Genetics and Epigenetics of Children's Behavior Problems

Eirini Pappa

ACKNOWLEDMENTS

The Generation R Study is conducted by Erasmus Medical Center Rotterdam in close collaboration with the Faculty of Social Sciences of the Erasmus University Rotterdam, the Municipal Health Service Rotterdam, and the Stichting Trombosedienst & Artsenlaboratorium Rijnmond (STAR), Rotterdam. We gratefully acknowledge the contribution of the children and parents, general practitioners, hospitals, midwives, and pharmacies in Rotterdam. The general design of the Generation R is made possible by the Erasmus Medical Center Rotterdam, the Netherlands Organisation for Health Research and Development (ZonMw), the Netherlands Organisation for Scientific Research (NWO), the Ministry of Health, Welfare, and Sport, and the Ministry of Youth and Families.

Cover: XXXX
Lay-out: Legatron Electronic Publishing, Rotterdam
Printing: Ipskamp Drukkers BV, Enschede

ISBN/EAN: 978-94-6191-XXX-X

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Genetics and Epigenetics of Children's Behavior Problems

Genetica en epigenetica van gedragsproblemen bij kinderen

Proefschrift

ter verkrijging van de graad 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 vrijdag 20 november 2015 om 11:30 uur

door Eirini Pappa
geboren te Athene, Griekenland

Erasmus University Rotterdam

Promotiecommissie

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Chapter 3

Pappa I, Mileva-Seitz VR, Bakermans-Kranenburg MJ, Tiemeier H, van IJzendoorn MH. **The Magnificent Seven: A Quantitative Review of Dopamine Receptor D4 and its Association with Child Behavior.** *Neuroscience and Biobehavioral Reviews, 2015, epub ahead of print.*

Chapter 4

Pappa I, Fedko IO, Mileva-Seitz VR, Hottenga JJ, Bakermans-Kranenburg MJ, Bartels M, van Beijsterveldt CEM, Jaddoe VWV, Middeldorp CM, Rippe RCA, Rivadeneira F, Tiemeier H, Verhulst FC, van IJzendoorn MH, Boomsma DI. **SNP heritability of behavior problems in childhood: Genome-wide Complex Trait Analysis.** *Journal of the American Academy of Child & Adolescent Psychiatry, 2015, 54(9) :737-744.*

Chapter 5

Pappa I, St Pourcain B, Benke K, Cavadino A, Hakulinen C, Nivard MG, Nolte IM, Tiesler CMT, Bakermans-Kranenburg MJ, Davies GE, Evans DM, Geoffroy M, Grallert H, Groen-Blokhuis MM, Hudziak JJ, Kemp JP, Keltikangas-Järvinen L, McMahon G, Mileva-Seitz VR, Motazedi E, Power C, Raitakari OT, Ring SM, Rivadeneira F, Rodriguez A, Scheet PA, Seppälä I, Snieder H, Standl M, Thiering E, Timpson NJ, Veenstra R, Velders FP, Whitehouse AJO, Davey Smith G, Heinrich J, Hypponen E, Lehtimäki T, Middeldorp CM, Oldehinkel AJ, Pennel CE, Boomsma DI, Tiemeier H. **A Genome-Wide Approach to Children's Aggressive Behavior. The EAGLE consortium.** *American Journal Medical Genetics Part B, 2015, epub ahead of print.*

Chapter 6

Pappa I, Szekely E, Mileva-Seitz VR, Luijk MPCM, Bakermans-Kranenburg MJ, van IJzendoorn MH, Tiemeier H. **Beyond the Usual Suspects: A Multidimensional Genetic Exploration of Infant Attachment Disorganization and Security.** *Attachment and Human Development. 2015, (3): 288-301.*

Chapter 7

Rijlaarsdam J, **Pappa I**, Mileva-Seitz VR, Rippe RC, Roza SJ, Tiemeier H, Bakermans- Kranenburg MJ, van IJzendoorn MH. **An Epigenome-wide Association Study of Prenatal Maternal Stress in Neonates.** *Manuscript in preparation.*

Chapter 8

Pappa I, Mileva-Seitz VR, Ghassabian A, van IJzendoorn MH, Tiemeier H. Early **Pregnancy Maternal Thyroid Function and Genome-wide DNA Methylation in Neonates- Evidence from the Generation R Study.** *Manuscript in preparation*.

1

Introduction

Child psychiatry and developmental psychopathology are fascinating, intellectually intriguing, and immensely rewarding fields. Similar to adults, children suffer from mental disorders, with a prevalence of 7% in preschoolers [1] and 13-20% in school-aged children and adolescents [2]. The most prevalent mental health problems in children are attention deficit/hyperactivity disorder (ADHD), behavior problems (e.g., oppositional defiant disorder, conduct problems), emotional problems anxiety and depression symptoms) and autistic spectrum disorders, all of which show some comorbidity with each other [3]. There is evidence of continuity between behavior problems in childhood and later psychopathology [4-6], indicating that the same risk factors may be responsible for the onset, development, and/ or persistence of mental health problems throughout the lifespan.

The latest school of thought in child psychiatry and developmental psychopathology emphasizes quantitative models for conceptualizing psychopathology [7,8]. In these models, "normal" differences among individuals reside on one end of the continuum, while psychiatric disorders lie at the opposing end. This differs from the conventional, categorical approach where a diagnosis is made based on the presence of a set of symptoms. A major research aim is to identify the various factors, processes, and mechanisms that influence the continuum of behavior problems in children and improve their diagnosis, prognosis and therapeutic options.

Behavior problems in childhood, much like other common medical conditions (e.g., heart disease, diabetes), are considered complex disorders. This means they are likely to be associated with multiple genetic, lifestyle, and environmental factors. Indeed, risk factors for children's behavior problems include *child characteristics* (i.e. age, gender, ethnicity, temperament, genetic variation), *family and parent characteristics* (parental education and psychiatric and medical history, family function, parental sensitivity), and *broader environmental influences* (i.e. infections and exposure to pollutants, school and neighborhood environment) [9]. One of the most consistent patterns/findings is the familial aggregation of problem behavior. This highlights the importance of genetic factors and non-shared environment in explaining individual differences among children [10].

It has long been recognized that behavior problems are to some extent inherited. It is common to say that a behavior problem "runs in the family", or that a child's behavior resembles their parents' behavioral patterns ("like mother, like daughter"). The first theories about the inheritance of common behavior problems are attributed to the ancient Greek scholars Hippocrates (460-377 B.C.) [11] and Euripides (484-406 B.C.) [12]. In more recent decades, developmental research in twin and family studies has provided estimates of moderate to high heritability (e.g. aggression [13], autistic-like traits [14], ADHD symptoms [15]). However, very little is known about the exact genetic underpinnings of these behaviors. Over the past 10 years, researchers have focused on the identification of specific genetics variants influencing behavior in children, giving rise to the expanding field of child psychiatric genetics.

Child Psychiatric Genetics

The candidate gene approach

Candidate gene studies were the first to explore the association of genetic variation with child behavior problems. The selection of the candidate genes is based on foreknowledge of the gene's biological function. Some of the most studied candidate genes are the serotonin transporter (*5-HTTLPR*), monoamine oxidase (*MAO*), dopamine receptor D4 (*DRD4*), and catechol-O-methyltransferase (*COMT*) gene [16].

Candidate gene studies have produced many findings - both positive and negative − but with few consistent replications [17,18]. This lack of replication has numerous possible explanations. Phenotypes are often heterogeneous, where different studies use different instruments, and different informants, to measure the traits of interest. There are age effects on problem behavior. Sample sizes are often small and populations stratified or ethnically heterogeneous without controlling for these factors. Statistical problems also plague the literature, such as the lack of control for multiple testing. Underlying all, there are a limited number of biological studies examining the functional consequences of genetic variation, which means that functions of human genetic variants are guesses at best [19].

Research on candidate genes thus faces numerous challenges. However, a careful choice of the selection of candidate genes, the minimization of arbitrary genotype classifications, the appropriate correction for multiple testing in well-powered studies, and the replication of initial findings in independent samples may help us to understand better the role of these genetic factors in the etiology of common behavior problems in childhood.

The genome-wide approach

We know from past studies that the genetic architecture of most psychiatric disorders is complex, with many contributing genetic variants [20]. Similarly, we can assume that problem behavior in children is associated with multiple independent genetic variants, each of which has small effects on phenotype. Genome-wide Complex Trait Analysis (GCTA) [21] can be used to identify the proportion of genetic variance that is attributed to common single nucleotide polymorphisms (SNPs).

Secondly, hypothesis-free, genome-wide association studies (GWAS) can be used to identify the genetic variants associated with individual differences in children's behavior. GWAS have been successfully used in adults, identifying novel genetic variants in psychiatric outcomes [22,23]. However, so far, large-scale GWAS have not been conducted for common behavioral problems in children (e.g. ADHD, aggressive behavior, anxiety and depression, and autistic spectrum problems). These studies require large sample sizes and necessitate the collaboration of international research groups within consortia. The associated SNPs themselves may not be causal, but they may be in linkage disequilibrium with the causal variants. More studies are needed to identify the functionality of novel findings. In addition to GWAS, gene-based [24] and pathway analyses [25] are used to investigate further the genetic architecture of the trait of interest.

The epigenome-wide approach

Epigenome-wide association studies (EWAS) provide a novel approach to identifying molecular mechanisms underlying common diseases [26]. The key concept is that that environmental influences could alter DNA methylation patterns, in critical periods of development, leading to negative health outcomes. For example, suboptimal fetal environment could result in long-lasting consequences for the offspring's psychological development, and this association could be mediated by differential methylation of the offspring's genes.

Previous studies in mammals have shown the importance of prenatal exposures for epigenetic programming [27,28]. In humans, most studies have focused on differential methylation of candidate genes [29-31]. It is not yet known whether prenatal environment is associated with genome-wide epigenetic differences in neonates.

Aims

The specific aims of this thesis were:

(1) To investigate the role of variation within a well-studied candidate gene (dopamine receptor D4, *DRD4*) in children's common behavior problems, and the associated limitations of such an approach.

(2) To estimate the proportion of genetic heritability attributed to common SNPs and identify novel genetic variants associated with children's aggressive behavior and infant attachment and disorganization.

(3) To investigate whether early prenatal environment influences genome-wide methylation patterns in neonates. We focused specifically on prenatal maternal stress and early pregnancy maternal thyroid function as indices of prenatal environment.

General design

The studies described in this thesis were embedded in the Generation R Study. This is a population-based prospective cohort study from fetal life onward, based in Rotterdam, the Netherlands [32]. The Generation R Study offers a unique opportunity to identify early environmental and genetic causes of normal and abnormal growth, development, and health [33]. Data collection in children and their parents ranges from questionnaires to observations and biological measures. Genome-wide data are also available in the participating children.

In this thesis, we also used data from a subsample of the Generation R Study, called the Generation R Focus study [34], where additional detailed behavioral observations are available. This subsample is ethnically homogenous (all children, their parents, and grandparents were born in the Netherlands), in order to exclude possible confounding and effect modification due to ethnicity.

The GWAS studies reported in this thesis were performed in collaboration with other birth cohorts, as part of the Early Genetics and Longitudinal Epidemiology (EAGLE) consortium [http://research.lunenfeld.ca/eagle/]. The aim of the EAGLE consortium is to identify genetic variants related to common behavior problems and cognition in children.

Outline of this thesis

We performed studies to identify genetic determinants of children's behavior problems. We have also aimed to identify epigenetic modifications in cord blood associated with variation in prenatal environment.

In **section A**, we focus on the candidate gene approach. We examined whether the number of variable number tandem repeats (VNTRS) within the *DRD4* gene is associated with ADHD symptoms in childhood, and whether this relationship is mediated by levels of stranger fear (**chapter 2**). Secondly, we performed a systematic review of the literature, summarizing the biological studies on *DRD4* VNTRs. We also systematically reviewed and meta-analyzed studies on *DRD4* VNTRs and behavioral traits in population-based, non-clinical samples of children and adolescents (**chapter 3**).

In **section B**, we focus on the genome-wide approach. We estimated SNP heritability of multiple phenotypes of children's behavior problems and cognition. For this purpose, we combined data from two independent population-based Dutch cohorts (**chapter 4**). Furthermore, we conducted a GWAS meta-analysis of aggressive behavior in childhood within the framework of EAGLE consortium (**chapter 5**). We also performed a GWAS on the Generation R sample to investigate genetic determinants of infant attachment disorganization and security (**chapter 6**).

In **section C**, we focus on the epigenome-wide approach. We estimated a global maternal prenatal stress score and examined whether increased levels of prenatal maternal stress were associated with epigenome-wide differences in neonates (**chapter 7**). Furthermore, we investigated whether impaired maternal thyroid function in early pregnancy was associated with different epigenome-wide patterns in cord blood (**chapter 8**).

In **chapter 9**, we provide general discussion of the findings presented in this thesis. We discuss possible underlying mechanisms, new insights and directions for future studies, and possible clinical implications based on our findings.

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SECTION A

The candidate gene approach

2

DRD4 VNTRs, Observed Stranger Fear in Preschoolers and Later ADHD Symptoms

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Psychiatry Research, 2014; 220(3): 982-6

Abstract

Fear of strangers is a developmental milestone in childhood that encompasses behavioral inhibition and decreased novelty seeking. Children with attention deficit/hyperactivity disorder (ADHD) often exhibit fearless and impulsive behaviors, similar to those observed in children with atypically low levels of stranger fear. It is currently unknown whether these behaviors share common underlying biological mechanisms. Polymorphisms in the dopamine receptor 4 gene (*DRD4*) have been implicated in the risk for developing ADHD symptoms in childhood. Here we investigate whether (1) *DRD4* variable number tandem repeats (VNTRs) are associated with both stranger fear and ADHD symptoms, and (2) stranger fear in preschoolers mediates the link between *DRD4* VNTRs and ADHD in later childhood. Stranger fear was observed in a large sample (N = 589) of 3-year-old Caucasian children and ADHD symptoms were assessed by a validated, mother-rated questionnaire at 6 years. We found evidence that longer *DRD4* variants were associated with increased ADHD symptoms at 6 years, and that this relationship was partially mediated by lower levels of observed stranger fear at 3 years. Our results suggest a common underlying neurobiological mechanism in the association between low stranger fear and ADHD symptoms; variation in *DRD4* may be an important contributor to this mechanism.

Introduction

Preschoolers show significant individual variation in expressed fear when facing unfamiliar adults [1]. These patterns of behavior are often attributed to child temperament, and are suggested to be heritable [2,3] and relatively stable over time [4]. The development of stranger fear is an important milestone, emerging at 6 months of age and increasing before 12 months [5]. Lack of stranger fear has been associated with inattention and low inhibitory control in later childhood [6], and extreme stranger fear has been associated with greater social anxiety in adulthood [7]. Stranger fear is a multidimensional trait that encompasses behavioral inhibition, lack of impulsivity and novelty seeking, and shares conceptual overlap with attention deficit/hyperactivity (ADHD), a common neurodevelopmental disorder characterized by inattention, impaired inhibitory control and hyperactive/impulsive behavior [8]. Yet the underlying biological link between stranger fear and ADHD has not been previously established.

The *DRD4* gene contains a widely-studied 48-bp variable number tandem repeat (VNTR), with 2-11 repeats on exon III. The 7-repeat allele is a known risk factor of ADHD [9] and children with ADHD are often characterized as being fearless and impulsive. "Long" *DRD4* variants have also been associated with increased novelty seeking in healthy 3-year olds [10]. Children with long *DRD4* VNTRs who are at risk for ADHD might also exhibit a less fearful response to facing a stranger during a laboratory setting. Only two small studies [Auerbach et al. [11] and Lakatos et al. [12]] have examined this association, showing no main genetic effects.

The aims of the present study are: (1) to examine the association between different *DRD4* VNTR groups and observed levels of stranger fear during a well- validated laboratory task in a typically developing, non-clinical sample (N = 589) of 3-year old Caucasian children, and (2) to assess the association of *DRD4* VNTR with ADHD symptoms at 6 years and examine whether stranger fear at 3 years mediates the link between *DRD4* VNTRs and later ADHD symptoms.

Methods

Setting

This study was conducted in a subsample of children participating in the Generation R Study, referred to as the Generation R Focus Study [13]. The design and sample characteristics of the Generation R Study have been described in detail elsewhere [14]. The current study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from parents of the participating children.

Study Population

All children of the Generation R Focus Study are of Dutch origin. DNA was obtained from cord blood samples at birth. At age 36 months (M = 37.5, SD = 1.5), N = 589 children (52% boys) visited

our lab where stranger fear was assessed using the Stranger Approach episode from the Laboratory Temperament Assessment Battery (Lab-TAB, Goldsmith et al. [15]).

Genotyping

The *DRD4* 48 bp VNTR was amplified using primers D4-F-GCGACTACGTGGTCTACTCG and D4-R-AGGACCCTCATGGCCTTG. Reactions were performed in a 384-wells format in a total reaction volume of 10 ml containing 10 ng DNA, 1 pmol/ml of each primer, .4 mM dNTPs, 1 M betaine, 1x GC buffer I (Takara Bio, Inc., Otsu, Japan) and 0.5 U/ml LA Taq (Takara Bio, Inc.). PCR cycling consisted of initial denaturation of 1 min at 94°C, and 34 cycles with denaturation of 30 s at 95°C, annealing of 30 s at 58°C and extension of 1 min at 72°C. PCR fragments were size-separated on the Labchip GX (Caliper Life Sciences, Hopkinton, MA) using a HT DNA 5K chip (Caliper Life Sciences). The number of *DRD4* repeats was determined using the size of the PCR fragments. Ambiguous genotypes were re-genotyped. The main genotypes (2/4, 4/4 and 4/7-repeats) were in Hardy-Weinberg equilibrium ($\chi^2 = 0.01$, $p = .91$).

DRD4 VNTRS classifications

Due to ongoing uncertainty about *DRD4* VNTR classification [16], we assessed the most common classifications found in developmental research: (1) 7-repeat carriers vs. non carriers, (2) (2-5) vs. (6-11)-repeat carriers, (3) (2-4) vs. (5-11)-repeat carriers, and (4) (2-6) vs. (7-11)-repeat carriers. We also used a more conservative classification, based on evidence of biochemical differences only between 2-, 4- and 7- repeat variants [17]. For this functional approach, we used the classification (5) (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes.

Stranger Fear

Stranger fear was measured using the Stranger Approach episode of the Lab- TAB Preschool version [15]. The rationale was to measure social fear when a stranger approaches the child in a laboratory setting. The situation was modelled after real-life events. Children visited our research center when they were 3 year old. During the observation the child was left alone in a specially designed room, with toys. After some time, the task began and was divided into nine epochs during which a female stranger (disguised with a hat, sunglasses, and a coat) approached the child and asked standardized questions (i.e. "Are you having a good time here today?", "Are you playing with a lot of toys?"). Intensity of fear expression, distress vocalizations, activity decrease, approach, avoidance, gaze aversion, verbal hesitancy and nervous fidgeting were scored in each epoch from DVD recordings according to the original coding system by trained coders, blind to children's genetic information and ADHD status (mean intra-class correlation coefficients ranging from .70-.99, $n = 25$). Averages were computed for each child response or parameter across epochs. We computed a stranger fear composite score by first reversing the approach scale average, converting all scale averages into z-scores, and taking the mean of all standardized averages.

ADHD symptoms

ADHD symptoms begin in childhood but establishing a precise diagnosis at an early age is questionable [18]. Thus, we used mother-reported data on children's ADHD symptoms at 6 years (mean age, M = 5.9, SD = 0.20) measured by the Attention Deficit/Hyperactivity Problem subscale (six items, alpha = .70) of the Child Behavior Checklist [CBCL 1½-5, Achenbach and Rescorla [19]]. The six items of the Attention Deficit/Hyperactivity Problem subscale are: (a) cannot concentrate, cannot pay attention for long, (b) cannot sit still, restless, or hyperactive, (c) cannot stand waiting, wants everything now, (d) demands must be met immediately, (e) gets into everything, and (f) quickly shifts from one activity into another. The reliability and validity of the Dutch version of CBCL 1½-5 has been previously demonstrated [20]. Although the CBCL Attention Deficit/Hyperactivity Problem subscale is not a diagnostic tool, it shows high diagnostic and discriminating power for ADHD in population-based, non- clinical samples [21].

For N = 581 children both stranger fear and ADHD symptoms scores were available. Loss to follow-up children did not significantly differ in the levels of stranger fear [$t(8)$ = 0.66, p = .51].

Statistical analysis

We ran one-way ANOVAs in SPSS Version 20.0 for Windows to compare the mean levels of observed stranger fear across the different *DRD4* VNTR genotype groups. We used a four-step mediation analysis approach [22], based on several regression analyses to evaluate the role of observed stranger fear as a potential mediator of the association between *DRD4* VNTR genotypes and ADHD symptoms.

Results

Genotypic and phenotypic sample characteristics

In the Generation R sample, allele frequencies were similar to those reported for Caucasian populations [23]. The most common genotypes were 4/4 (41.1%), 4/7 (25.5%) and 2/4 (13.4%). Total stranger fear scores ranged from -1.11 to 1.76 (M = 0.00, SD = 0.40), with higher scores indicating greater fear. The scores on the ADHD symptoms scale ranged from 0.00 to 8.00 (M = 1.35, SD = 1.55), with higher scores indicating more mother-reported ADHD symptoms.

In our sample, levels of stranger fear were not associated with child's sex [$F(1, 588)$ = 3.11, p = .08)]. However, there was a significant association of child's sex with ADHD symptoms [$F(2, 579)$ = 18.72, p = .00] after adjusting for child's age, with girls showing lower ratings.

DRD4 VNTRs and Stranger Fear

We used five common genotypic classifications found in the literature and tested for associations with levels of stranger fear in our sample of 3-year-old children. For two genotypic classifications (1) (2-5) vs. (6-11)-repeat carriers and (2) (2-4) vs. (5-11)-repeat carriers, the 'long' *DRD4* alleles were associated with lower levels of stranger fear (see Table 1). In all other classifications, there was a consistent trend for an association between longer *DRD4* VNTRs and lower stranger fear.

Table 1 | Summary of the one way-ANOVA for stranger fear and alternative groupings of *DRD4* VNTRs,

Empirical Approach	*N* (589)	Mean	*SD*	r^2	*p*-value
1. 7-repeat carriers vs. non- carriers					
7-repeat non-carriers	393	0.02	0.48		
7-repeat carriers	196	-0.04	0.73	0.005	.09
2. (2-5) vs. (6-11)-repeat carriers					
(2-5) carriers	382	0.02	0.48		
(6-11) carriers	207	-0.05	0.73	0.008	.03
3. (2-4) vs. (5-11)-repeat carriers					
(2-4) carriers	367	0.02	0.48		
(5-11) carriers	222	-0.04	0.48	0.006	.05
4. (2-6) vs. (7-11)-repeat carriers					
(2-6) carriers	385	0.02	0.48		
(7-11) carriers	204	-0.04	0.73	0.005	.06
Functional approach					
5. (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes [a]					
(2/2, 2/4, 4/4) genotypes	323	0.01	0.48		
(2/7, 4/7, 7/7) genotypes	185	-0.04	0.73	0.000	.13

[a] In this group, total *N* = 508 children

DRD4 VNTRs, ADHD, and Stranger Fear

Formal mediation analysis procedures were used to determine whether the association of "long" *DRD4* VNTRs [(2-5) vs. (6-11)-repeat variants, the contrast that showed the strongest relation to stranger fear] with mother-rated ADHD symptoms at age 6 years were mediated by levels of stranger fear at age 3 years.

First, the direct effect (path a) from *DRD4* VNTRs to stranger fear at age 3 years was significant (*beta* = -0.09, p = .03); "long" alleles were associated with lower levels of stranger fear. Second, the direct effect (path b) from stranger fear and ADHD symptoms at age 6 years was also significant (*beta* = -0.08, p = .04); lower levels of stranger fear at age 3 years were associated with more ADHD symptoms at age 6 years. Third, the direct effect (path c) from *DRD4* VNTRs to ADHD symptoms at age 6 years was significant (*beta* = 0.10, p = .03, R^2 = .012); "longer" alleles were associated with more ADHD symptoms at age 6 years. However, the path became non-significant (path c') when controlling for levels of stranger fear (*beta* = 0.08, p = .053, R^2 = .021). The proportion of explained variance of the association of "long" *DRD4* VNTRs and ADHD symptoms at 6 years, although modest, was significantly increased when stranger fear at 3 years was also included in the model (R^2-change = .009, p = .03). All analyses were adjusted for age and sex.

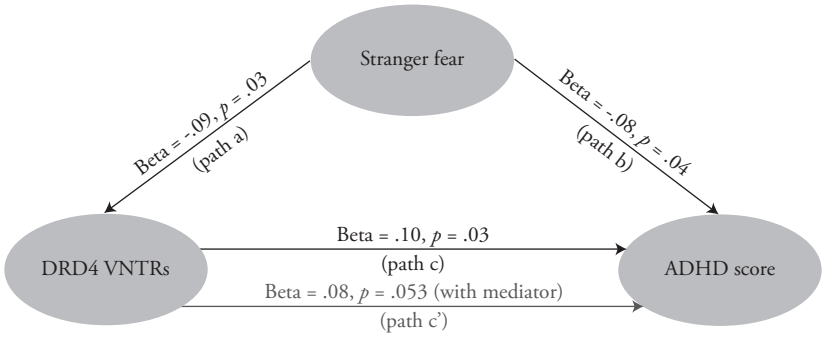

Figure 1 | Partial mediator effect of stranger fear at 3 years on the association between *DRD4* VNTRs [(2-5) vs. (6-11) repeat carriers] and ADHD scores at 6 years.

Discussion

In the current study we firstly investigated the association between *DRD4* VNTR genotypes and stranger fear in preschoolers by systematically exploring previously used genotype classifications. More specifically, we found a significant association between "long" *DRD4* genotypes (>5-repeat variants) and lower levels of stranger fear. Secondly, we replicated prior reports of an association between *DRD4* genotype and ADHD symptoms in our sample of 6-year old children (N = 589). Recent research shows that individuals with ADHD have altered neuronal activation patterns related to fear recognition in social contexts [24]. Our results suggest that less fearful behavior towards strangers may be an early indicator of later ADHD symptoms, and that *DRD4* variation explains part of this association. Controlling for stranger fear indicated partial mediation of the association between *DRD4* and ADHD symptoms. Although *DRD4* genotypes and levels of stranger fear explained only a modest proportion of the variance in ADHD symptoms, our results suggest a significant biological link between "temperamental" behaviors and a neurodevelopmental disorder, in a non-clinical, population-based sample of children. It is possible that developmental deficits beginning in early childhood and manifest the ADHD symptomatology are also responsible for the lower levels of stranger fear, an important developmental milestone [5].

ADHD is a common but complex disorder, and as such, it is likely that multiple genes with relatively small effects will be associated with ADHD symptoms [25]. Indeed, previous molecular genetic studies have explored the association of common genetic variation with increased ADHD symptoms [26]. The dopaminergic pathway has been involved and several candidate genes have been identified, including *DRD2*, *DRD5*, and *DAT1* [27]. In this study, we were specifically interested in the association of *DRD4* VNTRs with ADHD symptoms. It is possible however that more variation

within this gene or other genes of the same pathway would explain a larger proportion of the individual differences in ADHD symptoms among children.

We investigated the hypothesis that individual variation in stranger fear partially mediates the association between "long" *DRD4* VNTRs and ADHD symptoms in children. It is possible that other temperamental (i.e. high neuroticism and surgency, Martel et al. [28]), parental (i.e. parenting behavior and parent depression, Tung et al. [29]) and environmental (i.e. media use, Nikkelen et al. [30] and family income, Larsson et al. [31]) factors mediate the link between genetic variation and ADHD symptoms. The integration of all these factors in one viable model of ADHD is the ultimate goal, but lies beyond the scope of this study, addressing the various factors previously associated with ADHD symptoms.

The two previous studies on the genetics of stranger fear in small samples of children have failed to show a main effect of *DRD4* on stranger fear (Lakatos et al. [12], Auerbach et al. [11]). It is possible that lack of power and/ or the use of different genotypic classifications of *DRD4* VNTRs may have influenced these findings. Our study illustrates that although effect sizes can be very consistent across different classifications of the same underlying group of *DRD4* variants, the interpretation of the significance tests can depend on the choice of the classification. The use of the functional approach (i.e. comparing only the *DRD4* VNTRs for which evidence of biological differences exist), was also in line with the classical "short" vs. "long" classifications, although it yielded no statistical significant results. One explanation could be the smaller sample size of this group (N = 508), comparing to the total group (N = 589). Another possibility could be that there is true biological difference of the rare *DRD4* VNTRs and that this can be better captured by the "short" vs. "long" classifications. More studies are required before we can draw definitive conclusions about the functionality of rare *DRD4* VNTRs.

In our sample, girls show lower levels of mother-rated ADHD symptoms at 6 years. Previous research supports this finding, in both clinical [32] and population- based samples [33]. Furthermore, we found no evidence of gender differences in levels of stranger fear among 3-year-olds, which is consistent with a recent meta-analysis in children [34]. Given that our study indicates stranger fear as a partial mediator of the link between *DRD4* VNTRs and ADHD symptoms, it is possible that other dimensions of temperament (i.e. low inhibitory control), may be responsible for the gender differences in ADHD symptoms. Indeed, previous research has associated lower levels of effortful control with increased attention problems in boys [35].

A strength of this study is that it was conducted with a large and ethnically homogenous sample of children. Second, we replicated previous findings on *DRD4* VNTRs and ADHD symptoms and expanded this association, based on the conceptual overlap between stranger fear and ADHD symptoms. Third, by using observational measures of stranger fear at 3 years and mother-rated ADHD symptoms at 6 years, we reduced the risk of potential biases that may arise due to shared method variance.

