 16 positively predicted the probability of a lifetime diagnosis of internalizing disorder, as well as internalizing symptom scores three years later. Methylation of *SLC6A4* was not associated with internalizing disorders, while the association with internalizing symptom scores only showed a tendency for association.

Table 5. Associations between methylation levels of *NR3C1* and *SLC6A4* and internalizing symptom scores at follow-up.

Methylation	Internalizing symptom scores at follow-up			
	B	**SE**	**p**	**N**
*NR3C1*_1	**0.11**	**0.03**	**<.001**	454
*NR3C1*_1[a]	0.04	0.02	.059	
*NR3C1*_2	0.00	0.01	.862	904
*NR3C1*_2[a]	-0.01	0.01	.615	
*NR3C1*_3	-0.04	0.03	.123	412
*NR3C1*_3[a]	-0.02	0.02	.397	

Methylation	Internalizing symptom scores at follow-up			
	B	**SE**	**p**	**N**
SLC6A4	0.01	0.01	.054	939
SLC6A4[a]	0.01	0.01	.085	

Results were derived from linear regression analyses; bold numbers indicate significant results. All models were adjusted for sex and age. [a] Additionally adjusted for internalizing problems at baseline. *NR3C1*=Glucocorticoid receptor gene; *SLC6A4*=Serotonin transporter gene.

DNA methylation has scarcely been investigated in relation to internalizing disorders, particularly anxiety disorders. The association between *NR3C1* methylation and risk of internalizing disorders found in this study may provide a first step in unraveling the role of epigenetic modification in the pathophysiology of internalizing disorders. *SLC6A4* methylation was not associated with internalizing disorders as a whole in this study, but post hoc analyses suggested that it might be related to anxiety disorders only. In contrast, *NR3C1* methylation seemed to increase the risk of both anxiety and depression disorders. Our exploratory analyses on sex differences did not reveal convincing evidence that the relation between methylation and internalizing symptom scores is modified by sex. We did find a significant interaction with *NR3C1*_3 methylation, but only when adjusted for baseline symptom levels, and the effect was only just significant (p=0.049). Therefore, this interaction effect could well be a chance finding and requires replication before justifying speculation about mechanisms.

The prospective relationship between *NR3C1* methylation and internalizing symptom scores at follow-up appeared to be largely explained by the cross-sectional association with symptom scores at baseline. When we compared the first model with the second model, in which we additionally adjusted for baseline symptom scores, we noticed a difference in effect size for both genes. For *NR3C1* the value of the regression coefficient was lower in the second model, yet it is still somewhat elevated. This may suggest that

although most of the effect is explained by baseline symptom scores, there is still some vulnerability for internalizing symptom scores 3 years later. For *SLC6A4*, this drop in the regression coefficient is not observed. The association between *SLC6A4* and internalizing symptom scores at follow-up is not affected by internalizing symptom scores at baseline. Possibly, these differences in decrease of effect size may reflect gene-specific differences in the temporal stability of methylation, with less stable methylation for *NR3C1* than for *SLC6A4*.

The post hoc analyses for *NR3C1* showed that associations with internalizing symptom scores could be attributed to both anxiety and depression symptoms, and for *SLC6A4* only to depression symptoms. Together with the post hoc results on internalizing disorders, *NR3C1* methylation seems to have a more generic role in internalizing problems, whereas the role of *SLC6A4* may be less consistent across phenotypes of internalizing problems. However, drawing a firm conclusion from this single study is opportunistic, as replication of the current findings and extension the current findings to more specific phenotypic internalizing problems is warranted. Still, our findings may be useful in the search to unravel the physiological processes that underlie the different internalizing problems.

Further investigation of the link between DNA methylation and internalizing disorders may lead to a better understanding of the etiology, but may also have clinical implications, such as the potential for diagnostic biomarkers, which have already been suggested for the BDNF gene in relation to MDD [37]. The association between *NR3C1* methylation and internalizing problems in the current study makes it a likely target for a potential biomarker. For *SLC6A4*, the association with internalizing problems is not so straight forward, thus reducing its potential as a biomarker. However, a recent study by Okada *et al* [38] suggests that potential biomarkers for *SLC6A4* should be CpG unit specific, as they also did not find an association between overall *SLC6A4* methylation and early adversity or the severity of depression.

Associations between *NR3C1* methylation and internalizing problems were only found for DNA region 1 (positioned at the 3' end of the CpG island). This particular DNA region covers an area that extends beyond the CpG island, into the so-called CpG island shores (regions up to 2kb outside of the CpG island borders) [39]. More methylation in these shores has been strongly associated with a decrease in expression of the gene, which indicates an important functional role of CpGs in these shores [39, 40]. It is tempting to speculate that the associations between methylation in our other DNA regions (2 and 3) and gene expression were not sufficiently strong to influence any physiological process that may lead to the development of internalizing problems. However, more research is needed on the potential influence of individual methylated CpG sites or locations on the development of these disorders.

Several limitations should be acknowledged. Because blood was collected only at baseline, changes in methylation levels could not be analyzed. We could therefore not establish a causal link between methylation level and the onset of internalizing problems. Also, we were unable to analyze gene expression levels of *NR3C1* and *SLC6A4* in this study, although it should be noted that for both *NR3C1* and *SLC6A4*, higher methylation levels have been associated with lower expression levels before [11, 12, 28, 41].

Nonetheless, our large sample size and the use of multiple outcome measures (i.e. symptom scores and DSM-IV diagnoses) strengthen our findings. The association between stress-related environmental factors and methylation in previous TRAILS studies [8, 23], and the associations with internalizing problems in the current TRAILS study, also support the theory of the mediating effect of methylation in the development of internalizing disorders [42]. Although the functional role of gene methylation in the development of internalizing problems is still unclear, our findings provides suggestive evidence that gene methylation, of *NR3C1* in particular, may be involved in the development of internalizing problems in adolescence.

REFERENCES

1. Carr CP, Martins CM, Stingel AM, Lemgruber VB, Juruena MF. The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes. *J Nerv Ment Dis* 2013; **201**(12)**:** 1007-1020.
2. Etain B, Mathieu F, Henry C, Raust A, Roy I, Germain A *et al.* Preferential association between childhood emotional abuse and bipolar disorder. *J Trauma Stress* 2010; **23**(3)**:** 376-383.
3. van Grootheest DS, Cath DC, Beekman AT, Boomsma DI. Twin studies on obsessive-compulsive disorder: a review. *Twin Res Hum Genet* 2005; **8**(5)**:** 450-458.
4. Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. *Am J Psychiatry* 2000; **157**(10)**:** 1552-1562.
5. Bienvenu OJ, Davydow DS, Kendler KS. Psychiatric 'diseases' versus behavioral disorders and degree of genetic influence. *Psychol Med* 2011; **41**(1)**:** 33-40.
6. Roth TL. Epigenetic mechanisms in the development of behavior: advances, challenges, and future promises of a new field. *Dev Psychopathol* 2013; **25**(4 Pt 2)**:** 1279-1291.
7. Szyf M. DNA methylation, the early-life social environment and behavioral disorders. *J Neurodev Disord* 2011; **3**(3)**:** 238-249.
8. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ *et al.* Glucocorticoid receptor gene (*NR3C1*) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry* 2014; **4:** e381.
9. Klengel T, Pape J, Binder EB, Mehta D. The role of DNA methylation in stress-related psychiatric disorders. *Neuropharmacology* 2014.
10. Na KS, Chang HS, Won E, Han KM, Choi S, Tae WS *et al.* Association between Glucocorticoid Receptor Methylation and Hippocampal Subfields in Major Depressive Disorder. *PLoS One* 2014; **9**(1)**:** e85425.
11. Philibert RA, Sandhu H, Hollenbeck N, Gunter T, Adams W, Madan A. The relationship of 5HTT (*SLC6A4*) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B**(5)**:** 543-549.
12. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR *et al.* Epigenetic programming by maternal behavior. *Nat Neurosci* 2004; **7**(8)**:** 847-854.
13. Zhao J, Goldberg J, Bremner JD, Vaccarino V. Association between promoter methylation of serotonin transporter gene and depressive symptoms: a monozygotic twin study. *Psychosom Med* 2013; **75**(6)**:** 523-529.
14. Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB. The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology* 2008; **33**(6)**:** 693-710.
15. Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. *PLoS One* 2012; **7**(1)**:** e30148.
16. Heim C, Nemeroff CB. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* 2001; **49**(12)**:** 1023-1039.
17. Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S *et al.* Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 1996; **274**(5292)**:** 1527-1531.

18. Owens MJ, Nemeroff CB. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clin Chem* 1994; **40**(2)**:** 288-295.
19. Alt SR, Turner JD, Klok MD, Meijer OC, Lakke EA, Derijk RH *et al.* Differential expression of glucocorticoid receptor transcripts in major depressive disorder is not epigenetically programmed. *Psychoneuroendocrinology* 2010; **35**(4)**:** 544-556.
20. Kang HJ, Kim JM, Stewart R, Kim SY, Bae KY, Kim SW *et al.* Association of *SLC6A4* methylation with early adversity, characteristics and outcomes in depression. *Prog Neuropsychopharmacol Bol Psychiatry* 2013; **44:** 23-28.
21. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. *Annu Rev Physiol* 2005; **67:** 259-284.
22. Kessler RC, Avenevoli S, Ries Merikangas K. Mood disorders in children and adolescents: an epidemiologic perspective. *Biol Psychiatry* 2001; **49**(12)**:** 1002-1014.
23. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MMPJ, Verhulst FC, Oldehinkel AJ *et al.* Adverse life events and allele-specific methylation of the serotonin transporter gene (*SLC6A4*) in adolescents. The TRAILS study. *Psychosom Med* in press.
24. van IJzendoorn MH, Caspers K, Bakermans-Kranenburg MJ, Beach SR, Philibert R. Methylation matters: interaction between methylation density and serotonin transporter genotype predicts unresolved loss or trauma. *Biol Psychiatry* 2010; **68**(5)**:** 405-407.
25. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, Huizink AC *et al.* Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. *Int J Epidemiol* 2008; **37**(6)**:** 1227-1235.
26. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, Veenstra R *et al.* The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. *J Am Acad Child Adolesc Psychiatry* 2012; **51**(10)**:** 1020-1036.
27. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3)**:** 1215.
28. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2009; **12**(3)**:** 342-348.
29. Nederhof E, Bouma EM, Oldehinkel AJ, Ormel J. Interaction between childhood adversity, brain-derived neurotrophic factor val/met and serotonin transporter promoter polymorphism on depression: the TRAILS study. *Biol Psychiatry* 2010; **68**(2)**:** 209-212.
30. Hu XZ, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD *et al.* Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *Am J Hum Genet* 2006; **78**(5)**:** 815-826.
31. Achenbach TM. Manual for the Youth Self-Report and 1991 profile. Burlington, VT: University of Vermont; 1991.
32. Achenbach TM, Rescorla LA. Manual for ASEBA Adult Forms & Profiles. Burlington, VT: University of Vermont; 2003.
33. Achenbach TM, Dumenci L, Rescorla LA. DSM-oriented and empirically based approaches to constructing scales from the same item pools. *J Clin Child Adolesc Psychol* 2003; **32**(3)**:** 328-340.
34. Kessler RC, Ustun TB. The World Mental Health (WMH) Survey Initiative Version of the World Health Organization (WHO) Composite International Diagnostic Interview (CIDI). *Int J Methods Psychiatr Res* 2004; **13**(2)**:** 93-121.
35. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol* 2013; **14**(10)**:** R115.

36. Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S *et al.* Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Arch Gen Psychiatry* 1994; **51**(1)**:** 8-19.
37. Fuchikami M, Morinobu S, Segawa M, Okamoto Y, Yamawaki S, Ozaki N *et al.* DNA methylation profiles of the brain-derived neurotrophic factor (BDNF) gene as a potent diagnostic biomarker in major depression. *PLoS One* 2011; **6**(8)**:** e23881.
38. Okada S, Morinobu S, Fuchikami M, Segawa M, Yokomaku K, Kataoka T *et al.* The potential of *SLC6A4* gene methylation analysis for the diagnosis and treatment of major depression. *J Psychiatr Res* 2014; **53:** 47-53.
39. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P *et al.* The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet* 2009; **41**(2)**:** 178-186.
40. Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R *et al.* Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet* 2009; **41**(12)**:** 1350-1353.
41. Philibert R, Madan A, Andersen A, Cadoret R, Packer H, Sandhu H. Serotonin transporter mRNA levels are associated with the methylation of an upstream CpG island. *Am J Med Genet B Neuropsychiatr Genet* 2007; **144B**(1)**:** 101-105.
42. Szyf M. The early life environment and the epigenome. *Biochim Biophys Acta* 2009; **1790**(9)**:** 878-885.

SUPPLEMENTARY INFORMATION

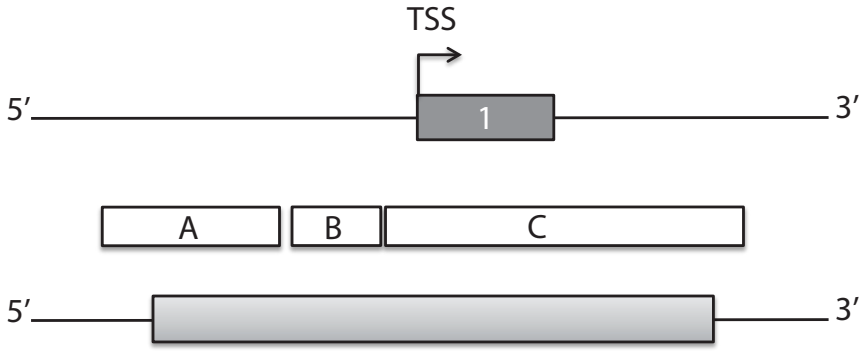


Figure S1. Schematic representation of the position of the serotonin transporter gene (*SLC6A4)* CpG island (Chr17:28562388-28563186; represented by the grey box, based on the UCSC human Feb. 2009 assembly GRCh37/hg19) relative to the transcription start site (TSS) in exon 1 (dark grey box; based on Ensembl Transcript ID ENST00000394821). The relative position of the genomic fragments of the gene used in methylation analyses in previous studies are represented by the lined boxes with letters A [1], B [2] and C ([3][primer set B] and this study).

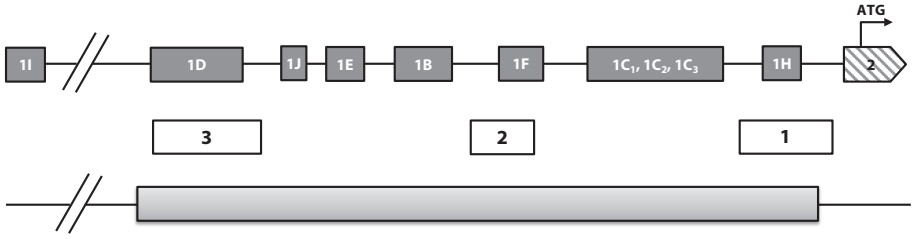


Figure S2. Schematic representation of the glucocorticoid receptor gene (*NR3C1*) and CpG island (chr5:142782072-142785071, represented by the grey box, based on the UCSC human Feb. 2009 assembly GRCh37/hg19). Genomic fragments (1=*NR3C1*_1, 2=*NR3C1*_2 and 3=*NR3C1*_3, white lined boxes) are shown in relation to the *NR3C1* CpG-island (grey box) and untranslated first exons (dark grey boxes) upstream of exon 2 (striped box). Image based on Labonte *et al* [4] and Turner *et al* [5] and adapted from van der Knaap *et al* [6].

Table S1. Regression analyses of internalizing symptom scores at follow-up, *5HTTLPR* genotype and the interaction between *SLC6A4* methylation and *5HTTLPR* genotype, on internalizing symptom scores at follow-up.

Methylation	Internalizing symptom scores at follow-up			
	B	**SE**	**P**	**N**
SLC6A4	0.00	0.01	0.927	
SLC6A4 X SL[a]	0.01	0.02	0.476	853
SLC6A4 X LL[a]	0.02	0.02	0.332	
SLC6A4 [b]	-0.01	0.01	0.522	
SLC6A4 X SL[a b]	0.02	0.01	0.094	830
SLC6A4 X LL[a b]	0.01	0.01	0.331	

Analyses performed on non-imputed dataset. *5HTTLPR* S-allele includes S and L_g alleles, the L-allele includes the L_a allele (see method section for further details). [a] = SS is the reference category, [b] = additionally adjusted for internalizing symptom scores at baseline.

Table S2. Regression analyses of internalizing symptom scores at follow-up, and the interaction between DNA methylation and sex, on internalizing symptom scores at follow-up.

Methylation	Internalizing symptom scores at follow-up			
	B	**SE**	**P**	**N**
*NR3C1*_1	**0.13**	**0.04**	**0.000**	426
*NR3C1*_1 X sex	-0.05	0.05	0.331	
*NR3C1*_1[a]	0.03	0.03	0.330	419
*NR3C1*_1 X sex[a]	0.03	0.05	0.477	
*NR3C1*_2	0.00	0.02	0.869	834
*NR3C1*_2 X sex	0.00	0.03	0.895	
*NR3C1*_2[a]	-0.01	0.01	0.519	813
*NR3C1*_2 X sex[a]	0.00	0.02	0.936	
*NR3C1*_3	-0.04	0.04	0.345	389
*NR3C1*_3 X sex	-0.02	0.06	0.753	
*NR3C1*_3[a]	0.03	0.03	0.408	385
*NR3C1*_3 X sex[a]	**-0.09**	**0.05**	**0.049**	

Methylation	Internalizing symptom scores at follow-up			
	B	**SE**	**P**	**N**
SLC6A4	**0.02**	**0.01**	**0.021**	856
SLC6A4 X sex	-0.02	0.01	0.133	
SLC6A4[a]	**0.02**	**0.01**	**0.017**	841
SLC6A4 X sex[a]	-0.02	0.01	0.102	

Note: Results were derived from linear regression analyses; bold numbers indicate significant results. All models were adjusted for age. [a]Additionally adjusted for internalizing symptom scores at baseline. *NR3C1*=Glucocorticoid receptor gene; *SLC6A4*=Serotonin transporter gene.

Table S2a. Associations between methylation rates of *NR3C1*_3 and internalizing symptom scores at follow-up for boys and girls separately.

	Boys				Girls			
Methylation	**Internalizing symptom scores at follow-up**				**Internalizing symptom scores at follow-up**			
	B	**SE**	**P**	**N**	**B**	**SE**	**P**	**N**
*NR3C1*_3[a]	**-0.07**	**0.03**	**0.022**	203	0.02	0.04	0.674	209

Note: Results were derived from linear regression analyses; bold numbers indicate significant results. The model was adjusted for age. [a]Additionally adjusted for internalizing problems at baseline. *NR3C1*=Glucocorticoid receptor gene.

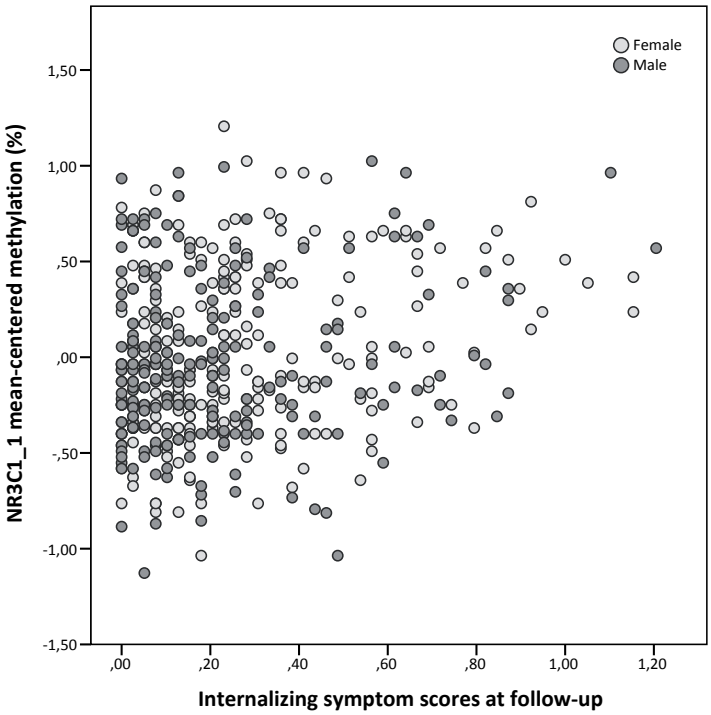


Figure S3. Graphical representation of *NR3C1*_1 centered methylation and internalizing symptom scores at follow-up.

REFERENCES SUPPLEMENTARY INFORMATION

1. Kang HJ, Kim JM, Stewart R, Kim SY, Bae KY, Kim SW *et al.* Association of *SLC6A4* methylation with early adversity, characteristics and outcomes in depression. *Prog Neuropsychopharmacol Bol Psychiatry* 2013; **44:** 23-28.
2. Zhao J, Goldberg J, Bremner JD, Vaccarino V. Association between promoter methylation of serotonin transporter gene and depressive symptoms: a monozygotic twin study. *Psychosom Med* 2013; **75**(6)**:** 523-529.
3. Philibert RA, Sandhu H, Hollenbeck N, Gunter T, Adams W, Madan A. The relationship of 5HTT (*SLC6A4*) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B**(5)**:** 543-549.
4. Labonte B, Yerko V, Gross J, Mechawar N, Meaney MJ, Szyf M *et al.* Differential glucocorticoid receptor exon 1(B), 1(C), and 1(H) expression and methylation in suicide completers with a history of childhood abuse. *Biol Psychiatry* 2012; **72**(1)**:** 41-48.
5. Turner JD, Alt SR, Cao L, Vernocchi S, Trifonova S, Battello N *et al.* Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. *Biochem Pharmacol* 2010; **80**(12)**:** 1860-1868.
6. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ *et al.* Glucocorticoid receptor gene (*NR3C1*) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry* 2014; **4:** e381.

Chapter 5

Glucocorticoid receptor gene methylation and HPA-axis regulation in adolescents. The TRAILS study

L.J. van der Knaap
A.J. Oldehinkel
F.C. Verhulst
F.V.A. van Oort
H. Riese

Psychoneuroendocrinology (2015) ***58****, 46—50*

ABSTRACT

Early life adversity and psychopathology are thought to be linked through HPA-axis deregulation. Changes in methylation levels of stress reactivity genes such as the glucocorticoid receptor gene (*NR3C1*) can be induced by adversity. Higher *NR3C1* methylation levels have been associated with a reduced *NR3C1* expression, possibly leading to impaired negative feedback regulation of the HPA-axis. In this study we tested whether methylation levels of *NR3C1* were associated with HPA-axis regulation, operationalized as cortisol responses. In 361 adolescents (mean age 16.1, SD = 0.6), salivary cortisol samples were collected before, during, and after a social stress task, from which response measures (cortisol activation and recovery) were calculated. Higher *NR3C1* methylation levels were associated with a flattened cortisol recovery slope, indicating a delayed recovery time. Cortisol response activation was not associated with *NR3C1* methylation. These results suggest that methylation of *NR3C1* may impair negative feedback of the HPA-axis in adolescents.

INTRODUCTION

Early life adversity and later psychopathology have been commonly linked with impairments in HPA-axis feedback regulation [1, 2]. Studies in rats showed that variations in maternal care (i.e. pup licking and grooming [LG] and arched-back nursing [ABN] behavior) can alter offspring HPA-axis responses to stress through epigenetic modifications such as DNA methylation. High methylation levels in the glucocorticoid receptor gene (*NR3C1*) of offspring from mothers exhibiting low levels of LG and ABN behavior were associated with a decreased *NR3C1* expression [3]. Decreased *NR3C1* expression could impair the feedback sensitivity of the HPA-axis and so result in higher levels of glucocorticoids (corticosterone in rats, cortisol in humans) in response to stress, a finding previously reported in offspring from low LG/ABN mothers. In humans, early life adversity is known to be an important risk factor for the development of psychopathology. Early life adversity has been associated with epigenetic modification of *NR3C1* (e.g., [4, 5]), which may mediate the association with later psychopathology by influencing the HPA-axis responses to stress. Only a few human studies explored the association between *NR3C1* methylation and the cortisol response [6-8]. These studies focused on a region of the *NR3C1* CpG island homologous to the region investigated in the rat studies, the exon 1_F promoter (1_7 in rats), but the results reported are inconclusive. Oberlander *et al* [7] reported an association between high methylation and increased salivary stress response in infants, whereas Tyrka *et al* [8] reported attenuated cortisol response to a dexamethasone/ corticotrophin-releasing hormone test in adults with high *NR3C1* methylation. Edelman *et al* [6] reported an association between *NR3C1* methylation and cortisol area under the curve (AUC) following the Trier Social Stress Task (TSST), but only in adult women. We investigated the association between *NR3C1* methylation and HPA-axis regulation, operationalized as the activation and recovery of the cortisol response following social stress, in a large sample of adolescents. Assuming comparable HPA-axis regulation mechanisms in rats and humans, we hypothesized that higher levels of methylation would be associated with an impaired feedback sensitivity, represented by a delayed recovery following social stress. As the activation of the HPA-axis is independent of glucocorticoid receptor availability, we further hypothesized that the cortisol response activation would not be related to the methylation level.

MATERIALS AND METHODS

Sample and procedure

This study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective population study of Dutch adolescents with biennial or triennial assessment

waves from age 11 to at least age 25. A detailed description of sampling and methods can be found in Oldehinkel *et al* [9]. For this study, data from the third assessment wave (T3, N=1816, mean age=16.3, SD=0.71) were used, which involved a blood draw and a series of laboratory tasks (for which 715 adolescents were selected to participate, see Figure S1 for a flow diagram of selection procedures), hereafter referred to as the experimental session. The experimental sessions took place on weekdays, lasted about 3 hours and 15 minutes, and started between 08:00 and 09:30 a.m. (morning sessions, 49%) or between 01:00 and 02:30 p.m. (afternoon sessions, 51%). Although cortisol levels may be higher in the morning due to the circadian rhythm of cortisol production, morning and afternoon cortisol responses to social stress were comparable in our sample [10], analog to prior reports [11]. The participants were asked to refrain from smoking and consuming coffee, milk, chocolate, and other sugar containing foods in the 2 hours before the experimental session. At the start of the session, the test assistant explained the procedure and administered a short checklist on current medication use (including oral contraceptives), and adherence to the smoking and food restrictions. One of the tasks of the experimental session was the Groningen Social Stress Test (GSST), a standardized protocol for the induction of moderate performance-related social stress. A detailed description of the GSST can be found in Bouma *et al* [10]. In short, participants were given 7 minutes to prepare a 6-min speech about themselves and their lives, which they had to present in front of a video camera. They were told that their videotaped performance would be judged on content of speech as well as on use of voice and posture, and rank-ordered by a panel of peers after the experiment. Participants had to speak continuously and the test assistant watched the performance critically, without showing empathy or encouragement. After 6 min of speech, a 3 minute interlude was introduced in which the participants were not allowed to speak. After this interlude, participants were instructed to perform mental arithmetic and subtract 17 repeatedly, starting with 13,278, whilst receiving negative feedback from the test assistant. The mental arithmetic challenge lasted for 6 min, again followed by a 3-min period of silence. In total, this test lasted about 30 minutes, after which the participants were debriefed about the experiment. Readministration of (an adapted version of) the social stress test after three years in a subsample (n=177) of TRAILS revealed test-retest correlations of cortisol levels during stress of about .40 (O.M. Laceulle, E. Nederhof, M.A.G. van Aken and J. Ormel, unpublished observations). Information about how the cortisol stress responses were related to other physiological responses and subjectively experienced stress can be found in Oldehinkel *et al* [12].