However, the present study is not without limitations. First, we assessed stranger fear with a laboratory observation task, which, although well-validated, was brief. Second, we were specifically

interested in main genetic effects of *DRD4* on stranger fear, yet it is possible that environmental factors moderate the genetic influences on fearful behavior [36]. Environmental factors were not addressed in the present study. Third, our findings of mediation of *DRD4* and ADHD symptoms via levels of stranger fear have to be replicated in independent samples, before generalizing them to larger populations. However, the current study is the largest of its kind to date and provides the first evidence for a common biological underpinning of stranger fear in preschoolers and ADHD symptoms in later childhood. Common genetic variation explaining the association of ADHD symptoms via individual variation in levels of stranger fear during early life may help us better understand the neuropsychology of ADHD etiology.

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3

The Magnificent Seven: A Quantitative Review of Dopamine Receptor D4 and its Association with Child Behavior

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Neuroscience and Biobehavioral Reviews, 2015, epub ahead of print.

Abstract

A large volume of behavioral research has explored the variable number of tandem repeat (VNTR) polymorphism on the dopamine receptor D4 gene (*DRD4*). However, findings are inconsistent and there is no agreement about what constitutes "functional" and "less functional" variants at this locus. First, we systematically review studies exploring biological differences between *DRD4* VNTRs (k = 21). Second, we systematically review studies relating *DRD4* variation to behavioral traits in population-based, non-clinical samples of children and adolescents (k = 46; N = 13,195), highlighting the various genotypic classifications previously used. Third, we use meta-analyses to examine associations of *DRD4* VNTRs with five broadly-defined behavioral outcomes (externalizing and attention problems, executive function, social/emotional development, and "reactive" temperament). We identify a significant association of "longer" *DRD4* variants with lower levels of executive function and social/emotional development, but not independent of the choice of genotypic classification. We suggest that until the functionality of *DRD4* VNTRs is established, researchers should report all genotypic classifications to ensure full transparency and allow for further meta-analytic work.

Introduction

With the advent of genotyping, thousands of association studies have used candidates genes – genes selected based on *a priori* biological knowledge – to explore genetic associations with behavioral problems in childhood and adolescence. The dopamine receptor D4 (*DRD4*) gene has become a fashionable choice, due to its important role in the prefrontal cortex [1], and it has generated a substantial literature showing either significant associations or lack of associations with just about every complex trait of interest in child behavior [2-6].

The *DRD4* gene codes for the dopamine D4 receptor, which is most expressed in specific areas of the brain, such as the frontal cortex and amygdala [7]. This gene contains a widely-studied 48-bp variable number tandem repeat (VNTR), with 2-11 repeats on its third exon [8]. In Caucasian populations, the three most common alleles at this locus are: the 4-repeat (mean allele-frequency = 0.71), the 7-repeat (0.12) and the 2-repeat (0.08). However, there are considerable differences in allele frequencies within European and other populations [9]. Additional identified variation in the amino sequence within this VNTR region makes *DRD4* one of the most variable receptors in humans [10].

There are established associations between *DRD4* VNTRs and variation in novelty seeking [11], attention deficit/hyperactivity disorder (ADHD) [12], and reward system function [13] in adults. A growing literature has also begun implicating *DRD4* VNTRs in a variety of child behavioral outcomes, such as executive function, temperament, and social development. Yet findings are often inconsistent, due to multiple reasons including lack of power, differing definitions and tools used to assess the same theoretical constructs, the use of both population-based and clinical samples, and the use of varying genotypic classifications of the *DRD4* VNTRs.

The classification and functionality of *DRD4* VNTRs is a considerable challenge for two major reasons. First, there is no conclusive evidence about the functionality of common variants [14]. Second, there is no widely accepted classification approach for the *DRD4* VNTRs genotypes [15-19]. For this reason, common variants are often classified in multiple ways in the literature (i.e. 7-repeat carriers vs. non-carriers or "short" vs. "long" carriers). Selecting the appropriate classification of genotypes is of great importance for the generalizability of individual findings from association studies.

In the present report we first conducted a systematic review of studies on the biological differences between *DRD4* VNTRs. Second, we conducted a systematic review of studies on the association with child and adolescent behavioral outcomes, selecting studies on population-based and non-clinical samples, and summarizing the multiple classifications previously used. Third, we performed meta-analyses to assess the combined effect size for the association between *DRD4* VNTRs and five broadly-defined child behavioral outcomes: (a) externalizing problems, (b) attention problems, (c) executive function, (d) social/emotional development, and (e) "reactive" temperament. Wherever possible, we computed combined effect sizes for each genotypic classification. To our knowledge, this is the first study of this magnitude examining the as yet poor biological understanding of the functionality of *DRD4* VNTRs in population-based samples of children and adolescents.

Methods

Systematic Literature Search

We conducted a systematic search of the literature, using the databases PubMed, PsycInfo, Embase and the search engine Google Scholar, from the first publication available in 1992 until January 2014. Our first objective was to collect all existing evidence of functional differences between *DRD4* VNTRs. Thus, we searched the terms ("*DRD4*" OR "dopamine") AND "VNTR*" AND ("biology" OR "function"). The exclusion and selection process of the first systematic review is illustrated in Figure 1. Our second objective was to summarize the studies assessing main genetic effects of *DRD4* VNTRs and behavioral outcomes in childhood or adolescence. The terms ("*DRD4*" OR "dopamine") AND "VNTR*" AND "behavior" AND ("child*" OR "adolescent*") were used. The reference lists of the resulting articles were manually checked for relevant publications. The exclusion and selection process of the second systematic review is illustrated in Figure 2.

Exclusion Criteria

We excluded abstracts, book chapters, review and meta-analytical articles, studies on adults (> 18 years), studies on psychiatric phenotypes and their pharmacological response (schizophrenia, mood/eating disorders, addictions, obsessive-compulsive disorder (OCD), autistic spectrum disorders (ASD), attention deficit/hyperactivity disorder (ADHD), intellectual disabilities (ID) etc.), and articles written in a language other than English. When different studies were performed on the same sample of individuals, we selected the study with the largest sample size. In studies with repeated measures, we selected the biggest sample or, if the samples were of equal size, we extracted data from the first wave. Studies for which main effects of *DRD4* VNTRs could not be referred by any statistical measurement were excluded from further analysis. We also excluded studies that did not clearly report the classification system of genotypes used.

Data Extraction

We extracted data on *DRD4* VNTR classification systems, phenotypes, sample size, size of genotype subgroups, age, sex, and ethnicity of the participants. Five genotypic classification were identified: (a) 7-repeat carriers vs. non carriers, (b) (2-5) vs. (6-11)-repeat carriers, (c) (2-6) vs. (7-11)-repeat carriers, (d) (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes, and (e) (2-4) vs. (5-11)-repeat carriers. We categorized phenotypes into five broadly-defined groups: (a) externalizing problems (Table 2) (b) attention problems (Table 3), (c) executive function (Table 4), (d) social/emotional development (Table 5), and (e) "reactive" temperament (Table 6). In the case of temperament, the multi-dimensionality of the constructs encompassing this broadly- defined phenotype made it challenging to report them in a straight-forward fashion. However, since *DRD4* VNTRs have been previously associated with susceptibility to environmental stimuli [20], we made the decision to only use the dimension of temperament associated with individual differences in reactivity (two studies were excluded). Thus, we followed the more unified distinction of "reactive" temperament (including negative affect, extraversion/ surgency and lack of effortful control), as previously described [21,22].

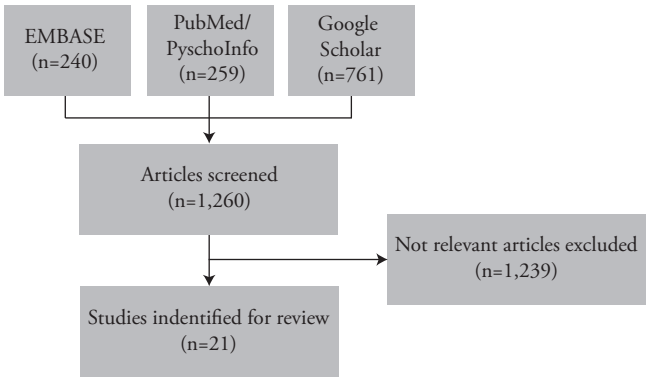

Figure 1 | Flowchart of selection of biological/functional studies of *DRD4* VNTRs.

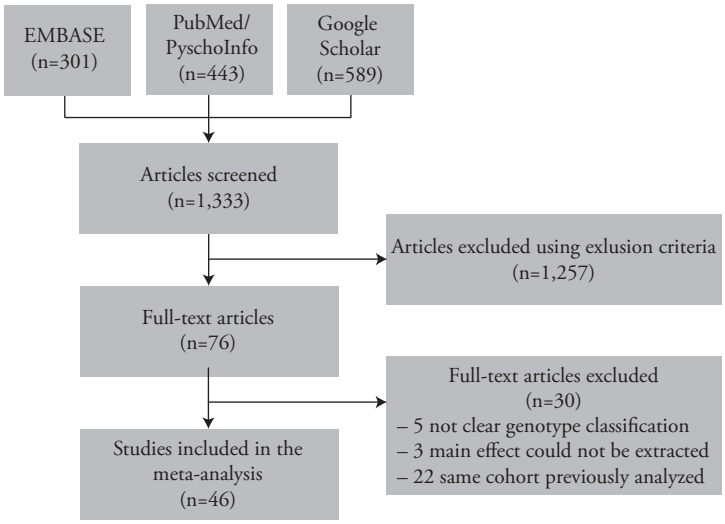

Figure 2 | Flowchart of selection of behavioral/association studies of *DRD4* VNTRs in population-based samples of children and adolescents.

Effect sizes (Cohen's *d*) and 95% confidence intervals (CI) were calculated using an online calculator [23]. For each study, we estimated effect sizes of all constructs related to the above-mentioned broadly-defined behavioral outcomes. A weighted average of all relative constructs was estimated for each study, taking into account the directionality of these constructs (i.e. for social/emotional development, attachment security and attachment disorganization were considered opposite constructs, thus the effect size estimated for attachment disorganization was reversed before estimating the total effect). In studies of gene-gene or gene-environment interactions, we extracted only the data on *DRD4* VNTRs

main effects, since the interaction effects were not relevant to the objectives of this study. In studies with clinical case-control design, we extracted data only on controls/unaffected individuals. Data extraction was performed independently by two coders (I.P. and V.R.M-S.) and inter-rated reliability was high (intra-class correlation coefficient, ICC = .86, k = 15).

Overall meta-analyses

Random effects model meta-analyses were performed using the Comprehensive Meta-Analysis (CMA) program [24]. In five separate meta-analyses we assessed the overall effect size of the association between *DRD4* VNTRs and five outcomes in children and adolescents, using all available studies derived from our systematic review. Within each meta-analysis participants were never included more than once. In studies where multiple genotypic classifications were presented, we used only the most common (7-repeat carriers vs. non-carriers) for the overall meta-analysis. We used the Cochran's Q- statistic to assess heterogeneity among studies.

Specific meta-analyses

We included genotypic classification as a moderator to test whether the overall effect size of the association with *DRD4* VNTRs would vary significantly between different groupings. In moderator analyses, we included only groups with four or more studies in order to enhance robustness [25]. Thus, the restricting number of available studies made impossible to test the effect of other potential moderators (i.e. age, sex and ethnicity). An overview of these potential moderators can be found in Tables 2 to 6. A comprehensive summary of the studies and the combined effect sizes of the association between *DRD4* VNTRs and behavioral outcomes is presented in Table 7.

Results

Description of studies

Biological/functional studies

Twenty-one studies published between 1992 and 2014 fulfilled the inclusion criteria and examined the potential biological differences among *DRD4* VNTRs. Table 1 provides a comprehensive summary of these studies. We grouped the studies according to their main methodology, as: (1) *in vitro*, (2) *in vivo* and (3) *in silico* studies. Since these studies measure *DRD4* VNTRs functionality in a variety of experimental conditions, not directly comparable, it was impossible to estimate an overall effect of *DRD4* VNTRs in a meta-analytical way. Thus it can only be concluded that eight of the studies in Table 1 show evidence of decreased functionality of the 7-repeat compared with the 2-repeat and the 4-repeat, seven studies indicate no functional differences between these variants, and six more studies show inconclusive results.

Table 1 | Summary of the studies assessing functional differences among *DRD4* VNTRs

References	Function	Technique[$]	Main finding
***In vitro* studies**			
Gonzalez et al. (2012)	↓[a]	BRET assay*	The 2- and 4-repeata form functional heterodimers with dopamine D2 receptor short allele (D2s) while 7- repeat/ D2s heterodimers are non- functional
Borroto-Escuela et al. (2011)	↓	BRET assay	The long form of *DRD2* receptor (D2L) forms heterodimers with all 3 main *DRD4* variants but is less active with the 7-repeat
Van Craenenbroeck et al. (2011)	↓	BRET assay	Oligomerization of the 2-, 4- and 7-repeats with different affinities. Folding efficiency regulates *DRD4* biogenesis
Woods (2010)	↓	interaction synthetic peptides	The 7-repeat has more a-helices structure than the 4-repeat. Differential interaction of the 4- and 7- repeats with kinases and phosphatases (second- messenger cascade)
Czermak et al. (2006)	↔[b]	[^{35}S] GTPγS binding assay	Two-fold higher stimulation of G protein binding for the 2-repeat, compared to the 4- and 7-repeats. 7- repeat's structural conformation is more sensitive to sodium concentrations. Similar responses to norepinephrine and epinephrine for 2-, 4- and 7- repeats
Van Craenenbroeck et al. (2005)	↓	immuno-fluorescence	Similar baseline expression levels for the 2-, 4- and 7-repeats. The 2-repeat may be less susceptible to pharmacological chaperone effect and normally more effectively folded than the 4- and 7-repeats
Schoots and Van Tol (2003)	↓	expression in luciferase reporter vectors	The 7-repeat has suppressed expression of reporter genes in vectors, compared to the 2- and 4-repeats, possible differences in RNA stability or translational efficiency
Vallone et al. (2000)	↔	expression levels in cell lines	Different sequence variants of *DRD4* VNTRs have unknown functional role
Kazmi et al. (2000)	•[c]	[^{35}S] GTPγS binding assay	No quantitative differences in G-protein coupling for the 2-, 4- and 7-repeats
Gilliland and Alper (2000)	•	[^{35}S] GTPγS binding assay	Similar function of atypical antipsychotics to the 2- and 7-repeats
Oak et al. (2000)	•	expression levels in cell lines	Number of SH3 binding domains at the polymorphic VNTR region may be responsible for subtle functional differences between the 2-, 4-, 7- and 10-repeats. No biochemical evidence between receptor function and length of VNTRs
Jovanovic et al. (1999)	•	cAMP radio-immunoassay	Slightly more potency for dopamine binding at the 10-repeat, compared to the 2-repeat. No direct association of functionality and length of VNTRs
Oldenhof et al. (1998)	•	[^{35}S] GTPγS binding assay	SH3 binding sites in the third intracellular loop, interacting with adapter proteins. The repeat sequence is not essential for the interaction
Asghari et al. (1995)	↔	cAMP radio-immunoassay/ ligand binding assays	Two-fold decreased potency of dopamine to inhibit cAMP formation for the 7-repeat, compared to the 2-and 4-repeat. Deletion of VNTRs does not affect functionality of *DRD4*

Table 1 | *(Continued)*

References	Function	Technique[$]	Main finding
Asghari et al. (1994)	↔	ligand binding assays	Subtle differences in responses of the 2-, 4- and 7-repeats to pharmacological agonists and antagonists
Lichter et al. (1993)	↔	DNA sequencing & haplotyping	Hypervariable protein with three possibilities: a. alterations in ligand binding, b. variation in signal transduction and c. no functional differences
Van Tol et al. (1991)	↓	ligand binding assays	Differential pharmacological responses of the 2- and 4-repeats and the 7-repeat to clozapine and spiperone
***In vivo* studies**			
Simpson et al. (2010)	•	mRNA expression levels	mRNA levels in 28 human post-mortem brain tissue, no differences between *DRD4* VNTRs and expression levels, weak trend of reduced mRNA levels for the 7-repeat
***In silico* studies**			
Naka et al. (2011)	•	LD structure	The 7-repeat has originated through multiple mutational events and has not been subjected to strong, recent positive selection
Wang et al. (2004)	↓	LD & population analysis	Positive selection of the 7-repeat based on LD patterns
Ding et al. (2002)	↔	allele age calculations	Linkage disequilibrium (LD) pattern of *DRD4* VNTRs, origin of the 7-repeat in upper Paleolithic era (40,000-50,000 years)

Annotations: [a] (↓) the7-repeat variant shows decreased functionality; [b] (↔) study is inconclusive; [c] (•) no effect of *DRD4* VNTRs in functionality

Behavioral/association studies

Forty-six studies published between 1998 and 2014 fulfilled the inclusion criteria and examined main genetic effects of *DRD4* VTNR genotypes and behavioral outcomes in children and adolescents (see Tables 2 to 6 for effect sizes). The studies are ordered according to the groupings of *DRD4* VNTRs. In the majority of the studies (k = 30), carriers of one or more copies of the 7-repeat were compared with non-carriers. There is a large amount of variance among the studies in the age ranges (< 1 to 18 years) and the methods for measurement of behavioral traits (e.g. for executive function, one study used parental questionnaires, two studies used teachers' reports, and eight studies used lab-based tasks).

Table 2 | Overview of association studies examined main effects of *DRD4* VNTRs on externalizing problems in children and adolescents, sorted by genotypic classification (significant associations are reported in bold)

#	Author	Year	Genotype [a]	Cohen's *d*	95 % CI [b] b	N_{total}	N_{risk}	N_{other}	Age (years)	Female (%)	Ethnicity [d]
1	Lavigne et al.	2013	1	0.07	(-0.29, 0.43)	173	37	136	4	44.6	2
2	O'Brien et al. (girls)	2013	1	-0.01	(-0.42, 0.40)	92	42	50	15	100.0	1
3	O'Brien et al. (boys)	2013	1	-0.02	(-0.5, 0.46)	79	24	55	15	0.0	1
4	**Marsman et al.**	**2013**	**1**	**-0.13**	**(-0.23, -0.02)**	**1,451**	**522**	**929**	**11**	**51.0**	**1**
5 [$]	**Hohman et al. (aggression)**	**2009**	**1**	**0.31**	**(0.07, 0.54)**	**298**	**120**	**178**	**15**	**51.7**	**1**
5	Hohman et al. (deliquency)	2009	1	0.16	(-0.07, 0.39)	298	120	178	15	51.7	1
6 [$]	Marino et al. (aggression)	2004	1	0.08	(-0.03, 0.45)	120	43	77	10	19.0	1
6	Marino et al. (deliquency)	2004	1	0.03	(-0.34, 0.41)	120	43	77	10	19.0	1
7	**Nobile et al.**	**2007**	**2**	**0.58**	**(0.09, 1.06)**	**589**	**184**	**405**	**12**	**49.1**	**1**
8	Schimdt et al.	2007	2	0.00	(-0.58, 0.58)	108	40	68	7	NA [e]	NA
9	Propper et al.	2007	2	0.11	(-0.20, 0.42)	169	64	105	2	50	2
10	Bakermans-Kranenburg	2006	2	0.00	(-0.38, 0.38)	47	14	33	< 1	51.1	1
11	Beaver et al.	2007	3	0.02	(-0.12, 0.15)	872	314	558	15	0.0	2
12	Barkley et al.	2006	3	0.00	(-0.49, 0.49)	66	26	40	9	9.0	1
13	Kretschmer et al.	2013	4	0.01	(-0.10, 0.13)	1,151	441	710	16	53.3	1
14	Dmitrieva et al. (girls)	2011	4	-0.26	(-0.74, 0.21)	92	23	69	15.5	100.0	1
15	Dmitrieva et al. (boys)	2011	4	0.40	(-0.06, 0.86)	101	24	77	15.5	0.0	1

Annotations: [a] according to the schema: 1 = 7-repeat carriers vs. non carriers, 2 = (2-5) vs. (6-11)-repeat carriers, 3 = (2-6) vs. (7-11)-repeat carriers and 4 = (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes, [b] 95% confidence intervals for Cohen's *d* estimates, [c] effect sizes were estimated according to risk allele (the "longer" *DRD4* VNTRs carriers according to the genotypic classification schema), [d] according to 1 = mainly Caucasian (≥ 80%), 2 = mixed population (20-80% Caucasian), [e] NA-not available, [$] for studies with the same number indicator, the weighted average of Fisher's Z was estimated from similar constructs (for more details, see Methods) and used in the meta-analysis.

Table 3 | Overview of association studies examined main effects of *DRD4* VNTRs on attention problems in children and adolescents, sorted by genotypic classification (significant associations are reported in bold)

#	Author	Year	Genotype [a]	Cohen's	95 % CI [b]	N_{total}	N_{risk} [c]	N_{other}	Age (years)	Female (%)	Ethnicity [d]
1	Berry et al.	2013	1	-0.02	(-0.20, 0.15)	666	168	498	4.5	49.9	1
2	**Becker et al.**	**2010**	**1**	**0.29**	**(0.06, 0.52)**	**300**	**122**	**178**	**11.5**	**51.7**	**1**
3	Neuman et al.	2007	1	0.00	(-0.16, 0.16)	644	248	396	13	37.3	1
4	Marino et al.	2004	1	0.06	(-0.31, 0.43)	120	43	77	10	19.0	1
5	**Curran et al.**	**2001**	**1**	**0.40**	**(0.13, 0.67)**	**216**	**39**	**177**	**10**	**33.0**	**1**
6	**Schimdt et al.**	**2001**	**2**	**0.36**	**(0.04, 0.67)**	**161**	**64**	**97**	**5.5**	**53.5**	**NA [e]**
7	Barkley et al.	2006	3	0.00	(-0.49, 0.49)	66	26	40	14	9.0	1

Annotations: [a] according to the schema: 1 = 7-repeat carriers vs. non carriers, 2 = (2-5) vs. (6-11)-repeat carriers and 3 = (2-6) vs. (7-11)-repeat carriers, [b] 95% confidence intervals for Cohen's d estimates, [c] effect sizes were estimated according to risk allele (the "longer" *DRD4* VNTRs carriers according to the genotypic classification schema), [d] according to 1 = mainly Caucasian (≥ 80%), [e] NA = not available.

Table 4 | Overview of association studies examined main effects of *DRD4* VNTRs on executive function in children and adolescents, sorted by genotypic classification (significant associations are reported in bold)

#	Author	Year	Genotype [a]	Cohen's	95 % CI [b]	N_{total}	N_{risk} [c]	N_{other}	Age (years)	Female (%)	Ethnicity [d]
1 [$]	Berry et al. (stroop test)	2013	1	0.18	(0.00, 0.36)	666	168	498	4.5	49.9	1
1	Berry et al. (snack delay)	2013	1	0.07	(-0.11, 0.24)	666	168	498	4.5	49.9	1
1	Berry et al. (snack delay time)	2013	1	-0.05	(-0.22, 0.13)	666	168	498	4.5	49.9	1
1	Berry et al. (commission errors)	2013	1	-0.21	(-0.38, -0.03)	666	168	498	4.5	49.9	1
1	**Berry et al. (ommission errors)**	**2013**	**1**	**-0.16**	**(-0.34, 0.01)**	**666**	**168**	**498**	**4.5**	**49.9**	**1**
2 [$]	Kegel et al. (stroop test)	2013	1	-0.22	(-0.54, 0.10)	159	59	100	7	NA[e]	NA
2	Kegel et al. (digid span)	2013	1	-0.29	(-0.61, 0.03)	159	59	100	7	NA	NA
2	**Kegel et al. (backword digit)**	**2013**	**1**	**-0.44**	**(-0.76, -0.11)**	**159**	**59**	**100**	**7**	**NA**	**NA**
2	**Kegel et al. (EF)**	**2013**	**1**	**-0.42**	**(-0.74, -0.09)**	**159**	**59**	**100**	**7**	**NA**	**NA**
3	Altink et al. (neurocognition)	2012	1	0.00	(-0.41, 0.41)	94	40	54	11.5	45.6	2
4 [$]	Gilsbach et al. (IC reaction time)	2012	1	-0.06	(-0.85, 0.73)	26	10	16	12	34.6	1
4	Gilsbach et al. (IC task error time)	2012	1	0.11	(-0.68, 0.90)	26	10	16	12	34.6	1
4	Gilsbach et al. (TT reaction time)	2012	1	-0.11	(-0.90, 0.67)	26	10	16	12	34.6	1
4	Gilsbach et al. (TT task error time)	2012	1	-0.09	(-0.88, 0.70)	26	10	16	12	34.6	1
5	Johnson et al. (sustained attention)	2008	1	0.00	(-0.52, 0.52)	60	15	45	11.5	NA	NA
6 [$]	Froehlich et al. (spatial memory/total)	2007	1	0.15	(-0.15, 0.47)	176	60	116	6	49	2
6	Froehlich et al. (spatial memory/tasks)	2007	1	0.15	(-0.16, 0.5)	176	60	116	6	49	2
6	Froehlich et al. (rules/stages)	2007	1	-0.04	(-0.35, 0.28)	176	60	116	6	49	2
6	Froehlich et al. (rules/total)	2007	1	0.02	(-0.29, 0.34)	176	60	116	6	49	2
6	Froehlich et al. (spatial span/length)	2007	1	0.10	(-0.22, 0.41)	176	60	116	6	49	2
6	Froehlich et al. (spatioan span/total)	2007	1	0.03	(-0.28, 0.34)	176	60	116	6	49	2
6	Froehlich et al. (planning/problems)	2007	1	-0.06	(-0.37, 0.25)	176	60	116	6	49	2

Table 4 | *(Continued)*

#	Author	Year	Genotype [a]	Cohen's	95 % CI [b]	N_{total}	N_{risk} [c]	N_{other}	Age (years)	Female (%)	Ethnicity [d]
6	Froehlich et al. (planning/time)	2007	1	0.08	(-0.24, 0.39)	176	60	116	6	49	2
7	**Auerbach et al. (info processing)**	**2001**	**1**	**-0.61**	**(-1.16, -0.07)**	**61**	**20**	**41**	**1**	**48.7**	**1**
8	Propper et al. (EF/girls)	2012	2	-0.25	(-0.75, 0.25)	81	21	60	7	100.0	2
9	**Propper et al. (EF/boys)**	**2012**	**2**	**0.84**	**(-1.35, -0.33)**	**85**	**21**	**64**	**7**	**0.0**	**2**
10	**Lackner et al. (EF)**	**2012**	**2**	**-0.54**	**(-1.03, -0.06)**	**71**	**28**	**43**	**4**	**47.9**	**1**
11	**Barkley et al. (EF)**	**2006**	**3**	**0.84**	**(-1.35, -0.33)**	**66**	**26**	**40**	**9**	**9.0**	**1**

Table 5 | Overview of association studies examined main effects of *DRD4* VNTRs on social/emotional development in children and adolescents, sorted by genotypic classification (significant associations are reported in bold).

#	Author	Year	Genotype [a]	Cohen's *d*	95 % CI [b]	N_{total}	N_{risk}	N_{other}	Age (years)	Female (%)	Ethnicity [d]
1 [$]	Knafo et al. (slef-initiated)	2011	1	-0.12	(-0.41, 0.16)	211	71	140	4	NA[e]	NA
1	**Kanfo et al. (compliance)**	**2011**	**1**	**0.40**	**(0.11, 0.69)**	**211**	**71**	**140**	**4**	**NA**	**NA**
1	Kanfo et al. (mother-rated)	2011	1	0.13	(-0.15, 0.42)	211	71	140	4	NA	NA
2	Bakermans-Kranenburg et al.	2011	1	0.32	(-0.17, 0.80)	91	22	69	7	52.7	1
3 [$]	Luijk et al. (attachment security 1)	2011	1	0.10	(-0.08, 0.29)	512	176	336	1.5	48.3	1
3	Luijk et al. (attachment disorganization 1)	2011	1	0.10	(-0.08, 0.29)	512	176	336	1.5	48.3	1
4 [$]	Luijk et al. (attachment security 2)	2011	1	0.12	(-0.10, 0.34)	478	101	377	1	45.5	1
4	Luijk et al.(attachment disorganziation 2)	2011	1	-0.19	(-0.03, 0.41)	478	101	377	1	45.5	1
5 [$]	Spangler et al. (attachment security)	2009	1	0.10	(-0.30, 0.52)	95	24	71	1	50.0	1
5	Spangler et al. (attachment disorganization)	2009	1	-0.21	(-0.20, 0.64)	95	24	71	1	50.0	1
6	van IJzendoorn et al. (att.disorganization)	2006	1	0.39	(-0.11, 0.89)	63	14	49	< 1	46.0	1
7	Bakermans-Kranenburg et al.	2004	1	0.17	(-0.17, 0.51)	132	55	77	1	NA	1
8 [$]	**Marino et al. (withdrawn)**	**2004**	**1**	**0.45**	**(0.07, 0.82)**	**120**	**43**	**77**	**10**	**19.0**	**1**
8	Marino et al. (social problems)	2004	1	0.35	(-0.03, 0.73)	120	43	77	10	19.0	1
9	**Lakatos et al. (att.disorganization)**	**2000**	**1**	**0.63**	**(0.17, 1.09)**	**90**	**33**	**57**	**1**	**39.6**	**1**
10	Rubin et al. (withdrawn)	2013	2	-0.05	(-0.34, 0.24)	394	95	299	11	44.2	1
11	Propper et al. (withdrawn/girls)	2012	2	0.23	(-0.27, 0.73)	81	21	60	7	100.0	2
12	**Propper et al. (withdrawn/boys)**	**2012**	**2**	**-0.69**	**(-1.2, -0.18)**	**85**	**21**	**64**	**7**	**0.0**	**2**
13	**Lackner et al. (theory of mind)**	**2012**	**2**	**-0.70**	**(-1.18, -0.20)**	**71**	**28**	**43**	**4**	**47.9**	**1**
14	**Daigle et al. (victimization)**	**2010**	**3**	**0.13**	**(0.11, 0.15)**	**949**	**325**	**624**	**16**	**NA**	**2**

Annotations: [a] according to the schema: 1 = 7-repeat carriers vs. non carriers, 2 = (2-5) vs. (6-11)-repeat carriers, and 3 = (2-6) vs. (7-11)-repeat carriers, [b] 95% confidence intervals for Cohen's *d* estimates, [a,c] effect sizes were estimated according to risk allele (the "longer" *DRD4* VNTRs carriers according to the genotypic classification schema), [d] according to 1=mainly Caucasian (≥ 80%), 2 = mixed population (20-80% Caucasian), [e] NA = not available, [$] for studies with the same number indicator, the weighted average of Fisher's Z was estimated from similar constructs (for more details, see Methods) and used in the meta-analysis.

Table 6 | Overview of association studies examined main effects of *DRD4* VNTRs on "reactive" temperament in children and adolescents, sorted by genotypic classification (significant associations are reported in bold).

#	Author (phenotype dimension)	Year	Genotype [a]	Cohen's *d*	95 % CI [b]	N_{total}	N_{risk} [c]	N_{other}	Age (years)	Female (%)	Ethnicity [d]
1	Smith et al. (inhibition)	2013	1	-0.06	(-0.27, 0.15)	407	134	273	3	52.0	1
2	Perez-Edgar et al. (inhibition)	2013	1	0.03	(-0.42, 0.48)	78	32	46	16	52.6	1
3 [$]	Nederhof et al. [excitement seeking (self)/boys]	2011	1	0.08	(-0.08, 0.24)	609	247	362	16	52.5	1
3	Nederhof et al. (excitement seeking (mother)/boys]	2011	1	0.04	(-0.12, 0.20)	609	247	362	16	52.5	1
3	Nederhof et al. (excitement seeking (father)/boys]	2011	1	-0.14	(-0.30, 0.02)	609	247	362	16	52.5	1
3	Nederhof et al. (high intesity pleasure/boys)	2011	1	0.06	(-0.10, 0.22)	609	247	362	16	52.5	1
4 [$]	Nederhof et al. [excitement seeking (self)/girls]	2011	1	0.14	(-0.02, 0.30)	673	232	441	16	52.5	1
4	Nederhof et al. (excitement seeking (mother)/girls]	2011	1	0.15	(-0.01, 0.30)	673	232	441	16	52.5	1
4	Nederhof et al. (excitement seeking (father)/girls]	2011	1	0.14	(-0.01, 0.30)	673	232	441	16	52.5	1
4	Nederhof et al. (high intesity pleasure/girls)	2011	1	0.02	(-0.13, 0.18)	673	232	441	16	52.5	1
5[$]	Holmboe et al. (surgency/extraversion)	2011	1	0.29	(-0.15, 0.74)	90	28	62	< 1	54.4	1
5	Holmboe et al. (orientation/regulation)	2011	1	0.29	(-0.15, 0.74)	90	28	62	< 1	54.4	1
5	**Holmboe et al. (negative effect)**	**2011**	**1**	**0.59**	**(0.13, 1.04)**	**90**	**28**	**62**	**< 1**	**54.4**	**1**
6.a [$]	Ivorra et al. (range of state)	2011	1	0.00	(-0.37, 0.37)	117	39	78	< 1	48.7	1
6.a	Ivorra et al. (regulations of state)	2011	1	0.00	(-0.37, 0.37)	117	39	78	< 1	48.7	1
6.b [$]	Ivorra et al. (range of state)	2011	3	0.00	(-0.37, 0.37	117	40	77	< 1	48.7	1
6.b	Ivorra et al. (regulations of state)	2011	3	0.00	(-0.37, 0.37)	117	40	77	< 1	48.7	1
6.c [$]	Ivorra et al. (reange of state)	2011	4	0.00	(-0.44, 0.44)	81	30	51	< 1	48.7	1
6.c	Ivorra et al. (regulations of state)	2011	4	0.00	(-0.44, 0.44)	81	30	51	< 1	48.7	1
7 [$]	Sheese et al. (sensation seeking)	2007	1	0.10	(-0.53, 0.73)	45	14	31	1.5	NA [e]	NA

Table 6 | *(Continued)*

#	Author (phenotype dimension)	Year	Genotype [a]	Cohen's *d*	95 % CI [b]	N_{total}	N_{risk} [c]	N_{other}	Age (years)	Female (%)	Ethnicity [d]
7	Sheese et al. (effortful control)	2007	1	0.06	(-0.57, 0.70)	45	14	31	1.5	NA	NA
8.a [$]	Lakatos et al. (latency to play)	2003	1	0.14	(-0.30, 0.57)	84	34	50	1	39.6	1
8.a	Lakatos et al. (anxiety to mother)	2003	1	-0.06	(-0.49, 0.36)	84	34	50	1	39.6	1
8.a	Lakatos et al. (latency to smile)	2003	1	0.17	(-0.25, 0.60)	84	34	50	1	39.6	1
8.a	Lakatos et al. (latency to accept toy)	2003	1	-0.12	(-0.55, 0.30)	84	34	50	1	39.6	1
8.a	Lakatos et al. (anxiety to stranger)	2003	1	-0.07	(-0.59, 0.45)	84	34	50	1	39.6	1
8.a	Lakatos et al. (interactionstranger)	2003	1	0.14	(-0.38, 0.66)	84	34	50	1	39.6	1
8.b [$]	Lakatos et al. (latency to play)	2003	4	0.46	(-0.09, 1.00)	55	22	33	1	39.6	1
8.b	Lakatos et al. (anxiety to mother)	2003	4	0.07	(-0.45, 0.59)	55	22	33	1	39.6	1
8.b	Lakatos et al. (latency to smile)	2003	4	0.20	(-0.32, 0.72)	55	22	33	1	39.6	1
8.b	Lakatos et al. (latency to accept toy)	2003	4	-0.15	(-0.67, 0.37)	55	22	33	1	39.6	1
8.b	Lakatos et al. (anxiety to stranger)	2003	4	-0.07	(-0.59, 0.45)	55	22	33	1	39.6	1
8.b	Lakatos et al. (interaction stranger)	2003	4	0.14	(-0.38, 0.66)	55	22	33	1	39.6	1
9	Arbelle et al. (shyness)	2003	1	-0.21	(-0.67, 0.26)	99	23	76	7.5	33.0	1
10	Noble et al. (novelty seeking)	1998	1	0.37	(0.00, 0.75)	119	45	74	12.0	0.0	1
11.a [$]	Ebstein et al. (range of state)	1998	1	0.28	(-0.22, 0.79)	81	28	53	< 1	51.2	NA
11.a	**Ebstein et al. (regulation of state)**	**1998**	**1**	**0.61**	**(0.14, 1.08)**	**81**	**28**	**53**	**< 1**	**51.2**	**NA**
11.b [$]	Ebstein et al. (range of state)	1998	2	0.37	(-0.10, 0.83)	81	28	53	< 1	51.2	NA
11.b	Ebstein et al. (regulation of state)	1998	2	0.47	(0.00, 0.93)	81	28	53	< 1	51.2	NA
11.c [$]	Ebstein et al. (range of state)	1998	4	0.30	(-0.22, 0.82)	64	21	43	< 1	51.2	NA
11.c	**Ebstein et al. (regulation of state)**	**1998**	**4**	**0.64**	**(0.11, 1.17)**	**64**	**21**	**43**	**< 1**	**51.2**	**NA**
12 [$]	Dmitrieva et al. (temper/girls)	2011	4	-0.06	(-0.53, 0.41)	92	23	69	15.5	100.0	1

Table 6 | *(Continued)*

#	Author (phenotype dimension)	Year	Genotype [a]	Cohen's *d*	95 % CI [b]	N_{total}	N_{risk} [c]	N_{other}	Age (years)	Female (%)	Ethnicity [d]
12	Dmitrieva et al. (thrill seeking/girls)	2011	4	0.04	(-0.43, 0.51)	92	23	69	15.5	100.0	1
13 [$]	**Dmitrieva et al. (temper/boys)**	**2011**	**4**	**0.62**	**(0.15, 1.08)**	**101**	**24**	**77**	**15.5**	**0.0**	**1**
13	**Dmitrieva et al. (thrill seeking/boys)**	**2011**	**4**	**0.72**	**(0.26, 1.19)**	**101**	**24**	**77**	**15.5**	**0.0**	**1**
14	Lee et al. (novelty seeking)	2003	5	0.37	(-0.22, 0.95)	243	31	212	14	71.1	3

Annotations: [a] according to the schema: 1 = 7-repeat carriers vs. non carriers, 2 = (2-5) vs. (6-11)-repeat carriers, 3 = (2-6) vs. (7-11)-repeat carriers, 4 = (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes and 5 = (2-4) vs.(5-11)-repeat carriers; [b] 95% confidence intervals for Cohen's *d* estimates; [c] effect sizes were estimated according to risk allele (the "longer" *DRD4* VNTRs carriers according to the genotypic classification schema), [d] according to 1 = mainly Caucasian (≥ 80%), 2 = mixed population (20-80% Caucasian), 3 = mainly non-Caucasian (< 20% Caucasians) [e] NA = not available; [$] for studies with the same number indicator, the weighted average of Fisher's Z was estimated from similar constructs (for more details, see Methods) and used in the meta-analysis.

Meta-analyses

Table 7 summarizes the meta-analytical results on the five broadly-defined behavioral outcomes. The meta-analysis of all studies on externalizing problems (k = 15, N = 5,408) showed no significant association with *DRD4* VNTRs. Similarly, no significant effect size was found for the association between *DRD4* VNTRs and attention problems in population-based studies (k = 7, N = 2,173), although the Q- statistic indicates significant heterogeneity among the studies. Neither were significant results obtained for the association of *DRD4* VNTRs with "reactive" temperament (k = 14, N = 3,048).

For executive function, meta-analysis of the total set of studies (k = 11, N = 1,545) suggested a significant association with *DRD4* VNTRs [Cohen's d = -0.21, 95% CI (- 0.40,-0.02), p = .04), indicating that "longer" DRD4 repeats were associated with lower levels of executive function in children and adolescents. However, there was significant heterogeneity among the studies (Q-*statistic* = 26.46, p < .01) and the estimates were attenuated in the meta-analysis of the 7-repeat carriers vs. non-carriers (k = 7, N = 1,242).

For social/emotional development, a significant combined effect size [Cohen's d = - 0.16, 95% CI (-0.27, -0.05), p = .04) was found in the total set of studies (k = 14, N = 3,372). When restricted to the studies of (2-5) vs. (6-11)-repeat carriers (k = 4, N = 631), the association remained significant, with an effect of Cohen's d = -0.38 [95% CI (-0.73,-0.03), p = .03], indicating that longer *DRD4* VNTRs were associated with less optimal social/ emotional outcomes. The meta-analysis of the most common classification (7-repeat carriers vs. non-carriers) indicated similar direction of the effect, but non-significant [Cohen's d = -0.12, 95% CI (-0.28, 0.03), p = .12]. In all meta-analyses of social/emotional development, the Q-statistic indicated significant heterogeneity among the studies (Q-*statistic* = 29.67, p < .01).

Table 7 | Combined effects of *DRD4* VNTRs for all studies and moderating role of genotypic classification

Outcome	*k* [a]	N [b]	Cohen's *d* [c]	95% CI [d]	*p*	Heterogeneity *Q* [e]
(a) Externalizing problems						
All studies	15	5,408	0.02	(-0.06, 0.10)	.52	18.29
Studies with 7-repeat carriers vs. non-carriers	6	2,213	-0.02	(-0.14, 0.10)	.88	6.28
Studies with (2-5) vs. (6-11)-repeat carriers	4	913	0.16	(-0.08, 0.39)	.20	3.94
(b) Attention problems						
All studies	7	2,173	0.16	(-0.03, 0.34)	.09	**13.52***
Studies with 7-repeat carriers vs. non-carriers	5	1,946	0.13	(-0.03, 0.30)	.12	**10.80***
(c) Executive function						
All studies	**11**	**1,545**	**-0.21**	**(-0.40, -0.02)**	**.04**	**26.46****
Studies with 7-repeat carriers vs. non-carriers	7	1,242	-0.08	(-0.27, 0.11)	.41	11.82
(d) Social/emotional development						
All studies	**14**	**3,372**	**-0.16**	**(-0.27, -0.05)**	**.04**	**29.67****
Studies with 7-repeat carriers vs. non-carriers	9	1,792	-0.12	(-0.28, 0.03)	.12	**17.40***
Studies with (2-5) vs. (6-11)-repeat carriers	**4**	**631**	**-0.38**	**(-0.73, -0.03)**	**.03**	**7.91***
(e) "Reactive" temperament						
All studies [f]	14	3,048	0.07	(-0.04, 0.19)	.22	21.54
Studies with 7-repeat carriers vs. non-carriers	11	2,402	0.03	(-0.07, 0.13)	.55	11.83
Studies with (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes	5	393	0.06	(-0.32, 0.45)	.77	**14.08****

Annotations: [a] number of studies; [b] total sample size; [c] combined effect size; [d] 95% confidence interval around the point estimate of the effect size; [e] heterogeneity *Q*-statistic, [f] for "reactive" temperament, studies which report multiple genotype classifications participate in the total meta-analysis only with the 7-repeat carriers vs. non-carriers classification (see Methods), * $p < .05$, ** $p < .01$

Discussion

One of the strongest arguments in favor of candidate gene studies is that the selection of genes would be based on biological and thus functional foreknowledge, maximizing the likelihood of finding a real association. In this review, we showed that this presumed advantage does not always hold up, as even for the *DRD4* VNTR – a highly variable and widely-used polymorphism in behavioral research on children and adolescents – there remains significant ambiguity about the functionality of alleles and

allele groupings. Subsequently, selection of appropriate categories for use in association analyses is difficult, and gives rise to a literature containing multiple classification approaches, an overall lack of consistent findings, and no ready way of comparing, grouping, or meta-analyzing individual findings across studies.

In this study, we identified 21 studies that examine the functional properties of *DRD4* VNTRs, of which eight report reduced functionality of the 7-repeat allele, seven report no functional differences of *DRD4* VNTRs, and another six are inconclusive. Thus although there is some evidence of structural differences among the most common alleles (i.e. the 2-repeat, the 4-repeat and the 7-repeat), there is no certainty that these differences result in biological, functional effects. Since most of the 21 studies were performed in highly controlled, experimental conditions (e.g., in cell lines, in solutions with defined salt concentrations), we can only speculate that any differences or absence of differences between *DRD4* variants holds true in the living human organism. Meta-analysis is an important tool to summarize evidence from different studies, but in biological sciences (e.g., molecular biology and biochemistry) specific methodological problems hinder meta-analytical efforts [26]. The use of different approaches (e.g. pharmacological, biochemical, and *in silico*) and measurements (e.g. molecular, physiological, and structural) introduce heterogeneity that cannot be resolved with current meta-analytical methods. The heterogeneity of the experimental conditions, the difficulty in obtaining an estimate of the size of the "sample" used in "*in vivo*" and "*in silico*" studies, and the inability to estimate directionality of the functional studies hamper a quantitative combination of these studies. Our study highlights the need for more biological studies, using comparable research methods, to demonstrate the functionality of rare and common *DRD4* VNTRs with the ultimate aim of pooling them to examine the robustness of the results.

Second, we systematically reviewed the literature for association studies of *DRD4* VNTRs and five broadly-defined behavioral outcomes in non-clinical samples of children and adolescents. We analyzed all relevant studies using a meta-analytical approach. Our results did not support the association between *DRD4* VNTRs and attention problems in childhood and adolescence, as has been previously reported in other meta-analytical efforts in adults [27,28]. The main reason for this discrepancy is likely to be that the previous meta-analyses have focused on case-controls studies and clinical cases of ADHD. In our study, the association of *DRD4* VNTRs was tested against normative differences of attention in population-based samples of children. Neurodevelopmental disorders, such as ADHD, can be best viewed as the extreme of a continuum with underlying genetic variation throughout the population [29]. However, genetic research indicates that the extremes of quantitative traits (i.e. ADHD cases), can differ from the rest of the distribution (i.e. normal variation in attention), a phenomenon referred to as genetic discontinuity [30]. Thus, it is possible that the relative contribution of *DRD4* VNTRs is larger in clinical cases of ADHD, with a greater genetic burden than population-based samples [31].

Similarly, our meta-analyses on externalizing problems and "reactive" temperament yielded no significant associations with any *DRD4* VNTRs classification. However, the overall meta-analysis of *DRD4* VNTRs and executive function indicated that "longer" variants are associated with poorer

executive functioning (i.e. lower sustained attention and planning). This association was attenuated in the meta- analysis of the studies that used the most common genotypic classification (7-repeat carriers vs. non-carriers) and heterogeneity in this set of studies remained significant. The restricted number of studies did not allow us to examine the effect of other potential moderators (i.e. age, sex, ethnicity, methods of measurement executive function) that could explain this heterogeneity.

Furthermore, the meta-analysis of the total set of studies on social/emotional development identified a significant effect as well, indicating that longer variants are associated with less optimal social and emotional development (i.e. prosocial behavior and secure attachment). Secure attachment, empathy, and prosocial behavior are considered related constructs leading to positive social/ emotional development [32], and potentially with an underlying neurobiological basis [33]. When we used the genotypic classification as a moderator, we identified that the association was mainly driven by the "short" vs. "long" classification [(2-5) vs. (6-11)-repeat carriers] and was attenuated in the most common classification (7-repeat carriers vs. non-carriers). In the first classification [(2-5 vs. 6-11)-repeat carriers], genotypes are classified according to length of the repeat, whereas in the second classification (7-repeat carriers vs. non-carriers), any number of length combinations are grouped together based solely on the presence of absence of the 7-repeat variant. One explanation could be that there are true biological differences of the rare DRD4 variants, which are better captured by the "short" vs. "long" classification. Another possibility is that the studies using this classification are significantly different from the studies included in the most common classification in a number of other ways, including the age, sex, and ethnicity of participants. However, the small number of studies did not allow us to formally test for these factors as potential moderators. Finally, the possibility of a chance finding cannot be ruled out.

Our systematic approach to the behavioral research in children and adolescents indicated that *DRD4* VNTR alleles are categorized according to multiple rationales, including the number of repeats, empirical results from previous association studies, geographic and population differences in allele frequencies, and other undefined approaches. In the limited number of population-based studies on child behavior, we identified five classification systems and although some of the classifications are more common (the 7-repeat carriers vs. non-carriers is the most commonly used classification), evidence supporting one classification over the other is inconclusive or lacking. We suggest results might differ depending on the initial choice of genotype groupings, which carries with it potential for bias if careful *a priori* hypotheses and groupings are not established.

A limitation of the available studies as summarized in this meta-analysis is that the effect sizes were rather heterogeneous for many of the outcomes. The use of alternative genotyping classifications of *DRD4* VNTRs, and/ or other potential sample moderators (i.e. age, sex and ethnicity) could be responsible for this heterogeneity. The role of these potential moderators can only be formally investigated when more studies are available, but we demonstrated that the use of various genotyping classifications by different research groups hinders meta-analytical efforts, and may even mask true developmental effects. For example, a growing body of research indicates that the effect of genes on behavior may change over time [34,35]. However, meta-analytical approaches to *DRD4* VNTRs and

behavior problems in childhood cannot be conducted without adequately addressing the problem of alternative genotyping classifications. Our study explicitly shows the consequences of this problem. Finally, although the moderating effect of other study characteristics could not be tested in this study, our systematic review and meta-analyses extend previous work by indicating how the use of alternative genotyping classifications affect associations of *DRD4* VNTRs with behavioral outcomes in children [15,36].

In summary, this systematic review indicates that it is not yet possible to draw definitive conclusions about the functionality of *DRD4* VNTRs polymorphisms. Despite the wealth of studies, we appear to remain in the discovery phase of behavioral research employing DRD4 VNTRs. A moratorium should be declared on the singling out of the 7-repeat – the "Magnificent Seven" – at least until the application of multidisciplinary principles shed light on the functionality of the different variants. We propose that until that time researchers analyze and present *all* classifications of *DRD4* VNTRs, even when the individual study sample size would not permit formal testing of contrasts and associations. This will ensure full transparency and eventually allow for the more powerful meta-analytic identification of patterns that might be currently masked by widely varying approaches to classification.

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SECTION B

The genome-wide approach

4

SNP heritability of Behavior Problems in Childhood: Genome-Wide Complex Trait Analysis

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Journal of the American Academy of Child & Adolescent Psychiatry, 2015, 54(9) :737- 744.

Abstract

Genetic factors contribute to individual differences in behavior problems. In children, genome-wide association studies (GWAS) have yielded the first suggestive results, when aiming to identify genetic variants which explain heritability, but the proportion of genetic variance that can be attributed to common single nucleotide polymorphisms (SNPs) remains to be determined, as only a few studies have estimated SNP heritability, with diverging results. Genomic-relationship-matrix restricted maximum likelihood (GREML) as implemented in the software Genome-wide Complex Trait Analysis (GCTA) was used to estimate SNP heritability (SNP h^2) for multiple phenotypes within four broad domains of children's behavioral problems (attention deficit/ hyperactivity symptoms, internalizing, externalizing, and pervasive developmental problems) and cognitive function. We combined phenotype and genotype data from two independent, population-based *Dutch* cohorts, yielding a total N = 1,495 to 3,175 of 3, 7 and 9-year-old children. Significant SNP heritability estimates were found for attention deficit/ hyperactivity symptoms (SNP h^2 =0.37- 0.71), externalizing problems (SNP h^2 = 0.44), and total problems (SNP h^2 = 0.18), rated by mother or teacher. Sensitivity analyses with exclusion of extreme cases and quantile normalization of the phenotype data decreased SNP h^2 as expected under genetic inheritance, but they remained statistically significant for most phenotypes. We provide evidence of the influence of common SNPs on child behavior problems in an ethnically homogenous sample. These results support the continuation of large GWAS collaborative efforts, to unravel the genetic basis of complex child behaviors.

Introduction

Complex behaviors are shaped by both genetic and environmental influences [1,2], and numerous twin, family and adoption studies have estimated significant contributions of genetic factors to individual differences in behavioral and psychiatric traits [3-5]. In addition, longitudinal population-based studies provide evidence of the genetic stability of common behavioral problems (e.g. anxiety and depression symptoms [6], attention problems [7]) across the lifespan, with higher heritability estimates in childhood (e.g. for attention problems, heritability estimates decreased from 0.70 in childhood to 0.40 in adulthood [7]).

In adult samples, Genome-Wide Association Studies (GWAS) identified genes and pathways related to complex traits [8,9]. This approach has also yielded positive findings in studies of important traits in children (e.g., birth weight [10] and length [11]). For childhood psychiatric traits and problem behaviors, successes have been limited [12-15], which can probably be ascribed to the very modest sample sizes in these studies [16]. The relatively small or absent genetic associations with complex traits of interest in GWAS [12-15] may seem in contrast to the large heritability estimates from twin and family studies, but are indeed in line with recent evidence that the small effect sizes of individual SNPs may be responsible for the non- replicability of these associations [17]. To assess whether GWA studies of child behavior problems can be expected to yield important findings regarding biological pathways, we address the question what part of the heritability of childhood behavior problems is captured by common (minor allele frequency > 1%) single-nucleotide polymorphisms (SNPs) included in standard genotyping arrays.

The genetic variance explained by genome-wide SNPs [18] can be estimated by using the genetic similarity among unrelated individuals as a predictor of their phenotypic resemblance. When individual level genotype data are available, these can be used to obtain a measure of genetic similarity between all possible pairs of (unrelated) individuals in the study. In a second step, this genetic relatedness matrix (GRM) is used to predict the phenotype similarity between individuals just as the different similarity of monozygotic (MZ) and dizygotic (DZ) twin pairs predicts their different phenotype resemblances. This approach has been implemented in the software package Genome-wide Complex Trait Analysis (GCTA) [18]. The heritability estimates from GCTA (SNP h^2) are commonly considered an indicator of the upper limit of the variance that can be explained by current GWAS efforts. Power estimations have indicated that for quantitative traits, a sample size of 3,000 individuals is required to detect a SNP h^2 of 0.30 with 80% power [19]. Thus, large sample sizes are required to reliably estimate the SNP heritability of complex behavioral traits, which can imply the need to pool data from multiple studies.

To date, few SNP heritability estimates are available for behavioral problems in childhood. Some studies indicate substantial additive genetic heritability of normative differences in children's social communication difficulties [20] and in clinical cases of Attention Deficit/Hyperactivity Disorder (ADHD) [21,22], and childhood onset Obsessive-Compulsive Disorder (OCD) [23]. However, other studies indicate modest, statistically non-significant SNP heritability estimates for children's

internalizing problems [12], anxiety [24], and callous-unemotional (CU) traits [13] in population-based samples. A study from the Twins Early Development Study (TEDS) indicated no significant SNP heritability for parent-, teacher-, and self-reported behavioral problems (i.e. attention problems, internalizing and externalizing problems) in contrast to cognitive and anthropomorphic traits in a population-based sample (N = 2,500) of 12-year-old children [25].

Here we focus on four domains of children's behavioral problems: attention deficit problems, externalizing, internalizing , and pervasive developmental problems. Genetic influences on non-verbal cognitive abilities were also estimated. To obtain sufficient power, we combined genotype and phenotype data from two independent, population-based Dutch cohorts: the Generation R Study (GEN-R) and the Netherlands Twin Register (NTR). Genotyped SNP data from both studies were used to construct a genetic relatedness matrix (GRM) (Fedko et al, unpublished manuscript). For both studies, behavior problems of a total N = 1,495 to 3,175 of 3, 7 and 9 year old-children were rated by mothers and/or teachers. We estimated the SNP heritability in each of these traits and we compared our findings to the SNP heritability estimates previously reported.

Methods

Participants

This study included data from children from two population-based Dutch cohorts, the Generation R Study (GEN-R) and the Netherlands Twin Register (NTR). GEN-R is a prospective cohort based in Rotterdam. The characteristics of the study have been previously described in detail [26]. NTR is a nationwide longitudinal sample of twins and their family members followed from birth onwards after voluntary registration [27]. In both studies parents gave informed consent for participation and also to approach teachers of the children. Study protocols were approved by the local ethics committees.

Measures

All phenotypes analyzed in this study have been described in detail in previous publications of GEN-R and NTR and twin-based heritabilities in the Dutch population were reported for these traits (see Supplementary Material, Table S1).

Conners' Parent Rating Scale (CPRS-R; ADHD-related symptoms)

ADHD symptoms and related co-morbid symptoms were assessed using the CPRS-R [28] completed by the mothers. Four scales of the CPRS-R were used: (i) ADHD combined, (ii) ADHD Inattentive, (iii) ADHD Hyperactive-Impulsive, and (iv) Oppositional Defiant Disorder (ODD) scale.

Child Behavior Checklist (CBCL; behavior problems)

We assessed child behavior problems using the well-validated Child Behavior Checklist (CBCL) [29], completed by the mother. Internalizing, externalizing and total problems were assessed using

the appropriate CBCL syndrome scales. For the CBCL internalizing, externalizing, and total problems scores, the GEN-R study used the CBCL for ages 1½-5 [30] and NTR used the CBCL for ages 6-18 years [31]. In the CBCL for ages 1½-5 years, the Internalizing scale consists of four scales (Emotionally Reactive, Anxious/ Depressed, Somatic Complaints, and Withdrawn) and the Externalizing scale consists of two scales (Attention Problems and Aggressive Behavior). In the CBCL for ages 6-18 years, the Internalizing scale consists of three scales (Anxious/ Depressed, Withdrawn/ Depressed and Somatic Complaints) and the Externalizing scale consists of two scales (Rule-Breaking Behavior and Aggressive Behavior). The Total Problems score is computed by summing the ratings of all problem items included in the CBCL. To avoid phenotypic heterogeneity in the combined data set due to differences in the items between the two CBCL versions, we selected only overlapping items to compute the scores (see Supplementary Material, Table S2).

Child Behavior Checklist (CBCL; pervasive developmental problems)

We assessed pervasive developmental problems using the Pervasive Developmental Disorder (PDD) subscale of the CBCL 1½-5 years [30]. The PDD subscale has been shown to be a valid screening tool for autism spectrum disorders (ASD) [32].

Teacher's Rating Form (TRF; ADHD-related symptoms and behavior problems)

The TRF for ages 6 to 18 years [31] was used to assess attention problems (Attention Problems scale) and behavioral problems (Externalizing scale), rated by the teacher. We used the teachers' ratings of externalizing and not internalizing problems, since it has been previously shown that they can better identify children with externalizing than internalizing problems [33]. The teacher reports were also selected to assess behavior in a different environment, and to avoid informant effects which could bias estimates of genetics contribution to common child behavior problems [34,35].

Non-verbal cognitive abilities

Non-verbal cognitive abilities were assessed with the Snijder-Oomen nonverbal intelligence test [36] (SON-R 2.5-7 years) in the GEN-R study and the non-verbal subtest of the Revised Amsterdam Children Intelligence Test [37] (RAKIT) in the NTR. Both measurements are well-validated and correlate substantially with the Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R) [38] and the Wechsler Intelligence Scale for Children (WISC) [39]. The non-verbal cognition scores in both samples were transformed to *Mean* = 100 and *SD* = 15.