Sample selection for the methylation analyses was based on the availability of blood, participation in the experimental session, availability of cortisol samples, Dutch ethnicity, and sufficient DNA concentration. We randomly excluded one of each sibling pair and removed DNA samples that were not suitable for analyses due to poor quality. From

the remaining sample (N=468), we excluded girls using oral contraceptives as they displayed no cortisol response during the GSST [10]; adolescents who used SSRIs, systemic corticosteroids, or analgesic (pain-relieving) drugs on the day of the experiment or the day before, because these medications can influence responses to stress; and adolescents who smoked before the experiments and thus failed to adhere with the protocol; and adolescent with laboratory detection failures in more than 2 of the 4 saliva samples. In total, data of 361 adolescents were used in the statistical analyses.

Cortisol responses

Details on cortisol assessment (expressed in nmol/l) and analyses are described in Bouma *et al* [10] and Janssens *et al* [13]. In short, free cortisol concentrations were measured directly in duplicate in 100 ml saliva using an in-house radioimmunoassay (RIA) applying a polyclonal rabbit cortisol antibody and 1,2,6,7 ^{3}H Cortisol (Amersham International Ltd., Amersham, UK) as tracer. After incubation for 30 min at 60°C, the bound and free fractions were separated using activated charcoal. Responses to the GSST were assessed by four cortisol samples, referred to as C1, C2, C3, and C4. C1 was taken just before the start of the GSST. Cortisol levels in saliva reflect HPA-axis activity circa 20 minutes earlier, as there is a time window between the production of cortisol by the adrenal cortices and the presence of cortisol in saliva [14]. Hence C1 reflects HPA-axis activity before the GSST, and is considered a pretest measure. C2 was collected directly after the end of the GSST and thus reflects HPA-axis responses during speech. C3, collected 20 minutes after the end of the GSST is considered a reflection of HPA-axis activity around the end of the GSST. C4 was collected 40 minutes after the end of the GSST and is considered a post-stress activity measure of the HPA-axis.

Cortisol response variables were computed from the response to the social stress task [15]. Response activation was computed by regressing cortisol levels during the task (C2) on cortisol levels before the task (C1) and saving the standardized residuals. Positive scores represent relatively high HPA-axis activation compared to other participants. Response recovery was computed by regressing cortisol levels measured 40 minutes after the task (C4) on cortisol levels during the task (C2) and saving the standardized residuals. A positive score represents a flat recovery slope compared to other participants.

NR3C1 methylation

DNA was extracted from whole-blood samples using a manual salting-out procedure as described by Miller *et al* [16]. Three regions in the *NR3C1* CpG island were analyzed, using a primer set previously used by McGowan *et al* [4] and two additional primer sets designed for this study (with EpiDesigner by Sequenom) to optimize coverage of the CpG island. DNA regions were numbered (*NR3C1*_1 to 3) according to their position in the CpG island (*NR3C1*_1 Chr5: 142782046-142782472, encompassing exon 1H; *NR3C1*_2 Chr5:

142783585-142783906, encompassing exon 1F; *NR3C1*_3 Chr5: 142784559-142784950, encompassing exon 1D; further details, a graphical representation on DNA regions and methylation assessment were published before [5]). DNA methylation was analyzed using bisulfite treated DNA, PCR, reverse transcription, base-specific cleavage of in vitro transcribed RNA product, and mass spectrometry (Sequenom EpiTYPER, San Diego, CA, USA). Bisulfite conversion of DNA (500ng) was performed using EZ-96 DNA Methylation Kit (Shallow) (Zymo Research, Irvine, CA, USA). It must be noted that bisulfite conversion required for methylation analyses does not differentiate between the different types of cytosine methylation (i.e., 5-hydroxycytosine) or other derivatives, such as 5-formylcytosine, and 5-carboxylcytosine. As such, our use of the term DNA methylation reflects multiple cytosine modifications, but to remain consistent with primary publications, we will adhere to the term DNA methylation. PCR, reverse transcription, cleavage and mass spectrometry was performed in triplicate, according to the EpiTYPER protocol. The generated mass signal patterns were translated to quantitative methylation levels (expressed in percentages) by the MassARRAY EpiTYPER analyzer software (v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA).

Statistical analyses

DNA methylation levels were mean-centered [5], and averaged over all CpG units within a DNA region. We used linear regression to study the associations between *NR3C1* methylation and HPA-axis responses to social stress. Statistical tests were performed in SPSS (v.21.0). We adjusted for multiple testing using the Bonferroni method; effects were regarded as significant if the p-value was smaller than .008. We included the covariates sex, age, daily smoking, and the start time of the experimental session in our analyses. Analyses without covariates are given in Supplementary Table S1.

RESULTS

Of the 361 participants, 222 were male (61.5%), and the mean age was 16.1 years (SD=0.6). Whereas no association was found between methylation of *NR3C1* and activation of the cortisol response in any of the three regions, methylation of *NR3C1* in region 2 was positively associated with cortisol response recovery (Table 1). Controlling for cortisol response activation in the analyses on cortisol response recovery resulted in highly similar outcomes (data not shown).

Table 1. Results of the linear regression analyses of *NR3C1* methylation and HPA-axis regulation in the Groningen Social Stress Task.

		cortisol response activation			cortisol response recovery		
	N	**B**	**SE**	**p**	**B**	**SE**	**p**
***NR3C1*_1**	339	-0.01	0.13	.96	0.01	0.13	.96
***NR3C1*_2**	337	0.00	0.10	.99	**0.38**	**0.10**	**<.001**
***NR3C1*_3**	307	0.05	0.13	.72	0.02	0.11	.88

Methylation variables are mean-centered. Cortisol variables are standardized. Significant results (p<0.008 after Bonferroni correction) are indicated with bold numbers. See Section 2 for details on the calculation of cortisol reactivity and cortisol recovery variables. Analyses are adjusted for sex, age, daily smoking, and start time of the experimental session.

DISCUSSION

In this study on the relationship between *NR3C1* methylation and cortisol responses during social stress in adolescents, a high *NR3C1* methylation level in region 2 was associated with a flattened cortisol response recovery slope, indicating a longer recovery time. There was no association between *NR3C1* methylation and cortisol response activation, nor between response recovery and methylation in the other *NR3C1* regions.

Increased levels of glucocorticoids act at the glucocorticoid receptor sites in the extrahypothalamic centers, hypothalamus, and pituitary gland to inhibit activation of the HPA-axis after the stressor has subsided [17, 18]. Our findings suggest that high *NR3C1* methylation is associated with a less functional HPA-axis feedback regulation mechanism. As glucocorticoids are downstream effectors of the HPA-axis which mediate the negative feedback control of CRH and ACTH secretion [18], it is not surprising that there was no association between *NR3C1* methylation and HPA-axis activation.

Comparing our findings with those of other studies is difficult due to differences in age range of the subjects included, differences in the experimental design and the way cortisol was assessed and used in statistical analyses. HPA-axis regulation methods reported by others include the use of the AUC of cortisol from blood of a dexamethasone/corticotropin-releasing hormone test [8], the AUC of salivary cortisol responses to the Trier Social Stress Test [6]. These methodological differences could account for inconsistencies between reported findings [19], e.g. the attenuated response to the DEX/CRH test reported by Tyrka *et al* [8] and the increased AUC reported by Edelman *et al* [6].

The association between high methylation and impaired recovery of the cortisol stress response was only found in a specific region of the *NR3C1* CpG island, the same in which high methylation levels were previously associated with childhood maltreatment in suicide victims [4]. This region encompasses the exon 1_F *NR3C1* promoter, the human homolog of the exon *NR3C1* 1_7 promoter in rats, which contains a nerve growth factor-inducible protein (NGFI-A) response element that is involved in regulating DNA

transcription [3]. Reduced transcription of *NR3C1,* and thus reduced expression of glucocorticoid receptors, can alter HPA-axis responses through diminished negative feedback sensitivity. In an earlier study in the present population, we showed that exposure to stressful life events or traumatic youth events was positively associated *NR3C1* methylation [5], and that methylation of *NR3C1* was associated with an increased risk of a lifetime internalizing disorder and more internalizing problems [20]. However, these associations involved another region, located at the 3'end of the CpG island (region 1 of *NR3C1*). We could speculate that the CpG sites that are vulnerable to stress exposure or linked with psychopathology are independent of the CpG sites involved in regulation of the HPA-axis, which would deny the postulated mediating role of the HPA-axis. This is unlikely, however, since a flattened cortisol recovery at age 16 has recently been associated with a higher risk of psychopathology at 3-year follow-up in TRAILS [15]. Replication of our findings is required to determine robustness and gain insight into the mediating role of DNA methylation in the development of psychopathology.

Several limitations of the study must be acknowledged. Because of the cross-sectional nature of the study and the single assessment of DNA methylation and cortisol responses, we are unable to determine whether *NR3C1* methylation is a cause or a possible consequence of altered HPA-axis functioning. Another limitation is the use of blood as a proxy of the target tissue, the brains. This seems justified, however, because methylation levels of CpG islands in the brain and blood have been found to be highly correlated [21], and the association between childhood adversity and *NR3C1* 1_F methylation that was initially described in postmortem human hippocampal tissue has repeatedly been replicated in studies using peripheral samples for review, see [22]. Also, for our DNA analysis we used whole blood, containing a heterogeneous mixture of cell type. Since we were unable to account for the cellular heterogeneity of the blood cells, we could not rule out that the associations may be in part a reflection of differences in cellular composition.

To conclude, our findings support the notion that methylation of *NR3C1* may be associated with an impaired negative feedback of the HPA-axis in adolescents. This could reflect a pathway through which exposure to stressful experience change stress responses and so contribute to a higher risk of developing psychopathology later in life.

REFERENCES

1. Bosch NM, Riese H, Reijneveld SA, Bakker MP, Verhulst FC, Ormel J *et al.* Timing matters: long term effects of adversities from prenatal period up to adolescence on adolescents' cortisol stress response. The TRAILS study. *Psychoneuroendocrinology* 2012; **37**(9)**:** 1439-1447.
2. Glover V, O'Connor TG, O'Donnell K. Prenatal stress and the programming of the HPA axis. *Neurosci Biobehav Rev* 2010; **35**(1)**:** 17-22.
3. Weaver IC, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S *et al.* The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. *J Neurosci* 2007; **27**(7)**:** 1756-1768.
4. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2009; **12**(3)**:** 342-348.
5. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ *et al.* Glucocorticoid receptor gene (*NR3C1*) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry* 2014; **4:** e381.
6. Edelman S, Shalev I, Uzefovsky F, Israel S, Knafo A, Kremer I *et al.* Epigenetic and genetic factors predict women's salivary cortisol following a threat to the social self. *PLoS One* 2012; **7**(11)**:** e48597.
7. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (*NR3C1*) and infant cortisol stress responses. *Epigenetics* 2008; **3**(2)**:** 97-106.
8. Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. *PLoS One* 2012; **7**(1)**:** e30148.
9. Oldehinkel AJ, Rosmalen JG, Buitelaar JK, Hoek HW, Ormel J, Raven D *et al.* Cohort Profile Update: The TRacking Adolescents' Individual Lives Survey (TRAILS). *Int J Epidemiol* 2014.
10. Bouma EM, Riese H, Ormel J, Verhulst FC, Oldehinkel AJ. Adolescents' cortisol responses to awakening and social stress; effects of gender, menstrual phase and oral contraceptives. The TRAILS study. *Psychoneuroendocrinology* 2009; **34**(6)**:** 884-893.
11. Kudielka BM, Schommer NC, Hellhammer DH, Kirschbaum C. Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. *Psychoneuroendocrinology* 2004; **29**(8)**:** 983-992.
12. Oldehinkel AJ, Ormel J, Bosch NM, Bouma EM, Van Roon AM, Rosmalen JG *et al.* Stressed out? Associations between perceived and physiological stress responses in adolescents: the TRAILS study. *Psychophysiology* 2011; **48**(4)**:** 441-452.
13. Janssens KA, Oldehinkel AJ, Verhulst FC, Hunfeld JA, Ormel J, Rosmalen JG. Symptom-specific associations between low cortisol responses and functional somatic symptoms: the TRAILS study. *Psychoneuroendocrinology* 2012; **37**(3)**:** 332-340.
14. Aardal-Eriksson E, Karlberg BE, Holm AC. Salivary cortisol--an alternative to serum cortisol determinations in dynamic function tests. *Clin Chem Lab Med* 1998; **36**(4)**:** 215-222.
15. Nederhof E, Van Oort F, Bouma EM, Laceulle OM, Oldehinkel AJ, Ormel H. Predicting mental disorders from hypothalamic-pituitary-adrenal axis functioning: a three-year follow-up in the TRAILS study. *Psychosom Med* In press.
16. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3)**:** 1215.

17. Nicolaides NC, Kyratzi E, Lamprokostopoulou A, Chrousos GP, Charmandari E. Stress, the stress system and the role of glucocorticoids. *Neuroimmunomodulation* 2015; **22**(1-2)**:** 6-19.
18. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. *Annu Rev Physiol* 2005; **67:** 259-284.
19. Rosmalen JG, Oldehinkel AJ. The role of group dynamics in scientific inconsistencies: a case study of a research consortium. *PLoS Med* 2011; **8**(12)**:** e1001143.
20. van der Knaap LJ, van Oort FV, Verhulst FC, Oldehinkel AJ, Riese H. Methylation of *NR3C1* and *SLC6A4* and internalizing problems. The TRAILS study. *J Affect Disord* 2015; **180:** 97-103.
21. Tylee DS, Kawaguchi DM, Glatt SJ. On the outside, looking in: A review and evaluation of the comparability of blood and brain "-omes". *Am J Med Genet B Neuropsychiatr Genet* 2013; **162**(7)**:** 595-603.
22. Daskalakis NP, Yehuda R. Site-specific methylation changes in the glucocorticoid receptor exon 1F promoter in relation to life adversity: systematic review of contributing factors. *Front Neurosci* 2014; **8:** 369.

SUPPLEMENTARY INFORMATION

Table S1. Results of the linear regression analyses of *NR3C1* methylation and HPA-axis regulation in the Groningen Social Stress Task.

		cortisol response activation			cortisol response recovery		
	N	**B**	**SE**	**p**	**B**	**SE**	**p**
NR3C1_1	341	-0.02	0.13	.88	0.05	0.13	.69
NR3C1_2	339	-0.08	0.10	.40	**0.45**	**0.10**	**<.001**
NR3C1_3	308	0.05	0.14	.69	0.00	0.11	.99

Methylation variables are mean-centered. Cortisol variables are standardized. Significant results (p<0.008 after Bonferroni correction) are indicated in boldface. See method section for details on the calculation of cortisol reactivity and cortisol recovery variables. Analyses are unadjusted for covariates.

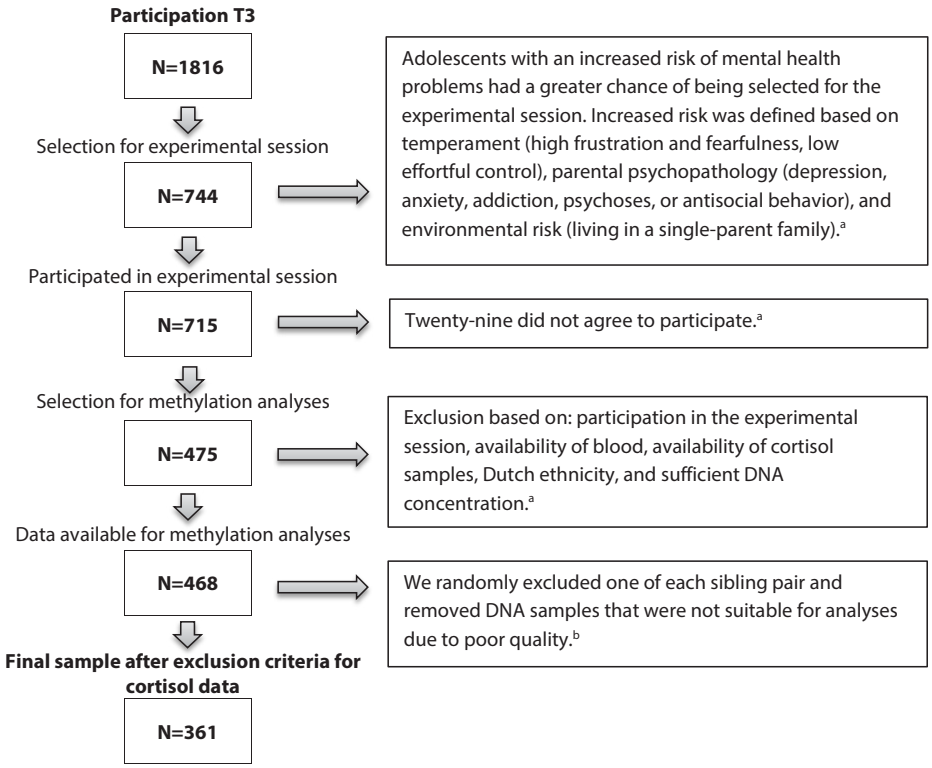


Figure S1. Flow diagram of selection procedures. [a] Described before in e.g. Bouma *et al* [1]. [b] Described before in van der Knaap *et al* [2]. See method section for details on exclusion criteria for cortisol data.

REFERENCES SUPPLEMENTARY INFORMATION

1. Bouma EM, Riese H, Ormel J, Verhulst FC, Oldehinkel AJ. Adolescents' cortisol responses to awakening and social stress; effects of gender, menstrual phase and oral contraceptives. The TRAILS study. *Psychoneuroendocrinology* 2009; **34**(6)**:** 884-893.
2. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ *et al.* Glucocorticoid receptor gene (*NR3C1*) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry* 2014; **4:** e381.

Chapter 6

Glucocorticoid receptor gene (*NR3C1*) and serotonin transporter gene (*SLC6A4*) methylation and obesity measures in adolescent boys and girls. The TRAILS study

L.J. van der Knaap
H. Riese
F.V.A. van Oort
F.C. Verhulst
A.J. Oldehinkel

Submitted for publication in Journal of Obesity

ABSTRACT

Background: In recent years epigenetics has gained interest as a possible mechanism underlying obesity. Particular targets for investigation are genes involved in appetite and energy regulation, such as the serotonin transporter gene (*SLC6A4*) and the glucocorticoid receptor gene (*NR3C1*). Thus far, only the association between *SLC6A4* methylation and obesity measures has been investigated, and only in males. We aimed to replicate this study by investigating the association between *SLC6A4* methylation and obesity measures in adolescent boys, and to extend the study by including girls and *NR3C1* methylation.

Subjects: In a population sample of 941 adolescents (age 14-18y), we investigated associations between *SLC6A4* and *NR3C1* methylation and obesity measures, i.e. weight, BMI, the sum of four skinfold thicknesses (S4SF), body fat percentage (%BF), waist circumference (WC), waist-hip-ratio and height. We assessed methylation levels from blood samples.

Results: *SLC6A4* methylation was positively associated with weight, BMI, S4SF, %BF and WC in boys. A 1% higher mean methylation was associated with a 2.27mm higher S4SF (p=0.006), a 0.54 higher %BF (p=0.002) and a 0.68cm higher WC (p=0.025). For girls, we did not find any associations between *SLC6A4* methylation and obesity measures. *NR3C1* methylation was only positively associated with height in boys. A 1% higher mean methylation was associated with a 1.60cm higher body height (p=0.009) in boys. There were no associations between *NR3C1* methylation and obesity measures in girls.

Conclusions: We replicated previous findings by reporting a positive association between *SLC6A4* methylation and obesity measures in boys. *SLC6A4* methylation may thus contribute to obesity risk in adolescent boys, but not in girls. *NR3C1* methylation does not contribute to an obese phenotype in adolescence, but may be involved in body height in adolescent boys.

INTRODUCTION

Human obesity is a worldwide public health concern that has been associated with increased health risks such as cardiovascular disease, type II diabetes and various cancers. Although the risk of obesity is partly genetically determined, effect sizes are small and a large portion of the heritability remains unexplained [1]. In recent years, epigenetic mechanisms have gained interest as a possible underlying mechanism that may contribute to obesity risk. Epigenetic modifications can be influenced by environmental triggers and regulate gene expression without changing the underlying DNA sequence. One type of epigenetic modification is DNA methylation, which regulates gene expression by chemically modifying cytosine-phosphate-guanine (CpG) dinucleotides in the DNA, and thereby altering the access to DNA for transcription factors. DNA methylation in CpG islands (i.e. regions with an increased frequency of CpG sites often located in the gene promoter) is generally associated with decreased gene expression [2].

Obesity generally results from an energy imbalance, which occurs when energy intake exceeds energy expenditure. Therefore, potential epigenetic targets for investigation are genes involved in appetite regulation and maintaining energy balance. The serotonergic system plays an important role in both the maintenance of energy balance [3] and regulating eating behavior [4]. Animal studies have shown that hyperphagia or obesity can be induced by manipulating the serotonergic synthesis, e.g. by creating lesions in the raphe nuclei, by creating genetic knock-outs of the serotonin transporter gene (*Slc6a4; Slc6a4*$^{-/-}$ mice have an obese phenotype [5]), or through pharmacological interventions (for review, see Donovan and Tecott [3]).

Another candidate gene involved in the pathogenesis of obesity is the glucocorticoid receptor gene (*NR3C1*), an important regulator of the stress response. Glucocorticoid synthesis is stimulated in response to stress, and glucocorticoids (cortisol in humans) also play an important role in maintaining HPA-axis homeostasis by providing negative feedback. Glucocorticoids exert metabolic effects and play an important role in appetite regulation and the regulation of lipid homeostasis through their effects on other hormones involved in feeding regulation, such as insulin, leptin and ghrelin [6, 7]. An excess of glucocorticoids has been associated with visceral fat accumulation [6]. This is also observed in individuals with Cushing's syndrome, who are exposed to extremely high levels of glucocorticoids, and develop rapid weight gain, as well as central obesity. Deregulated feedback of glucocorticoids in the HPA-axis can occur when *NR3C1* expression is impaired [8], a consequence of higher methylation levels [9]. This may also contribute to the risk of developing obesity. Genetic variations in the glucocorticoid receptor gene, and thus receptor functionality, have also been shown to be associated with obesity measures in men [10] and adolescent boys [11], but this association could not be replicated in an older cohort within the same study [11].

Although the number of studies investigating the relationship between epigenetics and obesity is increasing [12], candidate gene studies on *SLC6A4* and *NR3C1* are largely lacking. In a recent publication, Zhao *et al* [13] reported that *SLC6A4* promoter methylation was positively associated with obesity measures in a sample of adult male veteran monozygotic twins, with an overrepresentation of individuals suffering from major depression or post-traumatic stress disorder. To the best of our knowledge, the association between *NR3C1* methylation and obesity measures has not been reported before.

In the present study, we aimed to replicate the findings reported by Zhao *et al* [13] in a younger age group, by investigating the relationship between *SLC6A4* methylation and obesity in adolescent boys. In addition, we wanted to extend their findings by including female adolescents and methylation of *NR3C1*. For *SLC6A4*, we hypothesized a positive association between methylation and measures of obesity in adolescent boys, similarly to the findings reported by Zhao *et al* [13]. For girls, we expected to find an association in the same direction. Given that the glucocorticoid receptor is an important regulator of glucocorticoid levels and high methylation is likely to impair glucocorticoid receptor functioning, we hypothesized that high *NR3C1* methylation levels would also be positively associated with obesity measures.

SUBJECTS AND METHODS

Sample selection

This study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective population study in which Dutch adolescents are followed from preadolescence into adulthood. Assessment waves are conducted biennially or triennially, and five assessment waves have been completed so far. Written consent was obtained from each subject and their parents at every assessment wave. The present study involves data collected during the third assessment wave (T3, 2005-2007, N=1816, mean age 16.3, SD 0.71). The study was approved by the Dutch Central Medical Ethics Committee and subjects received compensation for their participation. A detailed description of sampling and methods can be found in Ormel *et al* [14] and Oldehinkel *et al* [15].

The T3 assessment involved a blood draw, from which DNA was successfully extracted in 1156 T3 participants. Selection for methylation analyses was based on the availability of blood, Dutch ethnicity, and sufficient DNA concentration. We randomly excluded one of each sibling pair and removed DNA samples that were not suitable for analyses. Together, this resulted in a total of 945 individuals (48.3% males) suitable for methylation analyses.

DNA methylation analyses

DNA selection and methylation analyses. DNA was extracted from whole-blood samples using a manual salting-out procedure as described by Miller *et al* [16]. We used primer sets previously used by McGowan *et al* [17] for *NR3C1* (Chr5: 142783585-142783906) and Philibert *et al* [18] (set B) for *SLC6A4* (Chr17: 28562358-28562783), which encompass regions of the CpG island (i.e., a region with a higher frequency of CpG sites than expected) in the promoter regions of both genes. Forward primers contained a 10 mer sequence tag (aggaagagag), and reverse primers are equipped with a T7-promoter tag (cagtaatacgactcactataggg) and an 8 base pair insert (agaaggct). DNA methylation levels were analyzed using the EpiTYPER method from Sequenom. Bisulfite conversion was followed by PCR amplification, reverse transcription and base-specific cleavage. Fragments were analyzed on a mass spectrometer (Sequenom EpiTYPER, San Diego, CA, USA). Bisulfite conversion of DNA was performed using EZ-96 DNA Methylation Kit (Shallow) (Zymo Research, CA, USA), according to manufacturers' protocol. PCR, reverse transcription, cleavage and mass spectrometry were performed in triplicate, according to EpiTYPER protocol. The mass signal patterns generated were translated to quantitative methylation levels for different CpG units by the MassARRAY EpiTYPER analyzer software from Sequenom (v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA). Fragments with CpG dinucleotides are referred to as CpG units. One CpG unit can contain one or more CpG dinucleotides. CpG units with a mass outside the range of the mass spectrometer or overlap in mass of another CpG unit could not be analyzed.