Genotyping and Imputation

3,102 Caucasian children from the GEN-R study and 2,826 Caucasian children from NTR were genotyped on Illumina (660W, 610K) and Affymetrix 6.0, platforms respectively. Because the number of overlapping SNPs between platforms was small ($N \sim 120K$) both cohorts were cross-platform imputed using MaCH-Admix imputation software [40] as described in Fedko et al. (unpublished manuscript). Cross-platform imputation supplies all participants from both cohorts with genetic

information from all SNPs genotyped on both platforms. To avoid population stratification between samples due to genotyping platform, the Genome of the Netherlands reference set [41] was used to phase and subsequently impute missing genotypes into both cohorts. The final data set consisted of 5,928 individuals where each individual had information for N = 989,757 SNPs expressed in dosage scores. Post-imputation quality control (QC) was performed to imputed data sets to check and control for possible residual imputation stratification due to genotyping platform or true genetic differences between cohorts. The overall imputation quality measure (R^2) was high (*mean* = 0.97, *median* = 0.99). Case-control analysis of the imputed sample, where GEN-R children were assigned as cases and NTR children as controls showed n = 4,340 SNPs that were significantly different in frequency ($p < 10^{-5}$). These SNPs were excluded from further analysis.

Genome-Wide Complex Trait Analysis (GCTA)

We built a genetic relationship matrix (GRM) based on cross-platform imputed data using GCTA version 1.20 [42]. Data for the GRM was filtered based on the following two criteria: 1) $R^2 > 0.8$ to allow SNPs with high imputation quality; 2) MAF > 0.01 to exclude SNPs with low minor allele frequency. We performed Principal Components Analysis (PCA) on the resulting GRM to check for possible residual stratification due to genotyping platform. We used the GREML (Genomic-relatedness- matrix restricted maximum likelihood) method to estimate SNP heritability in distantly related individuals from all genotyped and imputed SNPs in the data set. The convention excludes those subjects whose genetic relatedness exceeds the 0.025 threshold in GRM, which corresponds to third-fourth degree cousins. We applied such a cut-off while performing GREML analysis and one of each pair of closely related individuals was excluded from analysis resulting in N range from 1,495 to 3,175 depending on phenotype (see Supplementary Material, Table S4). For all phenotypes we included age and sex as covariates. We also adjusted for the cohort of origin (GEN-R or NTR) to control for residual imputation stratification due to genotyping platform, true genetic differences, and possible phenotype differences.

Statistical Analyses

In both GEN-R and NTR non-response analysis indicated no differences in the baseline characteristics of children whose assessment of child behavior problems was not completed at ages 7 and 9 years. As these are longitudinal studies, families are permitted to miss participation for a particular survey, allowing them to participate again in later surveys (27). To explore the effect of extreme cases, often found in ratings of children's behavior problems, we winsorized phenotypes used in this study when it was required. If the corresponding absolute z-score was more than 3.29 for a phenotype, we replaced the raw score with the less extreme value, i.e. with the next highest score plus one unit [43]. Additionally we checked for possible population stratification in combined dataset, adjusting for 10 principal components (PCs) in analysis of each scale.

Sensitivity analyses were also performed to explore the influence of 1) exclusion of extreme cases, 2) skewness on SNP-heritability estimates, and 3) the influence of study of origin. First, we estimated

SNP-heritability by excluding extreme scores below or above three standard deviations from the mean. If such cases represent extremes due to e.g. measurement errors, we expect SNP heritability to increase after exclusion of these cases. If, however, they represent genuine outliers, we expect them to be also outliers for heritable traits, and consequently the SNP heritability will decrease after exclusion of these cases. Second, we transformed the data to the quantile normalized scale, using the Van der Waerden transformation. This transformation reduces the extreme influence that outliers could have by ranking them as low or high within a normal distribution [44], although it results in some loss of phenotypic information. In addition, we performed GCTA separately on the two participating studies (i.e. GEN-R and NTR), to explore possible effects of the specific study. All transformations were conducted in SPSS 21.0 [45].

Results

Genotypic and Phenotypic Sample Characteristics

The sample characteristics of the children participating in each study and in the combined dataset, before and after exclusion of related individuals, are presented in Supplementary Material (Table S3 and S4). The distribution of age, sex, and behavior problems did not significantly differ between the two studies.

Estimates of SNP heritability

Table 1 summarizes the SNP heritability estimates using the combined GRM, adjusting for age, sex, and sample of origin. For the mother ratings of child problem behavior, estimates were substantial and statistically significant for the ADHD Combined scale (SNP h^2 = 0.40, SE = .14, p = .01), as well as for the ADHD Inattentive scale (SNP h^2=0.37, SE=.14, p=.003) and the Hyperactive-Impulsive scale (SNP h^2 = 0.45, SE = .14, p = .0006) measured by the Conners' Parent Rating Scale (CPRS). We also found significant SNP heritability estimates for the CBCL Total problems score (SNP h^2 = 0.18, SE = .10, p = .03). For the teacher ratings, we obtained significant SNP heritability estimates for both the Attention problems scale (SNP h^2 = 0.71, SE = .22, p = .0006) and the Externalizing scale (SNP h^2 = 0.44, SE = .22, p = .03).No significant estimates were found for the CPRS ODD scale, the CBCL PDD subscale, the CBCL Internalizing and Externalizing scales and the non-verbal cognition. When 10 PCs were used as covariates, we found non-significant difference in SNP heritability estimates (1-3% drop or 1-2% increase, data not shown) for all scales. Level of significance remains the same, except for CBCL Total problems score (*p-value* = 0.07 and *p-value* = 0.03 with and without PCs adjustment accordingly).

Table 1 | SNP heritability estimates in child behavior problems. All analyses were performed with the combined GRM and adjusted for age, sex, and sample of origin (GEN-R or NTR) on winsorized scores

	Age (*SD*)	SNP h^2	SE	95% CI*	*N*	*p-value*
Parent ratings						
CPRS ADHD Combined scale	8.34 (0.7)	0.40	0.14	(0.13, 0.67)	2,262	< 0.01**
CPRS ADHD Inattentive scale	8.34 (0.7)	0.37	0.14	(0.10, 0.64)	2,264	< 0.01**
CPRS Hyperactive-Impulsive scale	8.34 (0.7)	0.45	0.14	(0.18, 0.72)	2,260	< 0.001***
CPRS ODD scale	8.34 (0.7)	0.20	0.14	(0.00, 0.47)	2,262	0.07
CBCL Internalizing scale	6.57 (0.83)	0.12	0.10	(0.00, 0.32)	3,175	0.11
CBCL Externalizing scale	6.57 (0.83)	0.12	0.10	(0.00, 0.32)	3,174	0.13
CBCL Total problems score	6.57 (0.83)	0.18	0.10	(0.00, 0.38)	3,175	< 0.05*
CBCL PDD subscale	3.15 (0.23)	0.16	0.11	(0.00, 0.33)	3,015	0.07
Teacher ratings						
TRF Attention problems scale	6.82 (2.35)	0.71	0.22	(0.28, 1.00)	1,495	< 0.001***
TRF Externalizing scale	6.82 (2.35)	0.44	0.22	(0.01, 0.87)	1,495	< 0.05*
Observational ratings						
Non-verbal cognition	6.14 (0.42)	0.11	0.16	(0.00, 0.42)	1,974	0.23

Note: CPRS = Conners' Parent Rating Scale; ADHD = Attention Deficit/ Hyperactivity Disorder; ODD = Oppositional Defiant Disorder; CBCL = Child Behavior Checklist; PDD = Pervasive Developmental Disorder; TRF = Teacher's Rating Form; *SE* = standard error; *N* = final sample size; *SD* = standard deviation

Sensitivity Analyses

To examine the influence of extreme cases on SNP heritability estimates and to enable comparison with the TEDS results [25] that were based on deletion of extreme cases, we also performed GCTA analyses excluding individuals above or below three standard deviations from the mean. Overall, the exclusion of extreme cases decreased the SNP heritability estimates almost by half in most of the complex problem behaviors, suggesting that these children are genuine outliers and that their extreme phenotype values do not represent measurement errors or other artifacts. Even after removal of outliers, SNP heritability estimates for all scales of CPRS (ADHD combined, ADHD Inattentive, ADHD Hyperactive-Impulsive and ODD scale) were still substantial. The CBCL Total problems score and the teacher-reported Attention problems and Externalizing problems also remained significant after exclusion of extreme cases. The results are summarized in Table 2.

Table 2 | Impact of extreme cases on SNP heritability in child behavior problems. In all analyses, statistical outliers (mean ±3 sd) were excluded. All analyses were performed with the combined GRM and adjusted for age, sex, and sample of origin (GEN-R or NTR).

	Age (*SD*)	SNP h^2	SE	95% CI*	*N*	*p-value*
Parent ratings						
CPRS ADHD Combined scale	8.34 (0.7)	0.22	0.14	(0.00, 0.49)	2,240	0.05*
CPRS ADHD Inattentive scale	8.34 (0.7)	0.24	0.14	(0.00, 0.51)	2,229	< 0.05*
CPRS Hyperactive-Impulsive scale	8.34 (0.7)	0.33	0.15	(0.04, 0.62)	2,231	0.01*
CPRS ODD scale	8.34 (0.7)	0.28	0.14	(0.01, 0.55)	2,246	< 0.05*
CBCL Internalizing scale	6.57 (0.83)	0.04	0.10	(0.00, 0.24)	3,139	0.36
CBCL Externalizing scale	6.57 (0.83)	0.06	0.10	(0.00, 0.26)	3,136	0.28
CBCL Total problems score	6.57 (0.83)	0.16	0.10	(0.00, 0.36)	3,143	0.05*
CBCL PDD subscale	3.15 (0.23)	0.14	0.11	(0.00, 0.36)	2,999	0.10
Teacher ratings						
TRF Attention problems scale	6.82 (2.35)	0.49	0.22	(0.06, 0.92)	1,470	0.01*
TRF Externalizing scale	6.82 (2.35)	0.46	0.23	(0.01, 0.91)	1,463	< 0.05*
Observational ratings						
Non-verbal cognition	6.14 (0.42)	0.11	0.16	(0.00, 0.42)	1,968	.23

Note: CPRS = Conners' Parent Rating Scale; ADHD = Attention Deficit/ Hyperactivity Disorder; ODD = Oppositional Defiant Disorder; CBCL = Child Behavior Checklist; PDD = Pervasive Developmental Disorder; TRF = Teacher's Rating Form; *SE* = standard error; *N* = final sample size; *SD* = standard deviation.

Furthermore, similar to the TEDS study [25], we performed GCTA analyses on the quantile normalized scales, using the Van der Waerden transformation to examine the potential influence of skewness on SNP heritability estimates. The SNP heritability with the transformed scales were similar to those with untransformed scales, with substantial genetic effects contributing to the ADHD Combined scale (SNP h^2 = 0.30, SE = .14, p = .01), ADHD Inattentive scale (SNP h^2 = 0.30, SE = .14, p = .01), and Hyperactivity-Impulsive scale (SNP h^2 = 0.37, SE = .14, p = .004) rated by the mother using the CPRS. Also, the CBCL PDD subscale (SNP h^2 = 0.18, SE = .11, p = .05) and the teacher-reported Attention problems scale (SNP h^2 = 0.64, SE = .22, p = .002) and Externalizing scale (SNP h^2 = 0.60, SE = .22, p = .004) yielded significant SNP heritability estimates. The results are summarized in Supplementary Table S5.

Finally, we also provide SNP heritability estimates of the two samples independently. As expected, the smaller NTR sample shows estimates with larger SE values. Although variable, the SNP heritability estimates of the two samples did not differ significantly from each other. The results are summarized in Supplementary Table S6.

Discussion

The aim of this study was to provide estimates of SNP heritability of normative differences in attention deficit problems (measured at 7 and 9 years), externalizing and internalizing problems (measured at 7 years), pervasive developmental problems (measured at 3 years), and non-verbal cognitive function (measured at 7 years) in population-based samples. Our study provides evidence of significant SNP heritability for attention deficit/ hyperactivity problems, externalizing and total problems, rated by mother or teacher. We identified non-significant SNP heritability estimates for pervasive developmental and internalizing problems. These results are parallel to twin heritabilities previously reported on the same phenotypes, i.e. higher twin heritabilities were associated with higher SNP heritabilities. Sensitivity analyses showed that SNP heritability estimates decreased but remained significant for most phenotypes after exclusion of the extreme cases.

Previous studies on the heritability captured by common SNPs have yielded significant SNP heritability estimates for normative differences in autistic-like traits [20,46], and in clinical cases of childhood-onset OCD [23], and ADHD [21,22]. Quantifiable, although non-significant SNP heritability has also been reported for internalizing problems in population-based samples of preschoolers [12]. However, a recent TEDS study by Trzaskowski et al. [25] indicated no additive genetic effects for common child behavior problems. This discrepancy may be due to several factors. First, there are methodological differences between the two studies. In the present study we removed ethnic outliers instead of correcting for them by principal components. Moreover, we estimated heritability with and without extreme cases, showing that in some cases, treatment of outliers results in substantially different findings. Extreme cases might be biologically significant extremes or they might constitute statistical outliers. Our results indicate that extremes were more likely to be genetic extremes rather than statistical outliers and they suggest that in the TEDS study [25] the exclusion of extreme cases may have resulted in an underestimation of SNP heritability [47]. Winsorizing the extreme cases instead of excluding them may address the problem of extremely skewed distributions while still retaining information for all subjects. It should be noted, however, that even after exclusion of the extreme cases, we found significant additive genetic heritability for ADHD-related symptoms and children's behavior problems.

Secondly, the two studies involve different samples. The TEDS sample [25] involved 12-year-old children, whereas in our sample we analyzed data on behavior problems at 3, 7 and 9 years. Estimations of genetic effects may differ developmentally, although the direction depends on the phenotype of interest. For example, SNP heritability of autistic-like traits was shown to be low (SNP h^2 = 0.24, SE = .07, N = 5,204) but strongest in early childhood [20], whereas it increased from ages 7-to 12-years in the case of general cognitive ability [48]. Previous twin studies have also indicated an increase in the heritability for general cognitive ability [49], as well as for non-verbal IQ [50] from childhood to adulthood. In line with the low heritability estimates in 5 to 6-year-old twins for non-verbal IQ, we found no significant SNP heritability of non-verbal cognitive ability at 6 years, in a subsample of N = 1,974 unrelated individuals. Similarly, our study indicated non-significant

SNP heritability of pervasive developmental problems in 3-year old children (*N* = 3,015). Given the low overall heritability, larger samples may be needed to estimate modest SNP heritability of non-verbal cognition and pervasive developmental problems in early childhood. The perception of genetic heritability as time/age-dependent [51] could explain discrepancies between samples and between measurements at different time- points (e.g. CBCL measures at 7 years and CPRS measures at 9 years), and suggests that SNP heritability estimates cannot be easily generalized across age.

Thirdly, SNP heritability, as an estimation of the fraction of phenotypic variation explained by common SNPs, is dependent of sample characteristics [52]. Thus, as a population property, SNP heritability estimates can differ between samples, both because the GRM differs between populations and because environmental factors are different. Environmental influences may play a more important role in a sample derived from multiple, culturally diverse sites in the UK, while genetic effects would be more prominent in the geographically restricted and rather homogeneous Dutch society, in terms of socio-economic conditions. Parental reports of child problem behaviors might partly be determined by subjective criteria for what parents consider to be problem behavior and these criteria may be dependent on cultural norms or socio-economic circumstances such as crowding [53].

In this study, we found non-significant SNP heritability for parent-reported internalizing problems in 7-year-old children. One reason for this finding could be the difficulty in assessing internalizing symptoms in early childhood. Internalizing symptoms are often not overtly expressed in young children and thus not easily observed by the parents [54]. Another reason could be that since the prevalence of internalizing symptoms typically increases in middle-to-late adolescence [55,56], we are not yet able to identify all children who will develop internalizing symptoms later in life. The particularly high heterogeneity and the distinctive genetic architecture of internalizing problems have also been addressed in previous work [57].

A limitation of this study is the sample size. GCTA power calculations indicate that even with large sample sizes, the SEs of the SNP heritability estimates are large [58]. Thus, even larger samples are needed to estimate modest additive genetic effects. However, the sample size of the current study is for the most phenotypes comparable to the study of Trzaskowski et al. [25], indicating that sample size is not exclusively responsible for the discrepancies between studies. An inherent parameter to most behavior problems research, is the skewed distribution of the phenotypes. Nevertheless, sensitivity analyses with transformed distributions and winsorized extreme cases did not reduce the significant SNP heritability estimates to non- significance. This study is based on data from two longitudinal studies (GEN-R and NTR). Systematic attrition is a limitation inherent to longitudinal studies [59], potentially leading to selective dropout of high risk individuals, and thus to underestimation of the heritability of common behavior problems in children. However, previous research has shown that psychopathology of the participants has a small to moderate effect on attrition rates [60,61] and estimations from longitudinal studies are robust and generalizable [62,63]. Finally, the results of this study are derived from population-based samples of children. Although it is has been shown that additive effects of hundreds of SNPs are responsible for observed normal variation in most quantitative traits [64], it is possible that the genetic architecture of children diagnosed with severe

behavior problems differs from that of children in population-based samples (e.g. increased role of rare variants, and *de novo* mutations).

In summary, this study provides molecular genetic evidence of additive genetic influences on specific child behavior problems in an ethnically and socio-economically homogeneous sample. SNP heritability for other common behavior problems in children, or for the P factor as proposed by Caspi et al. (2014) [65], remains to be estimated. SNP heritability estimates may be influenced by diversity in socio-economic environment, developmental stage, and study design arguing for approaches that model GxE interactions, developmental information and possibly data from population-based and clinical samples in GCTA research. Our results provide support for and encourage the continuation of GWAS efforts by genetics consortia focusing on complex behavioral traits in search of elusive heritability.

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Supplementary Material

Table S1 | Twin-based heritability estimates in NTR for the phenotypes analyzed in this study (attentional deficit problems, externalizing problems, internalizing problems, and autistic-like traits), as previously published. When both mother and father reported estimates were available, the mother ratings were selected.

Phenotypes	Age (years)	*N* twin pairs	Estimations	Reference
Parent ratings				
CPRS ADHD Combined scale	7	1,595	0.78	[1]
CPRS ADHD Inattentive scale	9	3,470	0.38-0.78	[2]
CPRS Hyperactive-Impulsive scale	9	3,470	0.72-0.80	[2]
CPRS ODD scale	7	1,595	0.55	[3]
CBCL Internalizing scale	7	1,940	0.38	[4]
CBCL Externalizing scale	7	1,940	0.52	[4]
CBCL Total problems score	3	1,358	0.38	[5]
CBCL PDD subscale	NA	NA	NA	NA
Teacher ratings				
TRF Attention problems scale	7	2,259	0.39	[6]
TRF Externalizing scale	5	215	0.43	[7]
Observational ratings				
Non-verbal cognition	5	237	0.31	[8]

Note: CPRS = Conners' Parent Rating Scale; ADHD = Attention Deficit/ Hyperactivity Disorder; ODD = Oppositional Defiant Disorder; CBCL = Child Behavior Checklist; PDD = Pervasive Developmental Disorder; TRF =Teacher's Rating Form; NA = not available.

Table S2 | List of common CBCL 1½-5 years and CBCL 6-18 years items used to asses children's behavior problems in the two participating studies (GEN-R and NTR).

Scale	Common items in CBCL 1½- 5 and CBCL 6-18
Internalizing	
	– sulks a lot
	– worries
	– nervous, tense
	– self-conscious or easily embarrassed
	– too fearful or anxious
	– unhappy, sad or depressed
	– aches or pains (without medical cause)
	– constipated, doesn't move bowels
	– headaches (without medical cause)
	– nausea, feels sick (without medical cause)
	– stomachaches or cramps (without medical cause)
	– vomiting, throwing up (without medical cause)
	– withdrawn, doesn't get involved with others
Externalizing	
	– wanders away
	– destroys things belonging to his/ her family or other children
	– disobedient
	– doesn't seem to feel guilty after misbehaving
	– gets in many fights
	– hits others
	– physically attacks people
	– screams a lot
	– stubborn, sullen or irritable
	– sudden changes in mood or feelings
	– wants a lot of attention
	– temper tantrums or hot temper
Total problems	
	– aches or pains (without medical cause)
	– acts too young for age
	– can't concentrate, can't pay attention for long
	– can't sit still, restless or hyperactive
	– clings to adults or too dependent
	– constipated, doesn't move bowels
	– cries a lot
	– cruel to animals
	– destroys his/ her own things

Table S2 | *(Continued)*

Scale	Common items in CBCL 1½-5 and CBCL 6-18
Total problems	
	– destroys things belonging to his/her family or other children
	– disobedient
	– doesn't eat well
	– doesn't get along with other children
	– doesn't seem to feel guilty after misbehaving
	– easily jealous
	– fears certain animals, situations or places
	– gets hurt a lot, accident-prone
	– gets in many fights
	– headaches (without medical cause)
	– hits others
	– nausea, feel sick (without medical cause)
	– nervous movements or twitching
	– nervous, or tense
	– nightmares
	– overeating
	– overtired
	– physically attacks people
	– picks node, skin or other parts of body plays with own sex parts too much poorly coordinated or clumsy
	– problems with eyes (without medical cause)
	– rashes or other skin problems (without medical cause) screams a lot
	– self-conscious or easily embarrassed
	– shows little interest in things around him/ her too shy or timid
	– sleeps less than most kids during day and/or night speech problems
	– stares into space or seems preoccupied stomachaches or cramps (without medical cause) strange behavior
	– stubborn, sullen or irritable
	– sudden changed in mood or feelings sulks a lot
	– talks or cries out in sleep temper tantrums or hot temper too fearful or anxious
	– underactive, slow moving or lacks energy unhappy, sad or depressed
	– unusually loud
	– vomiting, throwing up (without medical cause) wakes up often at night
	– wanders away
	– wants a lot of attention whining
	– withdrawn, doesn't get involved with others
	– worries

Table S3 | Descriptive statistics for the distribution of common children's problem behavior, in the two genotyped samples and the combined dataset.

	GEN-R	NTR	Combined
CPRS ADHD Combined scale			
N	1,971	854	2,825
age (*sd*)	8.15 (0.21)	9.03 (1.24)	8.41 (0.81)
sex (% girls)	49	54	51
mean(*sd*)	7.27 (6.70)	8.16 (8.01)	7,53 (7.13)
age (*sd*)	8.15 (0.21)	9.03 (1.24)	8.41 (0.81)
sex (% girls)	49	54	51
mean(*sd*)	2.08 (2.71)	3.00 (3.76)	2.36 (3.10)
skewness (*se*)	1.91 (0.06)	1.67 (0.08)	1.94 (0.05)
kurtosis (*se*)	4.10 (0.11)	2.54 (0.17)	4.12 (0.09)
CPRS ODD scale			
N	1,973	856	2,829
age (*sd*)	8.15 (0.21)	9.03 (1.24)	8.41 (0.81)
sex (% girls)	49	54	51
mean(*sd*)	3.47 (2.87)	4.63 (3.80)	3.82 (3.22)
skewness (*se*)	1.15 (0.06)	1.05 (0.08)	1.23 (0.05)
kurtosis (*se*)	1.57 (0.11)	0.81 (0.17)	1.70 (0.09)
CBCL PDD problems scale			
N	2,030	2,084	4,144
age (*sd*)	3.04 (0.09)	3.32 (0.26)	3.18 (0.24)
sex (% girls)	49	54	51
mean(*sd*)	1.71 (1.89)	3.26 (2.86)	2.49 (2.55)
skewness (*se*)	1.70 (0.05)	1.17 (0.05)	1.50 (0.04)
kurtosis (*se*)	4.76 (0.11)	1.53 (0.11)	2.86 (0.08)
CBCL Internalizing scale			
N	2,175	2,126	4,301
age (*sd*)	5.99 (0.38)	7.49 (0.44)	6.74 (0.85)
sex (% girls)	49	54	52
mean(*sd*)	1.44 (1.94)	2.17 (2.33)	1.80 (2.17)
skewness (*se*)	2.15 (0.05)	1.77 (0.05)	1,94 (0.04)
kurtosis (*se*)	6.35 (0.11)	4.48 (0.11)	5.29 (0.08)
CBCL Externalizing scale			
N	2,174	2,126	4,300
age (*sd*)	5.99 (0.38)	7.49 (0.44)	6.74 (0.85)
sex (% girls)	49	54	52
mean(*sd*)	2.43 (2.85)	3.49 (3.55)	2.95 (3.26)
skewness (*se*)	2.15 (0.05)	1.48 (0.05)	1.78 (0.04)
kurtosis (*se*)	6.45 (0.11)	2.49 (0.11)	3.93 (0.08)

Table S3 | *(Continued)*

	GEN-R	NTR	Combined
CBCL Total problems score			
N	2,175	2,127	4,302
age (*sd*)	5.99 (0.38)	7.49 (0.44)	6.74 (0.85)
sex (% girls)	49	54	52
mean(*sd*)	9.15 (8.51)	12.31 (10.06)	10.71 (9.44)
skewness (*se*)	2.04 (0.05)	1.44 (0.053)	1.70 (0.04)
kurtosis (*se*)	6.71 (0.11)	2.70 (0.11)	4.12 (0.08)
TRF Attention problems scale			
N	1,419	358	1,777
age (*sd*)	6.72 (2.42)	7.32 (0.42)	6.83 (2.18)
sex (% girls)	47	48	47
mean(*sd*)	3.03 (4.71)	5.69 (6.32)	3.56 (5.19)
skewness (*se*)	2.17 (0.07)	1.52 (0.13)	2.02 (0.06)
kurtosis (*se*)	4.84 (0.13)	2.44 (0.26)	4.37 (0.12)
TRF Externalizing scale			
N	1,419	358	1,777
age (*sd*)	6.72 (2.42)	7.32 (0.42)	6.83 (2.18)
sex (% girls)	47	48	47
mean(*sd*)	2.39 (5.12)	4.75 (7.38)	2.86 (5.73)
skewness (*se*)	3.55 (0.07)	2.43 (0.13)	3.24 (0.06)
kurtosis (*se*)	15.25 (0.13)	6.59 (0.26)	12.52 (0.12)
Non-verbal cognition			
N	2,059	202	2,261
age (*sd*)	6.09 (0.39)	6.73 (0.33)	6.15 (0.43)
sex (% girls)	50	56	51
mean(*sd*)	100 (15)	100 (15)	100 (15)
skewness (*se*)	-0.16	-0.15	-0.16
kurtosis (*se*)	0.39 (0.11)	0.26 (0.44)	0.35 (0.10)

Note: CPRS = Conners' Parent Rating Scale; ADHD = Attention Deficit/ Hyperactivity Disorder; ODD = Oppositional Defiant Disorder; CBCL = Child Behavior Checklist; PDD = Pervasive Developmental Disorder; TRF = Teacher's Rating Form

Table S4 | Descriptive statistics for the distribution of common children's problem behavior, in the two genotyped samples and the combined dataset after applying cut-off 0.025 of relatedness to combined sample.

	GENR	NTR	Combined
CPRS ADHD Combined scale			
N	1,797	465	2,262
age *(sd)*	8.15 (0.22)	9.05 (1.24)	8.34 (0.7)
sex (% girls)	49	57	51
mean *(sd)*	7.27 (6.7)	8.23 (8.11)	7.47 (7.02)
skewness *(se)*	1.33 (0.06)	1.3 (0.11)	1.35 (0.05)
kurtosis *(se)*	1.66 (0.12)	1.27 (0.23)	1.72 (0.1)
CPRS ADHD Inattentive scale			
N	1,799	465	2,264
age *(sd)*	8.15 (0.22)	9.05 (1.24)	8.34 (0.7)
sex (% girls)	49	56	51
mean *(sd)*	3.08 (3.6)	3.89 (4.38)	3.25 (3.78)
skewness *(se)*	1.59 (0.06)	1.32 (0.11)	1.55 (0.05)
kurtosis *(se)*	2.48 (0.12)	1.12 (0.23)	2.21 (0.1)
CPRS Hyperactive-Impulsive scale			
N	1,798	462	2,260
age *(sd)*	8.15 (0.22)	9.05 (1.24)	8.33 (0.7)
sex (% girls)	49	57	51
mean *(sd)*	2.09 (2.74)	2.97 (3.77)	2.27 (3.00)
skewness *(se)*	1.92 (0.06)	1.76 (0.11)	1.99 (0.05)
kurtosis *(se)*	4.13 (0.12)	3.02 (0.23)	4.49 (0.1)
CPRS ODD scale			
N	1,798	464	2,262
age *(sd)*	8.15 (0.22)	9.05 (1.24)	8.34 (0.7)
sex (% girls)	49	56	51
mean *(sd)*	3.46 (2.86)	4.74 (3.91)	3.73 (3.15)
skewness *(se)*	1.13 (0.06)	1.1 (0.11)	1.25 (0.05)
kurtosis *(se)*	1.51 (0.12)	0.92 (0.23)	1.91 (0.1)
CBCL PDD problems scale			
N	1,823	1,192	3,015
age *(sd)*	3.04 (0.1)	3.32 (0.26)	3.15 (0.23)
sex (% girls)	49	54	51
mean *(sd)*	1.73 (1.9)	3.29 (2.87)	2.35 (2.45)
skewness *(se)*	1.67 (0.06)	1.17 (0.07)	1.57 (0.04)
kurtosis *(se)*	4.65 (0.11)	1.54 (0.14)	3.3 (0.09)

Table S4 | *(Continued)*

	GENR	NTR	Combined
CBCL Internalizing scale			
N	1,977	1,198	3,175
age *(sd)*	6.01 (0.38)	7.5 (0.44)	6.57 (0.83)
sex (% girls)	49	54	51
mean *(sd)*	1.46 (1.97)	2.23 (2.36)	1.75 (2.16)
skewness *(se)*	2.16 (0.06)	1.78 (0.07)	1.99 (0.04)
kurtosis *(se)*	6.34 (0.11)	4.62 (0.14)	5.57 (0.09)
CBCL Externalizing scale			
N	1,976	1,198	3,174
age *(sd)*	6.01 (0.38)	7.5 (0.44)	6.57 (0.83)
sex (% girls)	49	54	51
mean *(sd)*	2.46 (2.88)	3.5 (3.57)	2.86 (3.2)
skewness *(se)*	2.17 (0.06)	1.51 (0.07)	1.88 (0.04)
kurtosis *(se)*	6.56 (0.11)	2.7 (0.14)	4.57 (0.09)
CBCL Total problems score			
N	1,977	1,198	3,175
age *(sd)*	6.01 (0.38)	7.5(0.44)	6.57 (0.83)
sex (% girls)	49	54	51
mean *(sd)*	9.28 (8.6)	12.39 (10.2)	10.46 (9.36)
skewness *(se)*	2.05 (0.06)	1.52 (0.07)	1.81 (0.04)
kurtosis *(se)*	6.78 (0.11)	3.00 (0.14)	4.81 (0.09)
TRF Attention problems scale			
N	1,295	200	1,495
age *(sd)*	6.74 (2.51)	7.3 (0.43)	6.82 (2.35)
sex (% girls)	47	52	48
mean *(sd)*	3.05 (4.73)	5.9 (6.65)	3.43 (5.12)
skewness *(se)*	2.17 (0.07)	1.54 (0.17)	2.1 (0.06)
kurtosis *(se)*	4.89 (0.14)	2.68 (0.34)	4.86 (0.13)
TRF Externalizing scale			
N	1,295	200	1,495
age *(sd)*	6.74 (2.51)	7.3 (0.43)	6.82 (2.35)
sex (% girls)	47	52	48
mean *(sd)*	2.41 (5.13)	5.2 (8.01)	2.78 (5.68)
skewness *(se)*	3.52 (0.07)	2.28 (0.17)	3.31 (0.06)
kurtosis *(se)*	15.1 (0.14)	5.4 (0.34)	12.94 (0.13)

Table S4 | *(Continued)*

	GENR	NTR	Combined
Non-verbal cognition			
N	1,856	118	1,974
age *(sd)*	6.1 (0.4)	6.72 (0.35)	6.14 (0.42)
sex (% girls)	51	54	51
mean *(sd)*	99.85 (15.04)	100.26 (14.96)	99.87 (15.03)
skewness *(se)*	-0.12 (0.06)	-0.14 (0.22)	-0.12 (0.06)
kurtosis *(se)*	0.32 (0.11)	0.26 (0.44)	0.31 (0.11)

Note: CPRS = Conners' Parent Rating Scale; ADHD = Attention Deficit/ Hyperactivity Disorder; ODD = Oppositional Defiant Disorder; CBCL = Child Behavior Checklist; PDD = Pervasive Developmental Disorder; TRF = Teacher's Rating Form

Table S5 | Impact of skewness on GCTA estimations in child behavior problems. All scales have been normalized using the Van der Waerden method. All analyses were performed with the combined GRM and adjusted for age, sex, and sample of origin (GEN-R or NTR).