Data cleaning procedures. All samples were analysed in triplicate, and methylation levels of the triplicates were averaged for each CpG unit [19]. Samples with a standard deviation of ≥10% between replicates and CpG units with >25% missing values were removed (*SLC6A4*: CpGU17). We accounted for mass-change in CpG units by SNPs (only when minor allele frequency >5%) by removing CpG units containing SNPs (*SLC6A4*: CpGU18) equal in mass to non CpG units or other CpG units containing SNPs (none in our sample). In total, 10 CpG units remained eligible for *NR3C1* and 11 for *SLC6A4*. Overall, we obtained methylation levels of *NR3C1*_2 for 904 individuals and of *SLC6A4* for 939 individuals, with a combined total of 941 individuals with methylation data of at least one gene.

Obesity measures

We measured weight, height, hip and waist circumference and skinfolds using regularly calibrated equipment after removing shoes and heavy clothing. Weight was measured in kilograms using a calibrated analogue Seca balance scale (Model 770; SECA Corp., Hamburg, Germany). Height was measured in centimeters using a Seca stadiometer (model 214; SECA Corp.). BMI (kg/m^2) was calculated by dividing the weight by the

squared height in meters (cut-off points for BMI are based on [20]). We obtained triceps, biceps, subscapular, and suprailiac skinfold thicknesses in millimeters with a Harpenden skinfold caliper (CMS Instruments, London, United Kingdom); and calculated the sum of the four thicknesses (S4SF). Waist circumference (WC) in centimeters was measured at the midpoint between the lower costal margin and the iliac crest. All measurements were performed in duplicate, and a third measurement was added if the difference between these measurements exceeded a predefined value (i.e., >2mm for height, >0.5kg for weight, >0.5cm for WC, >2mm for all skinfold measurements). All available measurements were used to calculate means. Percentage body fat (%BF) was calculated on the basis of a hand-to-foot bioelectrical impedance analysis (type BIA 101; Akern, Pontassieve, Italy), using the Deurenberg equation [21].

Other measures

We asked how many days per week the adolescents participated in at least 60 minutes of moderate or vigorous physical activity, from which "sufficient physical activity" was determined as at least 5 days per week [22], in accordance with international recommendations [23]. Smoking and alcohol use were assessed with a self-report questionnaire. Adolescents who had not smoked in the past 4 weeks were categorized as non-smokers, adolescents who had smoked less than one cigarette a day as non-daily smokers, and those who had smoked one or more cigarettes per day as daily smokers. Adolescents who reported that they did not drink alcohol in the past 4 weeks were categorized as abstainers. Adolescents who reported drinking were categorized into two groups: those who had drunk alcohol up to 9 times were defined as low-frequent users and those who had drunk alcohol 10 times or more as high-frequent users. Depressive problems were assessed dimensionally using the mean item score of the DSM-IV Affective Problems scale from the Youth Self-Report (YSR: 13 items, α=.78; [24]). All items referred to the past six months.

Statistical analyses

The associations between methylation levels and obesity measures were analyzed in SPSS (v21) with generalized linear models. Methylation level was specified as predictor and the different obesity measures were specified as outcome variables in separate models for each gene and outcome variable. Each model contained age, smoking and alcohol use as covariates, identical to the covariates used by Zhao *et al* [13]. For girls, we also added the use of oral contraceptives (OC) as a covariate in the models, as OC use can affect DNA methylation and influence obesity associated parameters [25]. In a secondary analysis, we further included depressive symptoms and physical activity as additional covariates in the above mentioned statistical models. These covariates were also used by Zhao *et al* [13] in a sensitivity analyses to examine whether the high prevalence of depres-

sion and post-traumatic stress disorder in their sample confounded their results, and to examine the potential influence of physical activity on their results. They also included diet (daily energy intake in Kcal per day) in their sensitivity analyses, but this information was not available in the current study.

Given that Zhao's study [13] provided clear expectations regarding the direction of the association between *SLC6A4* methylation and obesity measures, we performed one-sided tests. Although the association between *NR3C1* methylation and obesity measures has not been described thus far, circumstantial evidence [8, 9] strongly suggested a positive association between methylation levels in this gene and obesity measures, hence these test were performed one-sidedly as well. Effects were regarded as significant if the p-value was smaller than 0.05. We chose not to adjust for multiple testing, because the obesity measures are not independent.

Table 1. Descriptive statistics.

	Boys		***Girls***	
	Mean ± SD or %	**N**	**Mean ± SD or %**	**N**
Age (years)	16.2 ± 0.6	454	16.2 ± 0.7	487
Oral contraceptive use (% of girls)			31.4	433
Body weight (kg)	68.3 ± 12.3	445	62.0 ± 9.2	472
Body height (cm)	180.3 ± 7.4	445	169.1 ± 6.4	475
Body mass index (BMI; kg/m^2)	21.0 ± 3.3	445	21.7 ± 3.0	472
Sum of four skinfolds (S4SF; mm)	40.0 ± 21.8	445	61.8 ± 20.9	472
Body fat percentage (%BF)	25.0 ± 4.6	444	31.3 ± 4.3	465
Waist circumference (WC; cm)	75.7 ± 8.7	445	75.4 ± 8.0	474
Waist to hip ratio (WHR)	0.8 ± 0.1	445	0.8 ± 0.1	473
Smoking		363		389
non-smokers	71.6	260	64.5	251
non-daily smokers	7.2	26	6.7	26
daily smokers	21.2	77	28.8	112
Alcohol use		435		466
abstainers	22.3	97	18.9	88
low-frequent users	61.4	267	71.5	333
high-frequent users	16.3	71	9.7	45
Sufficient physical activity	33.7	436	26.5	475
Depressive problems	0.2 ± 0.2	441	0.4 ± 0.3	478

Note: Details on the definitions of smoking categories, alcohol use categories, physical activity and depressive symptoms are given in the method section.

RESULTS

Demographic and clinical characteristics of our sample are presented in Table 1. Eighty-four percent of our sample (N=454) was male. Correlations between the different obesity measures in boys and girls are presented in Table 2. All obesity measures were highly correlated in both boys and girls (p<.001), except for height, which showed no correlation with BMI, S4SF and %BF in boys and with S4SF in girls. The associations between *SLC6A4* and obesity measures in boys and girls are presented in Table 3. *SLC6A4* methylation was positively associated with weight, BMI, S4SF, %BF and WC in boys.

Table 2. Correlations between obesity measures in boys and girls.

Boys		**Height (cm)**	**BMI (kg/m^2)**	**S4SF(mm)**	**BF (%)**	**WC (cm)**	**WHR**
Weight (kg)	**ρ**	.49***	.89***	.74***	.69***	.87***	.20***
	N	445	445	445	444	445	445
Height (cm)	**ρ**		0.04	0.04	0.00	.23***	-.12*
	N		445	445	444	445	445
BMI (kg/m^2)	**ρ**			.82***	.80***	.87***	.29***
	N			445	444	445	445
S4SF (mm)	**ρ**				.78***	.81***	.32***
	N				444	445	445
BF (%)	**ρ**					.76***	.28***
	N					444	444
WC (cm)	**ρ**						.54***
	N						445
Girls		**Height (cm)**	**BMI (kg/m^2)**	**S4SF (mm)**	**BF (%)**	**WC (cm)**	**WHR**
Weight (kg)	**ρ**	.38***	.86***	.73***	.65***	.83***	.13**
	N	472	472	469	465	471	471
Height (cm)	**ρ**		-.13**	-.03	-.16***	.18***	-.10*
	N		472	472	465	474	473
BMI (kg/m^2)	**ρ**			.81***	.78***	.81***	.19***
	N			469	465	471	471
S4SF (mm)	**ρ**				.73***	.74***	.23***
	N				464	472	471
BF (%)	**ρ**					.64***	.11*
	N					465	465
WC (cm)	**ρ**						.47***
	N						473

Note: Numbers shown are Spearman correlation coefficients. BMI= body mass index; S4SF= sum of four skinfold thicknesses; BF= body fat; WC= waist circumference; WHR= waist-hip-ratio. *p<.05, **p<.01, ***p<.001.

An absolute 1% higher mean methylation was associated with a 2.27mm higher sum of four skinfold thicknesses, a 0.54 higher body fat percentage and a 0.68cm higher waist circumference in boys. Adjustment for physical activity and depressive problems resulted in highly similar regression coefficients, but the associations between *SLC6A4* methylation and BMI and weight were no longer significant (B=0.75, p=0.063 for weight, B=0.20, p=0.068 for BMI, from Supplementary Table S1). For girls, we did not find any associations between *SLC6A4* methylation and obesity measures.

The associations between *NR3C1* methylation and obesity measures are presented in Table 4. In boys, *NR3C1* methylation was only positively associated with height. An absolute 1% higher mean methylation was associated with a 1.60cm higher body height

Table 3. Associations between *SLC6A4* methylation and obesity measures in boys and girls.

	Obesity measures			
	B	**95% LCB**	**p***	**N**
Weight				
Boys	**0.81**	**0.02**	**0.046**	**350**
Girls	-0.05	-0.69	0.553	339
BMI				
Boys	**0.22**	**0.001**	**0.049**	**350**
Girls	-0.08	-0.29	0.723	339
S4SF				
Boys	**2.27**	**0.80**	**0.006[a]**	**350**
Girls	0.08	-1.38	0.465	337
%BF				
Boys	**0.54**	**0.23**	**0.002[a]**	**349**
Girls	-0.04	-0.35	0.579	334
WC				
Boys	**0.68**	**0.11**	**0.025[a]**	**350**
Girls	-0.28	-0.85	0.794	339
WHR				
Boys	0.00	-0.004	0.469	350
Girls	0.00	-0.01	0.182	338
Height				
Boys	0.13	-0.37	0.334	350
Girls	0.21	-0.22	0.210	340

Note: BMI= body mass index; S4SF= sum of four skinfold thicknesses; %BF= body fat percentage; WC= waist circumference; WHR= waist-hip-ratio. LCB= lower confidence bound. Methylation values are mean-centered. All models adjusted for age, smoking and alcohol use. For girls, models are additionally adjusted for OC use. *p-value of the one-sided test. Values in bold script indicate that p<.05. [a]P-values that remained significant after additional adjustment for physical activity and depressive symptoms.

in boys. Additional adjustment for physical activity and depressive problems resulted in highly similar regression coefficients and p-values (Supplementary Table S2). There were no associations between *NR3C1* methylation and obesity measures in girls.

As a post-hoc analysis, we explored the associations between methylation in individual CpG units of *SLC6A4* and obesity measures in boys, similarly to Zhao *et al* [13]. The results of these analyses are largely in line with the main results and can be found in Supplementary Table S3. Methylation levels in CpG units 1, 3, 15, 16 and 22 were associated with one or more obesity measures.

Table 4. Associations between *NR3C1* methylation and obesity measures in boys and girls.

	Obesity measures			
	B	**95% LCB**	**p***	**N**
Weight				
Boys	0.08	-1.75	0.472	342
Girls	-0.33	-1.59	0.670	326
BMI				
Boys	-0.37	-0.87	0.895	342
Girls	-0.08	-0.50	0.623	326
S4SF				
Boys	-0.63	-4.06	0.620	342
Girls	-0.76	-3.62	0.669	324
%BF				
Boys	-0.08	-0.78	0.577	341
Girls	0.38	-0.22	0.149	321
WC				
Boys	-0.59	-1.89	0.775	342
Girls	-0.33	-1.45	0.686	326
WHR				
Boys	-0.01	-0.02	0.831	342
Girls	-0.01	-0.02	0.968	325
Height				
Boys	**1.60**	**0.50**	**0.009[a]**	342
Girls	-0.19	-1.05	0.645	327

Note: BMI= body mass index; S4SF= sum of four skinfold thicknesses; %BF= body fat percentage; WC= waist circumference; WHR= waist-hip-ratio. LCB= lower confidence bound. Methylation values are mean-centered. All models adjusted for age, smoking and alcohol use. For girls, models are additionally adjusted for OC use. *p-value of the one-sided test. Values in bold script indicate that p<.05. [a]P-values that remained significant after additional adjustment for physical activity and depressive symptoms.

DISCUSSION

SLC6A4 promoter methylation was significantly associated with obesity measures in adolescent boys, in line with the study by Zhao *et al* [13]. These associations were not found in adolescent girls. Additionally, *NR3C1* methylation was only significantly associated with height in boys, and there were no associations with any obesity measures in girls. In Zhao *et al* [13], associations between *SLC6A4* methylation and obesity measures were attenuated after adjustment for the additional covariates physical activity, diet and depressive symptoms, but remained statistically significant. In the current study, additional adjustment for physical activity and depressive symptoms also attenuated the results, but resulted in two non-significant associations with weight and BMI. However, these associations were already marginally significant prior to the additional adjustment and the regression coefficients remained highly comparable to the original results, indicating that additional adjustment for physical activity and depressive symptoms did not confound our results.

Despite sample and design differences, our findings are remarkably comparable to those reported by Zhao *et al* [13]. They reported an association between *SLC6A4* methylation and weight, BMI and WC, we found associations between *SLC6A4* methylation and S4SF, %BF and WC. Zhao *et al* [13] reported that "on average a 1% increase in mean methylation was associated with a 0.78cm increase in WC" in their sample, compared to an increase of 0.68cm in WC in adolescent boys in our study. This suggests that the association between *SLC6A4* methylation and obesity measures in males is quite robust, and can already be seen in adolescents.

The associations between *SLC6A4* methylation and weight and BMI were not very strong in our sample. Without accounting for differences in height, weight is not necessarily an indicator of overweight or obesity. Also, although BMI is widely used to determine overweight or obesity, it does not differentiate between fat or muscle weight. The body fat percentage in adolescents with a normal BMI may exceed normal ranges [26], and individuals with a normal weight and normal BMI might actually be metabolically overweight or obese [27], and run the risk of obesity-related diseases [28]. The prevalence of obese and overweight individuals in our sample was relatively low (12.9% overweight; 2.6% obese), and the percentage of body fat and other estimations of body fat percentage such as the skinfold measurements may therefore more accurately represent overweight or obesity in adolescents and better predict health risks.

For *NR3C1*, methylation levels in the promoter region were only associated with height in adolescent boys. Glucocorticoids are known to be involved in linear growth, but an excess of glucocorticoids is generally considered to be associated with growth impairment [28]. Our finding warrants replication and further study. The lack of associations with other obesity measures was unexpected. The relationship between glucocorticoids and obesity is complex, but evidence of this association has been reported extensively (e.g. [6, 29]). As we did not obtain gene expression data in the current study, we could not examine the effect of methylation on receptor functionality directly. In a previous study we found that higher methylation levels in the promotor region of *NR3C1* were associated with an impaired glucocorticoid response recovery following stress [30], so methylation does seem to affect HPA-axis functioning, but the effect may have been too weak to influence the likelihood of developing an overweight or obese phenotype.

Contrary to expectations, there were no associations between methylation levels and obesity measures in girls. Possibly, the lack of associations in girls can be attributed to sex hormones, in particular estrogens. Estrogens are known to be involved in appetite regulation and energy expenditure and considered protective against obesity [31]. To prevent natural cyclic fluctuations in estrogen levels we adjusted for the use of oral contraceptives, but this did not change the outcomes. Perhaps the higher basal level of estrogens affects the association between DNA methylation and obesity in girls. More research is needed to investigate this possible link.

Several limitations have to be acknowledged. Firstly, because of the cross-sectional nature of the study and the single assessment of DNA methylation, we cannot make statements on causality. Secondly, we cannot account for the cellular heterogeneity of the blood cells, and thus cannot rule out that the associations may be in part a reflection of differences in cellular composition. Thirdly, there was no direct overlap between the region studied by Zhao *et al* [13] (Chr17: 28562784-28562928) and the region investigated in the current study (Chr17: 28562358-28562783). Nonetheless, our results provide extra coverage of the *SLC6A4* CpG island and strengthens the association between *SLC6A4* methylation and obesity.

In conclusion, this replication study provides further support for involvement of epigenetic modifications in obesity in males. Deeper understanding of the involvement of *SLC6A4* in obesity may help target interventions for obesity and possibly even associated health risks. Epigenetics seems to be a promising field for investigating obesity, and a logical next step would be investigation of causality and gender differences to develop strategies to target the obesity epidemic.

REFERENCES

1. Apalasamy YD, Mohamed Z. Obesity and genomics: role of technology in unraveling the complex genetic architecture of obesity. *Hum Genet* 2015.
2. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; **16**(1)**:** 6-21.
3. Donovan MH, Tecott LH. Serotonin and the regulation of mammalian energy balance. *Front Neurosci* 2013; **7:** 36.
4. Halford JC, Boyland EJ, Lawton CL, Blundell JE, Harrold JA. Serotonergic anti-obesity agents: past experience and future prospects. *Drugs* 2011; **71**(17)**:** 2247-2255.
5. Murphy DL, Lesch KP. Targeting the murine serotonin transporter: insights into human neurobiology. *Nat Rev Neurosci* 2008; **9**(2)**:** 85-96.
6. Spencer SJ, Tilbrook A. The glucocorticoid contribution to obesity. *Stress* 2011; **14**(3)**:** 233-246.
7. Walker BR. Extra-adrenal regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1: physiological regulator and pharmacological target for energy partitioning. *Proc Nutr Soc* 2007; **66**(1)**:** 1-8.
8. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR *et al.* Epigenetic programming by maternal behavior. *Nat Neurosci* 2004; **7**(8)**:** 847-854.
9. Weaver IC, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S *et al.* The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. *J Neurosci* 2007; **27**(7)**:** 1756-1768.
10. Rosmond R, Chagnon YC, Holm G, Chagnon M, Perusse L, Lindell K *et al.* A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis. *Obesity Res* 2000; **8**(3)**:** 211-218.
11. Voorhoeve PG, van den Akker EL, van Rossum EF, Koper JW, van Mechelen W, Lamberts SW *et al.* Glucocorticoid receptor gene variant is associated with increased body fatness in youngsters. *Clin Endocrinol (Oxf)* 2009; **71**(4)**:** 518-523.
12. van Dijk SJ, Molloy PL, Varinli H, Morrison JL, Muhlhausler BS, Members of Epi S. Epigenetics and human obesity. *Int J Obes (Lond)* 2015; **39**(1)**:** 85-97.
13. Zhao J, Goldberg J, Vaccarino V. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *Int J Obes (Lond)* 2013; **37**(1)**:** 140-145.
14. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, Veenstra R *et al.* The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. *J Am Acad Child Adolesc Psychiatry* 2012; **51**(10)**:** 1020-1036.
15. Oldehinkel AJ, Rosmalen JG, Buitelaar JK, Hoek HW, Ormel J, Raven D *et al.* Cohort Profile Update: the TRacking Adolescents' Individual Lives Survey (TRAILS). *Int J Epidemiol* 2015; **44**(1)**:** 76-76n.
16. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3)**:** 1215.
17. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2009; **12**(3)**:** 342-348.
18. Philibert RA, Sandhu H, Hollenbeck N, Gunter T, Adams W, Madan A. The relationship of 5HTT (*SLC6A4*) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B**(5)**:** 543-549.

19. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ *et al.* Glucocorticoid receptor gene (*NR3C1*) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry* 2014; **4:** e381.
20. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* 2000; **320**(7244)**:** 1240-1243.
21. Deurenberg P, van der Kooy K, Leenen R, Weststrate JA, Seidell JC. Sex and age specific prediction formulas for estimating body composition from bioelectrical impedance: a cross-validation study. *Int J Obesity* 1991; **15**(1)**:** 17-25.
22. Liem ET, Vonk JM, Sauer PJ, van der Steege G, Oosterom E, Stolk RP *et al.* Influence of common variants near INSIG2, in FTO, and near MC4R genes on overweight and the metabolic profile in adolescence: the TRAILS (TRacking Adolescents' Individual Lives Survey) Study. *Am J Clin Nutr* 2010; **91**(2)**:** 321-328.
23. Butcher K, Sallis JF, Mayer JA, Woodruff S. Correlates of physical activity guideline compliance for adolescents in 100 U.S. Cities. *J Adolesc Health* 2008; **42**(4)**:** 360-368.
24. Achenbach TM. Manual for the Youth Self-Report and 1991 profile. Burlington, VT: University of Vermont; 1991.
25. Campesi I, Sanna M, Zinellu A, Carru C, Rubattu L, Bulzomi P *et al.* Oral contraceptives modify DNA methylation and monocyte-derived macrophage function. *Biol Sex Differ* 2012; **3:** 4.
26. Nooyens AC, Koppes LL, Visscher TL, Twisk JW, Kemper HC, Schuit AJ *et al.* Adolescent skinfold thickness is a better predictor of high body fatness in adults than is body mass index: the Amsterdam Growth and Health Longitudinal Study. *Am J Clin Nutr* 2007; **85**(6)**:** 1533-1539.
27. Ruderman N, Chisholm D, Pi-Sunyer X, Schneider S. The metabolically obese, normal-weight individual revisited. *Diabetes* 1998; **47**(5)**:** 699-713.
28. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G *et al.* Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology* 1998; **139**(7)**:** 3296-3305.
29. Lee MJ, Pramyothin P, Karastergiou K, Fried SK. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. *Biochim Biophys Acta* 2014; **1842**(3)**:** 473-481.
30. Van der Knaap LJ, Oldehinkel AJ, Verhulst F, Van Oort FVA, Riese H. Glucocorticoid receptor gene methylation and HPA-axis regulation in adolescents. The TRAILS study. *Psychoneuroendocrinology* (in press).
31. Palmer BF, Clegg DJ. The sexual dimorphism of obesity. *Mol Cell Endocrinol* 2015; **402:** 113-119.

SUPPLEMENTARY INFORMATION

Table S1. Associations between *SLC6A4* methylation and obesity measures in boys and girls, adjusted for additional covariates.

	Obesity measures			
	B	**95% LCB**	**P***	**N**
Weight				
Boys	0.75	-0.05	0.063	345
Girls	-0.07	-0.71	0.568	338
BMI				
Boys	0.20	-0.02	0.068	345
Girls	-0.08	-0.30	0.731	338
S4SF				
Boys	**2.26**	**0.77**	**0.006**	345
Girls	0.02	-1.44	0.492	336
%BF				
Boys	**0.53**	**0.23**	**0.002**	344
Girls	-0.04	-0.35	0.585	333
WC				
Boys	**0.63**	**0.06**	**0.036**	345
Girls	-0.32	-0.89	0.822	338
WHR				
Boys	0.00	-0.005	0.487	345
Girls	0.00	-0.01	0.170	337
Height				
Boys	0.12	-0.38	0.347	345
Girls	0.20	-0.23	0.220	339

Note: BMI= body mass index; S4SF= sum of four skinfold thicknesses; %BF= body fat percentage; WC= waist circumference; WHR= waist-hip-ratio. LCB= lower confidence bound. Methylation values are mean-centered. All models adjusted for age, smoking, alcohol use, physical activity and depressive symptoms. For girls, we additionally adjusted for OC use. *p-value of the one-sided test. Values in bold script indicate that p<.05.

Table S2. Associations between *NR3C1* methylation and obesity measures in boys and girls, adjusted for additional covariates.

	Obesity measures			
	B	**95% LCB**	**P***	**N**
Weight				
Boys	0.10	-1.70	0.463	338
Girls	-0.42	-1.68	0.709	325
BMI				
Boys	-0.38	-0.88	0.900	338
Girls	-0.08	-0.51	0.627	325
S4SF				
Boys	-0.64	-4.07	0.621	338
Girls	-0.88	-3.77	0.693	323
%BF				
Boys	-0.12	-0.81	0.613	337
Girls	0.40	-0.21	0.141	320
WC				
Boys	-0.62	-1.91	0.784	338
Girls	-0.47	-1.60	0.755	325
WHR				
Boys	-0.01	-0.02	0.841	338
Girls	-0.01	-0.02	0.964	324
Height				
Boys	**1.68**	**0.59**	**0.006**	338
Girls	-0.30	-1.16	0.716	326

Note: BMI= body mass index; S4SF= sum of four skinfold thicknesses; %BF= body fat percentage; WC= waist circumference; WHR= waist-hip-ratio. LCB= lower confidence bound. Methylation values are mean-centered. All models adjusted for age, smoking, alcohol use, physical activity and depressive symptoms. For girls, we additionally adjusted for OC use. *p-value of the one-sided test. Values in bold script indicate that p<.05.

Table S3 (continued on other page). Associations between *NR3C1* and *SLC6A4* methylation in individual CpG units and obesity measures in boys.

	Mean methylation (SD)	Weight				BMI				S4SF			
		B	95% LCB	p*	N	B	95% LCB	p*	N	B	95% LCB	p*	N
SLC6A4													
CpGU1	2.6% (0.9%)	1.01	-0.18	0.082	350	0.19	-0.13	0.165	350	1.49	-0.74	0.136	350
CpGU3	5.9% (1.6%)	**0.68**	**0.04**	**0.041**[a]	349	0.11	-0.06	0.143	349	0.60	-0.60	0.205	349
CpGU4	6.1% (2.4%)	0.04	-0.36	0.429	344	0.05	-0.07	0.251	344	-0.02	-0.79	0.514	344
CpGU6	1.5% (1.7%)	-0.32	-0.92	0.816	347	-0.11	-0.28	0.877	347	-0.64	-1.75	0.831	347
CpGU9	7.6% (2.0%)	0.20	-0.31	0.255	350	0.00	-0.14	0.484	350	0.28	-0.68	0.317	350
CpGU10	2.3% (1.5%)	0.09	-0.68	0.423	286	-0.01	-0.22	0.528	286	-0.08	-1.54	0.538	286
CpGU12	14.8% (3.3%)	0.23	-0.07	0.106	335	0.05	-0.04	0.183	335	0.36	-0.21	0.152	335
CpGU14	9.0% (3.1%)	-0.02	-0.36	0.543	336	0.02	-0.08	0.391	336	0.46	-0.16	0.111	336
CpGU15	8.1% ((2.3%)	0.06	-0.39	0.412	348	0.05	-0.08	0.266	348	0.43	-0.42	0.205	348
CpGU16	15.0% (3.6%)	**0.30**	**0.004**	**0.048**	321	**0.10**	**0.02**	**0.017**[a]	321	**1.17**	**0.63**	**0.000**[a]	321
CpGU22	25.2% (4.3%)	0.22	-0.03	0.071	326	0.06	-0.01	0.079	326	**0.66**	**0.19**	**0.010**[a]	326

Table S3 (continued). Associations between *NR3C1* and *SLC6A4* promoter methylation in individual CpG units and obesity measures in boys.

	%BF				WC				WHR				Height			
	B	**95% LCB**	**p***	**N**	**B**	**95% LCB**	**p***	**N**	**B**	**95% LCB**	**p***	**N**	**B**	**95% LCB**	**p***	**N**
SLC6A4																
CpGU1	**0.48**	**0.02**	**0.043**[a]	349	0.80	-0.06	0.063	350	-0.002	-0.01	0.707	350	0.67	-0.07	0.070	350
CpGU3	0.11	-0.14	0.236	348	0.35	-0.12	0.109	349	-0.001	-0.005	0.676	349	0.39	-0.01	0.053	349
CpGU4	-0.01	-0.17	0.550	343	-0.03	-0.33	0.574	344	-0.002	-0.004	0.879	344	-0.11	-0.36	0.769	344
CpGU6	-0.07	-0.30	0.704	346	-0.23	-0.65	0.808	347	-0.002	-0.004	0.803	347	0.08	-0.29	0.369	347
CpGU9	0.08	-0.12	0.255	349	0.04	-0.33	0.425	350	0.0002	-0.003	0.450	350	0.29	-0.02	0.064	350
CpGU10	-0.003	-0.30	0.507	285	-0.08	-0.64	0.594	286	-0.002	-0.01	0.756	286	0.13	-0.34	0.320	286
CpGU12	0.12	-0.001	0.052	334	0.13	-0.09	0.158	335	-0.0002	-0.002	0.566	335	0.10	-0.09	0.192	335
CpGU14	0.07	-0.06	0.191	335	0.14	-0.10	0.173	336	0.001	-0.001	0.155	336	-0.13	-0.33	0.846	336
CpGU15	**0.18**	**0.01**	**0.044**[a]	347	0.09	-0.24	0.324	348	-0.001	-0.004	0.746	348	-0.12	-0.41	0.762	348
CpGU16	**0.20**	**0.09**	**0.001**[a]	320	**0.35**	**0.14**	**0.003**[a]	321	**0.002**	**0.000**	**0.049**	321	-0.07	-0.25	0.743	321
CpGU22	**0.18**	**0.08**	**0.001**[a]	325	0.17	-0.01	0.056	326	0.0004	-0.001	0.318	326	0.06	-0.10	0.269	326

Note: BMI= body mass index; S4SF= sum of four skinfold thicknesses; %BF= body fat percentage; WC= waist circumference; WHR= waist-hip-ratio. LCB= lower confidence bound. All models are adjusted for age, smoking and alcohol use. [a]P-values that remained significant after additional adjustment for physical activity and depressive symptoms. *p-value of the one-sided test. Values in bold script indicate that p<.05.

Chapter 7

Catechol-O-methyltransferase gene methylation and substance use in adolescents: the TRAILS study

L. J. van der Knaap*
J. M. Schaefer*
I. H. A. Franken
F. C. Verhulst
F. V. A. van Oort
H. Riese

**Shared first authorship*

Genes Brain Behavior, 2014, 13(7), 618-625.

ABSTRACT

Substance use often starts in adolescence and poses a major problem for society and individual health. The dopamine system plays a role in substance use, and catechol-*O*-methyltransferase (COMT) is an important enzyme that degrades dopamine. The Val$^{108/158}$Met polymorphism modulates COMT activity and thus dopamine levels, and has been linked to substance use. *COMT* gene methylation, on the other hand, may affect expression and thus indirectly COMT activity. We investigated whether methylation of the *COMT* gene was associated with adolescents' substance use. Furthermore, we explored whether the *COMT* Val108/158Met polymorphism interacts with *COMT* gene methylation in association with substance use. In 463 adolescents (mean age=16, 50.8% girls), substance use (cigarette smoking, alcohol and cannabis use) was assessed with self-report questionnaires. From blood samples, *COMT* Val$^{108/158}$Met genotype and methylation rates of membrane bound (MB) and soluble (S) *COMT* promoters were assessed. *MB-COMT* promoter methylation was associated with non-daily smoking [odds ratio (OR)=1.82, *P* =0.03], but not with daily smoking (OR=1.20, *P* =0.34), *MB-COMT* promoter methylation was not associated with alcohol use. Adolescents with the Met/Met genotype and high rates of *MB-COMT* promoter methylation were less likely to be high-frequent cannabis users than adolescents with the Val/Val or Val/Met genotype. *S-COMT* promoter methylation was not associated with substance use. These results indicate that there is an association between substance use and *COMT* gene methylation. Although this association is complex, combining genetic and epigenetic variation of the *COMT* gene may be helpful in further elucidating the influence of the dopamine system on substance use in adolescence.

INTRODUCTION

Substance use (i.e. alcohol, cigarettes or cannabis) often starts in adolescence. Prolonged use can lead to poor health, and detrimental social and economic outcomes [1]. The dopaminergic reward system plays an important role in substance use and addiction [2]. Frequent substance use is associated with altered dopamine levels in the brain reward system [3]. Catechol-O-methyltransferase (COMT) degrades dopamine, and variations in COMT expression and activity could modify reward system functioning, thereby influencing vulnerability to substance use.

The *COMT* gene (chr:22, q11.21 [4]) encodes two different protein isoforms, each with its own promoter [5]: the membrane-bound isoform (*MB-COMT*, 271 amino acids), and the soluble isoform (*S-COMT*, 221 amino acids). The functional Val$^{108/158}$Met single nucleotide polymorphism (SNP) in the *COMT* gene, rs4680, has been associated with altered COMT activity [6]. The Val/Val genotype results in a three to fourfold increase in COMT activity and was more prevalent in substance users [7-11], albeit not consistently [12]. These findings indicate a higher COMT activity, hence faster dopamine degradation, in substance users, which arguably is associated with a drive for constant activation of the reward system.

While *COMT* genotypes influence COMT activity, epigenetic modifications (e.g. DNA methylation) of the *COMT* gene may affect gene expression. Indeed, increased *COMT* gene methylation was associated with decreased gene expression [13, 14], but very little is known about the association between *COMT* gene methylation and substance use. In the only general population study we know of, nicotine dependence was related to higher *MB-COMT* promoter methylation, suggesting lower *COMT* gene activity and thus less dopamine degradation in smokers [15]. In schizophrenia patients, alcohol use was associated with increased *MB-COMT* promoter methylation [13]. While studies on genetic variation suggest COMT hyperactivity in substance users, these first epigenetic results indicate lower *COMT* gene activity in substance users. No studies have yet investigated the relationship between cannabis use and *COMT* gene methylation.

In this study we investigated the association between substance use (i.e., cigarettes, alcohol and cannabis) and *COMT* gene methylation in the *MB-COMT* promoter (previously studied by Xu *et al* [15]), as well as the *S-COMT* promoter (not studied previously). We used DNA from a large general population sample of adolescents (14-18 years). Given the lack of studies so far, we carefully hypothesized that *COMT* gene methylation will not only be associated with tobacco and alcohol use, but also with cannabis use. Given the seemingly contradictory findings on *COMT* genotype and *COMT* gene methylation (increased activity vs lower expression of COMT in substance users), an interplay between the two may be present, with indirect oppositional effects on dopamine levels. Therefore, we explored whether the association between *COMT* gene methylation and substance use depended on the *COMT* Val$^{108/158}$Met polymorphism.

MATERIAL AND METHODS

Subjects

This study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective population study in which Dutch preadolescents (*N*=2230) are followed into adulthood. Assessment waves, involving interviews, biological measures and validated questionnaires, are conducted biennially or triennially, and five assessment waves have been completed so far. The present study involves data collected during the third assessment wave, which took place from September 2005 to December 2007 (*N*=1816, mean age 16.3 years, SD=0.71). Written consent was obtained from each subject and their parents at every assessment wave. The study was approved by the Dutch Central Medical Ethics Committee (CCMO) and all subjects received compensation for their participation. A detailed description of sampling and methods can be found in Huisman *et al* [16] and Ormel *et al* [17]. In short, the assessment at T3 included an extensive experimental session, in which 715 adolescents participated (focus sample, response rate 96.1%). Adolescents with a higher risk of mental health problems had a greater chance of being selected for the experimental session. Risk was defined based on T1 measures of temperament (high frustration and fearfulness, low effortful control), lifetime parental psychopathology, and living in a single-parent family. In total 66.0% of the focus sample had at least one of the above described risk factors; the remaining 34.0% were selected randomly from the 'low-risk' TRAILS participants. Although 'high-risk' adolescents were slightly oversampled, the sample included the total range of mental health problems present in a community population of adolescents. T3 also involved a blood draw. Selection for methylation analyses (*N*=475) was based on the participation in the extensive experimental session, availability of a blood sample with sufficient DNA concentration, Dutch ethnicity, and we randomly excluded one of each sibling pair. This selection of 475 adolescents did not differ significantly (p>.05) from the TRAILS focus sample (*N*=715) with regard to sex, socioeconomic status and age. Following drop-out after methylation analyses (for further explanation see section on methylation analyses), we obtained *MB-COMT* promoter methylation rates for 458 subjects, and *S-COMT* promoter methylation rates for 463 subjects.

Substance use

Substance use was assessed with a self-report questionnaire at T3, which was filled out at school or at the subjects' home. Confidentiality of the study was important and adolescents were reassured that their parents or teachers would not have access to the information they provided. Smoking was assessed with the question: "How many cigarettes did you smoke in the past 4 weeks?". Adolescents who had not smoked in the past 4 weeks were categorized as non-smokers. Adolescents who had smoked less

than one cigarette a day in the past 4 weeks were categorized as non-daily smokers and those who had smoked one or more cigarettes per day as daily smokers [18]. Cannabis use was assessed with the question: "How many times have you used weed (marijuana) or hash in the past 4 weeks?". Adolescents who had not used cannabis in the past 4 weeks were categorized as non-users. Adolescents who had used up to four times were categorized as low-frequent users and those who used more than four times as high-frequent users [19]. Alcohol use was assessed with the question: "How many times have you had alcohol in the past 4 weeks? By this, we mean the number of occasions, like going to a party, going out, or an evening at home". Adolescents who reported that they had not drunk alcohol in the past 4 weeks were categorized as abstainers. Adolescents who reported drinking were categorized into two groups: those who had drunk alcohol up to 9 times were defined as low-frequent users and those who had drunk alcohol 10 times or more were defined as high-frequent users [19].

DNA methylation

Analysis. DNA was extracted from whole-blood samples using a manual salting-out procedure [20]. Primer sets described previously [15, 21] were used to analyze regions of the CpG islands (regions containing high frequencies of CpG sites) in the *S-COMT* (S2, Chr 22: 19949993-19950393 [21]) and *MB-COMT* (Chr 22: 19928950-19929359 [15]) promoters (Figure 1).

DNA methylation rates were analyzed using the EpiTYPER method from Sequenom. Bisulfite conversion was followed by PCR amplification, reverse transcription and base-specific cleavage. Fragments were analyzed on a mass spectrometer (Sequenom EpiTYPER, San Diego, CA, USA). Bisulfite conversion of DNA was performed using EZ-96

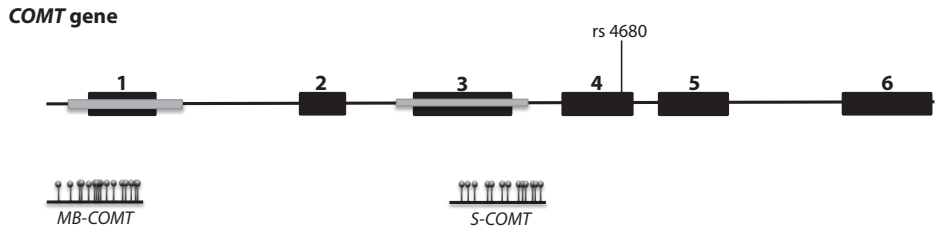


Figure 1. Schematic representation of the *COMT* gene (location: 22q11.21). Black numbered boxes represent exons 1-6 and the approximate position of the *COMT* Val$^{108/158}$Met polymorphism (rs4680, GuanineàAdenine, substituting the amino acid Valine (Val) for Methionine (Met) at position 108/158 in the amino acid chain for *S-COMT* and *MB-COMT* respectively) in exon 4 is shown. Grey boxes represent the CpG islands in the *MB-COMT* and *S-COMT* promoters in exon 1 and 3 respectively. The DNA fragments of *MB-COMT* and *S-COMT* promoters used for methylation analyses are shown in relative position to the CpG islands. Adapted from Zhao *et al* [21].

DNA Methylation Kit (Shallow; Zymo Research, CA, USA), according to manufacturers' protocol. PCR, reverse transcription, cleavage and mass spectrometry were performed in triplicate, according to EpiTYPER protocol. The mass signal patterns generated were translated to quantitative methylation rates for different CpG-units by the MassARRAY EpiTYPER analyzer software from Sequenom. (v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA). Fragments with CpG dinucleotides are referred to as CpG units. One CpG unit can contain one or more CpG dinucleotides. CpG units with a mass outside the range of the mass spectrometer, or with overlap in mass of another CpG unit, could not be analyzed (*MB-COMT*: 7 CpG units, *S-COMT*: 6 CpG units).

Data cleaning procedures. All samples were analyzed in triplicate and for each CpG unit, methylation rates of the triplicates were averaged [22]. Samples with a standard deviation of ≥10% between replicates were removed for analysis. CpG units with ≥25% missing values were not included in the analyses (two CpG units, CpGU2 and CpGU3, in the *S-COMT* promoter and one CpG unit, CpGU16, in the *MB-COMT* promoter). We accounted for mass-change in CpG units by SNPs (only when minor allele frequency >5%) by removing CpG units containing SNPs from analyses (1 CpG unit (*S-COMT* promoter, CpGU7)), and by removing units with the same mass as non-CpG units containing SNPs or other CpG units containing SNPs (none in our sample)). In total, eleven CpG units were available for the *MB-COMT* promoter region and five CpG units were available for the *S-COMT* promoter region.

Genotyping

The *COMT* Val$^{108/158}$Met SNP (rs4680) genotyping was performed on the Illumina BeadStation 500 platform (Illumina Inc., San Diego, CA) using Golden Gate assay and array technology (for details, see [23, 24]). Data on the Val$^{108/158}$Met genotype (Val/Val, Val/Met or Met/Met) was available for 1411 of the TRAILS subjects, of whom 452 had complete data on both genotype and methylation rates (Table 1). The lower number available for methylation analyses resulted from a pre-selection of subjects (see above). The genotyping call rate for rs4680 was 100%. A X^2 -test confirmed that rs4680 was in Hardy-Weinberg equilibrium (P=0.92).

Table 1: Descriptives

	N (%)	mean (SD)
Age	463 (100%)	16.1 (0.6)
Girls	235 (50.8%)	
Smoking (n=458)		
None	332 (72.5%)	
not daily	34 (7.4%)	
Daily	92 (20.1%)	
Cannabis use (n=452)		
None	391 (86.5%)	
Low-frequent	34 (7.5%)	
High-frequent	27 (6.0%)	
Alcohol use (n=449)		
None	89 (19.8%)	
Low-frequent	303 (67.5%)	
High-frequent	57 (12.3%)	
Val/Met polymorphism (n=452)		
Met/Met	141 (31.2%)	
Val/Met	222 (49.1%)	
Val/Val	89 (19.7%)	

Statistical analyses

Descriptives were computed and ANOVA was performed to compare *S-COMT/MB-COMT* promoter methylation rates between different genotypes. We used multinomial logistic regression to study the association between *COMT* genotype and substance use, using the Met/Met genotype (with the lowest enzyme activity) as the reference category. To avoid loss of power when comparing different substance use categories, we did not limit our sample to individuals with methylation data, but we used the genotype data of the 1411 TRAILS subjects.

As missing methylation values (1-6% *MB-COMT* promoter, 2-22% *S-COMT* promoter) in higher methylated units affect the average methylation rate, we mean-centered our methylation data for each CpG unit (resulting in a mean methylation of 0, with original standard deviation (SD)) thereby maintaining the individual variation in CpG-units. We then averaged mean-centered methylation over the CpG units within the *MB-COMT* and *S-COMT* promoter regions. This procedure was used previously in van der Knaap *et al* [22].

To test whether substance use was associated with methylation rates of the *MB-COMT* or *S-COMT* promoter regions, we used multinomial logistic regression analyses. The group of non-substance users was used as reference group in all analyses. In addition, we tested whether the interaction between the *COMT* Val$^{108/158}$Met genotype and meth-

ylation was significantly associated with substance use. If this was the case, analyses were stratified by *COMT* genotype.

As Xu *et al* demonstrated CpG-site specific associations of the *MB-COMT* promoter with nicotine dependence (for overlap with CpG units in the current study, see Table S1/Figure S1), we tested whether methylation rates differed between substance use categories for individual CpG units using multinomial logistic regression, with non-substance users as reference category. For these exploratory analyses, we adjusted for multiple testing using the Bonferroni method. The new p-value regarded as significant was 0.0045.

We adjusted all our analyses for age and sex, as age and sex are both related to DNA methylation [25] and substance use [26]. The sample size varied over analyses depending on the number of missing data (see Table 1).

RESULTS

Descriptives

Alcohol use was the most commonly reported type of substance use in our study population. Smoking was also frequently reported (Table 1). The ranges of methylation of the different CpG units are shown in Table S2. *S-COMT* promoter methylation was higher in adolescents with the Met/Met genotype ($F_{2,449}$=4.178, P<0.05, Figure 2).

Val/Val adolescents were less likely to be low-frequent cannabis users [odds ratio (OR)=0.57, confidence interval (CI)=0.32; 1.01, P=0.06) or high-frequent cannabis users (OR=0.45, CI=0.21; 0.96, P=0.04) than non-users, compared to adolescents with the Met/Met genotype. The Val/Met genotype did not have significantly different odds of cannabis use compared to the Val/Val genotype. There was no significant association between *COMT* genotype and smoking or alcohol use.

Substance use and *COMT* gene methylation

As shown in Table 2, *MB-COMT* promoter methylation was associated with non-daily smoking, but not with daily smoking. *MB-COMT* or *S-COMT* promoter methylation were not associated with cannabis use or alcohol use (Table 2). Due to differences in *S-COMT* promoter methylation according to *COMT* genotype (described above, Figure 2) we added genotype as a covariate in the analyses with *S-COMT* promoter methylation. This did not change the relationships between *S-COMT* promoter methylation and substance use.

In the individual CpG unit analyses (Figure 3), we found that *MB-COMT* promoter methylation in CpG unit 3 (OR=1.17, 95% CI= 1.05;1.30, P=0.004) and CpG unit 9 (OR=1.64, 95% CI= 1.06; 2.52, P=0.03) were associated with non-daily smoking, although only the effect for *MB-COMT*-promoter CpGU3 remained significant after correction for

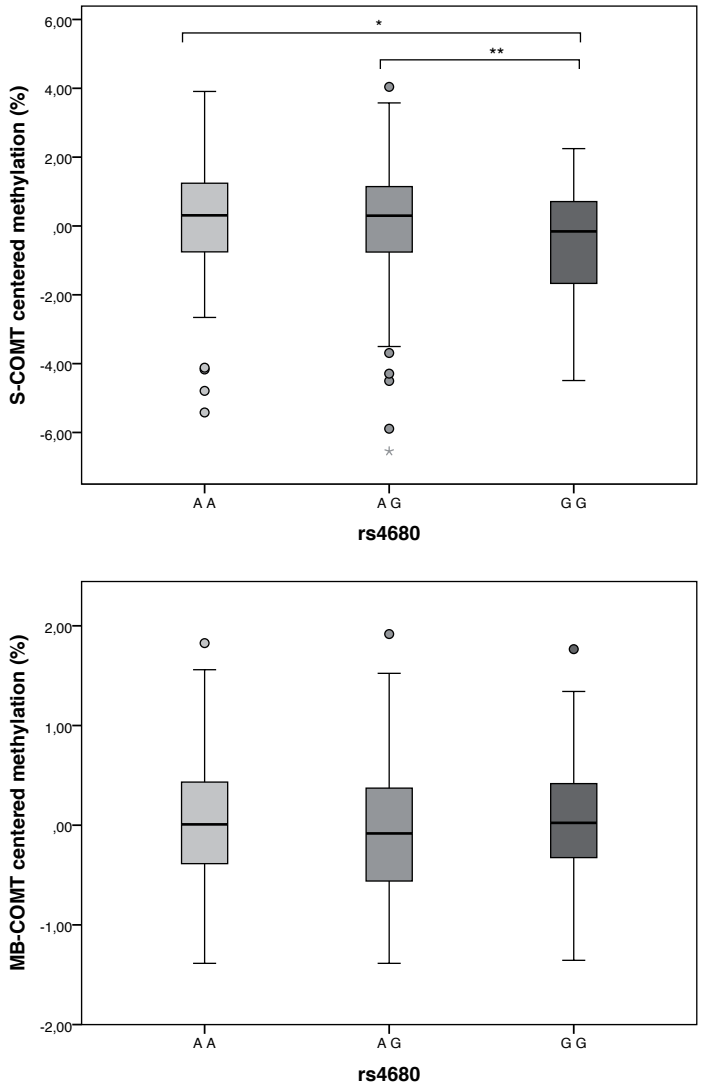


Figure 2. Centered methylation rates of the *S-COMT* and *MB-COMT* promoter for each genotype (A=Met, G=Val, bars: p25-p75). *p<0.05, **p<0.01

multiple testing. There were no associations between methylation rates of single CpG units and cannabis use. For alcohol use we found that *MB-COMT* promoter methylation was associated with low frequent alcohol use in CpGU5 (OR=0.76, 95% CI= 0.58; 0.995, *P*=0.046) and in CpGU19 (OR=0.79, 95% CI=0.64; 0.98, *P*=0.03). These associations were not significant after correction for multiple testing. No effects were found for *S-COMT* promoter methylation.

Moderation by COMT genotype

When included in our model, the interaction term 'COMT *genotype x* MB-COMT *promoter methylation*' was associated with cannabis use (Table 3). Therefore, we stratified the analyses for methylation and cannabis use by *COMT* Val$^{108/158}$Met genotype. In adolescents with the Met/Met genotype, methylation rates were associated with lower odds of high frequent cannabis use (OR=0.25, 95% CI=0.08; 0.82, *P*=0.02). The interaction term 'COMT *genotype x* MB-COMT *promoter methylation*' was not associated with smoking or alcohol use and we also did not find any significant association between the interaction term 'COMT *genotype x* S-COMT *promoter methylation*' and substance use.

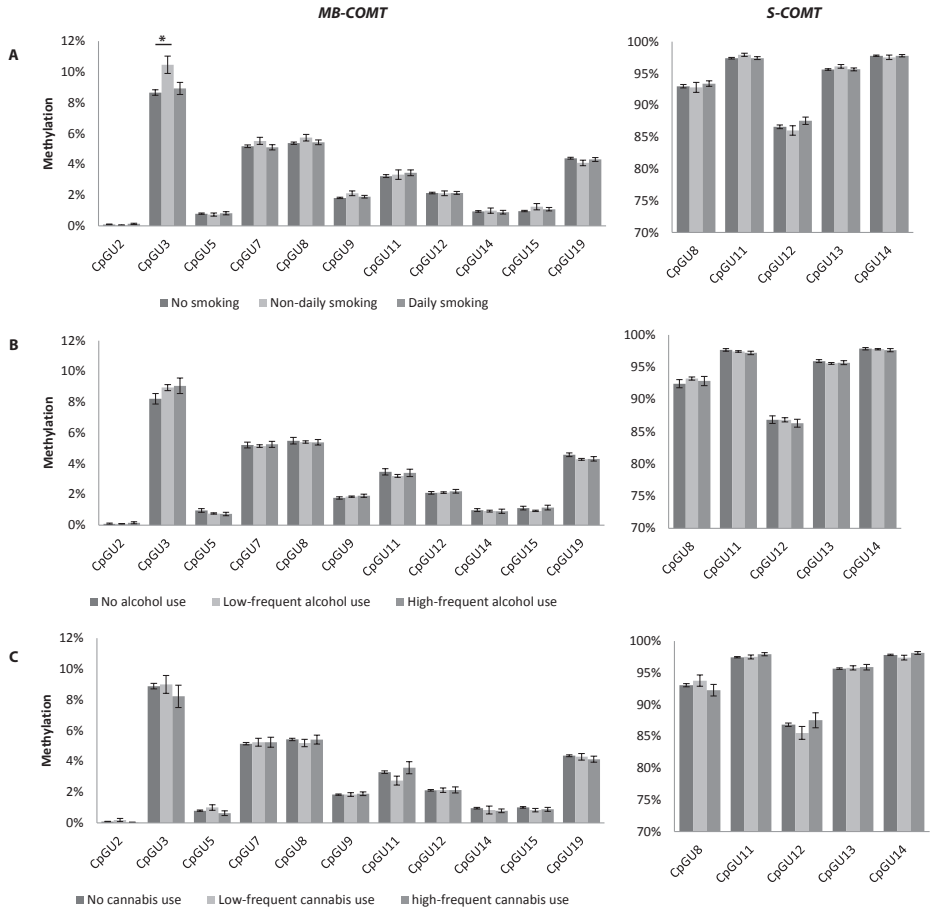


Figure 3. Methylation rates of individual CpG Units, divided into substance use categories. Separate graphs were used for the *MB-COMT* promoter (left) and *S-COMT* promoter (right). Mean methylation rates are presented for smoking categories (**A**), alcohol use categories (**B**), and for cannabis use categories (**C**). Error bars represent SEs. *p<0.0045

Table 2. Associations between methylation of the *COMT* gene and substance use

	OR	95% CI	p	OR	95% CI	p
Smoking	**Non daily**			**Daily**		
MB-COMT promoter methylation	1.82	1.07; 3.09	0.03	1.20	0.83; 1.73	0.34
S-COMT promoter methylation	1.04	0.83; 1.30	0.76	1.09	0.94; 1.27	0.27
Cannabis	**Low frequent**			**High frequent**		
MB-COMT promoter methylation	0.82	0.47; 1.42	0.48	0.70	0.38; 1.31	0.27
S-COMT promoter methylation	0.96	0.77; 1.19	0.68	1.06	0.82; 1.36	0.67
Alcohol	**Low frequent**			**High frequent**		
MB-COMT promoter methylation	0.91	0.63; 1.33	0.63	1.00	0.59; 1.67	0.99
S-COMT promoter methylation	0.99	0.85; 1.15	0.85	0.90	0.73; 1.10	0.30

Abbreviations: OR, odds ratio; CI, confidence interval. Reference categories: No smoking, no cannabis use and no alcohol use. Adjusted for sex and age.