	SNP h^2	*SE*	95% CI*	*N*	*P*-value
Parent ratings					
CPRS ADHD Combined scale	0.30	0.14	(0.03, 0.57)	2,262	0.01*
CPRS ADHD Inattentive scale	0.30	0.14	(0.03, 0.57)	2,264	0.01*
CPRS Hyperactive-Impulsive scale	0.37	0.14	(0.10, 0.64)	2,260	< 0.01**
CPRS ODD scale	0.19	0.14	(0.00, 0.46)	2,262	0.09
CBCL Internalizing scale	0.07	0.10	(0.00, 0.27)	3,175	0.26
CBCL Externalizing scale	0.12	0.10	(0.00, 0.32)	3,174	0.13
CBCL Total problems score	0.13	0.10	(0.00, 0.33)	3,175	0.10
CBCL PDD subscale	0.18	0.11	(0.00, 0.40)	3,015	0.05*
Teacher ratings					
TRF Attention problems scale	0.64	0.22	(0.21, 1.00)	1,495	< 0.01**
TRF Externalizing scale	0.60	0.22	(0.17, 1.00)	1,495	< 0.01**
Observational ratings					
Non-verbal cognition	0.11	0.16	(0.00, 0.42)	1,974	0.23

Note: CPRS = Conners' Parent Rating Scale; ADHD = Attention Deficit/ Hyperactivity Disorder; ODD = Oppositional Defiant Disorder; CBCL = Child Behavior Checklist; PDD = Pervasive Developmental Disorder; TRF = Teacher's Rating Form; *SE* = standard error; *N* = final sample size

Table S6 | Comparison of SNP heritability estimates in child behavior problems, in the two participating samples (GEN- R and NTR). All analyses are adjusted for age and sex.

	GEN-R sample					NTR sample				
	SNP h^2	*SE*	95% CI*	*N*	*p-value*	SNP h^2	*SE*	95% CI*	*N*	p-value
Parent ratings										
CPRS ADHD Combined scale	0.38	0.18	(0.03, 0.73)	1,798	0.01*	0.11	0.70	(0.00, 1.00)	477	0.44
CPRS ADHD Inattentive scale	0.36	0.17	(0.03, 0.69)	1,800	0.01*	0.01	0.70	(0.00, 1.00)	477	0.49
CPRS Hyperactive-Impulsive scale	0.52	0.18	(0.17, 0.87)	1,799	< 0.01**	0.00	0.70	(0.00, 1.00)	474	0.50
CPRS ODD scale	0.33	0.18	(0.00, 0.68)	1,799	< 0.05*	0.10	0.66	(0.00, 1.00)	476	0.44
CBCL Internalizing scale	0.33	0.17	(0.00, 0.66)	1,979	< 0.05*	0.21	0.26	(0.00, 0.72)	1,223	0.20
CBCL Externalizing scale	0.23	0.17	(0.00, 0.56)	1,978	0.08	0.00	0.26	(0.00, 0.51)	1,223	0.50
CBCL Total problems score	0.35	0.17	(0.02, 0.68)	1,979	< 0.05*	0.29	0.27	(0.00, 0.82)	1,223	0.13
CBCL PDD scale	0.29	0.18	(0.00, 0.64)	1,824	0.06	0.00	0.27	(0.00, 0.53)	1,217	0.50
Teacher ratings										
TRF Attention problems scale	0.73	0.25	(0.24, 1.00)	1,296	0.001**	.00	1.53	(0.00, 1.00)	203	0.50
TRF Externalizing scale	0.59	0.25	(0.10, 1.00)	1,296	< 0.01**	.00	1.50	(0.00, 1.00)	203	0.50
Observational ratings										
Non-verbal cognition	0.14	0.17	(0.00, 0.47)	1,856	0.19	.00	2.80	(0.00, 1.00)	122	0.50

Note: CPRS = Conners' Parent Rating Scale; ADHD = Attention Deficit/ Hyperactivity Disorder; ODD = Oppositional Defiant Disorder; CBCL = Child Behavior Checklist; PDD = Pervasive Developmental Disorder; TRF = Teacher's Rating Form; *SE* = standard error; *N* = final sample size.

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A Genome-wide Approach to Childhood Aggressive Behavior. The EAGLE Consortium

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American Journal Medical Genetics Part B: Neuropsychiatric Genetics, 2015 (ahead of print)

Abstract

Individual differences in aggressive behavior emerge in early childhood and predict persisting behavioral problems and disorders. Studies of antisocial and severe aggression in adulthood indicate substantial underlying biology. However, little attention has been given to genome-wide approaches of aggressive behavior in children. We analyzed data from nine population-based studies and assessed aggressive behavior using well-validated parent-reported questionnaires. This is the largest sample exploring children's aggressive behavior to date (N = 18,988), with measures in two developmental stages (N = 15,668 early childhood and N = 16,311 middle childhood/ early adolescence). First we estimated the additive genetic variance of children's aggressive behavior based on genome-wide SNP information, using genome-wide complex trait analysis (GCTA). Second, genetic associations within each study were assessed using a quasi-Poisson regression approach, capturing the highly right-skewed distribution of aggressive behavior. Third, we performed meta-analyses of genome-wide associations for both the total age-mixed sample and the two developmental stages. Finally, we performed a gene-based test using the summary statistics of the total sample. GCTA quantified variance tagged by common SNPs (10- 54%). The meta-analysis of the total sample identified one region in chromosome 2 (2p12) at near genome-wide significance (top SNP rs11126630, p = 5.30e-08). The separate meta-analyses of the two developmental stages revealed suggestive evidence of association at the same locus. The gene-based analysis indicated association of variation within AVPR1A with aggressive behavior. We conclude that common variants at 2p12 show suggestive evidence for association with childhood aggression. Replication of these initial findings is needed, and further studies should clarify its biological meaning.

Introduction

Aggressive behavior in childhood is often an antecedent to violent behavior in adolescence [1]. According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), childhood aggressive behavior is a criterion for disruptive behavior disorders such as oppositional defiant disorder (ODD) and conduct disorder (CD) [2]. Aggressive behavior is also implicated in neurodevelopmental disorders such as attention deficit hyperactivity disorder (ADHD) [3], autism spectrum disorder (ASD) [4], dysregulated behavior [5], substance use disorders [6], antisocial personality disorder [7], and schizophrenia [8] later in life. The above supports a cross- disorder prevalence, and to our knowledge this study is the first large-scale, genome-wide association study aiming to identify the specific factors that underlie the neurobiology of aggressive behavior in children.

Aggressive behavior emerges as early as infancy [9]. It typically increases during the first years of life and decreases before school entrance [10,11]. However, in population-based samples children exhibit heterogeneity in levels of aggressive behavior [12]. Developmental and psychological research has emphasized individual differences in trajectories of aggressive behavior over time [13,14], yet very little is known about the genetic underpinnings of these behaviors. Longitudinal twin studies show moderate to high sex- and informer-independent estimates of heritability (51-72%) of aggressive behavior between ages 3 and 12 [15]. Genetic factors account for approximately 65% of the total phenotypic stability in twins [16].

Prior studies of the genetic correlates of aggressive behavior have examined polymorphic variations in multiple candidate genes [17-20], across different ages (from childhood to adulthood) and types of samples (population-based, clinical), and using differing definitions of aggressive behavior. However, a recent meta-analysis of these studies found no substantive evidence for association between any of these polymorphisms and aggressive behavior [21].

In contrast, there are only a few genome-wide association studies (GWAS) of aggression-related behavior in humans. In adults, there are GWAS studies of aggression-related phenotypes of adult antisocial behavior [22] and behavioral disinhibition [23], but similar studies in children are scarce. A relatively small study of callous-unemotional behavior in children (N = 2,930) revealed no genome-wide significant hits [24], whereas a genome-wide linkage study of conduct problems in childhood (N = 1,295) identified suggestive regions at chromosomes 2 and 19 [25]. Similarly, a GWA study on hostility and anger traits in adolescents (N = 1,780) identified only suggestive hits [26].

In the present study, we first estimated the proportion of variance in childhood aggressive behavior accounted for by common genetic variants, using the approaches implemented in the Genome-wide Complex Trait Analysis (GCTA, [27]). It is expected that many genes, each with small effects, affect the complex aggressive behavior in childhood. Thus we used a genome-wide approach to test for genetic associations with parental reports of child's aggressive behavior. We specified a quasi-Poisson regression to model the highly right-skewed distribution of aggressive behavior during childhood. The association analyses were performed in the total sample and in two samples with partially overlapping subjects, characterized by different developmental stages (early childhood and

middle childhood/ early adolescence). Next, we performed gene- enrichment analysis, hypothesizing that candidate genes previously reported in the literature would be enriched in our genome-wide association study, In summary, data from nine population-based studies were analyzed, and results were combined in a meta-analysis, generating the largest sample used in childhood aggression research to date (N = 18,988).

Methods

Subjects

This study was performed in the framework of the Early Genetics and Lifecourse Epidemiology (EAGLE) consortium [28]. Information from the participating cohorts is summarized in Table 1 and a more detailed description of the individual cohorts is presented in the Supplementary Material. Cohorts could participate with data on two developmental stages (early childhood, range: 3-7 years, and middle childhood/ early adolescence, range: 8-15 years), whenever data were available. For the cohorts with data on both developmental stages, the largest sample was used for the further analyses (total sample). All children were of North European ancestry. Informed consent was obtained from all participants (parental consent, as appropriate) and study protocols were approved by the local ethics committees.

Table 1 | Descriptives of the cohorts participated in the GWAS meta-analysis of childhood aggression.

Study	Questionnaire	N_{total}	$N_{early\ childhood}$	$N_{middle\ childhood/\ early\ adolescence}$
ALSPAC	SDQ [a]	5,997	5,997	5,752
BC58-T1DGC	Other [b]	2,225	2,225	2,177
BC58-WTCCC2	Other	2,444	2,444	2,366
GENR	CBCL [c]	2,210	2,210	NA [d]
GINI+LISA	SDQ	950	NA	950
NTR	CBCL	1,081	1,081	987
RAINE	CBCL	1,366	1,363	1,366
TRAILS	CBCL	1,280	NA	1,280
YFS	Other	1,435	348	1,435
Total		**18,988**	**15,668**	**16,311**

[a] SDQ- Strengths and Difficulties Questionnaire; [b] comparable items measuring aggressive behavior in general parent-rated questionnaires; [c] CBCL- Childhood Behavioral Checklist ; [d] NA- not available

Genotyping, Quality Control and Imputation

DNA was extracted from whole blood or buccal cells. Comparisons of genotypes derived from whole blood and from buccal swabs in the same individuals showed excellent concordance indicating that

DNA from buccal swabs is of similar quality [29]. For ALSPAC, BC58-T1DGC, GENR, RAINE, TRAILS and YFS genotyping was performed on Illumina platforms, while for BC58-WTCCC2, GINI+LISA and NTR the Affymetrix platform was used. In all studies included in the meta-analyses, basic quality checks were performed (Table S1). Samples were also checked for excess heterozygosity, sex accuracy, relatedness, and missing data. Following these quality control steps, phased genotype data were imputed to build 36 (release 22) of the HapMap reference panel, resulting in more than 2.5 million SNPs for GWAS analysis.

Measurement of Aggressive Behavior

In all cohorts, well-validated questionnaires assessing aggressive behavior in children were mailed to parents of children. In eight out of the nine cohorts, maternal ratings of children's aggressive behavior were obtained. In GINI+LISA, the majority (> 80%) of the questionnaires were filled in by the mother. For GENR, NTR, RAINE and the TRAILS study, aggressive behavior was assessed with the Aggression scale of the Childhood Behavioral Checklist (CBCL). The CBCL 1½-5 [30] and CBCL 6-18 version [31] were administered for preschool and school-aged children, respectively. Mothers were asked to rate each problem item on a 3-point scale (0 = not true, 1 = somewhat true and 2 = very true) and the weighted total sum was scored, allowing < 25% missing data. Example items are: "My child gets in many fights", and "My child destroys others' things". ALSPAC and the GINI+LISA study used the conduct problem scale of the Strengths and Difficulties Questionnaire (SDQ), such as "My child often fights with other children or bullies them" and "My child often lies or cheats". As previously reported, scores derived from SDQ and CBCL questionnaires are highly correlated and are interchangeable for the assessment of children's behavior problems [32]. Finally, BC58-T1DGC, BC58-WTCCC2 and YFS studies used comparable items in general questionnaires rated by the mother, such as "My child's aggressive behavior frequently makes disciplinary action necessary". For YFS study, a 5-point Likert scale was used to rate children's aggressive behavior (1 = "totally disagree" to 5 = "totally agree"). A detailed list of all items used to assess children's aggressive behavior can be found in Table S2.

Genome-Wide Complex Trait Analysis (GCTA)

In ALSPAC (N = 5,997), GENR (N = 2,210) and NTR (N = 1,081) data, SNP-based heritability (SNP h^2) was estimated from observed genotypes using GCTA [27]. First, a genetic relatedness matrix between unrelated individuals was estimated. Participants whose relatedness equaled or exceeded 3rd-4th degree relatives (pairwise genetic relatedness > 0.025) were excluded. Second, a restricted maximum likelihood method (REML) was used to partition the phenotypic similarity between unrelated individuals into a genetic and residual component. SNP h^2 reflects the proportion of phenotypic variance that can be additively accounted for by common genetic variation [33] and indicates the upper limit of variance that can be explained by GWAS efforts. GCTA estimates were adjusted for covariates, i.e. sex, age, and the principal components of the genotype data to control for local ancestry and population stratification.

To test possible bias of the GCTA estimates due to the highly right-skewed distribution of aggressive behavior in children, we rank-transformed aggressive scores in the ALSPAC and GENR sample and performed GCTA as described above.

GWAS analyses, Quality Control (QC) and meta-analysis (GWAMAs)

GWAS analyses in unrelated participants were conducted within each cohort, excluding family members from each cohort prior to GWA analyses. Aggressive behavior was measured on a continuous scale (with higher scores indicating more aggressive behavior). In all cohorts, the non-standardized aggressive behavior scores showed a right-skewed distribution, with the majority of children scoring low on aggression. Association analyses were performed using quasi-Poisson regression, which can accommodate overdispersion [34], using the R Stats Package [35]. The quasi-Poisson regression model was preferred over a simple rank-transformation to obtain directly interpretable effect estimates from each cohort. Specifically, in every cohort the untransformed counts of aggressive behavior scores were regressed on age, sex and principal components of the genetic data to account for population stratification and allele dosage. SNP allele dosages were obtained from imputed data (for more details see Table S1) and used for GWAS analyses. Non-autosomal SNPs were excluded from GWAS analyses. All analyses were performed in R (Project for Statistical Computing [35], R script available upon request).

Prior to the GWAMAs, we conducted rigorous quality control (QC) to the summary data of each participating cohort, using the EasyQC software package [36]. Because of the different assessment instruments used to measure aggressive behavior (i.e. not all studies had the same scales to measure aggressive behavior in children) it is recommended to use the sample size-weighted z-score method for GWAMA [37]. This method is implemented in METAL (Meta-Analysis Helper) [38] and it was used to derive overall p values of the association of each SNP to children's aggressive behavior. Firstly, we meta-analyzed the data in the total sample (N = 18,988). Secondly, for studies with aggressive behavior measured at different developmental stages, we followed up with secondary analyses by meta-analyzing separately early childhood samples (range 3-7 years) and middle childhood/ early adolescence (range 8-15 years), a distinction that was based on developmental trajectories of aggression in children [14]. At the meta-analysis level, SNPs were filtered according to their frequency (minor allele frequency, MAF > 0.05) and imputation quality (MACH R^2 > 0.30 or IMPUTE INFO > 0.4). Results were corrected by genomic inflation factor (λ) at study level, for population stratification. We adopted a threshold of p < 1.00e-06 for genome-wide suggestive hits and a threshold of p < 5.00e-08 for genome-wide significant hits. Annotation and in silico functional analysis of our GWAS meta-analyses top hits was performed using SNPnexus [39].

Gene Enrichment Analysis & Candidate Gene Prioritization

We used two recent synopses [21,40] of candidate gene association studies and identified the candidate genes previously tested for association with aggressive behavior (in adults and/ or children), such as catechol-O-methyltransferase (*COMT*), serotonin receptor (*5-HTTLPR*), dopamine receptors and

brain-derived neurotrophic factor (*BDNF*). To evaluate their potential association with child aggressive behavior in a gene-based test, we performed the versatile gene-based association analysis (VEGAS) on the summary statistics of our total sample (N = 18,988). In summary, VEGAS accounts for the number of SNPs in a gene (± 50 kb form the start/ end of the gene) and the linkage disequilibrium between them to estimate an empirical *p-value* for the association of the gene and trait of interest [41]. No SNP information was available for candidate genes located on chromosome X, thus these genes were not included in the gene-enrichment analyses. For the 21 candidate genes tested, the Bonferroni-corrected threshold for multiple testing is 0.05/21 = 2.38e-03.

Results

GCTA

After filtering for common variants (MAF > 0.01), the ALSPAC sample (N_{total} = 5,997), GENR sample (N_{total} = 2,210) and NTR sample (N_{total} = 1,081) yielded more than 450,000 directly genotyped SNPs for analysis. After exclusion of close relatives, REML analyses were performed. Common genetic variation explained a quantifiable proportion of variance in the traits analyzed. Specifically, in the ALSPAC sample of 4- year old children SNP h^2 was 0.10 (SE = 0.06, p = .04, $N_{unrelated}$ = 5,505). In the GENR sample of 6-year old children, we estimated SNP $h2$ = 0.54 (SE = 0.19, p = .002, $N_{unrelated}$ = 2,101) and in the smaller NTR sample of 3-year old children SNP $h2$ was 0.46 (SE = 0.35, p = .09, $N_{unrelated}$ = 908). The 95% confidence intervals (CI) of the GCTA heritabilities in the ALSPAC, GENR and NTR samples overlap, and this indicates that differences should be interpreted carefully.

Similarly, we estimated GCTA heritability in children in middle childhood/ early adolescence participating in ALSPAC study. The estimations were similar to the ones estimated for the preschoolers, although not significant (SNP h^2 = 0.08, SE = 0.06, p = .10, $N_{unrelated}$ = 5,299).

Sensitivity analysis was performed to assess the effect of the right-skewed distribution of aggressive symptoms in the ALSPAC and GENR samples. GCTA estimations of the rank-transformed aggressive behavior in both samples indicated no substantial bias in our estimations, although the SNP h^2 was notable in the middle childhood/ early adolescence developmental stage of the ALSPAC study (SNP h^2 = 0.12, SE = 0.06, p = .02, $N_{unrelated}$ = 5,299).

GWAS meta-analysis

Nine cohorts contributed data for the total meta-analysis (N = 18,988 children, *mean* age = 8.44 years, SD = 4.16). EasyQC was implemented in all studies and after filtering and quality control, approximately 2.5 million genotyped and imputed SNPs were available per sample. Study-level and meta-level QC results are presented as Supplementary Material (Figures S1-S3). The meta-analysis revealed a near genome-wide significant locus on chromosome 2p12 (p = 5.30e-08) and several suggestive loci across other chromosomes (i.e. chromosome 3q26, 6p22, 10p12, 17q24). The top SNPs are summarized in Table 2 (more details can be found in Table S3). The Manhattan and

Table 2 | Top signals that reached suggestive genome wide significance (p < 1.00e-06), sorted by ascending p, in total sample (N = 18,988)

SNP	Chromosome	Position	Allele 1/2	Frequency 1	Direction [a]	# hits	*meta p*	heterogeneity *p* [b]	Nearest gene(s)
rs11126630	2	77796352	T/C	0.49	---------	14	5.30e-8	.78	*LRRTM4;SNAR-H*
rs9372149	6	107152014	A/T	0.32	+++++++++	2	1.18e-6	.38	*PDSS2*
rs2015436	6	28929137	T/C	0.73	--+------	12	1.57e-6	.58	*TRIM27*
rs10508552	10	17966561	C/G	0.65	-----+---	1	1.72e-6	.18	*MRC1*
rs7625357	3	169630044	T/C	0.33	+++++-+++	4	4.08e-6	.10	*MECOM*
rs2079515	17	70917365	C/G	0.47	+++++++++	2	4.29e-6	.21	*CASC17*

[a] order of participating studies: NTR, BC58-T1DGC, BC58-WTCCC2, GENR, ALSPAC, GINI+LISA, RAINE, TRAILS, YFS; [b] p-value showing heterogeneity between the participating studies

quantile-quantile (QQ) plot of GWAS meta-analysis in the total sample are presented in Figures 1 and 2, respectively. The strongest signal was observed for rs11126630 on chromosome 2 residing between LRRTM4 and SNAR-H. Consistently, across all participating studies, the T allele of the rs11126630 was related to lower levels of aggressive behavior in children (e.g. within the GENR study, the presence of the T allele was related to a decrease of 0.04 log counts in aggressive behavior). The effect estimates and standard errors at rs11126630 are presented for each study in Table S4. The regional association plot of our GWAS top hit is presented in Figure 3.

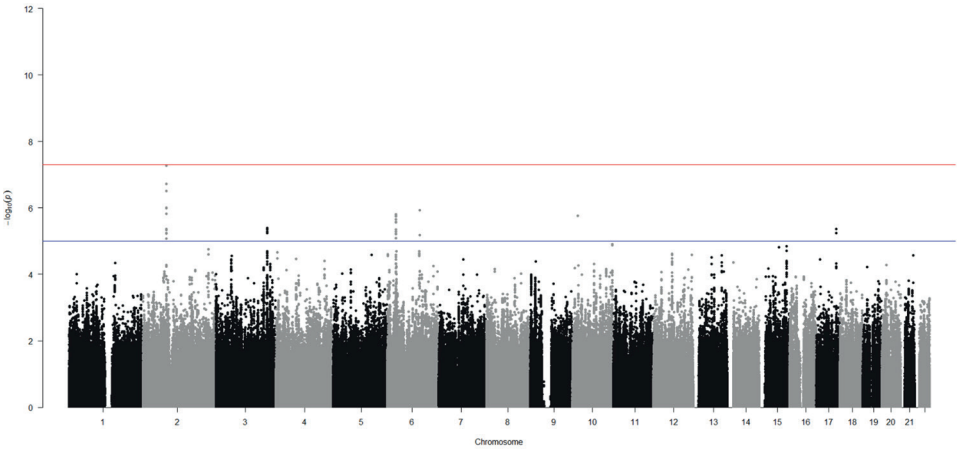

Figure 1 | Manhattan plot of the GWAS meta-analysis of childhood aggression in the total sample (N = 18,988). The x-axis represents the autosomal chromosomes and the y-axis shows the $-\log_{10}(P)$. The red line indicates the genome- wide significance level (p = 5.00e-08) and the blue line indicates the suggestive significance level (p = 1.00e-05)

For the two follow-up meta-analyses using two age-strata, we first analyzed data from children in early childhood (N = 15,668; *mean* age = 5.36 years, *SD* = 1.5). The meta-analysis did not show genome-wide significant results, although there were suggestive SNPs, as summarized in Table 3 (a more detailed table of the total suggestive hits can be found in Table S5). The Manhattan and quantile-quantile (QQ) plot of the GWAS meta-analysis for children in early childhood are presented in Figures S4 and S5, respectively.

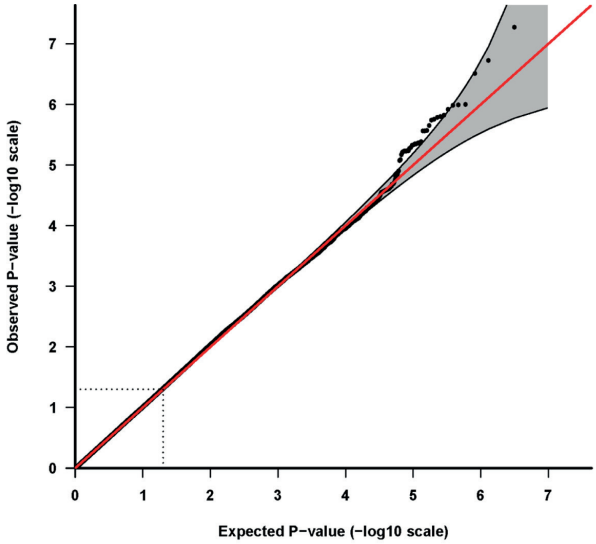

Figure 2 | Quantile- quantile (QQ) plot illustrating probability values from GWAS meta-analysis of aggression in the total sample (*N* = 18,988). The red line indicated the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

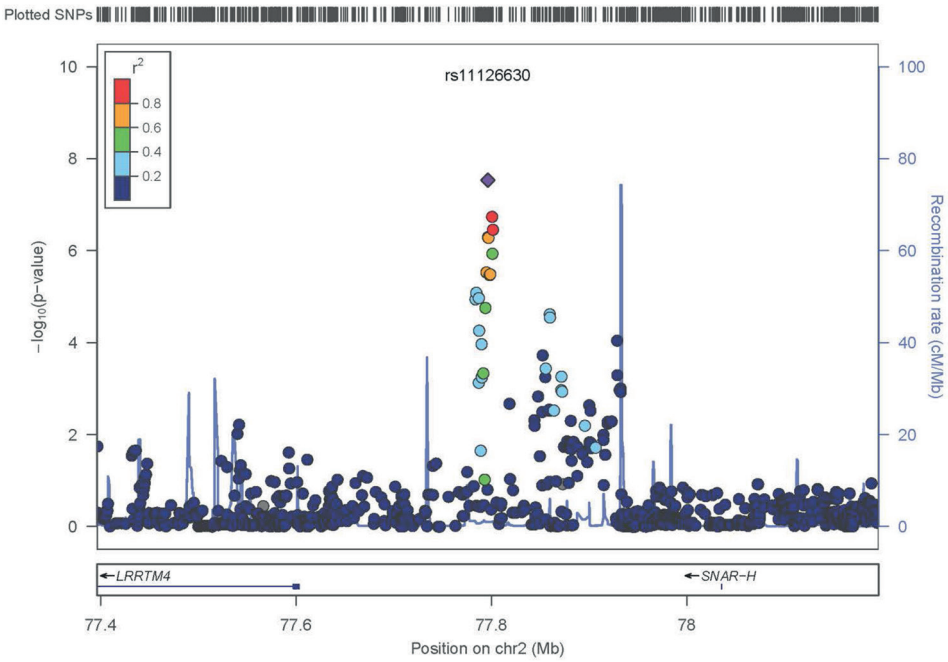

Figure 3 | Regional association plot of the genome-wide significant hit, in total sample. The top SNP is indicated with a diamond and the flanking SNPs, in circles, are colored according to their linkage disequilibrium (LD) with rs11126630. The plot was constructed by HapMap, CEU population (Northern and Western European ancestry).

Table 3 | Top signals that reached suggestive genome wide significance ($p < 1.00e-05$), sorted by ascending p, in early childhood ($N = 15,668$).