Table 3: Influence of Val$^{108/158}$Met genotype on association between *MB-COMT* promoter methylation and substance use.

	OR	95% CI	p	OR	95% CI	p
Smoking	**Non daily**			**Daily**		
Val/Met genotype	0.61	0.34; 1.08	0.09	0.92	0.65; 1.29	0.62
MB-COMT promoter methylation	1.10	0.10; 12.49	0.94	1.69	0.32; 8.97	0.54
Val/Met**MB-COMT* promoter methylation	1.16	0.49; 2.73	0.74	0.86	0.49; 1.51	0.61
Cannabis	**Low frequent**			**High frequent**		
Val/Met genotype	0.45	0.26; 0.79	0.01	0.60	0.33; 1.10	0.10
MB-COMT promoter methylation	1.33	0.12; 15.07	0.82	0.07	0.01; 0.75	0.03
Val/Met**MB-COMT* promoter methylation	0.83	0.34; 2.06	0.69	2.42	1.03; 5.66	0.04
Alcohol	**Low frequent**			**High frequent**		
Val/Met genotype	0.97	0.69; 1.37	0.87	1.00	0.62; 1.62	1.00
MB-COMT promoter methylation	1.11	0.21; 5.77	0.90	3.08	0.62; 35.51	0.34
Val/Met**MB-COMT* promoter methylation	0.95	0.55; 1.64	0.84	0.66	0.30; 1.43	0.29

Abbreviations: OR, odds ratio; CI, confidence interval. Reference categories: No smoking, no cannabis use and no alcohol use. Adjusted for sex and age.

DISCUSSION

This is the first study in which the association between *COMT* gene methylation and adolescents' substance use is analyzed. We found higher methylation rates in non-daily smokers compared to non-smokers and daily smokers. Also, in adolescents homozygous for the Met allele, methylation was associated with lower odds for cannabis use.

Higher rates of *MB-COMT* promoter methylation were associated with non-daily smoking in adolescents. It is difficult to explain why no association with daily smoking was found. We could speculate that specific, currently unknown, regulation mechanisms

are at work linking methylation of the *MB-COMT* promoter with non-daily smoking, which represents a more controlled form of smoking in adolescents. In the study by [15], no association between daily smoking and overall *MB-COMT* promoter methylation was found. However, differences in methylation between their daily smokers and controls became apparent when testing individual CpG sites: methylation rates were higher in daily smokers compared to non-smokers at CpG sites -193 and -39, which correspond with CpGU3 and CpGU12 in the current study (Table S1/Figure S1). Interestingly, in the unit specific analyses for smoking, we also found a higher methylation of CpGU3, but specific for non-daily smoking. We did not find differences in methylation of CpGU12 between the smoking groups. It is possible that during adolescence the relationship between methylation and smoking status is different from that later in life. The rate of methylation in the *MB-COMT* promoter in our study was relatively low compared to the methylation rates reported by [15], possibly due to chronic heavy smoking. Or, as DNA methylation rates increase with age [25], particularly in CpG islands [27], differences in methylation rates may reflect differences in age between the samples (~16-year-old adolescents vs. ~45-year-old adults). Our findings and those from Xu *et al* show that *COMT* gene methylation is associated with smoking status, but also raise many questions concerning the exact relationship. Since these are the first studies in this area this should not be surprising and obviously further research is necessary to gain insight into smoking habits and *COMT* gene methylation. Longitudinal studies with repeated measures of both methylation and smoking habits will be necessary to further increase our understanding of how both interrelate.

Although other studies have found relationships between methylation of genes in the dopamine system and alcohol dependence, e.g., higher rates of methylation in the dopamine transporter gene [28] and methylation of monoamine oxidase-A [29], we did not find a relationship between mean *MB-COMT* or *S-COMT* promoter methylation and alcohol use in adolescents. Neither did we find associations between methylation and alcohol use in our unit-specific analyses. We are not aware of any other study relating methylation of the *COMT* gene to alcohol use in adolescents.

In this study, the Val/Val variant was associated with lower odds of high frequent cannabis use in adolescents. A recent meta-analysis of the association between the *COMT* Val$^{108/158}$Met polymorphism and substance use identified the Val-allele as risk factor for smoking and for cannabis use [12], but the populations studied were highly heterogeneous. Adolescence is a phase in which novelty-seeking, impulsivity and peer behavior may play a major role in the initiation of substance use [30-32]. In line with this theory are findings from studies that have linked the Met/Met variant to increased novelty-seeking, which could drive cannabis use in adolescents [33-35]. Another study associated the Val/Val variant with novelty-seeking [36]. The relationship might be dependent on genetic varia-

tions in other genes in addition to the *COMT* polymorphism [37] and might be moderated by personality, stress or other environmental factors.

We found that adolescents with the Met/Met genotype and higher methylation rates had a lower risk for cannabis use. Hence, there seems to be an interaction between the *COMT* Val$^{108/158}$Met polymorphism and *MB-COMT* promoter methylation rates in relation to cannabis use. This is a novel finding which is in line with the anhedonia hypothesis of substance use [38].The combination of the low enzyme activity (Met/Met genotype) and reduced expression of the enzyme (higher methylation rates) might result in higher levels of dopamine through diminished dopamine degradation. It is known that low brain dopamine levels result in an under-active reward system, accompanied by anhedonia. Substance use could be explained as an attempt to alleviate this unfavorable anhedonic state [39, 40]. Arguably, individuals with a combination of the low enzyme activity Met/Met genotype have high dopamine levels - and do not have an anhedonic state - which might be preventive for substance use.

A strength of this study is the measurement of both genetic and epigenetic variations of the *COMT* gene, which provides a more complete picture of the role of COMT in substance use. In addition, we analyzed several types of substance use that are highly prevalent in adolescence and assessed recent use to minimize recall bias, thereby gaining reliable measures for substance use. Some limitations of our study have to be noted as well. The cross-sectional nature of this study prevented us from investigating whether methylation is a consequence of substance use, or whether methylation predisposes an individual to drug seeking behavior; a question with no definitive answer in the literature thus far [41]. To this end, repeated measurements of methylation status are needed. This paper includes a multiplicity of comparisons, which increases the risk of obtaining chance findings. This is especially relevant for the analyses of the single CpG unit data. To minimize this risk we applied a Bonferroni correction. However, for the analyses including methylation data, genotypes and substance use we did not correct for multiple testing. Therefore, we were cautious with interpreting our findings and would like to emphasize that replication is warranted. It should be noted that we studied adolescents who have had a relatively short exposure to substance use. Associations may be stronger in adults who have developed a substance addiction earlier in life or have a more intense and longer history of use. Adolescents in this study may still be experimenting with drugs, and this may be motivated by different brain mechanisms than drug addiction. We were interested in methylation of the *COMT* gene in the brain, but as this is impossible to determine in a cohort study of adolescents, we used DNA from blood cells to determine methylation rates. This is probably a valid approach as identical methylation patterns for the *COMT* gene in blood and the brain were reported previously [42], which indicates that *COMT* gene methylation in blood may be used as a proxy for *COMT* gene methylation in the brain.

To conclude, we showed that methylation of the *MB-COMT* promoter was associated with non-daily smoking in adolescents. This study further suggests that epigenetics, in combination with the *COMT* Val$^{108/158}$Met polymorphism, could be associated with cannabis use during adolescence. Maybe through altering COMT activity and gene expression, and thereby influencing the dopamine metabolism in the brain. However, this finding warrants replication in other populations, including adults and individuals who are addicted to substances. The findings of the study may also provide a first step in the prevention of substance use disorders. Epigenetic modifications may prove to be useful biomarkers to identify susceptibility or vulnerability for substance use, and, in time, our findings may even contribute to the development or improvement of effective behavioral or pharmacological interventions for substance use disorders. In order to obtain more insight into the mechanisms involved in substance use and abuse it may be helpful to include both genetic and epigenetic factors.

REFERENCES

1. Macleod J, Oakes R, Copello A, Crome I, Egger M, Hickman M *et al.* Psychological and social sequelae of cannabis and other illicit drug use by young people: a systematic review of longitudinal, general population studies. *Lancet* 2004; **363**(9421)**:** 1579-1588.
2. Robinson TE, Berridge KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev* 1993; **18**(3)**:** 247-291.
3. Wanat MJ, Willuhn I, Clark JJ, Phillips PE. Phasic dopamine release in appetitive behaviors and drug addiction. *Curr Drug Abuse Rev* 2009; **2**(2)**:** 195-213.
4. Grossman MH, Emanuel BS, Budarf ML. Chromosomal mapping of the human catechol-O-methyltransferase gene to 22q11.1----q11.2. *Genomics* 1992; **12**(4)**:** 822-825.
5. Tenhunen J, Salminen M, Lundstrom K, Kiviluoto T, Savolainen R, Ulmanen I. Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. *Eur J Biochem* 1994; **223**(3)**:** 1049-1059.
6. Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, Weinshilboum RM. Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. *Pharmacogenetics* 1996; **6**(3)**:** 243-250.
7. Beuten J, Payne TJ, Ma JZ, Li MD. Significant association of catechol-O-methyltransferase (*COMT*) haplotypes with nicotine dependence in male and female smokers of two ethnic populations. *Neuropsychopharmacology* 2006; **31**(3)**:** 675-684.
8. Horowitz R, Kotler M, Shufman E, Aharoni S, Kremer I, Cohen H *et al.* Confirmation of an excess of the high enzyme activity *COMT* val allele in heroin addicts in a family-based haplotype relative risk study. *Am J Med Genet* 2000; **96**(5)**:** 599-603.
9. Li T, Chen CK, Hu X, Ball D, Lin SK, Chen W *et al.* Association analysis of the DRD4 and *COMT* genes in methamphetamine abuse. *Am J Med Genet B Neuropsychiatr Genet* 2004; **129B**(1)**:** 120-124.
10. Redden DT, Shields PG, Epstein L, Wileyto EP, Zakharkin SO, Allison DB *et al.* Catechol-O-methyltransferase functional polymorphism and nicotine dependence: an evaluation of nonreplicated results. *Cancer Epidemiol Biomarkers Prev* 2005; **14**(6)**:** 1384-1389.
11. Vandenbergh DJ, Rodriguez LA, Miller IT, Uhl GR, Lachman HM. High-activity catechol-O-methyltransferase allele is more prevalent in polysubstance abusers. *Am J Med Genet* 1997; **74**(4)**:** 439-442.
12. Tammimaki AE, Mannisto PT. Are genetic variants of *COMT* associated with addiction? *Pharmacogenet Genomics* 2010; **20**(12)**:** 717-741.
13. Abdolmaleky HM, Cheng KH, Faraone SV, Wilcox M, Glatt SJ, Gao F *et al.* Hypomethylation of *MB-COMT* promoter is a major risk factor for schizophrenia and bipolar disorder. *Hum Mol Genet* 2006; **15**(21)**:** 3132-3145.
14. Sasaki M, Kaneuchi M, Sakuragi N, Dahiya R. Multiple promoters of catechol-O-methyltransferase gene are selectively inactivated by CpG hypermethylation in endometrial cancer. *Cancer Res* 2003; **63**(12)**:** 3101-3106.
15. Xu Q, Ma JZ, Payne TJ, Li MD. Determination of Methylated CpG Sites in the Promoter Region of Catechol-O-Methyltransferase (*COMT*) and their Involvement in the Etiology of Tobacco Smoking. *Front Psychiatry* 2010; **1:** 16.
16. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, Huizink AC *et al.* Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. *Int J Epidemiol* 2008; **37**(6)**:** 1227-1235.

17. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, Veenstra R *et al.* The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. *J Am Acad Child Adolesc Psychiatry* 2012; **51**(10)**:** 1020-1036.
18. Harakeh Z, de Sonneville L, van den Eijnden RJ, Huizink AC, Reijneveld SA, Ormel J *et al.* The association between neurocognitive functioning and smoking in adolescence: the TRAILS study. *Neuropsychology* 2012; **26**(5)**:** 541-550.
19. Creemers HE, Harakeh Z, Dick DM, Meyers J, Vollebergh WA, Ormel J *et al.* DRD2 and DRD4 in relation to regular alcohol and cannabis use among adolescents: does parenting modify the impact of genetic vulnerability? The TRAILS study. *Drug Alcohol Depend* 2011; **115**(1-2)**:** 35-42.
20. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**(3)**:** 1215.
21. Zhao AM, Cheng Y, Li XT, Li QL, Wang L, Xu JW *et al.* Promoter hypomethylation of *COMT* in human placenta is not associated with the development of pre-eclampsia. *Mol Hum Reprod* 2011; **17**(3)**:** 199-206.
22. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ *et al.* Glucocorticoid receptor gene (*NR3C1*) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry* 2014; **4:** e381.
23. Nijmeijer JS, Hartman CA, Rommelse NN, Altink ME, Buschgens CJ, Fliers EA *et al.* Perinatal risk factors interacting with catechol O-methyltransferase and the serotonin transporter gene predict ASD symptoms in children with ADHD. *J Child Psychol Psychiatry* 2010; **51**(11)**:** 1242-1250.
24. Stavrakakis N, Oldehinkel AJ, Nederhof E, Oude Voshaar RC, Verhulst FC, Ormel J *et al.* Plasticity genes do not modify associations between physical activity and depressive symptoms. *Health Psychol* 2013; **32**(7)**:** 785-792.
25. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol* 2013; **14**(10)**:** R115.
26. Becker JB, Hu M. Sex differences in drug abuse. *Front Neuroendocrinol* 2008; **29**(1)**:** 36-47.
27. Johansson A, Enroth S, Gyllensten U. Continuous Aging of the Human DNA Methylome Throughout the Human Lifespan. *PLoS One* 2013; **8**(6)**:** e67378.
28. Hillemacher T, Frieling H, Hartl T, Wilhelm J, Kornhuber J, Bleich S. Promoter specific methylation of the dopamine transporter gene is altered in alcohol dependence and associated with craving. *J Psychiatr Res* 2009; **43**(4)**:** 388-392.
29. Philibert RA, Gunter TD, Beach SR, Brody GH, Madan A. MAOA methylation is associated with nicotine and alcohol dependence in women. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B**(5)**:** 565-570.
30. Brook JS, Zhang CS, Brook DW. Developmental Trajectories of Marijuana Use From Adolescence to Adulthood Personal Predictors. *Arch Pediat Adol Med* 2011; **165**(1)**:** 55-60.
31. Marschall-Levesque S, Castellanos-Ryan N, Vitaro F, Seguin JR. Moderators of the association between peer and target adolescent substance use. *Addict Behav* 2014; **39**(1)**:** 48-70.
32. Teichman M, Barnea Z, Ravav G. Personality and Substance Use among Adolescents - a Longitudinal-Study. *Brit J Addict* 1989; **84**(2)**:** 181-190.
33. Golimbet VE, Alfimova MV, Gritsenko IK, Ebstein RP. Relationship between dopamine system genes and extraversion and novelty seeking. *Neurosci Behav Physiol* 2007; **37**(6)**:** 601-606.
34. Demetrovics Z, Varga G, Szekely A, Vereczkei A, Csorba J, Balazs H *et al.* Association between Novelty Seeking of opiate-dependent patients and the catechol-O-methyltransferase Val(158) Met polymorphism. *Compr Psychiatry* 2010; **51**(5)**:** 510-515.

35. Hosak L, Libiger J, Cizek J, Beranek M, Cermakova E. The *COMT* Val158Met polymorphism is associated with novelty seeking in Czech methamphetamine abusers: preliminary results. *Neuro Endocrinol Lett* 2006; **27**(6)**:** 799-802.
36. Lang UE, Bajbouj M, Sander T, Gallinat J. Gender-dependent association of the functional catechol-O-methyltransferase Val158Met genotype with sensation seeking personality trait. *Neuropsychopharmacology* 2007; **32**(9)**:** 1950-1955.
37. Strobel A, Lesch KP, Jatzke S, Paetzold F, Brocke B. Further evidence for a modulation of Novelty Seeking by DRD4 exon III, 5-HTTLPR, and *COMT* val/met variants. *Mol Psychiatry* 2003; **8**(4)**:** 371-372.
38. Wise RA. Catecholamine theories of reward: a critical review. *Brain Res* 1978; **152**(2)**:** 215-247.
39. Markou A, Koob GF. Postcocaine anhedonia. An animal model of cocaine withdrawal. *Neuropsychopharmacology* 1991; **4**(1)**:** 17-26.
40. Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F, Baler R. Addiction: decreased reward sensitivity and increased expectation sensitivity conspire to overwhelm the brain's control circuit. *Bioessays* 2010; **32**(9)**:** 748-755.
41. Nielsen DA, Utrankar A, Reyes JA, Simons DD, Kosten TR. Epigenetics of drug abuse: predisposition or response. *Pharmacogenomics* 2012; **13**(10)**:** 1149-1160.
42. Murphy BC, O'Reilly RL, Singh SM. Site-specific cytosine methylation in *S-COMT* promoter in 31 brain regions with implications for studies involving schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* 2005; **133B**(1)**:** 37-42.

SUPPLEMENTARY INFORMATION

For the *MB-COMT* genomic region, we used a primer set that was previously reported by Xu *et al.,* 2010. While Xu *et al* present the exact positions of CpG sites relative to the transcription start site, in the current study we present CpG units that contained one or multiple CpG sites. The overlap between the CpG sites as presented by Xu *et al* and the CpG units in the current study is shown in Table S1.

Table S1. Overlap between CpG units in the current study and CpG sites reported by Xu *et al*., 2010.

CpG units presented in our study	**CpG site positions relative to the transcription start site as presented by Xu *et al.***			
MB-COMT CpGU2	-211			
***MB-COMT* CpGU3**	-195[a]	-193[b]		
MB-COMT CpGU5	-161	-158	-156[a]	-154[a]
MB-COMT CpGU7	-131[a]	-127[a]	-125	-123[a]
MB-COMT CpGU8	-112[a]	-110		
MB-COMT CpGU9	-104[a]	-99[a]		
MB-COMT CpGU11	-55	-51		
MB-COMT CpGU12	-46[a]	-39[b]	-35[a]	
MB-COMT CpGU14	-23			
MB-COMT CpGU15	-8[a]			
MB-COMT CpGU19	+19[a]	+22[a]		

Note: most units contain more than one CpG. [a] No information on methylation in Xu *et al*., 2010. [b] Difference found between smokers and non smokers in Xu *et al*., 2010. Units printed in bold represent units that were significantly related to substance use (non-daily smoking) in the current analyses.

Table S2. CpG Unit descriptive statistics: methylation rates of the individual CpG units.

	N	**Minimum**	**Maximum**	**Mean**	**Std. Deviation**
MB-COMT CpGU2	458	0%	3%	0.1%	0.4%
MB-COMT CpGU3	445	1%	19%	8.9%	3.4%
MB-COMT CpGU5	457	0%	6%	0.8%	0.8%
MB-COMT CpGU7	456	2%	10%	5.2%	1.5%
MB-COMT CpGU8	436	2%	14%	5.4%	1.4%
MB-COMT CpGU9	458	0%	6%	1.9%	0.8%
MB-COMT CpGU11	458	0%	9%	3.3%	1.7%
MB-COMT CpGU12	456	0%	6%	2.1%	0.9%
MB-COMT CpGU14	456	0%	8%	0.9%	1.0%
MB-COMT CpGU15	458	0%	8%	1.0%	0.9%
MB-COMT CpGU19	455	2%	9%	4.4%	1.1%

Table S2. CpG Unit descriptive statistics: methylation rates of the individual CpG units. (continued)

	N	Minimum	Maximum	Mean	Std. Deviation
S-COMT CpGU8	362	70%	100%	93.1%	4.3%
S-COMT CpGU11	447	87%	100%	97.5%	2.0%
S-COMT CpGU12	366	65%	98%	86.8%	4.8%
S-COMT CpGU13	456	88%	100%	95.7%	2.2%
S-COMT CpGU14	437	89%	100%	97.8%	1.8%

MB-COMT

5'-

tggg|at|acc|agctctggg|ag|acc|ac|aggtgc|agtc|agc|ac|agc|agg|accttt|ag|ac|a|aggc|accc|agcccc|agtttcccc|acctggg|a|agggggct|ac ttgtggct|ag|a|agc|AGCC-243**CG**G1|actcctg|agc|a|ag|act|ag|acc|a|ag|AGGC-211**CG**GT2|atgtgg|ac|ACCCC-195**CG**-193**CG**TGGGC3|accccc| A-179**CG**GGG4|ac|accctggcc|AC-161**CG**C-158**CG**-156**CG**-154**CG**G5|ac|accctc|A-142**CG6**|agg|ac|ACCC-131**CG**GC-127**CG**-125**CG**-123**CG**G7|ac|acct| AC-112**CG**-110**CG**GGG8|A-104**CG**CCC-99**CG**9|acccc|atcct|ACCTGCTG-79**CG**CCC-74**CG**-72**CG**C-69**CG**-67**CG**CCC-62**CG**C10|ACCC-55**CG**CC-51**CG**C C11|A-46**CG**GCCTG-39**CG**TC-35**CG**CC12|AC-29**CG**G13|a|AG-23**CG**CCCTCCT14|a|ATCCC-8**CG**C15|AG-3**CG**C_16|AC+4**CG**CC17|ATTGC**CG**CC18|A T**CG**T**CG**TGGGGCTTCTGGGGC19|agct|agggctgcc -3'

S-COMT

5'-

gtgggtgctgc|agg|agg|agc|ac|ag|agc|ACTGG**CG**CCCCTCCCCTCC**CG**CCCTGC1|ag|ATGC**CG**G2|AGG**CG**CCTCTGCTGTTGGC3|ag ctgtgttgctgggcctggtgctgctggtggtgctgctgctgcttctg|aggc|actggggctggggcctgtgcctt|AT**CG**GCTGG4|a|A**CG5**|agttc|atcctgc|agccc |atcc|ac|a|acctgctc|atgggtg|ac|acc|a|agg|agc|AG**CG**C6|atcctg|a|acc|A**CG**TGCTGC7|agc|ATG**CG**G8|AGCC**CG**GG9|a|A**CG**C10|ac|ag|A G**CG**TGCTGG11|aggcc|attg|ac|acct|ACTG**CG**12|agc|ag|a|agg|agtgggcc|atg|a|A**CG**TGGG**CG**13|ac|a|ag|a|a|AGGTGGGGTC**CG**GGCC1 4|agc|aggtgctc|agctctggg|ac|agggg|accc|agg|acc|aggc|a -3'

Figure S1. The sequence and position of individual CpG sites and CpG units is shown for both *MB-COMT* (upper panel) and *S-COMT* (lower panel) DNA fragments. The vertical lines represent the position of splice sites on the complementary RNA strand (not shown), and any fragment containing one or more CpG sites (bold letters) is considered a CpG unit and are represented by uppercase letters and numbered at the right end side. For *MB-COMT*, the individual CpG sites are labeled by their relative position in relation to the transcription start site (+1, the underlined C in CpG unit 16) according to Xu *et al*., (2010) [1]. Methylation rates for grey CpG units could not be obtained or used for analyses (see method section for further details).

REFERENCES SUPPLEMENTARY INFORMATION

1. Xu Q, Ma JZ, Payne TJ, Li MD. Determination of Methylated CpG Sites in the Promoter Region of Catechol-O-Methyltransferase (*COMT*) and their Involvement in the Etiology of Tobacco Smoking. *Front Psychiatry* 2010; **1:** 16.

Chapter 8

General discussion

GENERAL DISCUSSION

In this thesis I investigated whether DNA methylation serves as an underlying biological mechanism that could predispose individuals with a history of adversity to poor health outcomes. Animal studies reported that an adverse early life environment was associated with high levels of methylation in the glucocorticoid receptor gene (*NR3C1)*, and consequently elevated stress responses and more fearful behavior. At the time I started this research, first studies in humans had partly replicated these findings, and extended them to other genes of interest, such as the serotonin transporter (*SLC6A4*). However, sample sizes were small and larger studies with a wider range of exposures and outcomes were necessary.

ADVERSE LIFE EVENTS AND DNA METHYLATION

In **Chapter 2 and 3** I investigated the relation between the type and timing of adverse life events and *NR3C1* and *SLC6A4* methylation. Firstly, the effect of type of adversity on methylation was studied, and a distinction was made between perinatal stress, stressful life events (SLEs), and traumatic youth experiences (TYEs). Secondly, the effect of timing of SLEs on methylation was studied by analyzing the independent associations between SLEs at different developmental periods (i.e., childhood and adolescence) and *NR3C1* and *SLC6A4* methylation.

For both genes there was no association between perinatal stress and DNA methylation in adolescence. This was somewhat surprising, as the lack of an association between *NR3C1* or *SLC6A4* methylation and perinatal stress reported in our studies contrasts with initial reports in animal studies. Several human studies have reported associations between perinatal stress and altered *NR3C1* or *SLC6A4* methylation in cord-blood [1-3], but the long-term effects of perinatal stress, however, had not been investigated. The results reported in **Chapter 2 and 3** of this thesis thus suggest that perinatal stress may not have long-term effects on *NR3C1* and *SLC6A4* methylation. Although DNA methylation is considered to be stable, the recent discovery of active removal of methyl groups[4, 5] indicates that DNA methylation is reversible and regulation might be more dynamic than was originally thought. This theory is in line with another result of our study; only SLEs experienced in adolescence were associated with *NR3C1* or *SLC6A4* methylation, and there were no associations between childhood SLEs and methylation in these genes. As our methylation data originated from blood collected in adolescence, methylation marks from perinatal stress or childhood SLEs may have been gradually lost over time. For the TYEs there was no data available on the age of the participants at the time of abuse. Because of this, we could not determine whether the association between TYEs

and methylation was due to recent traumatic experiences. It is possible that the effects of repeated exposure to TYEs on DNA methylation differs from the experience of multiple different SLEs on methylation, resulting in more stable methylation following repeated TYEs. However, this is highly speculative and more research is necessary combined with repeated measures of methylation to determine causality.