SNP	Chromosome	Position	Allele 1/2	Frequency 1	Direction[a]	# hits	*meta p*	heterogeneity *p* [b]	Nearest gene
rs2763339	10	69929889	T/G	0.36	+++++++	3	2.21e-6	.88	*COL13A1*
rs11760485	7	4361919	T/C	0.38	+++++++	2	1.18e-6	.48	*SDK1*
rs17086954	6	156016170	A/G	0.68	+++++++	1	5.91e-6	.45	*LOC101928923*
rs1577595	6	93694060	A/C	0.29	+++++++	3	9.63e-6	.87	*TSG1*
rs589804	2	4469035	T/G	0.51	++++++-	1	9.65e-6	.80	*LOC727982*

[a] order of participating cohorts: ALSPAC, BC58-T1DGC, BC58-WTCCC2, GENR, NTR, RAINE, YFS; [b] *p* value showing heterogeneity between the participating studies.

Table 4 | Top signals that reached suggestive genome wide significance ($p < 1.00e-05$), sorted by ascending p, in middle childhood/ early adolescence ($N = 16,311$).

SNP	Chromosome	Position	Allele 1/2	Frequency 1	Direction[a]	# hits	*meta p*	heterogeneity *p* [b]	Nearest gene
rs11126630	2	77796352	T/C	0.52	--------	10	6.32e-7	.70	*LRRTM4;SNAR-H*
rs11700808	21	19160726	A/C	0.54	-+-+++++	7	1.12e-6	.45	*LOC101927797*
rs9787796	11	131986383	T/C	0.15	++++++++	3	1.68e-6	.41	*OPCML*
rs3843585	10	71374757	T/C	0.62	+--+----	4	2.91e-6	.26	*COL13A1*
rs12153160	5	152931393	A/C	0.84	+-------	5	5.10e-6	.54	*GRIA1*
rs11977715	7	94955639	A/G	0.22	+++++++-	1	7.81e-6	.75	*ASB4*

[a] order of participating cohorts: GINI+LISA, ALSPAC, RAINE, TRAILS, YFS, BC58-T1DGC, BC58-WTCCC2, NTR; [b] *p* value showing heterogeneity between the participating studies

Secondly, we analyzed data from children in middle childhood/ early adolescence (*N* = 16,311), with a mean age of 11.39 years (*SD* = 1.86). We did not observe genome-wide significant results, although there were suggestive SNPs as summarized in Table 4 (more details in Table S6). The Manhattan and QQ plot for children in middle childhood/ early adolescence are presented in Figures S6 and S7, respectively.

Comparison of the results of the total mixed-age sample and the two developmental stages indicated that the locus on chromosome 2 (top SNP, rs11126630) showed also suggestive significance (p = 6.32e-07) in the middle childhood/ early adolescence stage. The same locus reached suggestive evidence for association in the early childhood stage (p = 1.27e-05). At the suggestive level, we also found overlapping signals between the two developmental stages on chromosome 10q22.

Gene Enrichment Analysis & Candidate Gene Prioritization

The association of aggression levels with candidate genes previously linked to individual differences in aggression as found in the VEGAS gene-based analysis is reported in Table 5. Only the AVPR1A (arginine vasopressin receptor 1A) gene was significantly associated with childhood aggression levels (p = 1.61e-03). The association persisted after correcting for multiple testing (Bonferroni correction; new threshold of significance p < 2.38e-03).

Table 5 | Association of candidate genes, as indicated in the literature, with levels of aggressive behavior, using a gene-based test (VEGAS).

Gene name	Chromosome	*N* SNPs	Start position (bp)	Stop position (bp)	Gene- based *p*
Serotonin pathway					
SLC6A4	17	63	25549031	25586841	.61
HTR1A	5	43	63292033	63293302	.40
HTR1B	6	134	78228666	78229839	.91
TPH1	11	79	17999113	18018885	.59
TPH2	12	179	70618892	70712488	.07
Dopaminergic pathway					
DRD1	5	144	174800280	174803769	.26
DRD2	11	195	112785526	112851211	.97
DRD3	3	119	115330246	115380589	.36
DRD4	11	50	627304	630703	.52
DBH	9	177	135491305	135514287	.30
COMT	22	147	18309308	18336530	.78
SLC6A3	5	140	1445909	1498538	.79
Adrenergic receptors					
ADRB1	10	94	115793795	115796657	.11
NET1	10	116	5444517	5490426	.75
SLC6A2	16	162	54248056	54295201	.78

Table 5 | *(Continued)*

Gene name	Chromosome	*N* SNPs	Start position (bp)	Stop position (bp)	Gene- based *p*
Stress response					
NR3C1	5	160	142637688	142795270	.42
FKBP5	6	82	35649344	35764692	.87
Other					
NOS1	12	243	116135361	116283965	.09
SLC2A1	1	135	43163632	43197434	.28
AVPR1A	12	91	61826482	61832857	1.61e-03
BDNF	11	104	27633017	27699872	.42

Discussion

Twin and family studies have suggested a significant heritability of aggressive behavior, while genome-wide association studies have indicated potential biological pathways leading to extreme aggression in adolescence and adulthood. This is the first large-scale, genome-wide approach attempting to discover novel genes implicated in children's aggressive behavior in population-based samples.

As part of the EAGLE consortium, we gathered the largest sample to date (total N = 18,988) from which to explore genetic correlates of children's aggressive behavior. We estimated the proportion of phenotypic variance explained by additive effects of common SNPs, using the GCTA approach. Our findings suggest that common genetic variation contributes to the phenotypic variation in aggressive behavior, with GCTA heritability estimates ranging between 10% and 54% in different cohorts during early childhood. The heterogeneity of the point GCTA estimates between cohorts could reflect that genotype data were obtained by different genotyping platforms (Illumina or Affymetrix), containing only partially overlapping SNPs (only for the GWAS analyses the genotype data were imputed). Second, GCTA heritability is an estimation of the ratio of phenotypic variation explained by common SNPs, thus is dependent on sample characteristics [42]. Environmental factors differentially influence gene expression in different samples, thereby changing phenotyping variance or the characteristics of the genetic relatedness matrix among cohorts. Finally, it is possible that heritability estimations change with age, as it has been previously shown for autistic like traits [43] and general cognitive ability [44].

Previously published heritability estimates based on twin studies of childhood aggressive behavior indicated moderate to high heritability (51-72%) [15] while GCTA estimates of adult antisocial behavior were not significantly different than zero (SNP h^2 = 0.55, SE = 0.41, p = .07, N_{cases} = 160 and $N_{controls}$ = 2,012) [22]. Our GCTA estimates however, support a polygenic contribution of common variants to children's aggressive behavior. Large sample sizes will be required to identify the responsible genes [45] in addition to in depth analysis of rare variation.

Our study revealed a near genome-wide significant locus on chromosome 2p12 (top SNP: rs11126630, p = 5.30e-08). This SNP is located near LRRTM4 (leucine rich repeat transmembrane neuronal 4) and SNAR-H (small ILF3/NF90-associated RNA H) genes. LRRTM4 regulates excitatory synapse development [46] while SNAR-H is implicated in the transcription process and is also expressed in neurons [47]. In silico studies showed that variation in 2p12 can affect regulatory transcription factor binding sites in the LRRTM4 gene promoter [48]. Another suggestive locus (p = 1.57e-06) was 6p22, near the TRIM27 gene. The top SNP rs9257616 (chromosome 6p22.1) has been previously associated with social communication traits, in a population sample of 8 year old children [49]. TRIM27 gene promotes apoptosis and it has been associated with neurotoxicity of dopaminergic neurons in mouse models [50]. How these genes may be implicated in the neurophysiology of children's aggressive levels should be a research priority. Replication efforts of our results in independent samples are needed, yet we note that this study is consistent with a previous smaller genome-wide linkage study on conduct disorder that pointed to similar regions on chromosome 2 [25].

The results of this GWA meta-analysis of aggressive behavior in children did not show overlap with the suggestive SNPs previously reported for aggression-related traits in adults [22,23]. The differences in the phenotypes measured in adults (behavioral inhibition and antisocial behavior, respectively) do not allow for direct comparisons with aggressive behavior in children.

Meta-analyses in the two developmental stages (early childhood and middle childhood/early adolescence) did not reveal genome-wide significant results. These analyses indicate that even small differences in sample size can impact on power to detect genetic associations at the genome-wide significance level. Furthermore, in the partially overlapping developmental stages, genetic variation at the 2p12 region is also enriched, as in the analyses with the total sample. This could imply that the associated region has a relatively stable and less age-dependent effect. At the suggestive level of significance, we also found association of the 10q22 region with levels of aggressive behavior in both developmental stages. Microdeletions in this region have been previously associated with cognitive and behavioral problems [51].

Finally, the *AVRP1A* gene was enriched in our gene-based analysis. It is possible that functional variation in the arginine vasopressin receptor is responsible for higher levels of aggressive behavior, as it has already been showed in rodents [52]. Variation in AVPR1A has also been of high interest for social behavior [53,54]. The results of our analyses on candidate genes previously associated with aggression also indicate that it is unlikely that common variants in these genes play a significant role in explaining individual variation in children's aggressive behavior. Since commercial genotyping arrays do not capture structural variation (i.e. copy number variation, variable number tandem repeats or micro-inversions) or the effect of rare SNPs, further studies on these candidate genes (such as *DRD4* and *5-HTTLPR*) can be valuable to investigate their potential influence on aggressive behavior in children [55].

This study has limitations. First, aggressive behavior was reported predominantly by mothers, which can be a source of reporting bias [56]. Future studies could use multiple reporters to estimate children's aggressive behavior more precisely. Second, although the sample size is the largest to date

for childhood aggression, compared to adults studies of psychiatric traits, the sample size is low, limiting the power to detect associations of common variants with small effects. In developmental and behavioral research, this is a considerable challenge. Third, this was a discovery study, thus we analyzed all available data in all cohorts, leaving no cohorts for replication. Future steps should include efforts to introduce additional cohorts to our consortium, increase the sample size and test whether our findings are replicated in independent samples.

In summary, we found evidence that common additive genetic variants explain variance in parent-rated, non-clinical aggressive behavior in children. Our discovery GWAS meta-analysis identified genes with potential biological links to aggression. The acquisition of even larger sample sizes through continued collaboration between groups and consortia will be crucial for the replication of these genetic loci and their contribution to child aggressive behavior.

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Supplementary Material

Individual Study Cohort Descriptions

Avon Longitudinal Study of Parents and Children (ALSPAC)

Avon Longitudinal Study of Parents and Children (ALSPAC) is a population based longitudinal pregnancy-ascertained birth-cohort in the Bristol area of the UK [2]. Specifically, recruitment sought to enrol all pregnant women with an estimated delivery date between 1st April 1991 and 31st December 1992, who where residents within three Health Districts of the former administrative county of Avon [1,2]. The initial cohort included 14,541 pregnancies and additional children eligible using the original enrolment definition were recruited up to the age of 18 years, increasing the total number of pregnancies to 15,247 (4.1% Non-White mothers). Information on the children from these pregnancies is available from questionnaires, clinical assessments, linkage to health and administrative records as well as biological samples including genetic and epigenetic information. Detailed information of all available data can be obtained online [4]. Ethical approval was obtained from the ALSPAC Law and Ethics Committee (IRB00003312) and the Local Research Ethics Committees, and written informed consent was provided by all parents.

British 1958 Birth Cohort (BC58-WTCCC2 and T1DGC)

The 1958 British Birth Cohort (1958BC) is a population based prospective study of all individuals born during one week in March 1958 in England, Scotland, and Wales (n = 17,638) [8]. Ethical approval for the 45 years survey was obtained from the South East Multicenter Research Ethics Committee (ref. 01/1/44) and the Joint UCL/UCLH Committees on the Ethnics of Human Research (Committee A) (ref. 08/H0714/40). Childhood follow-up waves were conducted when the cohort members were aged 7 and 11, when parents rated children's emotional difficulties using short forms of Rutter, Tizard & Whitmore's Health and Behaviour Checklists [10]. Genome- wide data for the 1958BC has been obtained through two sub-studies that had used the 1958BC as a source for population controls: 3,000 samples were randomly selected as part of the Wellcome Trust Case Control Consortium [WTCCC2 (International Multiple Sclerosis Genetics et al., 2011)] and 2,592 distinct samples were selected as part of the Type 1 Diabetes Genetics Consortium [T1DGC (Barrett et al., 2009)].

Generation R Study (GENR)

The Generation R Study is a population-based prospective cohort study. All children were born between April 2002 and January 2006. This study is designed to identify early environmental and genetic predictors of growth, development, and health from fetal life until young adulthood and has been described previously in detail [7]. DNA was extracted from cord blood taken at birth. Children of Northern European descent, as determined by principal component analyses of genome wide association (GWA) data, were selected. Of 5,908 children with DNA available, 2,210 children of Northern European descent (50.8% boys) had SNP data and available data on aggression. The

Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam has approved the study protocol, and participants have given informed consent in writing.

German Infant Study on the Influence of Nutrition Intervention and Lifestyle- Immune System- Allergy Study (GINI+ LISA)

A total of 5,991 mothers and their newborns were recruited into the German Infant study on the influence of Nutrition Intervention PLUS environmental and genetic influences on allergy development (GINIplus) between September 1995 and June 1998 in Munich and Wesel. 'The influence of Lifestyle factors on the development of the Immune System and Allergies in East and West Germany PLUS the influence of traffic emissions and genetics (LISAplus)' Study is a population based birth cohort study. A total of 3,097 healthy, mature (gestational age over 37 weeks) neonates with a birth weight over 2500 g were recruited between 1997 and 1999 in Munich, Leipzig, Wesel and Bad Honnef. Detailed descriptions of the LISA and GINI studies have been published elsewhere [3,13]. For both studies, approval by the local Ethics Committees and written consent from participant's families were obtained. Netherlands Twin Registry (NTR).

The Netherlands Twin Register (NTR) is a large population based study that includes approximately 40% of all multiple births in the Netherlands since 1986 [12]. The twins are followed from birth onwards. Data collection is ongoing. Parental ratings of problem behavior are available for ages 2, 3, 5, 7, 10 and 12 years. In the current analyses, measures collected at age 3 and age 10 were analyzed. Young twins were invited to participate in subprojects, and to provide a DNA sample [11]. In case individuals had multiple samples genotyped, the sample with the highest genotype quality was selected. In twin pairs, one individual was selected at random for inclusion in the GWA project. The study was approved by the Central Ethics Committee on Research involving human subjects of the VU University Medical Centre, Amsterdam, and an institutional review board certified by the U.S. Office of Human Research Protections (IRB number IRB-2991 under Federal-wide Assurance-3703; IRB/institute codes, NTR 03-180).

Tracking Adolescents' Individual Lives Survey (TRAILS)

Tracking Adolescents' Individual Lives Survey (TRAILS) is a large prospective population study of Dutch adolescents with bi- or triennial measurements from age 11 years. TRAILS participants were selected from five municipalities in the Northern part of the Netherlands. The cohort's characteristics are described in detail elsewhere [5]. DNA was extracted from blood samples or (in a few cases) buccal swaps, collected at about age 16. In total, 1,491 children of white European descent were genotyped, of whom 1,280 (49% boys, mean age 11.1, *SD* = 0.6) had available CBCL data on aggression. The study was approved by the Dutch Central Committee on Research Involving Human subjects (CCMO), and all measurements were carried out with participants' adequate understanding and written consent.

Western Australian Pregnancy Cohort Study (RAINE)

The Western Australian Pregnancy Cohort (RAINE) Study is a longitudinal study of 2900 pregnant women and their offspring consecutively recruited from maternity units between 1989 and 1991 [8]. The inclusion criteria were (i) English language skills sufficient to understand the study demands, (ii) an expectation to deliver at King Edward Memorial Hospital (KEMH), and (iii) an intention to remain in Western Australia to enable future follow-up of their child. Ninety percent of eligible women agreed to participate in the study. From the original cohort, 2868 children have been followed over two decades. The study was conducted with appropriate institutional ethics approval, and written informed consent was obtained from mothers at all follow-ups and participants at the year 17 follow-up. The RAINE sample is representative of the larger Australian population (88% Caucasian). DNA samples have been collected using standardized procedures at 14 or 16 years of age. Only those children with both biological parents of White European origin were included in the current analyses.

Young Finns Study (YFS)

The Young Finns study (YFS) is an on-going longitudinal population based cohort study that includes 3,596 healthy Finnish children and adolescents from six birth cohorts (aged 3, 6, 9, 12, 15, and 18 years at the study baseline in 1980). The study was approved by the ethical committee of the Varsinais-Suomi's hospital district's federation of municipalities. Detailed description of the YFS study has been published elsewhere [9].

Table S1 | Information on genotyping methods, quality control, imputation and statistical analysis of studies included in GWAS meta-analysis of children's aggressive behavior.

Study	Platform	Genotyping					Mutation			Association analyses	
			Inclusion criteria					Inclusion criteria			
		Genotype calling algorithm	MAF (%)	Call rate (%)	P for HWE	SNPs that met QC criteria	Imputation software	MAF (%)	Imputation quality	SNPs in analysis	Analysis software
ALSPAC	Illumina 550K	GenCall	> 1	> 97	> 10-7	488,325	MACH	> 1	r^2-hat > 0.30	2,543,887	quasi-Poisson R script
BC58-T1DGC	Illumina HumanHap 550 V.1	Illuminus	> 0	> 97	>1 0-7	539,458	MACH	≥ 0	r^2-hat > 0.30	2,552,990	quasi-Poisson R script
BC58-WTCCC2	Affymetrix GeneChip Human Mapping 500K	CHIAMO	> 0	> 97	> 10-7	490.032	IMPUTE	≥ 0	proper-info ≥ 0.40	3,856,236	quasi-Poisson R script
GENR	Illumina Human 610K/ 660W	GenomeStudio	≥ 0.1	> 97.5	> 10-7	518,245	MACH	≥ 1	r^2-hat > 0.30	2,540,729	quasi-Poisson R script
GINI+ LISA	Affymetrix Genome-Wide Human SNP Array 5.0	BRLMM-P	> 1	> 95	> 10-6	357,125	IMPUTE2	≥ 0	proper-info ≥ 0.40	2,623,614	quasi-Poisson R script
NTR	Affymetrix 600K	Perlegen proprietary	≥ 1	≥ 95	>1 0-5	435,291	MACH	≥ 1	proper-info ≥ 0.40	2,541,922	qquasi-Poisson R script
RAINE	Illumina 660 Quad	BeadStudio	≥ 1	> 95	> 10-7	535,632	MACH	> 1	r^2-hat > 0.30	2,543,887	quasi-Poisson R script
TRAILS	Illumina Cyto SNP12 v2	GenomeStudio	≥ 1	≥ 95	> 10-6	260,127	IMPUTE2	≥ 0	proper-info ≥ 0.40	2,543,887	quasi-Poisson R script
YFS	Illumina custom made BeadChip Human 670-Quad	Illuminus	≥ 5	≥ 95	> 10-6	546,667	MACH	≥ 0	r^2-hat > 0.30	2,543,887	quasi-Poisson R script

Table S2 | List of items used to assess children's aggressive behavior.

Questionnaire	Scale	Items		
CBCL 1½-5 [a]	Aggressive Behavior	– Can't stand waiting	– Lacks guilt	– Selfish
		– Cruel to animals	– Disturbs others	– Stubborn
		– Defiant	– Easily frustrated	– Temper
		– Demands must be met	– Gets into fights	– Wants attention
		– Destroys own things	– Hits others	– Not liked
		– Destroys other's things	– Hurts unintentionally	– Teases
		– Disobedient	– Angry moods	– Uncooperative
		– Mean to others	– Attacks people	– Screams
		– Punishment doesn't change behavior		
CBCL 6-18 [b]	Aggressive Behavior	– Argues a lot	– Defiant	
		– Mean to others	– Demands attention	– Mood changes
		– Destroys own things	– Destroys others' things	– Suspicious
		– Disobedient at home	– Disobedient at school	– Temper
		– Gets in fights	– Attacks people	– Loud
		– Screams a lot	– Explosive	– Sulks
		– Easily frustrated	– Stubborn, sullen	– Teases a lot
		– Threatens others		
SDC [c]		– Often has temper tantrums or hot tempers	– Generally disobedient	– Often lies or cheats
		– Often fights with other children	– Steals from home/ school/elsewhere	

Table S2 | *(Continued)*

Questionnaire	Scale	Items		
BC58 questionnaire	General Questions [d]	– Generally destructive	– Irritable	
		– Fights with other children	– Fights with other children	
		– Disobedient		
YFS questionnaire	General Questions	– Other children's parents often complain about the child's behavior	– Child easily gets into fights	– Child swears a lot
		– Aggressive behavior frequently makes disciplinary a ction necessary	– Child likes to argue	

[a] CBCL 1½-5-Childhood Behavioral Checklist for ages 1,5-5 years; [b] CBCL 6-18- Childhood Behavioral Checklist for ages 6-18 years; [c] SDQ- Strengths and Difficulties Questionnaire; [d] comparable items measuring aggressive behavior in general parent-rated questionnaires

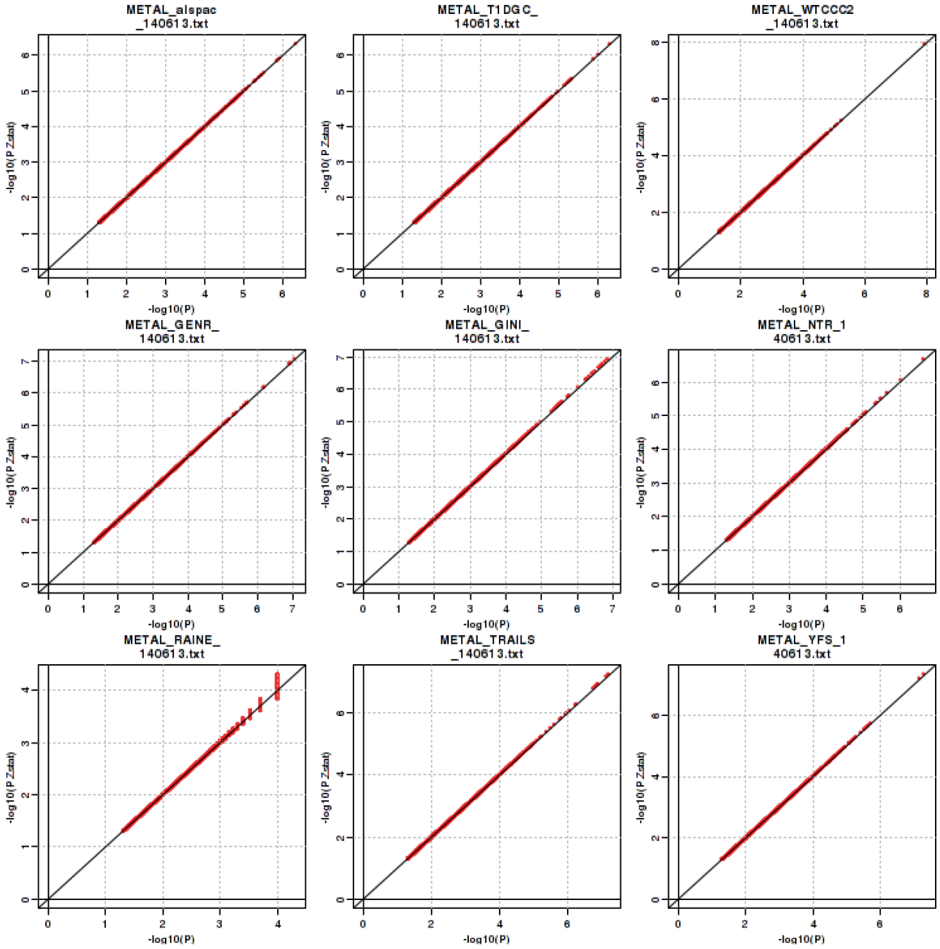

Figure S1 | P-Z plots for each participating cohort, in total sample (N = 18,988), revealing no major analytical issues with beta's, standard errors and P values (concordance with the identity line).

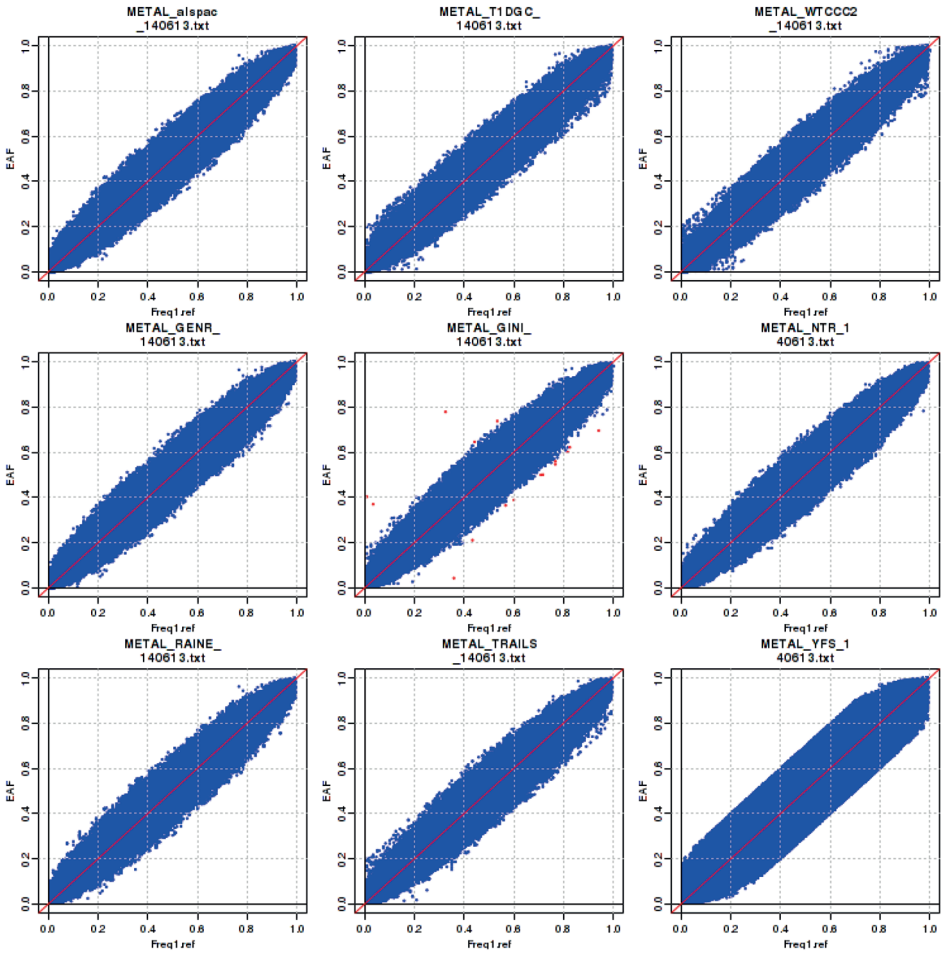

Figure S2 | EAF plot reporting study-specific allele frequencies plotted against allele frequencies expected from HapMap-CEU, for studies participating in total sample (N = 18,988).

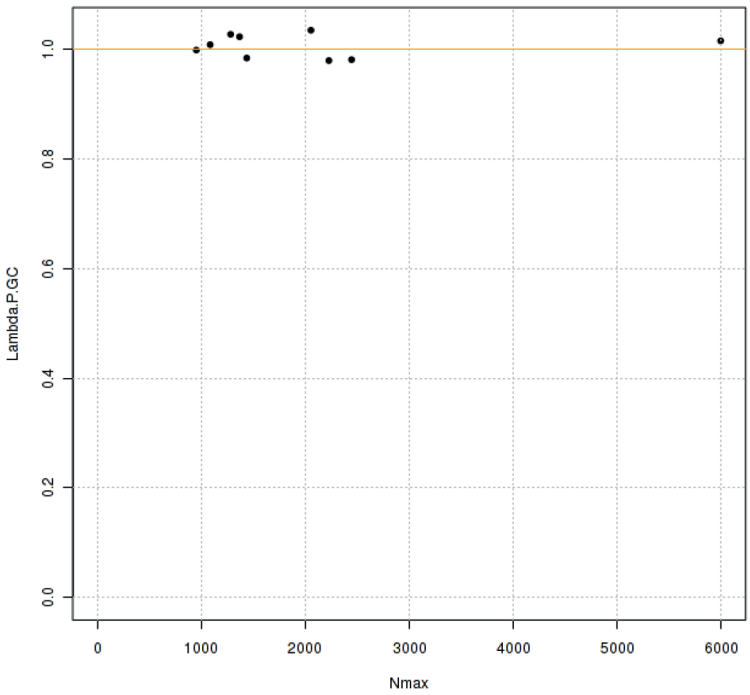

Figure S3 | Lambda-N plot revealing no issues with population stratification among the participating studies, in total sample (*N* = 18,988).

Table S3 | Results of all suggestive hits (p < 10-5) from GWAS meta-analysis on aggression in total sample (N = 18,988).