Exposure to TYEs was only associated with methylation of *NR3C1*, whereas associations between TYEs and methylation have been reported before for both *NR3C1* [6-8] and *SLC6A4* [9-11]. The lack of association between TYEs and *SLC6A4* methylation was therefore unexpected. Although we had a relatively large sample size, the number of individuals who had experienced TYEs was limited. Perhaps we did not have enough power in our sample to detect differences in *SLC6A4* methylation. On the other hand, small sample sizes in other similar studies may also have a reduced likelihood of reflecting a true effect [12]. Also, some of these studies made use of a clinical sample, which could also be related to high methylation levels (e.g. high *SLC6A4* methylation has been associated with more severe psychopathology [11]). Another possibility is that methylation by TYEs may be gene-specific, as we did find a positive association between TYEs and *NR3C1* methylation with a smaller sample size.

Another interesting finding in **Chapter 2** concerns the regional differences of *NR3C1* methylation. Exposure to multiple SLEs between birth and adolescence was positively associated with methylation in region 1 of the *NR3C1* CpG island (see page 22 for more information regarding *NR3C1* regions 1-3). Regions 1 and 3 were designed to optimize coverage of the CpG island study and had not been investigated before in relation to adverse life events. Methylation in region 2, on the other hand, had been investigated in relation to early life adversity in animal and human studies (e.g., [6, 13]). Region 2 was known to contain a transcription factor binding site that, when methylated, was known to inhibit the expression of this gene. Yet, most of the associations between stress and *NR3C1* methylation in our study were found in region 1. Since methylation in other regions of the CpG island is known to be highly variable between individuals [14], it is unlikely that the environmental influences on methylation are restricted to region 2. Indeed, childhood abuse has been related to methylation in other regions of the CpG island than regions 1, 2 or 3 in human post-mortem brain tissue [15]. A logical next step would be to investigate the presence of important transcription binding sites in region 1, to see how methylation in this region affects expression, and investigate biological pathways associated with changes in methylation.

For *SLC6A4,* I additionally investigated whether different *5HTTLPR* genotypes, are equally sensitive to be methylated after exposure to SLEs. The polymorphism *5HTTLPR* was thought to moderate the relation between SLEs and risk for depression [16]. The short (s)-allele was generally considered to be the susceptibility allele [17], with increased risk of depression in a high stress environment, and the long (l)-allele was considered to have

a protective function, but attempts at replication have resulted in mixed findings [18, 19]. One way to improve our understanding of gene-environment interactions could be to incorporate methylation patterns into these interactions. In **Chapter 3** we have seen that *5HTTLPR* genotype influenced the relationship between SLEs and *SLC6A4* methylation; only in carriers of two l-alleles methylation levels were higher when more SLEs were experienced. A highly methylated l-allele is considered to have a poor transcriptional activity, and methylation might nullify the protective function of the l-allele, which could contribute to the mixed findings mentioned earlier. This theory is supported by a study that showed more unresolved loss or trauma in highly methylated l-allele carriers and low methylated s-allele homozygotes, and less unresolved loss or trauma in high methylated s-allele homozygotes [20]. This suggests that epigenetic regulation may serve as an adaptive mechanism to adjust gene expression and compensate for changes in gene expression caused by genetic variation [20, 21].

DNA METHYLATION AND (MENTAL) HEALTH PROBLEMS

Internalizing problems

The relationship between *NR3C1* or *SLC6A4* methylation and internalizing problems (i.e., anxiety and depression problems) had only scarcely been investigated. Therefore, I studied whether *NR3C1* and *SLC6A4* methylation were associated with concurrent and future internalizing problems, i.e., clinical diagnoses of internalizing disorders and internalizing symptom scores.

Higher *NR3C1* methylation was associated with concurrent and prospective (3 years later) internalizing symptom scores, and lifetime internalizing disorders. Our findings also indicated that high *NR3C1* methylation is associated with an increased vulnerability to develop new internalizing problems over time. High *SLC6A4* methylation only showed a tendency for association with internalizing symptom scores. By investigating the relationship between methylation in these genes and anxiety and depression problems separately, I explored whether these associations were driven by one of these phenotypes. For depression, the relationship between *NR3C1* and *SLC6A4* methylation has been investigated, albeit scarcely, but the relationship between *NR3C1* or *SLC6A4* methylation and anxiety problems has not been studied before. However, this relationship seems plausible because associations between SLEs and anxiety disorders have been reported in the literature [22, 23]. Also, for some anxiety disorders an association with hyperactivity of the HPA axis has been reported [24], which suggests that *NR3C1* methylation may be related to anxiety. For *SLC6A4*, the effectiveness of selective serotonin reuptake inhibitors (SSRIs) in the treatment of anxiety disorders makes this gene a plausible candidate for the involvement in anxiety disorders.

NR3C1 methylation appeared to have a generic role in internalizing problems, as high methylation was associated with both more anxiety and depression problems. The role of *SLC6A4* methylation was more complex and somewhat less consistent across phenotypes; there was no significant main effect of high methylation and internalizing problems, but post hoc analyses indicated that there were significant associations with lifetime anxiety disorders, and concurrent and prospective depressive symptom scores. Before we can draw a firm conclusion from this study, replication is needed, and extension of the current findings to more specific phenotypic internalizing problems is warranted. Nonetheless, our findings may provide a first step towards understanding the physiological processes that underlie the different internalizing problems.

HPA-axis regulation

HPA-axis activity is regulated by glucocorticoids, cortisol in humans, through binding to glucocorticoid receptors, and mediating the negative feedback control of CRH and ACTH secretion. Studies on the relation between *NR3C1* methylation and HPA-axis responses to stress are scarce [1, 8, 25]. As high *NR3C1* methylation is considered to reduce *NR3C1* gene expression, the consequences of high methylation may specifically target the feedback inhibition or recovery of the HPA-axis, as HPA-axis activation is independent of glucocorticoid receptor availability. Therefore, I investigated the associations between *NR3C1* methylation and activation and recovery of the cortisol response following social stress.

High *NR3C1* methylation was associated with a delayed recovery following social stress. Cortisol response activation was not associated with *NR3C1* methylation. As DNA methylation generally reduces gene expression, a delayed recovery likely follows a reduced availability of glucocorticoid receptors and has no effect on HPA-axis activation. The positive association between *NR3C1* methylation and cortisol response recovery was only found in region 2 of the *NR3C1* CpG island, in which high methylation levels were previously associated with childhood maltreatment in suicide victims [6] and reduced *NR3C1* expression [26]. However, in our research on adverse life events and *NR3C1* methylation, and *NR3C1* methylation and internalizing problems, we found associations in region 1 of *NR3C1*. These findings suggest that regulation of the HPA-axis via methylation is independent of methylation associated with stress exposure or internalizing problems and may be related to regional differences in methylation. A first step towards expanding these findings would be to perform a replication study, to ensure that these results are not chance findings. In case of replication, it would be interesting to investigate whether these associations are indeed CpG-site specific, and whether regional differences in methylation have distinct effects on gene expression or gene splicing, which could lead to alternate biological pathways and health outcomes.

Obesity

The genes *NR3C1* and *SLC6A4* are not only involved in the development of mental health problems; they are also involved in the pathogenesis of obesity. Thus far, there has been only one report on the association between *SLC6A4* methylation and obesity measures (i.e. weight, BMI, WC, waist-hip ratio, and height), and research on *NR3C1* methylation and obesity is lacking. The study on *SLC6A4* methylation and obesity was limited to adult male veterans with a high prevalence of post-traumatic stress disorder and depression [27], which made generalization to the general population difficult. Therefore, I wanted to study whether these findings can be replicated in a population sample of adolescent boys, and extend the study by including adolescent girls, *NR3C1* methylation and additional measures of obesity, i.e., body fat percentage and skinfold thickness.

In line with the first publication on *SLC6A4* methylation and obesity measures by Zhao *et al* [27] , we also found associations between *SLC6A4* methylation and obesity measures. However, the type of obesity measures associated with high methylation differed somewhat between the first publication and our study. Zhao *et al* [27] reported associations with weight, BMI, and WC, whereas in our study we only found an association with WC and with body fat percentage, and the sum of skinfold thicknesses. No associations were found between weight or BMI and methylation, but weight, without factoring in height, is a poor indicator of overweight or obesity. Similarly, BMI does not differentiate between fat or muscle weight and a normal BMI may not always be indicative of good health. Some adolescents with a normal BMI have an elevated body fat percentage [28], and might actually be metabolically overweight or obese [29], which increases the risk of obesity-related diseases [30]. The prevalence of obese and overweight individuals in our sample based on BMI was relatively low (12.9% overweight; 2.6% obese), and the percentage of body fat and other estimates of body fat percentage, such as the skinfold measurements, may therefore be more accurately representations of overweight or obesity in adolescents and better predict health risks.

For *NR3C1*, high methylation levels were only associated with height in boys. Whereas glucocorticoids are known to be involved in linear growth, an excess of glucocorticoids is generally considered to be associated with growth impairment [30] and our finding thus warrants further study. The lack of associations with other obesity measures was unexpected. Although the relationship between glucocorticoids and obesity is complex, there are many reports on the existence of this association (e.g. [31, 32]). In our previous section we have shown that higher methylation in region 2 of *NR3C1* was associated with a delayed glucocorticoid response recovery following stress, but this effect on HPA-axis functioning may have been too weak to influence the likelihood of developing an overweight or obese phenotype, or may not have affected basal cortisol levels.

Another interesting finding was the lack of associations between *NR3C1* or *SLC6A4* methylation and any obesity measures in girls. This may be attributed to the sex hor-

mone estrogen, which is considered to be relatively protective against obesity [33]. Adjustment for oral contraceptives use, which prevents natural cyclic fluctuations in estrogen levels, did not change the outcomes; so perhaps higher basal level of estrogens affects the association between DNA methylation and obesity in girls.

Substance use

Besides psychiatric disorders and obesity, exposure to adverse life events has also been linked with substance use [34, 35]. Only a few studies have investigated the association between *COMT* gene methylation and substance use. While studies on genetic variation suggest COMT enzyme hyperactivity in substance users, these first epigenetic results indicate lower *COMT* gene activity in substance users. No studies have yet investigated the relationship between cannabis use and *COMT* gene methylation. Therefore, we investigated the association between the use of different substances (i.e., cigarettes, alcohol and cannabis) in adolescents and *COMT* gene methylation in the *MB-COMT* promoter and the *S-COMT* promoter. As the findings on *COMT* genotype and *COMT* gene methylation (increased activity vs lower expression of COMT in substance users) were contradictive, there might be interplay between genetic and epigenetic factors with indirect oppositional effects on dopamine levels. Therefore, we also explored whether the association between *COMT* gene methylation and substance use was dependent on the *COMT* Val$^{108/158}$Met polymorphism.

High levels of *MB-COMT* methylation were only associated with non-daily smoking. This finding was unexpected, but might suggest some regulatory mechanism linking *MB-COMT* methylation to a more controlled form of smoking, or, a perhaps more likely option, this could be a chance finding. In a previous study by Xu *et al* [36], no association was found between daily smoking and mean *MB-COMT* methylation, but associations were found in individual CpG sites of this promoter, with higher methylation levels in daily smokers compared to non-smokers at two CpG sites. These CpG sites were also investigated in our study, and we only found a higher methylation in one of these two CpG sites, but specifically for non-daily smoking. These inconsistencies between studies could be due to differences in age or smoking habits or a combination of both (i.e. long-term heavy smoking). For alcohol use and cannabis use, we did not find a relationship with *MB-COMT* mean or unit-specific methylation. For *S-COMT*, no associations were found between methylation and substance use. But methylation levels in this promoter were very high, which could indicate that this gene is silenced in DNA from blood, and therefore unresponsive to external environmental factors.

In our study, adolescents with the Val/Val genotype were less likely to be cannabis users than non-users, compared to Met-allele carriers. Thus far, there is no consensus on which risk allele is associated with substance use, as mixed findings have been reported, but this relationship might also be dependent on other environmental factors.

We therefore investigated the interaction between the *COMT* Val$^{108/158}$Met genotype and *MB-COMT* methylation, and found that adolescents with the Met/Met genotype and high methylation levels had low odds of high-frequent cannabis use. This combination of the low enzyme activity (Met/Met genotype) and reduced expression of the enzyme (higher methylation rates) might result in higher dopamine levels through diminished dopamine degradation, which may be preventative for substance use. Genetically, the Met allele was considered a risk allele for higher odds of cannabis use in our study, yet in combination with methylation, these odds of cannabis use were reduced. This may again be an indication that epigenetic regulation can compensate for changes in gene expression caused by genetic variation.

SYNTHESIS

In the introduction of this thesis I mentioned that I wanted to investigate whether DNA methylation is a plausible mechanism that may explain why adverse life events predispose individuals to adverse health outcomes. I introduced a mediation model, in which DNA methylation could act as mediator of the association between adversity and adverse health outcomes if we could find associations between adverse life events and DNA methylation, and between DNA methylation and adverse health outcomes. In **Chapters 2 and 3** we have seen that SLEs experienced between birth and adolescence were positively associated with both *NR3C1*_1 and *SLC6A4* methylation at age 16. In **Chapter 4** we found an association between *NR3C1*_1 methylation at age 16 years and internalizing problems at age 19 years and a tendency for such an association for *SLC6A4* methylation. As a next step, I will combine the variables used in **Chapters 2-4** to test the possibility of mediation by DNA methylation. Specifically for the synthesis of these results, I will test if the relationship between SLEs (0-15 years) and later internalizing symptom scores (19 years) is mediated by (mean-centered) methylation of *NR3C1*_1 (i.e. region 1 only, as the other two regions of *NR3C1* were not associated with either SLEs or internalizing symptom scores) and *SLC6A4*, using the PROCESS routine for SPSS (v21) developed by A.F. Hayes [37]. This proposed mediation model is presented in **Figure 1**.

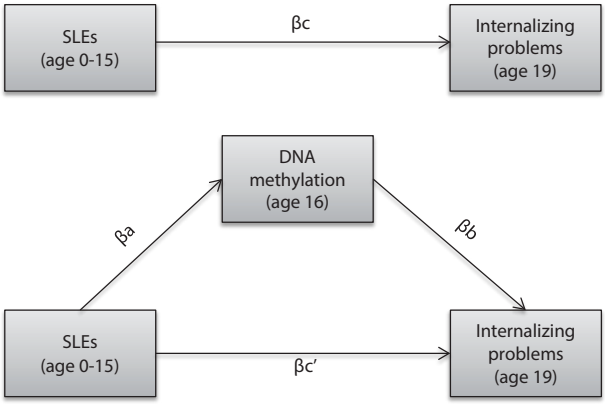


Figure 1. The proposed mediation model. βa: effect estimate for the association between SLEs and DNA methylation. βb: effect estimate for the association between DNA methylation and internalizing problems. βc: effect estimate for the association between SLEs and internalizing problems. βc': effect estimate for the association between SLEs + DNA methylation and internalizing problems.

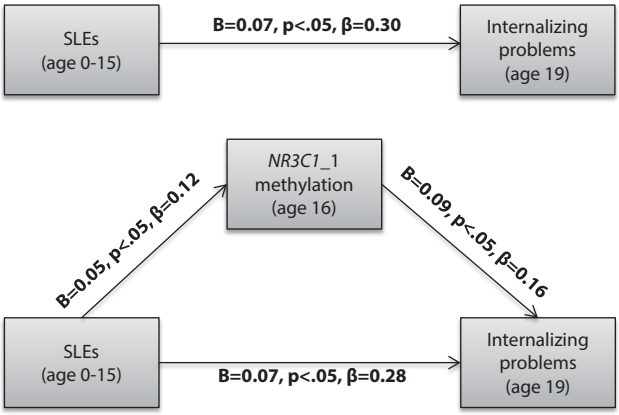


Figure 2. Results of testing the proposed mediation model for *NR3C1*_1. No covariates were added in this model.

The effect estimates of the mediation analyses for *NR3C1*_1 and *SLC6A4* are presented in and **Figures 2 and 3** respectively. For *NR3C1*_1 there was a mediation effect (B=0.005 (β=0.02), [95%CI: 0.001; 0.011], p<.05). This mediation effect persisted after adding the covariates sex and age (**βa**=0.12, p<.05**, βb**=0.16, p<.05**, βc**=0.29**,** p<.05, **βc'**=0.27, p<.05, **mediation effect:** β=0.02, p<.05).

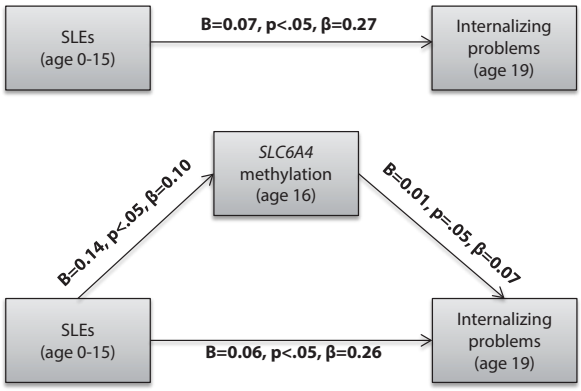


Figure 3. Results of testing the proposed mediation model for *SLC6A4*. No covariates were added in this model.

For *SLC6A4*, there also was a mediation effect (B=0.002 (β=0.007), [95%CI: 0.0001; 0.0043], p<.05). However, after adding sex and age as covariates, this mediation effect was no longer significant (**βa**=0.08, p<.05, **βb**=0.02, p=.55, **βc**=0.26, p<.05, **βc'**=0.26, p<.05, no mediation effect: β=0.002, p>.05). To find out whether the loss of the mediation effect was due to age or sex, these covariates were added to the model separately. From these analyses we can conclude that only sex affected the results.

These results confirm the proposed mediating role, albeit partial, of DNA methylation in the association between SLEs and internalizing problems. One thing to note about these mediation analyses is that the effect sizes are very small and the mediation effect is not complete, but partial. This is, however, not entirely unexpected as it is very likely that epigenetic modifications in other genes also link SLEs to later internalizing problems.

STRENGTHS AND LIMITATIONS

The use of the TRAILS database allowed for a large sample size, detailed accounts of SLEs between birth and adolescence, various outcome variables measured within TRAILS, and also genetic variation (e.g. Val$^{108/158}$Met and *5HTTLPR* genotypes). But some limitations also have to be acknowledged. As I have listed specific limitations for each study in their respective chapter, I will focus solely on the shared limitations in the next section.

Blood for the isolation of DNA and methylation analysis was only collected at the third assessment wave. As a consequence changes in methylation could not be analyzed, and statements about the causality of the associations could not be made. This limitation is

often encountered in epigenetic studies, possibly because many ongoing population studies were not originally designed for epigenetic research [38]. Another important limitation concerns the use of peripheral tissue (blood) as a proxy of the target tissue (the brain), which is often inaccessible in living humans. However, correlations between methylation levels in CpG islands in the brain and blood have been found to be high [39]. Also, associations between childhood adversity and *NR3C1* and *SLC6A4* methylation have repeatedly been replicated in studies using peripheral samples, despite differences in adversity measures [40, 41]. Another aspect to consider when using peripheral tissue, such as blood, is the cellular heterogeneity. Since we were unable to account for the cellular heterogeneity of the blood cells, which could be influenced by stress exposure (either acute or chronic), we could not rule out that the associations may in part be a reflection of differences in cellular composition. However, for *NR3C1*, it is likely that DNA methylation in blood is not associated with cellular heterogeneity [42]. Although cellular heterogeneity does not affect methylation levels in all genes, implementing cell count measures in whole blood (or other samples consisting of a mixture of cell types), or purification of cell populations is necessary to prevent confounding by cellular heterogeneity, particularly in studies with age related outcome measures (due to age-related changes in cell type proportions [43]) or immune related genes [44]. Also, we were unable to analyze gene expression levels of *NR3C1* and *SLC6A4*, although it should be noted that for both *NR3C1* and *SLC6A4*, higher methylation levels have been associated with lower expression levels before [6, 13, 21, 45]. Another limitation is the lack of a replication sample, but obtaining a suitable replication sample of adolescents with comparable measures of adverse life events and population characteristics is very challenging.

METHODOLOGICAL CONSIDERATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

In the last few decades the field of epigenetics has greatly expanded and new discoveries have been made, but our understanding of epigenetic processes in disease and causes of epigenetic change are still far from complete. Many questions still remain and technological advances have also introduced more questions over the last few years. The first studies on the impact of the social environment on DNA methylation have shown that environmental influences early in life may have long-term consequences on DNA methylation. This was later replicated in humans, most notably in a study reporting higher levels of methylation in victims of suicide who have experienced childhood abuse [6]. However, the studies performed in this thesis seem to contradict the importance of the early life environment, and point towards a more reactive form of DNA methylation with higher levels of methylation following more recent events, and thus

a more dynamic mechanism. Because earlier studies in humans have not incorporated repeated measures of methylation, it remains difficult to determine the stability of the stress-reactive epigenome. It is also unknown whether differences in methylation were already present at birth, or have been introduced later in life. Similarly, it is important to consider differences in environmental exposure during different developmental periods, such as childhood and adolescence. The transition from childhood to adolescence is accompanied with many differences in internal (e.g. hormonal) and external (e.g. transition from elementary school to high school) changes in environment. Therefore, adversities experienced in childhood and adolescence may differ, as was the case in two of the studies described in this thesis. These different SLEs were appropriate for each developmental phase, but may have accounted for the differences in methylation. However, this remains a difficult subject to investigate further, as humans are exposed to countless environmental exposures during their lifetime, and it is impossible to focus on specific events that may be related to DNA methylation. This also complicates reproduction of the results in a replication sample. A first step towards understanding the relation between SLEs and methylation could be to focus on a smaller time frame (e.g. 1 year), incorporate a subjective measure of experienced stress, and include any emotional support received after stress exposure (which might dampen the effect), combined with a repeated measure of methylation. Still, it may be an insurmountable feat to fully understand the effects of the social environment on methylation in humans. Another possible explanation for the discrepancy between our study and previous reports is that stress-induced epigenetic modifications may become gradually lost or recovered over time. The recent discovery of active demethylation mechanisms (e.g. by methyl-CpG-binding domain (MBD) protein 2b or ten-eleven translocation (TET) proteins) supports this theory of a more dynamic epigenome [5]. Indeed a recent study reported changes in individual CpG methylation following acute stress in adult rats [46]. Perhaps with a much longer lifespan in humans than rats, and thus a more variable environment, a more reactive and dynamic regulation in humans may be an evolutionary advantage.

Over the years, the techniques for analyzing DNA methylation and other epigenetic modifications have greatly improved and have become less expensive. The studies performed in this thesis focuses on specific candidate genes. The method that was used (mass spectrometry by Seqenom EpiTYPER) is roughly based on mass differences between methylated and non-methylated CpG units, i.e. fragments of nucleotides containing one or more CpG sites. This method allows for the analyses of relatively large fragments (200-600bp) that can cover larger regions of CpG islands in promoter regions than alternative approaches, such as pyrosequencing. In recent years, the popularity of the genome-wide DNA methylation has increased. Although this technique enables researchers to analyze a large amount of CpGs (450 000 sites with the Illumina 450K bead array), these CpGs are spread over thousands of genes and the coverage of CpG

sites per gene is very low. It does, however, give a more complete image of the epigenetic changes associated with disease. Genome-wide techniques would be preferable in order to identify the possible involvement of genes, but to gain more understanding of the contribution of individual genes to health outcomes, extensive methylation analyses in candidate gene studies (selected based on theory or previous mentioned genome-wide analyses) are necessary to discover which single CpG sites or small CpG units may play a role in the regulation of gene expression. As technology advances and becomes cheaper, new possibilities of whole-genome bisulfite sequencing are a relatively new option that combines the advantages of genome-wide and candidate gene studies. With this technique, it is possible to profile DNA methylation across the whole genome at a single nucleotide resolution. This allows for a more complete understanding of CpG methylation, also in regions other than CpG islands within gene promoters, such as gene bodies, where high levels of methylation have been associated with increased gene expression [47, 48], and may be preferable over the earlier mentioned techniques.

Once DNA methylation levels have been determined, there are different statistical approaches for DNA methylation analyses. Thus far, there is no consensus on the contribution or biological relevance of methylation in single CpG sites, multiple CpGs within a promoter region, or even on the promoter wide level. Because of this, there is no fixed method of using DNA methylation in statistical analyses. In our candidate gene studies, we have mostly studied mean (centered) methylation over the individual regions within each gene, and sometimes explored methylation in individual CpG units. The risk of analyzing CpG methylation over larger regions is that the contribution of a single CpG may become diluted and effects might be overlooked. However, exploring associations with multiple individual CpG sites may increase the risk of type I errors (chance findings). When methylation between CpGs is correlated, the mean methylation level may give a better overall picture and reduces the number of tests performed. Detailed investigation of methylation on the level of individual CpGs can then be done in a subsequent step. For example, in this thesis analyses with mean methylation indicated that one particular region in *NR3C1* (*NR3C1*_1) may be especially reactive to stress and health outcomes as this region was associated with adversity and adverse health outcomes. These results on a more regional level may be an indication that there are functionally relevant sites in this region and further investigation for transcriptionally active binding sites is necessary. Small effect sizes are not uncommon in methylation studies, and often raise questions on the functional relevance of these small methylation changes in specific regions of the genome, but they may also be an indication that that changes in methylation are only present in a selection of the sample.

Another new development in DNA methylation research is the discovery of 5-hydroxymethylcytosine (5hmC), also termed the sixth DNA base of the genome. Whereas 5hmC was initially seen as an intermediate process of demethylation, this sixth base may actu-

ally have an important function in regulating gene expression and cellular function. TET proteins catalyze the conversion of 5-methylcytosine to 5hmC followed by conversion to 5-formylcytosine and 5-carboxylcytosine, which can be converted back to cytosine [49]. Of these intermediate forms of cytosine, 5hmC is most common and may have an independent association with adverse health outcomes [50]. However, most methods used for DNA methylation analyses (including the Seqenom EpiTYPER technique used in this thesis) rely on bisulfite treatment to convert non-methylated cytosine bases into the base uracil (U), which does not distinguish between the different forms of cytosine methylation. Making this distinction is necessary, because the presence of intermediate forms of cytosine methylation may be an indication of removal or recovery of methylation marks. Several new techniques now exist that distinguish between 5mC and 5hmC in order to further understand the dynamics of demethylation, but further development is necessary. Perhaps, if this distinction could have been made in the studies presented in this thesis, we could have investigated whether the presence of more intermediate forms of cytosine methylation was related to a decrease in internalizing symptoms over time. Repeated measures of methylation could also provide new insights into this phenomenon of active demethylation.