SNP	Chromosome	Position	Effect Allele	Other Allele	Effect Allele frequency	*N*	meta *p*	heterogeneity *p* [a]	Direction [b]	Gene	Predicted function [c]
rs11126630	2	77796352	T	C	0.49	18,834	5.30e-8	.78	---------	*LRRTM4*	NA
rs10169036	2	77719996	T	C	0.49	18,833	1.86e-7	.82	+++++++++	*LRRTM4*	NA
rs11676317	2	77720862	A	G	0.49	18,832	3.05e-7	.82	+++++++++	*LRRTM4*	NA
rs12613157	2	77715693	T	C	0.43	18,834	9.84e-7	.58	+++++++++	*LRRTM4*	NA
rs1542677	2	77716233	C	G	0.43	18,834	1.00e-6	.57	+++++++++	*LRRTM4*	NA
rs1542678	2	77716259	T	G	0.57	18,834	1.02e-6	.57	---------	*LRRTM4*	NA
rs9372149	6	107152014	A	T	0.32	18,834	1.19e-6	.38	+++++++++	*PDSS2*	3'downstream
rs10192926	2	7772032:	A	T	0.41	18,833	1.48e-6	.66	+++++++++	*LRRTM4*	NA
rs2015436	6	28929137	T	C	0.73	18,834	1.57e-6	.58	--+------	*TRIM27*	NA
rs6912843	6	28936385	T	C	0.73	18,834	1.61e-6	.58	--+------	*TRIM27*	NA
rs10508552	10	17966561	C	G	0.65	18,825	1.73e-6	.18	-----+---	*MRC1*	intronic
rs9257425	6	28968779	A	G	0.73	18,834	1.81e-6	.58	--+------	*TRIM27*	NA
rs209121	6	28913883	T	C	0.73	18,833	2.20e-6	.56	--+------	*TRIM27*	NA
rs209146	6	28891073	A	G	0.27	18,834	2.68e-6	.57	++-++++++	*TRIM27*	NA
rs209140	6	28895692	A	G	0.27	18,834	2.69e-6	.57	++-++++++	*TRIM27*	NA
rs209131	6	28899978	A	G	0.73	18,834	2.71e-6	.57	--+------	*TRIM27*	NA
rs7625357	3	169630044	T	C	0.33	18,834	4.08e-6	.10	+++++-+++	*MECOM*	intronic
rs2079515	17	70917365	C	G	0.47	18,834	4.29e-6	.21	+++++++++	*CASC17*	NA
rs17014427	2	77714149	A	G	0.43	18,833	4.31e-6	.61	+++++++++	*LRRTM4*	NA
rs7750338	6	28885380	T	C	0.27	18,834	4.41e-6	.57	++-++++++	*TRIM27*	NA
rs10513679	3	169635396	T	C	0.32	18,834	4.46e-6	.12	+++++-+++	*MECOM*	intronic
rs11126631	2	77717455	T	C	0.42	18,834	4.63e-6	.48	+++++++++	*LRRTM4*	NA

Table S3 | *(Continued)*

SNP	Chromosome	Position	Effect Allele	Other Allele	Effect Allele frequency	*N*	meta *p*	heterogeneity *p* [a]	Direction [b]	Gene	Predicted function [c]
rs994214	2	77718116	A	G	0.42	18,834	4.65e-6	.60	+++++++++	*LRRTM4*	NA
rs7652404	3	169634670	T	C	0.68	18,834	5.12e-6	.11	-----+---	*MECOM*	intronic
rs429369	6	28884131	T	C	0.74	18,834	5.22e-6	.56	--+------	*TRIM27*	NA
rs2079514	17	70917323	A	C	0.46	18,834	5.73e-6	.21	+++++++++	*CASC17*	NA
rs6744785	2	77703052	T	C	0.55	18,827	5.73e-6	.55	--------	*LRRTM4*	NA
rs1421005	3	169633989	T	C	0.32	18,834	5.81e-6	.10	+++++-+++	*MECOM*	intronic
rs1016472	6	28884131	T	C	0.74	18,834	5.87e-6	.55	--+------	*TRIM27*	NA
rs13426167	2	77706397	T	C	0.71	18,832	5.87e-6	.68	+++++-+++	*LRRTM4*	NA
rs13415782	2	77703757	C	G	0.55	18,829	5.92e-6	.77	---------	*LRRTM4*	NA
rs209151	6	28878947	C	G	0.26	18,834	6.10e-6	.53	++-++++++	*TRIM27*	NA
rs13202332	6	107145426	T	G	0.34	18,834	6.63e-6	.20	-++++++++-	*PDSS2*	3'downstream
rs209177	6	28835326	A	G	0.26	18,834	8.06e-6	.42	++-++++++	*TRIM27*	NA
rs729756	2	77713147	A	G	0.31	18,833	8.31e-6	.67	-----+---	*LRRTM4*	NA

[a] *P* value showing heterogeneity between the participating studies; [b] order of participating cohorts: NTR, BC58-T1DGC, BC58-WTCCC2, GENR, ALSPAC, GINI+LISA, RAINE, TRAILS, YFS, [c] according to Ensembl (produced by SNPnexus), NA- not available.

Table S4 | Effect estimates of the near genome-wide significant SNP (rs11126630) in all participating studies of the total sample (*N* = 18,988).

Study	Effect Allele	Other allele	Effect allele frequency	Effect (*b*)	SE	N	study-level *p*
ALSPAC	C	T	0.50	0.03	0.01	5,997	.02
BC58-T1DGC	T	C	0.49	-0.04	0.02	2,225	.11
BC58-WTCCC2	T	C	0.47	-0.05	0.02	2,444	.02
GENR	T	C	0.49	-0.04	0.03	2,210	.23
GINI+LISA	T	C	0.50	-0.05	0.04	950	.27
NTR	C	T	0.51	0.09	0.04	1,081	.009
RAINE	C	T	0.51	0.05	0.04	1,366	.22
TRAILS	T	C	0.49	-0.05	0.03	1,280	.10
YFS	T	C	0.55	-0.03	0.01	1,435	.005

SE = standard error; *N*-sample size for every participating cohort.

Table S5 | Results of all suggestive hits ($p < 10^{-5}$) from GWAS meta-analysis on aggressive behavior in early childhood (N = 15,668).

SNP	Chromosome	Position	Effect Allele	Other Allele	Effect Allele frequency	N	meta p	heterogeneity p [a]	Direction [b]	Gene	Predicted function [c]
rs2763339	10	69929889	T	G	0.36	15,513	2.21e-6	.88	+++++++	*COL13A1*	intronic
rs2683571	10	69930719	C	G	0.38	15,514	2.60e-6	.93	+++++++	*COL13A1*	intronic
rs11760485	7	4361919	T	C	0.33	15,511	3.03e-6	.48	+++++++	*SDK1*	NA
rs2763337	10	69928411	A	G	0.65	15,512	3.57e-6	.88	-------	*COL13A1*	intronic
rs17086954	6	156016170	A	G	0.68	15,514	5.91e-6	.45	+++++++	*LOC101928923*	intronic
rs5021673	7	4360007	C	G	0.67	15,514	8.95e-6	.59	-------	*SDK1*	NA
rs1577595	6	93694060	A	C	0.29	15,514	9.63e-6	.87	+++++++	*TSG1*	NA
rs589804	2	4469035	T	G	0.51	15,514	9.65e-6	.80	++++++-	*LOC727982*	NA
rs937044	6	93702534	A	G	0.71	15,514	9.90e-6	.87	-------	*TSG1*	NA
rs1953598	6	93700332	C	G	0.29	15,514	9.91e-6	.87	+++++++	*TSG1*	NA

[a] *P* value showing heterogeneity between the participating studies; [b] order of participating studies: ALSPAC, BC58-T1DGC, BC58-WTCCC2, GENR, NTR, RAINE; YFS; [c] according to Ensembl (produced by SNPnexus), NA = not available.

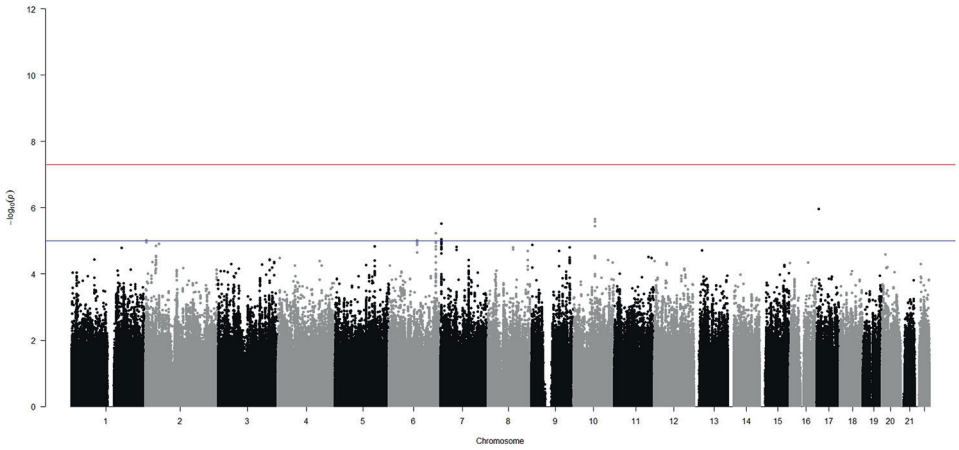

Figure S4 | Manhattan plot of the GWAS meta-analysis of childhood aggressive behavior in early childhood (N = 15,668). The x- axis represents the autosomal chromosomes and the y-axis shows the -$\log_{10}(p)$. The red line indicates the genome- wide significance level (p = 5 x 10^{-8}) and the blue line indicates the suggestive significance level (p = 10^{-5}).

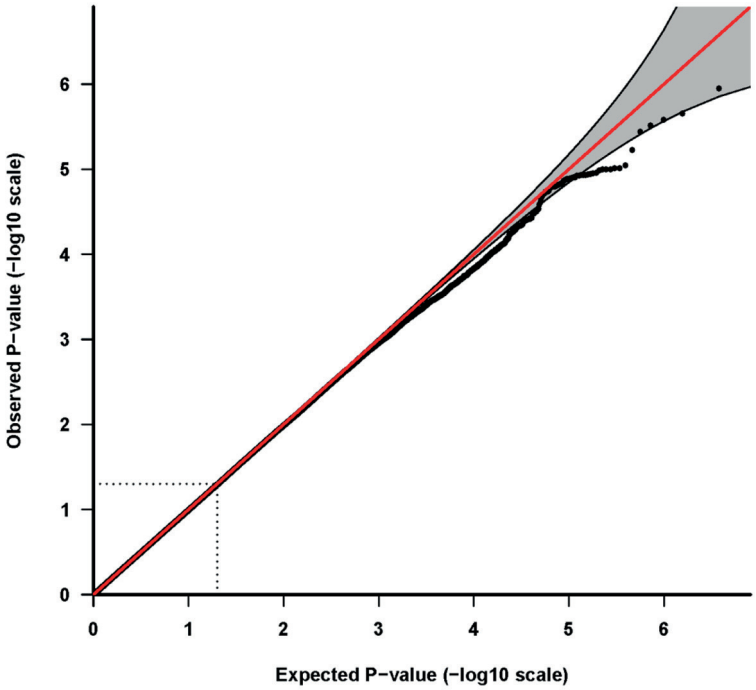

Figure S5 | Quantile-quantile (QQ) plot illustrating probability values from GWAS meta-analysis of aggression in early childhood (N = 15,668). The red line indicated the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

Table S6 | Results of all suggestive hits ($p < 10^{-5}$) from GWAS meta-analysis on aggressive behavior in middle childhood/ early adolescence (N = 16,311).

SNP	Chromosome	Position	Effect Allele	Other Allele	Effect Allele frequency	*N*	meta *p*	heterogeneity p [a]	Direction [b]	Gene	Predicted function [c]
rs11126630	2	77796352	T	C	0.52	16,409	6.32e-7	.70	--------	*LRRTM4*	NA
rs1542678	2	77796893	T	G	0.57	16,409	6.69e-7	.62	--------	*LRRTM4*	NA
rs1542677	2	77796867	C	G	0.42	16,409	6.83e-7	.62	++++++++	*LRRTM4*	NA
rs12613157	2	77796327	T	C	,4285	16,409	7.88e-7	.64	++++++++	*LRRTM4*	NA
rs11700808	21	19160726	A	C	,5406	16,408	1.12e-6	.45	-+-+++++	*LOC101927797*	NA
rs2825187	21	19124927	A	G	,2314	16,404	1.28e-6	.83	+-------	*LOC101927797*	NA
rs9787796	11	131986383	T	C	,1520	16,409	1.68e-6	.41	++++++++	*OPCML*	intronic
rs1040352	21	19162658	T	G	,4606	16,408	1.69e-6	.40	+-+-----	*LOC101927797*	NA
rs7130651	11	131985547	T	C	,1514	16,409	1.93e-6	.38	++++++++	*OPCML*	intronic
rs7130397	11	131985386	A	G	,8468	16,409	2.11e-6	.48	--------	*OPCML*	intronic
rs10169036	2	77800630	T	C	,4675	16,408	2.12e-6	.62	++++++++	*LRRTM4*	NA
rs994214	2	77798750	A	G	,4168	16,409	2.25e-6	.56	++++++++	*LRRTM4*	NA
rs17014427	2	77794783	A	G	,4176	16,408	2.67e-6	.53	++++++++	*LRRTM4*	NA
rs11126631	2	77798089	T	C	,4152	16,409	2.82e-6	.44	++++++++	*LRRTM4*	NA
rs11676317	2	77801496	A	G	,4664	16,407	2.90e-6	.58	++++++++	*LRRTM4*	NA
rs3843585	10	71374757	T	C	,6189	16,405	2.91e-6	.26	+--+----	*COL13A1*	intronic
rs3793814	10	71380589	A	G	,6339	16,408	3.92e-6	.55	---+----	*COL13A1*	intronic
rs10762324	10	71380268	A	G	,3658	16,401	4.11e-6	.56	+++-++++	*COL13A1*	intronic
rs3793815	10	71377693	T	C	,6421	16,408	4.78e-6	.34	---+----	*COL13A1*	intronic
rs950365	21	19134833	A	G	,4230	16,406	5.48e-6	.49	+-+-----	*LOC101927797*	NA
rs2212860	21	19125490	A	T	,5825	16,404	6.16e-6	.49	-+-+++++	*LOC101927797*	NA
rs12153160	5	152931393	A	C	,8386	16,407	6.73e-6	.54	+-------	*GRIA1*	intronic

Table S6 | *(Continued)*

SNP	Chromosome	Position	Effect Allele	Other Allele	Effect Allele frequency	*N*	meta *p*	heterogeneity p [a]	Direction [b]	Gene	Predicted function [c]
rs11977715	7	94955639	A	G	,2189	16,408	7.81e-6	.75	+++++++-	*ASB4*	intronic
rs1988787	21	19159049	A	G	,4211	16,407	7.83e-6	.53	+-+-----	*LOC101927797*	NA
rs4685500	3	2239609	T	C	,1875	16,409	8.31e-6	.57	-----+--	*CNTN4*	intronic
rs2825207	21	19149262	A	T	,5841	16,406	8.33e-6	.53	-+-+++++	*LOC101927797*	NA
rs12522802	5	152922416	T	C	,8616	16,409	8.99e-6	.49	+-+-----	*GRIA1*	intronic
rs12520252	5	152923019	C	G	,8616	16,409	9.21e-6	.49	+-+-----	*GRIA1*	intronic
rs12522937	5	152923029	T	C	,8616	16,409	9.21e-6	.49	+-+-----	*GRIA1*	intronic
rs12520227	5	152922945	A	C	,1384	16,409	9.25e-6	.49	-+-+++++	*GRIA1*	intronic
rs13415782	2	77784391	C	G	,5628	16,404	9.36e-6	.62	--------	*LRRTM4*	NA

[a] *P* value showing heterogeneity between the participating studies; [b] order of participating studies: GINI+LISA, ALSPAC, RAINE, TRAILS, YFS, BC58 -T1DGC, BC58-WTCCC2, NTR; [c] according to Ensembl (produced by SNPnexus), NA = copy number variation; NA = not available.

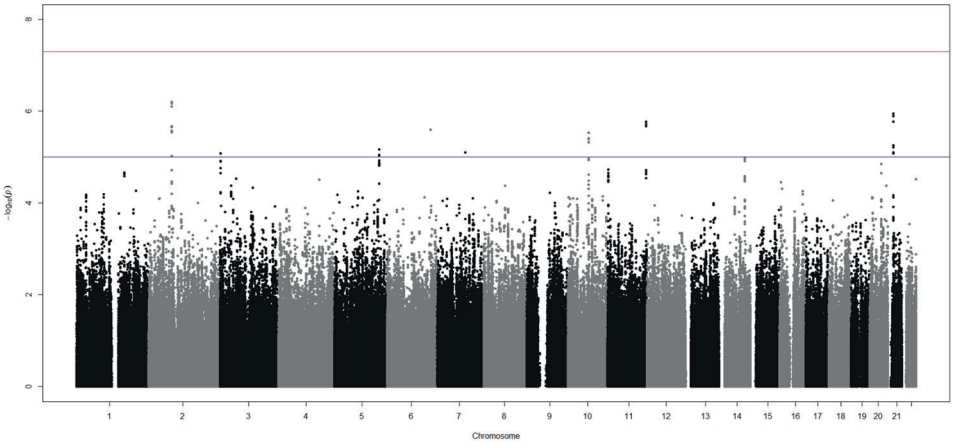

Figure S6 | Manhattan plot of the GWAS meta-analysis of childhood aggressive behavior in middle childhood/ early adolescence (N = 16,311). The x-axis represents the autosomal chromosomes and the y-axis shows the -$\log_{10}(p)$. The red line indicates the genome- wide significance level (p = 5 x 10^{-8}) and the blue line indicates the suggestive significance level (p = 10^{-5}).

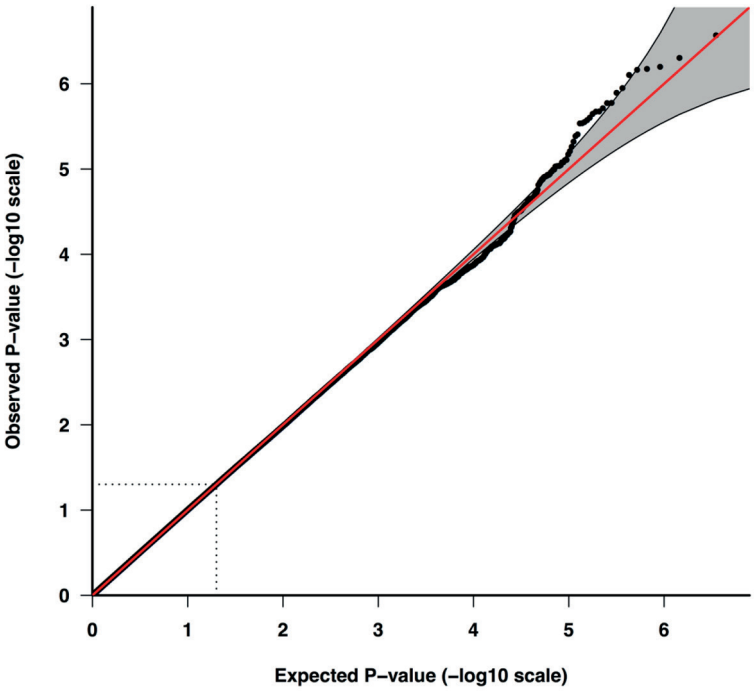

Figure S7 | Quantile-quantile (QQ) plot illustrating probability values from GWAS meta-analysis of aggressive behavior in middle childhood/ early adolescence (N = 16,311). The red line indicated the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

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Beyond the Usual Suspects: a Multidimensional Genetic Exploration of Infant Attachment Disorganization and Security

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Attachment and Human Development. 2015 (3): 288-301

Abstract

Although the environmental influences on infant attachment disorganization and security are well-studied, little is known about their heritability. Candidate gene studies have shown small, often non-replicable effects. In this study, we gathered the largest sample (N = 657) of ethnically homogenous, 14-month-old children with both observed attachment and genome-wide data. First, we used a Genome-Wide Association Study (GWAS) approach to identify single nucleotide polymorphisms (SNPs) associated with attachment disorganization and security. Second, we annotated them into genes (Versatile Gene-based Association Study) and functional pathways. Our analyses provide evidence of novel genes (*HDAC1, ZNF675*, *BSCD1*) and pathways (synaptic transmission, cation transport) associated with attachment disorganization. Similar analyses identified a novel gene (*BECN1*) but no distinct pathways associated with attachment security. The results of this first extensive, exploratory study on the molecular-genetic basis of infant attachment await replication in large, independent samples.

Introduction

The formation of a secure attachment to a primary caregiver in infancy is one of the most important milestones in human development [1,2]. Compromised quality of attachment relationships, in particular disorganized attachment, negatively affects children's later health and well-being, predicting hostile and aggressive behavior [3], internalizing [4] and externalizing problems [5], lower self-regulation [6], and peer problems [7]. Thus understanding the etiology of the development of disorganized and secure attachment is crucial. Whereas the environmental and early family influences on attachment disorganization and security are well explored [8,9], little is known about the heritable components of attachment. Here we present results from a genome wide association study (GWAS), and from gene-based and pathway analyses, aiming to elucidate the genetic components of infant attachment disorganization and security.

Twin studies have been the major source providing heritability estimates of infant attachment. These studies included relatively small samples of infants and young children, and nearly all indicated no genetic effect on attachment disorganization and security [10-13]. However, a recent twin study found significant genetic influences (40%) on attachment security in a sample of adolescents [14], suggesting that the contribution of genetic factors to attachment may be larger in older children than previously expected. Furthermore, there is some evidence of early neurodevelopmental influences of infant attachment disorganization [15].

Molecular genetic studies of attachment have primarily focused on main genetic effects of common polymorphic variations in dopaminergic [16,17], serotonergic [18] and oxytonergic [19] genes. Some of these studies provide evidence for an association between single gene polymorphisms and attachment. However, a large study of over 1,000 children from two cohorts found no evidence of single genetic markers associated with infant attachment [20]. On the contrary, there is evidence of common dopamine D4 receptor polymorphisms moderating the association between maternal unresolved loss or trauma and infant disorganization [21]. These findings support a notion that infant attachment is complex and multi-dimensional, being susceptible to both genetic and environmental effects.

In sum, the genetic architecture of attachment is still unclear: heritability estimates from twin studies range from negligible to moderate, whereas molecular genetic studies point to small influences of single genes but suffer from lack of replication. The combined influence of multiple genetic factors acting together has not been tested. Furthermore, the availability of new methods to analyze genes and functional pathways associated with specific phenotypes has not yet been applied to attachment quality.

In the current study, we observed infant attachment using the Strange Situation Procedure (SSP, [22]) in an ethnically homogenous group of 14-month-old infants, generating the largest sample with both attachment and genome-wide data (N = 657) to date. We used a multidimensional genetic approach, combining hypothesis-free genome-wide analyses with gene-based and pathway analyses. To our knowledge, this is the most extensive exploratory study and the first of its kind to explore the genetic components of infant attachment disorganization and security using state-of- the-art methods.

Methods

Setting

The study was conducted on children participating in the Generation R Focus Study [23]. This is a subsample of the Generation R Study, a population-based cohort in which children are followed from fetal life onwards. The design and sample characteristics of the Generation R Study have been described in detail elsewhere [23]. The current study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from parents of the participating children.

Study Population

All children of the Generation R Focus Study are of Dutch origin. DNA was collected from cord blood samples at birth. At age 14 months (M = 14.7, SD = 0.9), infants and their mothers visited the research center where they participated in the Strange Situation Procedure (SSP, [22]). For N = 657 infants (51% boys), information on both genotype and attachment was available.

Genotyping, Quality Control and Imputation

DNA was extracted from whole blood cells and genotyped on Illumina 610K/660W platforms. Basic quality checks for each SNP included sample call rates (≥ 97.5%), SNP call rates (≥ 90%), minor allele frequency (MAF) ≥ 1% and deviation from the Hardy Weinberg equilibrium (p < 1e-07). Samples were also checked for excess heterozygosity, gender accuracy, relatedness, and missing data. Following the quality control steps, phased genotype data were imputed to build 37 (hg19) of HapMap reference panel, using the MACH software [24]. After filtering on imputation quality ($r^2 > 0.30$), more than 2.5 million SNPs remained for further analysis.

Strange Situation Procedure

Mother-infant dyads were observed in the Strange Situation Procedure (SSP). The SSP is a well-validated, widely used procedure to measure attachment quality [1]. The procedure consists of seven episodes of 3 minutes each and is designed to evoke mild stress in the infant to trigger attachment behavior evoked by the unfamiliar lab environment, a female stranger entering the room and engaging with the infant, and the parent leaving the room twice [22]. A slightly shortened version of the SSP was used (pre-separation and separation episodes were shortened by 1 min each), with the critical reunion episodes remaining intact [15,20].

Attachment behavior was coded from recordings according to established coding systems by trained coders [22]. Using discriminant analysis on a large set of SSPs, an algorithm was developed to transform the SSP behavioral ratings into a continuous attachment security score that is highly predictive of the secure versus insecure classifications. Van IJzendoorn and Kroonenberg [25] adapted the algorithm leaving out crying behaviors, resulting in a valid Attachment Security Scale based on the interactive scales, which has been widely used because of the advantages of conducting

statistical analyses on continuous instead of categorical variables [26]. For purposes of the current analysis, continuous scores for disorganization were derived directly from coding, with higher scores indicating more disorganized behavior [27]. Higher security scores indicate a more secure attachment relationship.

Intercoder reliability [intraclass correlation coefficients (ICCs)] for the continuous attachment security and disorganization scores were .88 and .88, respectively (N = 70).

Single-variant and gene-based association analysis

First, GWAS analyses were performed for both attachment disorganization and security scores, treating them continuously and using a linear regression model on allele-dosage, age, sex and the first four principal components of the genotype data, to control for subtle population stratification. SNP allele dosages were obtained from the imputed data and used for the GWAS analyses. Analyses were performed using GRIMP [28].

Second, gene enrichment analysis was performed on the summary statistics of the GWAS outcomes for both attachment disorganization and security scores, using the versatile gene-based association analysis (VEGAS). The principles of VEGAS have been described by Liu et al. [29]. In brief, we used a computational method to determine whether polymorphisms in predefined set of genes show statistically significant differences within the distribution of attachment disorganization and security scores. VEGAS accounts for the different SNPs in a gene (± 50 kb from the start/ end of the gene) and the linkage disequilibrium (LD) between them to estimate a gene-based *p-value* for the association of each gene with the trait of interest. Genes located on the X-chromosome were excluded from this analysis.

Genome-wide pathway analysis

For both attachment disorganization and security scores, genome-wide pathway analyses were performed using the PANTHER 9.0 release database, as described in detail in Mi et al. [30]. Briefly, the principle is to identify the most probable candidate genes resulting from the GWAS efforts, and sort them into pathways using the PANTHER pathway-classification tool. For this purpose, all SNPs with GWAS p < 1e-04 were selected, an empirical threshold commonly used in genome-wide pathway analyses, resulting in N_{SNPs} = 328 for attachment disorganization and N_{SNPs} = 202 for attachment security scores. Subsequently, these SNPs were annotated to genes using the SCAN (SNP and Copy number ANNotation) database [31], resulting in N = 34 genes for both attachment disorganization and attachment security. In both cases, we estimated the overrepresentation of these genes in specific pathways using the PANTHER Gene Ontology (GO)-Slim Biological Process annotation dataset.

Results

Sample characteristics

For the N = 657 children with both attachment and genotype data, attachment disorganization scores ranged from 1.00 to 9.00 (M = 3.40, SD = 1.89) and attachment security scores ranged from -8.23 to 5.81 (M = 0.28, SD = 2.57). As expected, attachment disorganization and security were negatively correlated (r = -.29, N = 657, p < .01).

GWAS analyses

After filtering and quality control, approximately 2.5 million genotyped and imputed SNPs remained for genome-wide analyses. GWAS on attachment disorganization scores revealed a suggestive locus on chromosome 3p21 (p = 1.25e-07) and several suggestive hits across other chromosomes (i.e. chr12q24, chr5q15, chr3q23, chr7q11). The suggestive SNPs are summarized in Table 1. The Manhattan and quantile-quantile QQ plots of GWAS on attachment disorganization scores are presented in Figures 1 and 2, respectively.

GWAS on attachment security scores revealed also three suggestive loci, on chromosome chr2q31, chr3p25 and chr6q12. These loci are summarized in Table 2. The Manhattan and QQ plots of GWAS on attachment security are presented in Figure 3 and 4 respectively.

Gene-based analyses

The association between attachment disorganization scores and genes was assessed with the VEGAS gene-based analysis. Bonferroni correction for multiple testing (N = 17,876 genes) indicated as new threshold of significance p = 2.80e-06. Three genes (*HDAC1*, *ZNF675* and *BSDC1*) were significantly associated with attachment disorganization and seven genes showed suggestive associations. The results are summarized in Table 3.

Table 1 | Top signals that reached suggestive genome wide significance (p < 1.00e-05), sorted by ascending p, for disorganized attachment (N = 657).

SNP	Chromosome	Position	Allele 1/2	Frequency 1	# hits	B [a]	SE [b]	p	Nearest gene(s)
rs9825336	3	54282122	T/C	0.30	13	-0.30	.06	1.25e-07	*CACNA2D3*
rs7972837	12	115258353	G/A	0.54	1	0.26	.06	5.77e-07	*MED13L*
rs11742025	5	8552567	C/A	0.43	2	0.27	.06	7.53e-07	*MIR4485HG; LOC729506*
rs16849396	3	140959719	T/C	0.30	6	0.27	.06	5.46e-07	*CLSTN2; NMNAt3*
rs4728533	7	75424472	C/T	0.15	1	-0.45	.10	9.00e-06	*POR*

[a] B = effect of allele 1; [b] SE = standard error

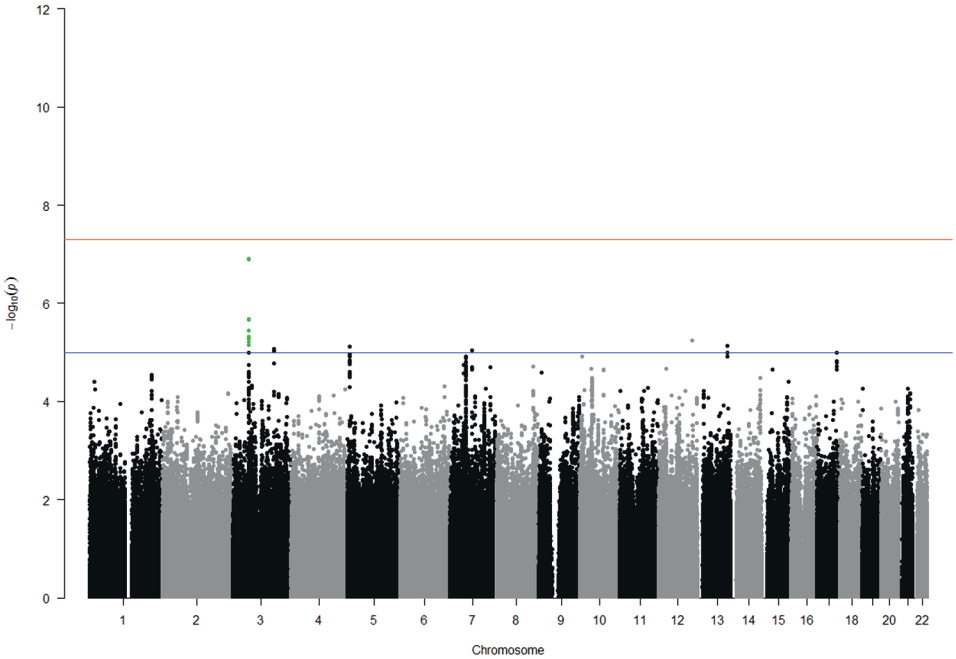

Figure 1 | Manhattan plot of the GWAS on attachment disorganization (N = 657). The x-axis represents the autosomal chromosomes and the y-axis shows the -$\log_{10}(p)$. The red line indicates the genome-wide significance level (p = 5.00e-08) and the blue line indicates the suggestive significance level (p = 1.00e-05). The green spots highlight the top locus (chromosome 3p21).