Although the focus in this thesis is on DNA methylation, it is important to remember that other epigenetic modifications may co-occur. DNA methylation is the easiest epigenetic modification to study, and is therefore most often used in research. Other epigenetic modifications include various histone modifications (e.g. acetylation, methylation, phosphorylation, ubiquitination), non-coding RNA, and micro RNA. Some epigenetic modifications are connected, such as histone modifications and DNA methylation. As mentioned in the introduction of this thesis, gene silencing by DNA methylation occurs through direct prevention of transcription factor binding, or by recruiting methyl-CpG-binding domain proteins (MBDs). These proteins recruit other proteins that modify histones (histone deacetylases), which results in a structural change that makes the DNA inaccessible for other transcriptional proteins [51]. On the other hand, histone modifications may also be a prerequisite for DNA methylation [52]. Although the focus on DNA methylation only may be highly informative, effects may be strengthened by incorporating other epigenetic mechanisms, which might also help with understanding the small but significant effect sizes seen in methylation studies.

The field of epigenetics keeps developing rapidly, but we are still a long way removed from fully understanding its implications in clinical practice. However, some discoveries may help with the development of strategies for diagnosis, treatment or intervention. Correlations between blood and brain methylation that have been reported may open a window for the use of blood as a biomarker for disorders. Pharmacological manipulation of DNA methylation has also been investigated. For several psychotropic drugs there have been reports of epigenetic changes in either DNA methylation or histone modi-

fications, but the full extent of the changes induced by medication is still unknown [53]. Thus far the association between adversity and methylation seems quite robust, but it is not yet known whether positive experiences may also affect the epigenome or possibly undo the effects of a negative environment in humans. In mice, there have been reports of a reduction of brain 5hmC when exposed to an enriched environment, and improvements in memory and learning behaviors [54]. Although some of these prospects seem promising, we first need a better understanding of epigenetics in adverse health outcomes before we can think of implementations. The research described in this thesis provides valuable new insights into the epigenetics of stress and adverse health outcomes. However, incorporating repeated measures of methylation (on single-nucleotide level) and gene expression data is necessary in subsequent research to provide a better understanding on causality and the pathways leading to disease.

We must also keep in mind that there are two sides of the epigenome; on one hand the vulnerability for adverse environments, the ability of negative experiences to silence proper biological functioning, but on the other hand it provides the ability to adapt to changing environments despite the rigidity of the genome and to improve one's endowment. Thus, a responsive epigenome might be either a blessing or a curse, but understanding the dynamics of epigenetic modifications might one day lead towards intervention or possibly treatment strategies to rectify the negative effects instilled by an adverse environment. Although epigenetic research still faces many challenges, with improving techniques, statistical analyses, repeated measures of methylation and large samples we may be able to clarify the role of epigenetics in health and disease.

REFERENCES

1. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (*NR3C1*) and infant cortisol stress responses. *Epigenetics* 2008; **3**(2)**:** 97-106.
2. Mulligan CJ, D'Errico NC, Stees J, Hughes DA. Methylation changes at *NR3C1* in newborns associate with maternal prenatal stress exposure and newborn birth weight. *Epigenetics* 2012; **7**(8)**:** 853-857.
3. Devlin AM, Brain U, Austin J, Oberlander TF. Prenatal exposure to maternal depressed mood and the MTHFR C677T variant affect *SLC6A4* methylation in infants at birth. *PLoS One* 2010; **5**(8)**:** e12201.
4. Branco MR, Ficz G, Reik W. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. *Nat Rev Genet* 2012; **13**(1)**:** 7-13.
5. Bhutani N, Burns DM, Blau HM. DNA demethylation dynamics. *Cell* 2011; **146**(6)**:** 866-872.
6. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M *et al.* Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* 2009; **12**(3)**:** 342-348.
7. Perroud N, Paoloni-Giacobino A, Prada P, Olie E, Salzmann A, Nicastro R *et al.* Increased methylation of glucocorticoid receptor gene (*NR3C1*) in adults with a history of childhood maltreatment: a link with the severity and type of trauma. *Transl Psychiatry* 2011; **1:** e59.
8. Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. *PLoS One* 2012; **7**(1)**:** e30148.
9. Beach SR, Brody GH, Todorov AA, Gunter TD, Philibert RA. Methylation at *SLC6A4* is linked to family history of child abuse: an examination of the Iowa Adoptee sample. *Am J Med Genet B Neuropsychiatr Genet* 2010; **153B**(2)**:** 710-713.
10. Beach SR, Brody GH, Todorov AA, Gunter TD, Philibert RA. Methylation at 5HTT mediates the impact of child sex abuse on women's antisocial behavior: an examination of the Iowa adoptee sample. *Psychosom Med* 2011; **73**(1)**:** 83-87.
11. Kang HJ, Kim JM, Stewart R, Kim SY, Bae KY, Kim SW *et al.* Association of *SLC6A4* methylation with early adversity, characteristics and outcomes in depression. *Prog Neuropsychopharmacol Bol Psychiatry* 2013; **44:** 23-28.
12. Button KS, Ioannidis JP, Mokrysz C, Nosek BA, Flint J, Robinson ES *et al.* Power failure: why small sample size undermines the reliability of neuroscience. *Nat Rev Neurosci* 2013; **14**(5)**:** 365-376.
13. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR *et al.* Epigenetic programming by maternal behavior. *Nat Neurosci* 2004; **7**(8)**:** 847-854.
14. Turner JD, Pelascini LP, Macedo JA, Muller CP. Highly individual methylation patterns of alternative glucocorticoid receptor promoters suggest individualized epigenetic regulatory mechanisms. *Nucleic Acids Res* 2008; **36**(22)**:** 7207-7218.
15. Labonte B, Yerko V, Gross J, Mechawar N, Meaney MJ, Szyf M *et al.* Differential glucocorticoid receptor exon 1(B), 1(C), and 1(H) expression and methylation in suicide completers with a history of childhood abuse. *Biol Psychiatry* 2012; **72**(1)**:** 41-48.
16. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 2003; **301**(5631)**:** 386-389.
17. Belsky J, Pluess M. Beyond diathesis stress: differential susceptibility to environmental influences. *Psychol Bull* 2009; **135**(6)**:** 885-908.
18. Karg K, Burmeister M, Shedden K, Sen S. The serotonin transporter promoter variant (5-HTTLPR), stress, and depression meta-analysis revisited: evidence of genetic moderation. *Arch Gen Psychiatry* 2011; **68**(5)**:** 444-454.

19. Risch N, Herrell R, Lehner T, Liang KY, Eaves L, Hoh J *et al.* Interaction Between the Serotonin Transporter Gene (5-HTTLPR), Stressful Life Events, and Risk of Depression A Meta-analysis. *Jama-Journal of the American Medical Association* 2009; **301**(23)**:** 2462-2471.
20. van IJzendoorn MH, Caspers K, Bakermans-Kranenburg MJ, Beach SR, Philibert R. Methylation matters: interaction between methylation density and serotonin transporter genotype predicts unresolved loss or trauma. *Biol Psychiatry* 2010; **68**(5)**:** 405-407.
21. Philibert R, Madan A, Andersen A, Cadoret R, Packer H, Sandhu H. Serotonin transporter mRNA levels are associated with the methylation of an upstream CpG island. *Am J Med Genet B Neuropsychiatr Genet* 2007; **144B**(1)**:** 101-105.
22. Beesdo-Baum K, Knappe S. Developmental epidemiology of anxiety disorders. *Child Adolesc Psychiatr Clin N Am* 2012; **21**(3)**:** 457-478.
23. Broeren S, Newall C, Dodd HF, Locker R, Hudson JL. Longitudinal investigation of the role of temperament and stressful life events in childhood anxiety. *Dev Psychopathol* 2014; **26**(2)**:** 437-449.
24. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response. *Annu Rev Physiol* 2005; **67:** 259-284.
25. Edelman S, Shalev I, Uzefovsky F, Israel S, Knafo A, Kremer I *et al.* Epigenetic and genetic factors predict women's salivary cortisol following a threat to the social self. *PLoS One* 2012; **7**(11)**:** e48597.
26. Weaver IC, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S *et al.* The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. *J Neurosci* 2007; **27**(7)**:** 1756-1768.
27. Zhao J, Goldberg J, Vaccarino V. Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *Int J Obes (Lond)* 2013; **37**(1)**:** 140-145.
28. Nooyens AC, Koppes LL, Visscher TL, Twisk JW, Kemper HC, Schuit AJ *et al.* Adolescent skinfold thickness is a better predictor of high body fatness in adults than is body mass index: the Amsterdam Growth and Health Longitudinal Study. *Am J Clin Nutr* 2007; **85**(6)**:** 1533-1539.
29. Ruderman N, Chisholm D, Pi-Sunyer X, Schneider S. The metabolically obese, normal-weight individual revisited. *Diabetes* 1998; **47**(5)**:** 699-713.
30. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G *et al.* Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology* 1998; **139**(7)**:** 3296-3305.
31. Spencer SJ, Tilbrook A. The glucocorticoid contribution to obesity. *Stress* 2011; **14**(3)**:** 233-246.
32. Lee MJ, Pramyothin P, Karastergiou K, Fried SK. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. *Biochim Biophys Acta* 2014; **1842**(3)**:** 473-481.
33. Palmer BF, Clegg DJ. The sexual dimorphism of obesity. *Mol Cell Endocrinol* 2015; **402:** 113-119.
34. Anda RF, Croft JB, Felitti VJ, Nordenberg D, Giles WH, Williamson DF *et al.* Adverse childhood experiences and smoking during adolescence and adulthood. *JAMA* 1999; **282**(17)**:** 1652-1658.
35. Dube SR, Anda RF, Felitti VJ, Edwards VJ, Croft JB. Adverse childhood experiences and personal alcohol abuse as an adult. *Addict Behav* 2002; **27**(5)**:** 713-725.
36. Xu Q, Ma JZ, Payne TJ, Li MD. Determination of Methylated CpG Sites in the Promoter Region of Catechol-O-Methyltransferase (*COMT*) and their Involvement in the Etiology of Tobacco Smoking. *Front Psychiatry* 2010; **1:** 16.
37. Hayes AF. *Introduction to Mediation, Moderation, and Conditional Process Analysis*. Guilford press: New York, New York, 2013.

38. Mill J, Heijmans BT. From promises to practical strategies in epigenetic epidemiology. *Nat Rev Genet* 2013; **14**(8)**:** 585-594.
39. Tylee DS, Kawaguchi DM, Glatt SJ. On the outside, looking in: A review and evaluation of the comparability of blood and brain "-omes". *Am J Med Genet B Neuropsychiatr Genet* 2013; **162**(7)**:** 595-603.
40. Daskalakis NP, Yehuda R. Site-specific methylation changes in the glucocorticoid receptor exon 1F promoter in relation to life adversity: systematic review of contributing factors. *Front Neurosci* 2014; **8:** 369.
41. Turecki G, Meaney MJ. Effects of the Social Environment and Stress on Glucocorticoid Receptor Gene Methylation: A Systematic Review. *Biol Psychiatry* 2014.
42. Talens RP, Boomsma DI, Tobi EW, Kremer D, Jukema JW, Willemsen G *et al.* Variation, patterns, and temporal stability of DNA methylation: considerations for epigenetic epidemiology. *FASEB J* 2010; **24**(9)**:** 3135-3144.
43. Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol* 2014; **15**(2).
44. Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlen SE, Greco D *et al.* Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. *PLoS One* 2012; **7**(7)**:** e41361.
45. Philibert RA, Sandhu H, Hollenbeck N, Gunter T, Adams W, Madan A. The relationship of 5HTT (*SLC6A4*) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B**(5)**:** 543-549.
46. Witzmann SR, Turner JD, Meriaux SB, Meijer OC, Muller CP. Epigenetic regulation of the glucocorticoid receptor promoter 1(7) in adult rats. *Epigenetics* 2012; **7**(11)**:** 1290-1301.
47. Hellman A, Chess A. Gene body-specific methylation on the active X chromosome. *Science* 2007; **315**(5815)**:** 1141-1143.
48. Wagner JR, Busche S, Ge B, Kwan T, Pastinen T, Blanchette M. The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts. *Genome Biol* 2014; **15**(2)**:** R37.
49. Maiti A, Drohat AC. Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: potential implications for active demethylation of CpG sites. *J Biol Chem* 2011; **286**(41)**:** 35334-35338.
50. Wang J, Tang J, Lai M, Zhang H. 5-Hydroxymethylcytosine and disease. *Mutat Res Rev Mutat Res* 2014; **762:** 167-175.
51. Urnov FD, Wolffe AP. Chromatin remodeling and transcriptional activation: the cast (in order of appearance). *Oncogene* 2001; **20**(24)**:** 2991-3006.
52. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C *et al.* The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006; **439**(7078)**:** 871-874.
53. Boks MPM. Epigenetic Effects of Currently Used Psychotropic Drugs. In: Peedicayil J, Grayson DR, Avramopoulos D (eds). *Epigenetics in Psychiatry*. Elsevier: Amsterdam, 2014, p 664.
54. Irier H, Street RC, Dave R, Lin L, Cai C, Davis TH *et al.* Environmental enrichment modulates 5-hydroxymethylcytosine dynamics in hippocampus. *Genomics* 2014; **104**(5)**:** 376-382.

Chapter 9

Summary/Samenvatting
TRAILS dissertations

SUMMARY

Adverse life events are strong risk factors for adverse health outcomes, such as psychiatric problems, obesity and related cardiovascular disease and diabetes. The pathways through which stressful events can promote the development of such divergent disorders in humans were largely unknown, but in recent years, a promising explanation has risen from the field of epigenetics (derived from the Greek "epi", which means "on top of"). Epigenetics refers to modifications of DNA or associated histone proteins, which alter gene expression and may result in adverse health outcomes. Epigenetic modifications can be actively remodeled by environmental signals, and may therefore be considered as a candidate mechanism for the environmental 'programming' of gene expression. In this thesis the epigenetic modification DNA methylation is studied, which involves the addition of a methyl group (CH_3) to a cytosine base to form 5-methylcytosine in a cytosine-phosphate-guanine (CpG) dinucleotide combination. Higher levels of methylation in CpG sites is generally associated with gene silencing or reduced gene expression.

In this thesis I have investigated whether DNA methylation may serve as an underlying biological mechanism that predisposes individuals with a history of adversity to adverse health outcomes. DNA methylation could be an eligible mediator of the association between adversity and adverse health outcomes when there is an association between adverse life events and DNA methylation, and between DNA methylation and adverse health outcomes. I therefore studied the associations of DNA methylation (in adolescents) with both possible predictors and possible health outcomes.

In **Chapter 2 and 3** I investigated whether stressful life events (SLEs), during childhood and adolescence, are associated with higher CpG methylation in the genes encoding the glucocorticoid receptor (*NR3C1*) and the serotonin transporter (*SLC6A4*). Firstly, the effect of type of adversity on methylation was studied, and a distinction was made between perinatal stress, SLEs, and traumatic youth experiences (TYEs). Secondly, the effect of timing of SLEs on methylation was studied by analyzing the independent associations between SLEs at different developmental periods (i.e., childhood [0-11 years] and adolescence [12-15 years]) and *NR3C1* and *SLC6A4* methylation. For *NR3C1*, high levels of methylation were associated with more SLEs, particularly when experienced in adolescence, and exposure to TYEs. The results on TYEs are consistent with prior studies, but we did not find support for the notion of a sensitive period (perinatal and childhood period) for *NR3C1* methylation in adolescents. The results of these studies seem to contradict the importance of the early life environment, and point towards a more reactive form of DNA methylation with higher levels of methylation following more recent events, and thus a more dynamic mechanism. For *NR3C1*, most associations between methylation and adverse life events were found in a region that had not been studied before. It would be meaningful to further investigate how methylation in this

region affects *NR3C1* expression, and investigate biological pathways associated with changes in methylation. For *SLC6A4*, high levels of methylation were only associated with more SLEs, again with a more pronounced association for SLEs in adolescence than in childhood. The lack of association between TYEs and *SLC6A4* methylation, seemed to contradict earlier findings. This contradiction may be due to differences in sample characteristics (i.e., size or the use of a clinical sample). For *SLC6A4*, we have seen that the *5HTTLPR* genotype influenced the relationship between SLEs and *SLC6A4* methylation; only in carriers of two l-alleles methylation levels were higher when more SLEs were experienced. This suggests that epigenetic regulation may serve as an adaptive mechanism to adjust gene expression and compensate for changes in gene expression caused by genetic variation

In **Chapter 4** I investigated whether higher methylation levels of *NR3C1* and *SLC6A4* were associated with concurrent and future (3 years later) internalizing problems (anxiety and depression), operationalized as clinical diagnoses of internalizing disorders and internalizing symptom scores. Higher *NR3C1* methylation was associated with higher concurrent and future internalizing symptom scores, and higher odds of lifetime internalizing disorders. *NR3C1* methylation also appeared to have a more generic role in internalizing problems, as high methylation was associated with both more anxiety and depression problems. High *SLC6A4* methylation only showed a tendency for association with internalizing symptom scores, and the role of *SLC6A4* methylation across phenotypes was more complex and somewhat less consistent; post hoc analyses indicated that there were significant positive associations with lifetime anxiety disorders, and concurrent and future depressive symptom scores. Replication and extension of the current findings to more specific phenotypic internalizing problems is needed, but our findings may provide a first step towards understanding the physiological processes that underlie the different internalizing problems.

In **Chapter 5** I investigated whether *NR3C1* methylation was associated with HPA-axis regulation, more specifically; activation and recovery of the cortisol response following social stress. High *NR3C1* methylation was associated with a delayed recovery following social stress, but not with cortisol response activation. As DNA methylation generally reduces gene expression, a delayed recovery likely follows a reduced availability of glucocorticoid receptors and has no effect on HPA-axis activation. The association between *NR3C1* methylation and cortisol response recovery was only found in region 2 of the *NR3C1* CpG island, in which high methylation levels were previously associated with childhood maltreatment in suicide victims and reduced *NR3C1* expression.

In **Chapter 6**, I investigated whether methylation of *SLC6A4* and *NR3C1* was associated with measures of obesity. This study was intended as a replication study on the association between *SLC6A4* methylation and obesity measures (i.e. weight, body mass index, waist circumference, waist-hip ratio, and height), which had only been investigated

in adult males veterans. Also, research on *NR3C1* methylation and obesity is lacking. Therefore, I wanted to replicate the findings of the previously mentioned *SLC6A4* study in adolescent boys and extend the study by including adolescent girls, methylation of *NR3C1*, and additional measures of obesity, i.e., body fat percentage and skinfold thickness. *SLC6A4* promoter methylation was significantly associated with obesity measures in adolescent boys, in line with the results of the original study, but the associations were not found in adolescent girls. For *NR3C1*, methylation levels in the promoter region were only associated with height in adolescent boys. Glucocorticoids are known to be involved in linear growth, but an excess of glucocorticoids is generally considered to be associated with growth impairment, thus replication of our findings is necessary. Possibly, the lack of associations in girls can be attributed to sex hormones, in particular estrogens. Estrogens are known to be involved in appetite regulation and energy expenditure and considered protective against obesity.

In **Chapter 7**, I investigated whether *COMT* methylation (in the *MB-COMT* and *S-COMT* promoter) was associated with substance use (i.e., cigarettes, alcohol and cannabis) in adolescents, and explored whether the association between *COMT* methylation and substance use was dependent on the *COMT* Val$^{108/158}$Met polymorphism. High levels of *MB-COMT* methylation were only associated with non-daily smoking. Perhaps this is an indication of a regulatory mechanism that links *MB-COMT* methylation to a more controlled form of smoking, or, perhaps more likely, this could be a chance finding. There was no relationship between mean *MB-COMT* or *S-COMT* promoter methylation and alcohol or cannabis use in adolescents. Adolescents with the Met/Met genotype and high methylation levels were at reduced risk of cannabis use, while genetically the Met allele was considered a risk allele for higher odds of cannabis use in this study. This may again be an indication that epigenetic regulation can compensate for changes in gene expression caused by genetic variation.

In **Chapter 8**, I further discuss the main findings of the studies presented in this thesis, and the developments in epigenetic research. In addition, I combined the information from **Chapters 2-4**, to test whether DNA methylation acts as a mediator in the association between SLEs and internalizing symptom scores. These results of the mediation analyses indicate that there is indeed a partial mediation effect of DNA methylation in the association between SLEs and internalizing symptom scores. The research described in this thesis provides valuable new insights into the epigenetics of stress and adverse health outcomes, but incorporating repeated measures of methylation (on single-nucleotide level) and gene expression data is necessary in subsequent research to provide a better understanding on causality and the pathways leading to disease.

SAMENVATTING

Het meemaken van negatieve levenservaringen is een sterke risicofactor voor het ontwikkelen van nadelige gezondheidsuitkomsten, zoals psychische problemen, obesitas en gerelateerde cardiovasculaire aandoeningen en diabetes. Het achterliggende mechanisme waardoor stressvolle gebeurtenissen de ontwikkeling van deze gevarieerde gezondheidsuitkomsten kunnen bevorderen was lange tijd onbekend, maar in de afgelopen jaren is er een mogelijk veelbelovende verklaring ontstaan vanuit het onderzoeksveld van de epigenetica (afkomstig van het Griekse prefix 'epi', dat staat voor 'bij' of 'op'). De term epigenetica doelt op onderzoek naar modificaties van het DNA, of de geassocieerde histonen, die de gen expressie kunnen beïnvloeden en hierdoor mogelijk tot nadelige gezondheidsuitkomsten kunnen leiden. Deze epigenetische modificaties kunnen actief herstructureerd worden door signalen uit de omgeving, en worden daardoor gezien als kandidaat mechanisme dat zou kunnen verklaren hoe de omgeving de gen expressie programmeert. In dit proefschrift is onderzoek gedaan naar de epigenetische modificatie 'DNA methylering'. Bij DNA methylering wordt er een methyl groep (CH_3) aan een cytosine base van het DNA toegevoegd wanneer deze zich in een cytosine-fosfaat-guanine (CpG) combinatie bevind. Hierdoor veranderd cytosine in 5-methylcytosine. Een hogere mate van methylering in CpGs wordt over het algemeen geassocieerd met het uitschakelen van genen (ook wel *gene silencing* genoemd) of het verminderen van de gen expressie.

In dit proefschrift heb ik onderzocht of DNA methylering kan dienen als een onderliggend mechanisme dat de relatie tussen negatieve levenservaringen en latere nadelige gezondheidsuitkomsten kan verklaren. DNA methylering zou deze relatie kunnen verklaren wanneer er een associatie is tussen negatieve levenservaringen en DNA methylering, en wanneer er een associatie is tussen DNA methylering en nadelige gezondheidsuitkomsten. Daarom heb ik onderzoek gedaan naar mogelijke voorspellers van DNA methylering (in adolescenten) en naar mogelijke gezondheidsuitkomsten van DNA methylering.

In **Hoofdstuk 2 en 3** heb ik onderzocht of stressvolle levensgebeurtenissen (SLEs), meegemaakt tijdens de kindertijd en de adolescentie, geassocieerd zijn met hogere waarden van DNA methylering in de genen die coderen voor de glucocorticoïd receptor (*NR3C1*) en de serotonine transporter (*SLC6A4*). Als eerste is onderzoek gedaan naar het type stress en DNA methylering, waarbij onderscheid gemaakt werd tussen perinatale stress, SLEs en traumatische gebeurtenissen (TYEs). Ten tweede is onderzoek gedaan naar de blootstelling van SLEs gedurende verschillende periodes van de ontwikkeling, tijdens de kindertijd (0-11 jaar) en tijdens de adolescentie (12-15 jaar), en methylering in de genen *NR3C1* en *SLC6A4*. Voor *NR3C1* werd er gevonden dat het meemaken van meer SLEs geassocieerd was met hogere methylering waarden, voornamelijk wanneer

deze SLEs meegemaakt waren in de adolescentie. Ook was het meemaken van TYEs geassocieerd met hogere *NR3C1* methylering waarden. De bevindingen van de TYEs analyses komen overeen met eerdere studies, maar bieden geen steun aan de notie dat er een gevoelige periode is (in de perinatale periode en de kindertijd) voor methylering van het *NR3C1* gen in adolescenten. De resultaten van onze studie lijken het belang van de vroege blootstelling aan omgevingsfactoren tegen te spreken en lijken te wijzen op een meer reactieve vorm van DNA methylering. We vinden namelijk hogere waarden van methylering na recente gebeurtenissen, wat kan duiden op een meer dynamisch mechanisme. De meeste associaties tussen stressvolle gebeurtenissen en methylering zijn gevonden in een specifiek regio van *NR3C1* dat nog niet eerder onderzocht is. Het zou waardevol zijn om verder onderzoek te doen naar de effecten van methylering in dit gebied op de gen expressie en andere biologische processen. Hoge methylering waarden in het *SLC6A4* gen waren alleen geassocieerd met het meemaken van meer SLEs, voornamelijk tijdens de adolescentie. De afwezigheid van een associatie tussen TYEs en *SLC6A4* methylering lijkt tegenstrijdig te zijn aan eerdere bevindingen. Deze tegenstrijdigheid wordt mogelijk veroorzaakt door verschillen in eigenschappen van steekproef (bijvoorbeeld de grootte van de steekproef, of het gebruik van een klinische steekproef). Voor *SLC6A4* bleek ook dat het *5HTTLPR* genotype de relatie tussen SLEs en *SLC6A4* methylering beïnvloedde. Alleen in dragers van twee l-allelen waren methylering waarden hoger wanneer er meer SLEs meegemaakt zijn. Dit houdt mogelijk in dat epigenetische regulatie een adaptief mechanisme is dat veranderingen in gen expressie door genetische variatie kan compenseren.

In **Hoofdstuk 4** heb ik onderzocht of hogere methylering waarden van *NR3C1* en *SLC6A4* geassocieerd waren met huidige en toekomstige (3 jaar later) internaliserende problemen (angst en depressie), geoperationaliseerd als klinische diagnoses van internaliserende stoornissen en internaliserende symptoomscores. Hogere waarden van *NR3C1* methylering waren geassocieerd met hogere huidige en toekomstige internaliserende symptoomscores en een hogere kans verhouding op het ontstaan van een internaliserende stoornis. Methylering van *NR3C1* bleek tevens een meer algemene rol te spelen bij het ontstaan van internaliserende problemen, aangezien hogere methylering waardes geassocieerd waren met zowel angst- als depressieve problemen. Bij hogere methylering waarden in *SLC6A4* bleek er alleen een trend te zijn naar meer internaliserende symptoomscores. Ook was de rol van *SLC6A4* methylering bij angst en depressie wat minder duidelijk en inconsistent: uit *post hoc* analyses bleek dat er een significantie positieve associatie was tussen methylering in dit gen en het ontwikkelen van een angststoornis, en tussen methylering en huidige en toekomstige depressieve symptoomscores. Het is nodig om deze bevindingen te repliceren en uit te breiden naar meer specifieke fenotypes, maar desondanks leveren deze analyses een eerste bijdrage

om de processen te begrijpen die ten grondslag liggen aan het ontstaan van verschillende internaliserende problemen.

In **Hoofdstuk 5** heb ik onderzocht of *NR3C1* methylering geassocieerd is met HPA-as regulatie, dat wil zeggen, de activatie en het herstel van de cortisol response na sociale stress. Hogere waarden van *NR3C1* methylering waren geassocieerd met een vertraagd herstel van de cortisol respons na sociale stress, maar niet met de activatie van de cortisol response. Aangezien DNA methylering over het algemeen de gen expressie verminderd, volgt een vertraagd herstel waarschijnlijk uit een verminderde beschikbaarheid van glucocorticoïd receptoren. De associatie tussen *NR3C1* methylering en het herstel van de cortisol response is alleen gevonden in regio 2 van het *NR3C1* CpG eiland, waarin eerder hogere methylering waardes zijn gerelateerd aan kindermisbruik in slachtoffers van zelfdoding en aan verminderde *NR3C1* expressie.

In **Hoofdstuk 6** heb ik onderzocht of methylering in de genen *SLC6A4* en *NR3C1* geassocieerd is met maten van obesitas. Deze studie is bedoeld als replicatie studie naar de associatie tussen *SLC6A4* methylering en maten van obesitas (namelijk gewicht, body mass index [BMI], taille omtrek, taille-heup ratio, en lengte), dat alleen onderzocht was in volwassen oorlogsveteranen (enkel mannen). Er is nog niet eerder onderzoek gedaan naar de relatie tussen *NR3C1* methylering en obesitas. Om deze redenen wilde ik de eerder genoemde associatie tussen *SLC6A4* methylering en obesitas maten repliceren in adolescente jongens en de studie uitbreiden door adolescente meisjes te includeren, en zowel methylering van *NR3C1* als additionele obesitas maten (dat wil zeggen, vetpercentage en huidplooimetingen) aan de studie toe te voegen. *SLC6A4* methylering bleek significant gerelateerd te zijn aan maten van obesitas in jongens, wat overeenkomt met de resultaten van de originele studie. Deze associaties waren afwezig in meisjes. Voor het *NR3C1* gen waren hogere methylering waarden alleen gerelateerd aan lengte in jongens. Van glucocorticoïden is bekend dat ze betrokken zijn bij lineaire groei, maar een overdaad aan glucocorticoïden is over het algemeen geassocieerd met een verminderde groei en dus is het nodig deze bevindingen te repliceren. De afwezigheid van een associatie tussen methylering en obesitas maten in meisjes zou mogelijk verklaard kunnen worden door het hormoon oestrogeen. Oestrogenen zijn betrokken bij de regulatie van het hongergevoel en energieverbruik en worden over het algemeen beschouwd als beschermend tegen obesitas.

In **Hoofdstuk 7** heb ik onderzocht of methylering in het *COMT* gen (in zowel de *MB-COMT* als de *S-COMT* promotor) geassocieerd was met middelengebruik (hieronder vallen sigaretten, alcohol en cannabis) in adolescenten. Ook heb ik gekeken of de associatie tussen *COMT* methylering en middelengebruik afhankelijk was van het *COMT* $Val^{108/158}$Met polymorfisme. Hogere waarden van *MB-COMT* methylering waren alleen geassocieerd met niet-dagelijks roken. Dit zou mogelijk kunnen wijzen op een regulatie mechanisme dat *MB-COMT* methylering relateert aan een gecontroleerde vorm van ro-

ken, of, een logischere optie, er is hier sprake van een kansbevinding. Er was geen relatie tussen *MB-COMT* of *S-COMT* methylering en alcohol of cannabis gebruik. Adolescenten met het Met/Met genotype en hogere methylering waarden hadden een verminderd risico om cannabis te gebruiken, terwijl genetisch gezien het Met allel gerelateerd was aan een hoger risico op cannabis gebruik in deze studie. Dit zou mogelijk opnieuw een indicatie zijn dat epigenetische mechanisme kunnen compenseren voor veranderingen in gen expressie door generische variatie.

In **Hoofdstuk 8** beschrijf ik de belangrijkste bevindingen van de onderzoeken die in dit proefschrift gepresenteerd zijn en de nieuwe ontwikkelingen in het epigenetische onderzoek. Ook heb ik de informatie uit **Hoofstukken 2-4** gecombineerd om te testen of DNA methylering de associatie tussen SLEs en internaliserende symptoomscores medieert. Uit de resultaten van deze mediatie analyses blijkt dat er inderdaad een partieel mediatie effect is van DNA methylering in de associatie tussen SLEs en internaliserende symptoomscores. Het onderzoek dat beschreven is in dit proefschrift biedt waardevolle nieuwe inzichten in de epigenetica van stress en nadelige gezondheidsuitkomsten, maar voor vervolg onderzoek is het nodig om herhaalde metingen van methylering (op het niveau van individuele CpGs) toe te passen en onderzoek te doen naar genexpressie om een beter beeld te krijgen van de oorzaken en gevolgen van methylering en de biologische processen die kunnen leiden tot gezondheidsproblemen.

TRAILS DISSERTATIONS

Sondeijker, F.E.P.L. (2006) Neuroendocrine and autonomic risk factors for disruptive behaviors in adolescents. Promotores: Prof.dr. F.C. Verhulst, Prof.dr. J. Ormel. Copromotor: Dr. R.F. Ferdinand. Erasmus University Rotterdam.

Brunnekreef, J.A. (2007) Information processing and problem behavior in preadolescents. Promotores: Prof.dr. J. Ormel, Prof.dr. R.B. Minderaa. Copromotores: Dr. M. Althaus, Dr.ir. L.M.J. de Sonneville. University of Groningen.

Dietrich, A. (2007) Autonomic nervous system function and behavioral characteristics in (pre)adolescents from a general population cohort. Promotores: Prof.dr. J. Neeleman, Prof.dr. J. Ormel. Copromotor: Dr. J.G.M. Rosmalen. University of Groningen.

Greaves-Lord, K. (2007) Roots of Anxiety. The role of cardiovascular regulation and cortisol in the development of anxiety in early adolescence. Promotores: Prof.dr. F.C. Verhulst, Prof.dr. J. Ormel. Copromotor: Dr. A.C. Huizink. Erasmus University Rotterdam.

Dijkstra, J.K. (2007) Status and affection among (pre)adolescents and their relation with antisocial and prosocial behavior. Promotor: Prof.dr.S. Lindenberg. Copromotor: Dr. R. Veenstra. University of Groningen.

Amone, K.P. (2009) Examining the link between socio-economic position and mental health in early adolescents. Promotores: Prof.dr. J. Ormel, Prof.dr. A.J. Oldehinkel. Copromotor: Dr. H. Burger. University of Groningen.

Noordhof, A. (2010) In the absence of a gold standard. Promotores: Prof.dr. J. Ormel, Prof.dr. A.J. Oldehinkel. University of Groningen.

Sentse, M. (2010) Bridging contexts: the interplay between family, child, and peers in explaining problem behavior in early adolescence. Promotor: Prof.dr. S. Lindenberg. Copromotor: Dr. R. Veenstra. University of Groningen.

Creemers, H.E. (2010) High Times - The role of temperament and other risk factors in the onset and continuation of cannabis use during adolescence. Promotores: Prof. F.C. Verhulst, Prof. A.C. Huizink. Erasmus University Rotterdam.

Bouma, EMC (2010) The sensitive sex. Depressive symptoms in adolescence and the role of gender, genes and physiological stress responses. Promotores: Prof.dr. A.J. Oldehinkel, Prof.dr. J. Ormel. Co-promotor: Dr. H.Riese. University of Groningen.

Liem, ET (2010) Development of overweight in adolescence. Genes, growth & mood. Promotores: Prof. dr. R.P. Stolk, Prof. dr. P.J.J. Sauer. University of Groningen.

Bakker, MP (2010) Stressful life events and adolescents' mental health: the TRAILS study. Promotores: Prof. dr. A.J. Oldehinkel, Prof.dr. J. Ormel. University of Groningen.

Buschgens, C.J.M. (2010). It runs in the family – Early biological factors and family environment in children with ADHD symptoms. Promotores: Prof. dr. J.K. Buitelaar, Prof. dr. M.A.G. van Aken. The Radboud University Nijmegen Medical Centre.

Sijtsema, JJ (2010) Adolescent aggressive behavior - Status and stimulation goals in relation to the peer context. Promotor: Prof.dr. S. Lindenberg. Co-promotor: Dr. R. Veenstra. University of Groningen.

Bosch, NM (2011) Adolescents in stress: The ups and downs of the psychophysiological stress response. Promotores: Prof.dr. A.J. Oldehinkel, Prof.dr. J. Ormel. Co-promotor: Dr. H. Riese. University of Groningen.

Wigman, JTW (2011) Persistance of the extended psychosis phenotype in young people: Link between vulnerability and clinical need. Promotores: Prof.dr. W.A.M. Vollebergh, Prof.dr. J. van Os. University of Utrecht.

Janssens, KAM (2011) The etiology of functional somatic symptoms in adolescents. A new perspective on lumping and splitting. Promotores: Prof.dr. J.G.M. Rosmalen, Prof.dr. A.J. Oldehinkel. University of Groningen.

Ivanova, KO (2012) From parents to partners : the impact of family on romantic relationships in adolescence and emerging adulthood. Promotores: Prof.dr. M.C. Mills, Prof.dr. R. Veenstra. University of Groningen.

Jaspers, M (2012) Prediction of psychosocial problems in adolescents : do early childhood findings of the preventive child healthcare help? Promotores: Prof.dr. S.A. Reijneveld, Dr. A.F. de Winter. University of Groningen.

Verboom, CE (2012) Depression and role functioning. Their relation during adolescence and adulthood. Promotores: Prof. dr. J. Ormel, Prof. dr. W.A. Nolen, Prof. dr. B.W.J.H. Pennix. Copromotor: Dr. J.J. Sijtsema. University of Groningen.

Marsman, H (2013) HPA-axis, genes and environmental factors in relation to externalizing behaviors. Promotor: Prof. dr. J.K. Buitelaar. The Radboud University Nijmegen Medical Centre.

Griffith-Lendering, MFH (2013) Cannabis use, cognitive functioning and behaviour problems. Promotores: Prof. dr. H. Swaab. Copromotor: Dr. S.C.J. Huijbregts. University of Leiden.

Vink, NM (2013) The role of stress in the etiology of asthma. Promotores: Prof.dr. H.M. Boezen, Prof.dr. J.G.M. Rosmalen, Prof.dr. D.S. Postma. University of Groningen.

Laceulle, OM (2013) Programming effects of adversity on adolescent adaptive capacity. Promotores: Prof. dr. J. Ormel, Prof.dr. M.A.G. van Aken. Copromotor: Dr. E. Nederhof. University of Groningen.

Prince van Leeuwen, AL (2013) Blunt vulnerabilities. Identifying risks for initiation and continued use of cannabis in a Dutch adolescent population. Promotores: Prof.dr. A.C. Huizink, Prof.dr. F.C. Verhulst. Copromotor: Dr. H.E. Creemers. Erasmus University Rotterdam.

Mathyssek, CM (2014) The development of anxiety symptoms in adolescents. Promotor: Prof.dr. F.C. Verhulst. Copromotor: dr. F.V.A. van Oort. Erasmus University Rotterdam.

Visser, L (2014) Early detection and prevention of adolescent alcohol use. Parenting and psychosocial factors. Promotor: Prof.dr. S.A. Reijneveld. Copromotor: dr. A.F. de Winter. University of Groningen.

Boelema, SR (2014) Alcolhol use in adolescence. A longitudinal study of its effect on cognitive functioning. Promotor: Prof.dr. W.A.M. Vollebergh. Copromotores: dr. Z. Harakeh, dr. M.J.E. van Zandvoort. Utrecht University.

Langenhof, MR (2015) Living in a changing world. How early-life development influences animal and human ability to cope with change. Promotores: Prof.dr.ir. J. Komdeur, Prof.dr. A.J. Oldehinkel. University of Groningen.

Stavrakakis, N (2015) Physical activity and depressive symptoms. Is a healthy body necessary for a healthy mind? Promotores: Prof.dr. A.J. Oldehinkel, Prof.dr. P. de Jonge. Copromotor: dr. A.M. Roest. University of Groningen.

Jeronimus, BF (2015) Environmental influences on neurotisicm: a story about emotional (in)stability. Promotores: Prof.dr. J. Ormel, Prof.dr. A.J. Oldehinkel. Copromotor: dr. H. Riese. University of Groningen.

Rüschoff, B (2015) Peers in carreers: Peer relationships in the transition from school to work. Promotores: Prof.dr. R. Veenstra, Prof.dr. S.M. Lindenberg. Copromotor: dr. J.K. Dijkstra. University of Groningen.

Chapter 10

Curriculum Vitae
PhD portfolio
Dankwoord
Acknowledgements

CURRICULUM VITAE

Lisette Jacoline van der Knaap was born on October 18th, in 1986 in Hulst (the Netherlands). In 2004 she obtained her Atheneum degree at the Sint Laurenscollege in Rotterdam. In the same year she started the study Biology at Utrecht University. After obtaining her Bachelor degree in Biology in 2008, she started her Master's degree in Neuroscience and Cognition (2008-2010) at Utrecht University, where she followed the Behavioral Neuroscience track. Her broad interest in different biological subjects can be seen in the diversity of her research topics. For her major research internship she investigated the relation between trait anxiety and decision-making in female Wistar rats and adolescent girls at the Department of Animals in Science and Society of Utrecht University, and her minor research internship was performed at the Rotterdam Zoo (Blijdorp), where she studied the visual discrimination ability of blacknose sharks using food-reinforced instrumental conditioning. Her Master thesis '*How does the corpus callosum mediate interhemispheric transfer? A review*' was published in *Behavioral Brain Research*. From 2011 until 2015 she was employed as a PhD-student at the Erasmus MC – Sophia Children's Hospital, Department of Child and Adolescent Psychiatry/Psychology (Head of Department: Prof. Dr. F.C. Verhulst) in Rotterdam (the Netherlands), where she worked on her PhD thesis entitled "Epigenetics and adverse health outcomes, *silenced by the past?*". Her research was embedded within TRAILS (TRacking Adolescents' Individual Lives Survey).

PHD PORTFOLIO

Name of PhD student:	Lisette Jacoline van der Knaap
PhD period:	June 2011 – June 2015
Erasmus MC department:	Child and Adolescent Psychiatry/Psychology
Promotors:	Prof. dr. F.C. Verhulst
	Prof. dr. A.J. Oldehinkel
Copromotors:	Dr. F.V.A. van Oort
	Dr. H. Riese

Workshops	**Year**	**ECTS**
Biobase training course: Principles of NGS Data Analysis and Interpretation	2012	0.3
The New Statistics: Estimation and Meta-Analysis for Better Research, by Geoff Cumming	2012	0.3
Erasmus MC PhD days	2012-2014	0.9
Courses		
Biostatistical Methods I: Basic Principles (CC02)	2011	5.7
Erasmus Summer Programme:	2012	
Principles of Genetic Epidemiology (ESP43)		0.7
Genome Wide Association Analysis (ESP29)		1.4
Causal Inference (ESP48)		0.7
Genomics in Molecular Medicine (ESP57)		1.4
English Biomedical Writing and Communication	2013-2014	4
Schrijven voor een groter publiek/Writing for a wider public	2015	1.4
Conference presentations and posters		
International Expert Meeting *'Novel Directions in Research on Gene-Environment Interplay'*, Utrecht, the Netherlands, poster presentation	2012	1
XXth World Congress of Psychiatric Genetics (WCPG), Hamburg, Germany, poster presentation	2012	1.4
DOHaD 2012 Satellite Meeting: New Developments in Developmental Epidemiology, Rotterdam, the Netherlands, poster presentation	2012	1
Society for Research in Child Development (SRCD) Biennial Meeting, Seattle (WA), USA, oral presentation	2013	2
Sophia Research Days, Rotterdam, the Netherlands, oral presentation	2014	1.4
XXIIth World Congress of Psychiatric Genetics (WCPG), Copenhagen, Denmark, poster presentation	2014	1.4

Conference attendance

ASEBA symposium: Standardized Assessment of Child Psychopathology: New Developments, Rotterdam, the Netherlands	2011	0.3
International Society of Psychoneuroendocrinology (ISPNE), Leiden, the Netherlands	2013	1
Symposium 'Hans Ormel: A life in Epidemiology and Beyond', Groningen (UMCG), the Netherlands	2014	0.3

Teaching

Vaardigheidsonderwijs 'VO.3: Ontwikkeling van 0-18 jaar' medical students, Erasmus MC, Rotterdam	2013	1

Other

(Bi)weekly Research Work Meetings	2011-2015	1
Monthly TRAILS Scientific Meetings	2011-2015	1

DANKWOORD

Dit proefschrift had niet tot stand kunnen komen zonder de betrokkenheid en hulp van de mensen om mij heen, waar ik dan ook graag mijn dank voor uit wil brengen in onderstaand gedeelte.

Allereerst wil ik graag de TRAILS jongeren en ouders bedanken voor hun deelname en inzet. Over de afgelopen jaren hebben zij met regelmaat vragenlijsten ingevuld, deelgenomen aan experimenten en interviews. Zonder de toewijding van de deelnemers had dit onderzoek niet mogelijk geweest.

Graag wil ik mijn promotoren, prof. dr. Frank Verhulst en prof. dr. Tineke Oldehinkel bedanken voor hun inzet en begeleiding de afgelopen jaren. Beste Frank, bedankt voor je ondersteuning, je vlotte reacties op vragen en papers, en de mogelijkheden die je mij geboden hebt, zoals de presentatie bij het SRCD in Seattle met de groep van prof. dr. Megan Gunnar. Beste Tineke, bedankt voor het delen van je expertise, je snelle feedback en kritische blik. Ook de meest lastige vragen van reviewers waar ik mee worstelde wist jij tot een goed eind te brengen.

Mijn copromotoren ben ik zeer dankbaar voor hun steun en begeleiding tijdens mijn promotietraject. Beste Floor, bedankt voor je altijd snelle en duidelijke feedback, statistische hulp en je toegankelijkheid; ik kon altijd binnen lopen met vragen. De afgelopen 2 jaar heb je zelfs vanaf een andere werkplek en stad altijd klaargestaan voor vragen en mij ook tijdens de laatste loodjes gesteund. Beste Harriette, bedankt voor je enorme enthousiasme, je inzet en behulpzaamheid. Je maakte de Groningen meet-ups altijd erg gezellig en het was enorm prettig om met jou samen te werken!

Graag wil ik de leden van de kleine commissie, prof. dr. Régine Steegers-Theunissen, dr. Bas Heijmans, hartelijk danken voor het lezen en beoordelen van het manuscript en voor jullie aanwezigheid tijdens mijn promotie. Prof. dr. Steven Kushner, thank you kindly for reading and evaluating the manuscript and for being present at my thesis defense. Ook de leden van de grote commissie, prof. dr. Anja Huizink, prof. dr. Jan Buitelaar, wil ik hartelijk bedanken voor het plaatsnemen in de grote commissie. Prof. Jim Hudziak, I would like to thank you for being part of the thesis defense committee. I would also like to thank you for your enthusiasm, advice and for sharing your ideas about the project during meetings in the Netherlands.

De eerste anderhalf jaar van mijn promotietraject heb ik doorgebracht in het genetica lab. Michael, ik ben jou enorm dankbaar voor je geduld en goede uitleg in het lab bij het leren van nieuwe technieken en vooral ook voor je Brabantse gezelligheid. Naamgenootje Lisette, ik wil jou ook graag bedanken voor het helpen opstarten in het lab en dat ik altijd bij je terecht kon voor (epi)genetica gerelateerde vragen. Nina en Marieke, ik volgde een beetje in jullie voetstappen in het lab, ook jullie bedankt voor de samenwerking!

Prof. dr. Ingmar Franken, bedankt voor het delen van uw kennis over middelengebruik en verslaving en de prettige en vlotte samenwerking bij de *COMT* paper.

Tevens wil ik van TRAILS enkele mensen bedanken. Aukelien en Tom, jullie hebben mij geholpen met de start en het verloop van de interviews en het was altijd gezellig als ik tussendoor de gegevens kwam brengen. Samen met Catharina (aan het begin van het project) hebben jullie ook geholpen met de biobank. Het was niet altijd even makkelijk om in de donkere kelder van het UMCG alle DNA samples te controleren en klaar te maken voor transport naar Rotterdam!

Mijn (oud) eilandgenootjes van de KJPP; Sylvana, Christina, Floor, Ilse, MP, Johanna, Sabine, Laura en Nathalie; bedankt voor de wetenschappelijke discussies en vooral voor de gezelligheid op het eiland! Alle andere (oud)collega's van de KJPP; Linda, Jorieke, Geerte, Nita, Suzanne, Hanan, Luuk, Karolijn, Willem, Vandhana, Esther, Gerbrich, Anneke en Mart, ieder van jullie is altijd enorm behulpzaam, ook jullie bedankt voor de gezelligheid op de afdeling! Stafleden; Mireille, Hélene, Gwen, Jan, Kirstin, bedankt voor jullie bijdragen aan de wetenschappelijke discussies van de RWM en voor de bereidwilligheid om te helpen met andere onderzoek gerelateerde zaken. Laureen, bedankt voor je hulp bij de laatste loodjes van het promotieproces, en de gezellige ratten-, en vakantiepraatjes!

Ook wil ik mijn dank uitbrengen aan mijn paranimfen Johanna en Steven. Johanna, wat een reis is de afgelopen 4 jaar ook voor jou geweest, ik heb bewondering voor je inzet tijdens jouw promotietraject, en wat was het prettig samenwerken aan de *COMT* paper! Ik heb het altijd erg gezellig gevonden om met jou op het eiland te zitten en te sparren over TRAILS en alle biomedische onderwerpen, en ik voel me vereerd dat ik over enkele maanden naast je mag komen te staan op jouw promotie! Steven, lieve broer, wij hebben altijd al een sterke familieband gehad. Ik heb enorme bewondering voor je doorzettingsvermogen en je positieve houding in het leven, ondanks de soms moeilijke carrière keuzes die je hebt moeten maken. Ik ben in ieder geval enorm trots op je, en ik hoop dat jouw humor mij een beetje tot rust kan brengen op de spannende dag van de promotie!

Heel veel dank ook aan mijn vrienden en vriendinnen; Carina, Lydia, Loes, Stéphanie, Anke, Sabine, Sarah en Olivier voor alle gezelligheid op reis, tijdens dagjes Rotterdam/Amsterdam/Haarlem/Lamswaarde, middagjes shoppen, skaten, bezoekjes aan de Efteling, Holland Casino, concerten, en vele andere leuke bezigheden!

ARC clubgenootjes, te veel om op te noemen, bedankt voor de gezellige trainingsavonden! Vikashj, Damiaan en Geoffrey bedankt voor het meeslepen in de competities, het was soms wat tijdrovend, maar wel erg leuk. René, Martin, Jaco, Tim, Kees en Werner, bedankt voor de gezellige 3D dagjes door de bossen, zelfs in Duitsland! En Werner, bedankt voor je gezelligheid, je advies en luisterend oor tijdens het schieten en de

materiaal roadtrips, want je kan nooit genoeg pijlen hebben, toch? Zo niet dan wel en waarom dan wel niet!

Als laatste wil ik graag mijn familie bedanken. Lieve opa, bedankt voor uw interesse in het onderwerp van mijn proefschrift. Lieve Oma, pas uw 90ste verjaardag gevierd (ten tijde van de promotie), maar nog altijd kwiek van geest en nog graag overal bij betrokken! Onze verlengde familie, tante Inge en oom Jos, bedankt dat jullie er altijd voor ons zijn, en voor de gezelligheid in de Zeeuwse polder en aan de grachten van Amsterdam. Cora, mede hobby-fotograaf, bedankt voor de gezelligheid in Terneuzen en op vakanties! Lieve papa, bedankt dat je het mede mogelijk hebt gemaakt voor ons om een universitaire studie te volgen zonder financiële problemen, voor je interesse in de epigenetica en mijn onderzoek, en voor het meedenken over een carrière na dit promotieonderzoek! Lieve mama, bedankt voor je steun en voor je onvoorwaardelijke liefde, dit is niet in woorden uit te drukken. De afgelopen jaren zijn niet altijd even makkelijk geweest (niet alleen voor mij), maar gelukkig weet ik dat jij altijd voor mij klaar staat!

Lisette

ACKNOWLEDGEMENTS

This research is part of the TRacking Adolescents' Individual Lives Survey (TRAILS). Participating centers of TRAILS include various departments of the University Medical Center and University of Groningen, the Erasmus University Medical Center Rotterdam, the University of Utrecht, the Radboud Medical Center Nijmegen, and the Parnassia Bavo group, all in the Netherlands. The work in this thesis was supported by the Sophia Children's Hospital Foundation (project number 634) and the 'Jan Dekkerstichting en dr. Ludgardine Bouwmanstichting' (project no. 2012-01). TRAILS has further been financially supported by various grants from the Netherlands Organization for Scientific Research (NWO), ZonMW, GB-MaGW, the Dutch Ministry of Justice, the European Science Foundation, BBMRI-NL, the participating universities, and Accare Center for Child and Adolescent Psychiatry.