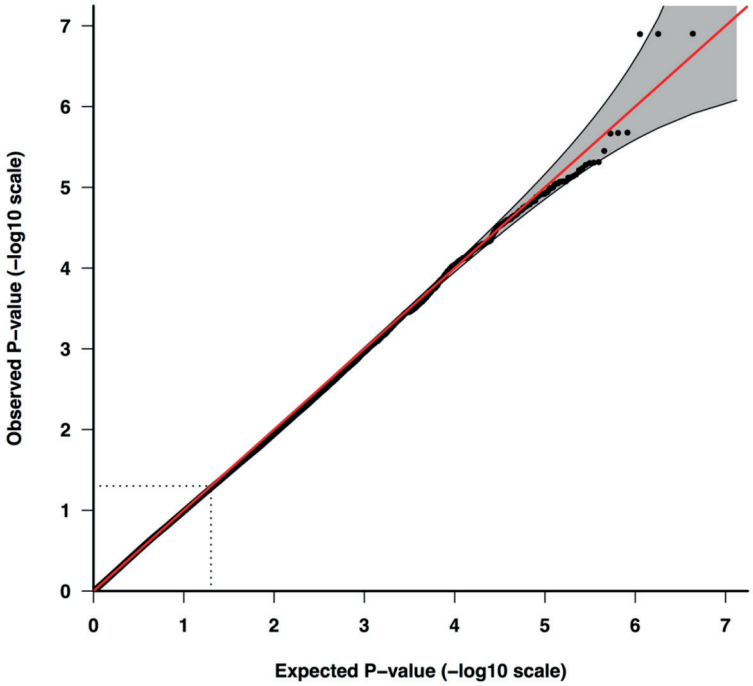

Figure 2 | Quantile-quantile (QQ) plot illustrating probability values from GWAS on attachment disorganization. The red line indicates the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

Table 2 | Top signals that reached suggestive genome wide significance (p < 1.00e-05), sorted by ascending p, for attachment security (N = 657).

SNP	Chromo-some	Position	Allele 1/2	Frequency 1	# hits	*B* [a]	SE [b]	*p*	Nearest gene(s)
rs17405552	2	187108194	A/G	0.10	1	0.43	.09	1.80e-06	*ITGAV; ZC315*
rs17041841	3	16076986	A/G	0.17	5	-0.36	.08	2.00e-06	*GALNT15*
rs9363340	6	66052728	C/G	0.13	1	-0.44	.10	6.20e-06	*EYS*

[a] *B* = effect of allele 1; [b] *SE* = standard error

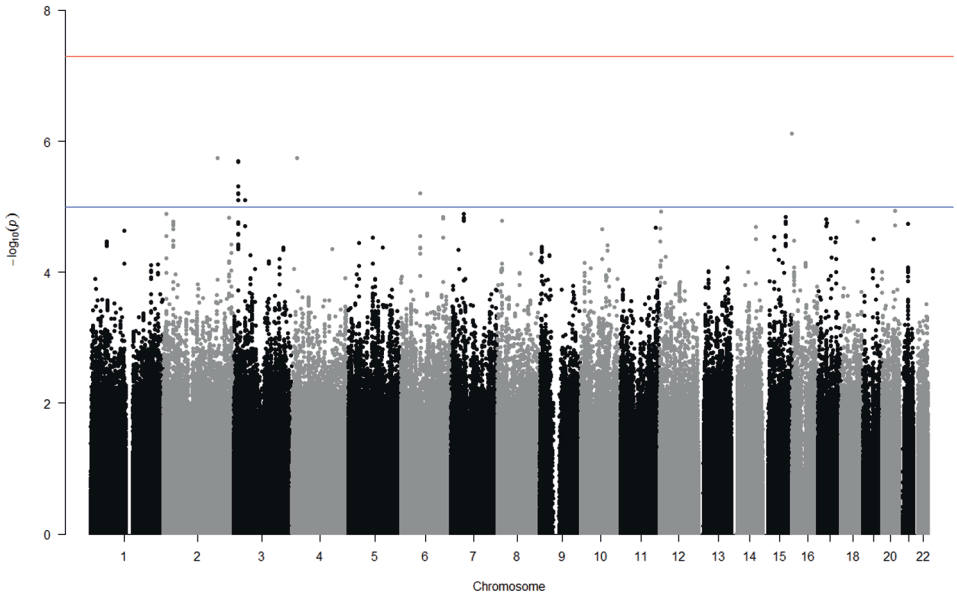

Figure 3 | Manhattan plot of the GWAS on attachment security (N = 657). The x-axis represents the autosomal chromosomes and the y-axis shows the -$\log_{10}(p)$. The red line indicates the genome-wide significance level (p = 5.00e-08) and the blue line indicates the suggestive significance level (p = 1.00e-05).

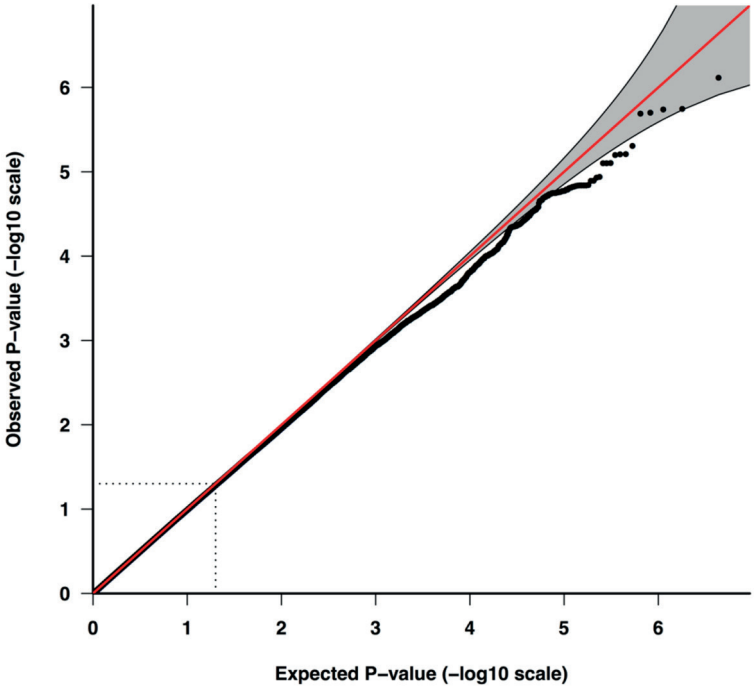

Figure 4 | Quantile- quantile (QQ) plot illustrating probability values from GWAS on attachment security. The red line indicates the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

Table 3 | Top genes associated with attachment disorganization, using VEGAS gene-based test (Bonferroni-corrected threshold, $p < 2.80e-06$).

Gene name	Chromosome	N_{SNPs}	Start position (bp)	Stop position (bp)	Gene-based *p*
HDAC1	**1**	**43**	**32530294**	**32571811**	**1.00e-06**
ZNF675	**19**	**74**	**23627547**	**23661857**	**1.00e-06**
BSDC1	**1**	**47**	**32603362**	**32632614**	**2.00e-06**
CCDC56	17	41	38203177	38204230	1.00e-05
CNTD1	17	46	38204379	38217131	1.10e-05
AOC2	17	40	38250134	38256251	1.30e-05
ZNF681	19	151	23713836	23733533	2.10e-05
RXRA	9	190	136358230	136472252	3.40e-05
NAPEPLD	7	90	102527258	102576805	4.20e-05
DDX19A	16	48	68938324	68964782	5.70-05

For the attachment security score, we found one gene significantly associated after Bonferroni correction (*BECN1*, $p = 2.00e-06$) and seven genes with suggestive associations. Note that two of these suggestive genes (*ZNF675* and *CNTD1*) were also identified as significantly or suggestively associated with attachment disorganization. The results are summarized in Table 4.

Table 4 | Top genes associated with attachment security, using VEGAS gene-based test (Bonferroni-corrected threshold, $p < 2.80e-06$).

Gene name	Chromosome	N_{SNPs}	Start position (bp)	Stop position (bp)	Gene-based *p*
BECN1	**17**	**45**	**38215675**	**38229836**	**2.00e-06**
LRRC37A3	17	19	60280949	60345365	7.00e-06
MYEF2	15	127	46218920	46257850	1.10e-05
DPYSL3	5	215	146750563	146813453	3.10e-05
ZNF675	19	74	23627547	23661857	3.30e-05
PDPK1	16	31	2527970	2593190	5.20e-05
STK32A	5	437	146594771	146743961	6.90e-05
CNTD1	17	46	38204379	38217131	7.20e0-5

Genome-wide pathway analyses

For attachment disorganization, two pathways (as annotated by the PANTHER pathway annotation methods) were significantly enriched after conservative Bonferroni correction for not completely independent pathways. Specifically, the synaptic transmission ($p = .006$) and the cation transport ($p = .04$) pathway were overrepresented in the suggestive GWAS results. The top pathways associated with disorganized attachment are presented in Table 5.

For attachment security, no pathway was significantly enriched according to the PANTHER annotation methods.

Table 5 | Top pathways overrepresented in suggestive GWAS hits (GWAS p < 1.00e-04) for attachment disorganization, estimated by PANTHER version 9.0. Note that genes can participate in multiple pathways.

PANTHER GO-Slim Biological Process	Expected N_{genes}	Observed Ng_{enes}	Fold Enrichment	Bonferroni-correct p
synaptic transmission	**0.61**	**6**	**> 5**	**.006**
cation transport	**0.87**	**6**	**> 5**	**.04**
neuron-neuron synaptic transmission	0.13	3	> 5	.07
ion transport	1.01	6	> 5	.09

Discussion

We conducted a GWAS as well as gene-based and gene-pathway analyses on observed infant attachment disorganization and security, encouraged by the successful recent application of similar approaches in medical research to identify new groups of genes and gene networks relevant for a specific trait or disorder [32]. We found no genome-wide significant associations between single SNPs and attachment disorganization. However, gene-based analyses using the VEGAS tool provided evidence of three genes associated with attachment disorganization (i.e. *HDAC1*, *ZNF675* and *BSDC1*). Further analyses with the PANTHER tool showed overrepresentations of the synaptic transmission and cation transport pathways related to higher attachment disorganization scores. Similar analyses for attachment security yielded evidence of one significantly associated gene (*BECN1*) but no overrepresentation of pathways related to higher scores on attachment security. It is possible that genetic influences are more prominent in atypical phenotypes, such as attachment disorganization, than in normative phenotypes, such as attachment security [18].

The nature of this study was exploratory, thus we used the hypothesis-free GWAS approach to identify SNPs in association with attachment disorganization and security scores. The quantile-quantile plot of attachment disorganization indicated a possible association with the *CACNA2D3* gene, on chromosome 3p21, which codes for a voltage-dependent calcium channel [33,34]. Other members of the same family of voltage-dependent calcium channels have been consistently associated with schizophrenia symptoms in the general population [35]. The role of *CACNA2D3* in attachment should be further investigated.

In addition to *CACNA2D3*, our gene-based analyses (with the VEGAS tool) indicated *HDAC1* as a possible gene associated with attachment disorganization. *HDAC1* is a histone deacetylase and regulates other genes that are expressed in the brain, such as *BDNF* [36]. It has been also suggested that

HDAC1 epigenetically mediates the effect of emotional stimuli to behavior in animal models [37]. We identified two other genes that were significantly associated with infant attachment disorganization – *ZNF675* and *BSCD1* – but little is known about their function.

The pathway analysis (with the PANTHER tool) indicated two biological processes (synaptic transmission and cation transport) overrepresented in association with attachment disorganization. Although many genes are involved in these pathways, the results point to pathways involved in neuronal function and synaptic plasticity. A previous study found that early structural differences in the gangliothalamic ovoid, comprising the basal ganglia and the thalamus, were involved in the etiology of infant attachment disorganization [15], which might be the corollary of the genetic associations with attachment disorganization.

Similar analyses for attachment security also revealed only suggestive and no significant loci in our GWAS analysis. However, gene-based tests indicated an association between *BECN1* and attachment security. *BECN1* is an autophagy related gene and has been implicated in neurodevelopmental disorders, such as schizophrenia [38] and autism [39]. The gene-based analysis indicated two suggestive genes (*ZNF675* and *CNTD1*) associated with both attachment disorganization and security, indicating the possibility of partially shared genetic etiology. Attachment disorganization and security are related and partially overlapping constructs, with a moderate negative correlation in the current sample, and perhaps it is this overlap that has a common genetic influence. However, the pathway analysis revealed no significant pathways associated with attachment security.

This study is not without limitations. Although our sample is the largest to date, our main limitation remains the sample size. Arguably, high quality observational measurements (such as the Strange Situation Procedure) and the use of continuous measurements (attachment scores instead of classifications) may substantially reduce the number of participants needed [40]. However, larger samples are needed to improve the power to find common SNPs associated with infant attachment in the general population. Another limitation of this study is the lack of replication in an independent sample of children. To our knowledge, there is no other study available with genome-wide data and observational measures of infant attachment. Finally, in this study we were particularly interested in exploring polymorphisms with main genetic effects on infant attachment. It is possible that some polymorphisms associated with attachment disorganization or security cannot be identified without considering the role of the environment. In fact, it is a common misconception that candidate gene moderating the influence of the environment would have to emerge in GWAS or other approaches aiming at main genetic effects. Dopamine-system related genes have been shown to moderate environmental influences in a differential susceptibility fashion in a series of experimental gene-environment (GxE) studies, without having main effects on the phenotypes [41].

The current study represents a first attempt to explore the genetic architecture of infant attachment, using multiple approaches to probe associations at different levels: at the level of the individual SNP (GWAS), at the level of genes (VEGAS), and at the level of gene pathways (PANTHER). We used a hierarchical, multi-method approach to first identify SNPs associated with attachment, annotate them to the relevant genes and finally sort these genes into functional pathways. Our findings indicate

that attachment disorganization may be partially influenced by multiple genes with small effects. The novel genes and pathways that have been found here could be the basis for further research, also in animal models [42]. For attachment security, our study showed weaker evidence of genetic influences, indicating distinct neurobiological pathways for attachment disorganization and security.

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SECTION C

The epigenome-wide approach

7

An Epigenome-wide Association Study of Global Prenatal Maternal Stress in Neonates

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Manuscript in preparation

Abstract

Adverse early life social and physical environments have been associated with unfavorable neurodevelopmental outcomes in children. DNA methylation patterns could mediate the effect of these adverse environmental cues by regulating gene expression during fetal and early life. In this study, we investigated the effects of exposure to maternal stress *in utero* on DNA methylation patterns at birth. To this aim, we constructed a prenatal maternal stress exposure (PMSE) score, which incorporates different aspects of stress (i.e., stressful life events, contextual stress, maternal psychopathology, and interpersonal stress). We performed an hypothesis-free epigenome-wide association study (EWAS), using data from a population-based sample of $N = 912$ Caucasian mothers and their neonates. Follow-up epigenome-wide pathway analysis and novel differential methylated regions (DMRs) discovery methods were also performed. We found no significant Bonferroni-corrected effects of PSME in DNA methylation in neonates. Pathway analysis indicated over-representation of specific pathways among our EWAS-suggestive results, related to cell cycle regulation, metabolic processes, and immunity. Finally, DMRs analysis in cord blood identified only suggestive regions of differential methylation associated with levels of PMSE score. This study did not identify any large effects of prenatal exposure to common forms of maternal stress in neonatal DNA methylation. However, subtle effects cannot be ruled out and larger, collaborative efforts are needed to test this hypothesis.

Introduction

Exposure of the fetus to maternal stress *in utero* can negatively affect development in later life [1-4]. For example, prenatal exposure to maternal depressive symptoms [5] and contextual stress (e.g., economic disadvantage) [6] have been associated with increased risk for offspring problem behavior, beyond variance attributable to postnatal exposures. It is increasingly recognized that epigenetic mechanisms, such as DNA methylation, might help explain the link between prenatal exposure to maternal stress and adverse developmental consequences [7,8]. The extent to which prenatal maternal stress exposure relates to offspring genome-wide DNA methylation at birth is yet to be evaluated.

The vast majority of studies investigating the association between prenatal exposure to maternal stress and offspring methylation at birth have focused on candidate genes [9-13]. For example, Cecil et al. (2014) demonstrated that neonates who were exposed to maternal stress (e.g., maternal psychopathology, criminal behaviors, substance use) in the prenatal period had higher methylation levels of the oxytocin receptor (*OXTR*) gene than non-exposed neonates [9]. Higher neonatal *OXTR* methylation, in turn, showed temporal stability (birth to age 9 years) and was associated with callous-unemotional traits 13 years later [9]. Similarly, prenatal exposure to maternal depressive symptoms has been associated with altered methylation of the serotonergic *SLC6A4* gene [10], the glucocorticoid receptor (*NR3C1*) gene [11], and imprinted maternally expressed genes [12,13] in neonates or infants. Overall, the findings of these candidate gene studies support the "fetal programming hypothesis" [14], suggesting that exposure of the fetus to maternal stress in utero may have lasting effects on DNA methylation in genes involved in fundamental developmental processes.

Since strong prior biological knowledge on the complex association between prenatal maternal stress exposure and offspring differential DNA methylation is lacking, it is critical to perform hypothesis-free, epigenome-wide association studies (EWASs) [15]. A previous EWA study provides evidence that prenatal exposure to extreme stressors, such as a natural disaster [i.e., the Quebec ice storm in 1998 [16]], is associated with offspring differential methylation in multiple genes. Although this study contributed significantly to the understanding of prenatal maternal stress exposure and offspring epigenetic modifications measured at eight years of age, the extent to which prenatal maternal exposure to other, more common, stressful events associates with neonatal differential genome-wide DNA methylation is not yet known.

To this aim, we used data from a population-based study, the Generation R Study (N = 912) to (i) extract a broad prenatal maternal stress composite that incorporates life stress (e.g., death in family, illness, work problems), contextual stress (e.g., financial difficulties, housing problems), maternal stress (e.g., psychopathology, substance abuse), as well as interpersonal stress (e.g., family relationship difficulties, arguments with friends); and (ii) examine the association between prenatal maternal stress exposure and offspring genome-wide cord blood methylation using single probe analysis, follow-up epigenome-wide pathway analysis, and novel region discovery methods [i.e., differentially methylated regions (DMRs) analyses] tailored to the Illumina 450K BeadChip array [17].

Methods

Study Design and Participants

This study used data from a population-based cohort, the Generation R (GEN-R) Study. GEN-R is a prospective study of children born from pregnant women residing in Rotterdam, the Netherlands, with expected delivery dates between April 2002 and January 2006. The design and sample characteristics of the Generation R Study have been described in detail elsewhere [18]. The current research used a subsample of 969 Caucasian neonates and their parents nested within the Generation R cohort who had epigenetic data at birth.

Except for factor analysis, in which we used data from all participants, the present study only included participants who had complete data for prenatal maternal stress exposure (N = 912). In all cases, parents signed written consent for participation. Study protocols were approved by the local ethics committee.

Genome-wide DNA methylation

500 nanograms of DNA from cord-blood (birth, N = 979) underwent bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, USA). We used the Illumina Infinium HumanMethylation450 BeadChip Kit (Illumina Inc., San Diego, USA) to measure DNA methylation at 485,577 CpG probes. Following standard criteria, quality control of data was performed by determining sample call rate, color balance, staining efficiency, extension efficiency, hybridization performance, stripping efficiency after extension, and bisulfite conversion. The present study included the 969 neonates who had DNA methylation data that passed quality control. All 49,564 probes identified as having (i) a single nucleotide polymorphism in the single base extension site with a frequency of > 1% in the GoNLv4 reference panel [19] or (ii) a non-optimal binding (non-mapping or mapping multiple times to either the normal or the bisulfite-converted genome) were removed from the dataset, leaving a total of 436,013 CpG probes for analysis. Samples were normalized using the 'dasen' method described by Pidsley et al. [20] and dye bias corrected [21]. Normalized values were converted into beta-values, which represent the methylation level at a CpG probe for each neonate.

Prenatal Maternal Stress Exposure

A prenatal maternal stress exposure (PMSE) score had been previously created based on maternal reports [9], covering four stress domains: (i) life stress (e.g., death in family, illness, work problems), (ii) contextual stress (e.g., financial difficulties, housing problems), (iii) maternal stress (e.g., psychopathology, substance abuse), and (iv) interpersonal stress (e.g., family relationship difficulties, arguments with friends). In this study, we used similar measures to create a PMSE score, based on maternal reports. For each domain, items were summed and divided by the number of completed items. Inter-correlations between the risk domain scores were positive and statistically significant (all $p < .001$). Confirmatory factor analysis (CFA) in Mplus 7.11 [22] was used to assess the internal

reliability of the stress domains and to extract one PMSE score in the whole Generation R cohort, showing good model fit (RMSEA; acceptable fit ≤ 0.08; CFI and TLI; acceptable fit ≥ 0.90) [23,24]. See Supplementary Material for full item descriptions (Table S1), inter-correlations between the stress domains (Table S2), and the confirmatory factor analysis model and fit indices (Figure S1).

The PMSE score was logarithmic (Log 10) transformed to approximate a normal distribution. Additionally, the transformed PMSE score was translated into z-scores and screened for outliers (values were winsorized when z-score ≥ 3.29, affecting n = 8 measurements . These analyses were conducted in SPSS 21.0 statistical package [25].

Covariates

We adjusted for technical covariates, including the sample's array number and position on the array. Following the methods developed by Houseman et al. [26], we included estimated proportions of cells in whole blood [% of CD8 T-cells, CD4 T-cells, natural killer (NK) cells, B-cells, monocytes and granulocytes] to adjust for different cell type compositions.

Furthermore, we included as covariates child gender, gestational age at birth, and maternal smoking during pregnancy. Information on child gender was obtained from midwife and hospital registries at birth. Gestational age at birth was established by fetal ultrasound examination. Information on maternal tobacco smoking was obtained by postal questionnaires in early, mid-, and late pregnancy. Maternal smoking was categorized on the basis of all three questionnaires into "never smoked during pregnancy", "quitted as soon as pregnancy was known", and "continued smoking during pregnancy".

Epigenome-wide association study (EWAS) analysis

Single probe analysis investigating the association between the PMSE score and neonatal DNA methylation was performed for all CpG probes that passed quality control (N = 436,013) using a linear mixed effects model, adjusting for fixed (i.e., gestational age, sex, maternal smoking during pregnancy, cell types estimation) and random (i.e., batch effects) covariates. The strict EWAS Bonferroni-corrected threshold for significance was estimated as p = 0.05 / 436,013 = 1.15e-07, while the level of suggestive significance was set to p < 1.00e-05.

Epigenome-wide pathway analysis

To investigate whether specific pathways are over-represented in our EWAS results, epigenome-wide pathway analyses were performed. For this purpose, all suggestive CpG sites (N_{CpGs} = 16) from the EWAS analysis (p < 1.00e-05) were selected and annotated to genes (N_{genes} = 15), using the IlluminaHumanMethylation450kCHR36 file. We used GeneMANIA [27] pathway tool sort the suggestive genes into pathways, based on the Gene Ontology (GO)-Slim Biological Function annotation dataset. Since in pathway analyses, the same genes are allowed to participate in different pathways, resulting in over-representation of not completely independent pathways, the false discovery rate (FDR) was used to adjust for multiple testing.

Differentially Methylated Regions (DMRs) analyses

Differentially Methylated Regions (DMRs) are defined as at least two spatially contiguous probes within 1 kb distance of each other and with a differential statistic consistently less than the 5th (for negative associations) or more than the 95th percentile (for positive associations) [28]. To perform DMRs analyses, we first used the *clusterMake*r function of the *bumphunter* package [29] to assign CpG probes into clusters, separated with a maximum distance of 1kb. For each cluster, we used the *GlobalTest* statistical package [30], to test the association of the PMSE score to differential methylation of each of these clusters, using a linear regression model, while adjusting for covariates.

Results

Sample Characteristics

The sample characteristics for all mothers and children participating in this study are presented in Table 1. The mean methylation values (β-values) in the cord blood showed a bimodal distribution, with 42% of the CpG probes being hypomethylated (i.e. β-value < 0.3), 11% of the CpG probes showing intermediate levels of methylation (i.e. β-value ≥ 0.3-0.7), and 47% of the CpG probes being hypermethylated (i.e. β-value ≥ 0.7).

Table 1 | Sample characteristics of mothers and children participating in this study.

	GEN-R (*N* = 912)
Child characteristics	
– Gender, % girls	48.4
– Gestational age, wk (*sd*)	40.2 (1.43)
Maternal characteristics	
– age (*sd*)	31.7 (4.13)
– PMSE score, log transformed (*sd*)	0.15 (.11)
– Smoking during pregnancy, %	
never	77.5
until pregnancy was confirmed	7.7
continued	14.8

Epigenome-wide association analysis

The EWAS analysis (N = 912) revealed no epigenome-wide significant association of the PMSE score with neonatal DNA methylation. However, several suggestive hits (p < 1.00e-05) showing differential methylation in multiple genes across the genome were observed. The top CpG probes derived from this EWAS analysis are presented in Table 2. The Manhattan and quantile-quantile plot are presented in Figures 1 and 2, respectively.

Table 2 | Top CpG probes that reached suggestive epigenome-wide Bonferroni- corrected significance (p < 1.00e-05), sorted by ascending p (N = 912).

Probe Name	Chromosome	Position	Effect (*SE*)	*P*	Nearest gene(s)
cg25937149	5	175788949	-.01 (.001)	2.10e-07	*KIAA1191*
cg19510604	15	74901076	-.03 (.006)	1.83e-06	*CLK3*
cg13529437	6	43607635	-.03 (.008)	3.31e-06	*MAD2L1BP*
cg27331471	19	45809725	.07 (.015)	5.34e-06	*CKM*
cg18094366	22	24237343	.01 (.001)	5.90e-06	*MIF*
cg24822696	6	125283245	.01 (.002)	6.48e-06	*RNF217*
cg26251101	2	46768944	.01 (.003)	6.64e-06	*RHOQ*
cg21888241	11	14541921	-.01 (.001)	1.39e-05	*PSMA1*
cg25829945	3	159706383	.03 (.005)	1.40e-05	*IL12A*
cg20969194	2	223163175	.04 (.008)	1.50e-05	*PAX3*
cg27106909	16	30106897	.04 (.009)	1.63e-05	*YPEL3*
cg18721212	19	36827430	-.02 (.004)	1.67e-05	*ZFP14*
cg03166236	2	7062351	-.05 (.010)	1.89e-05	*RNF144A*
cg15423613	7	66093349	.01 (.001)	2.07e-05	*KCTD7*
cg20026939	18	44788898	.04 (.010)	2.13e-05	*NA*
cg18026026	10	131265411	-.01 (.002)	2.35e-05	*MGMT*

NA = not available

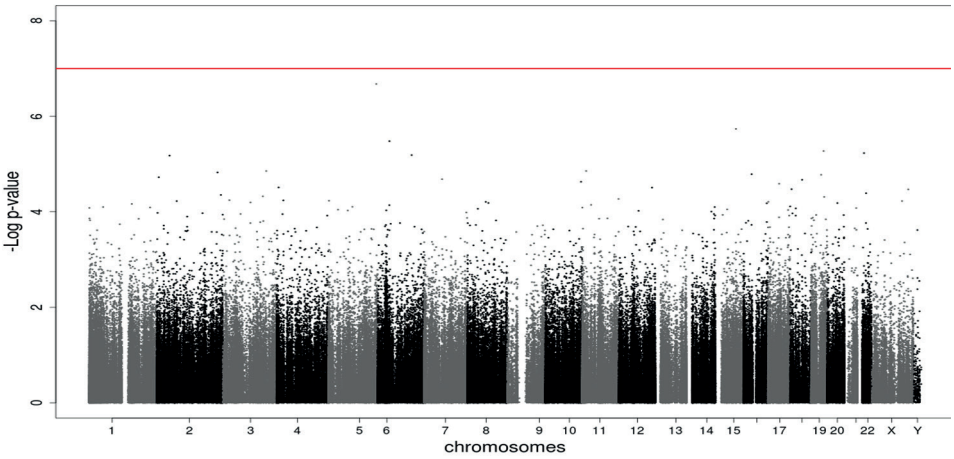

Figure 1 | Manhattan plot of EWAS of PMSE score in cord blood, in the GEN-R sample (N = 912). The x-axis represents the autosomal (1-22) and sex (X,Y) chromosomes and the y-axis shows the -$\log_{10}(p)$. The red line indicates the Bonferroni-corrected epigenome-wide significance level (p = 1.00e-07).

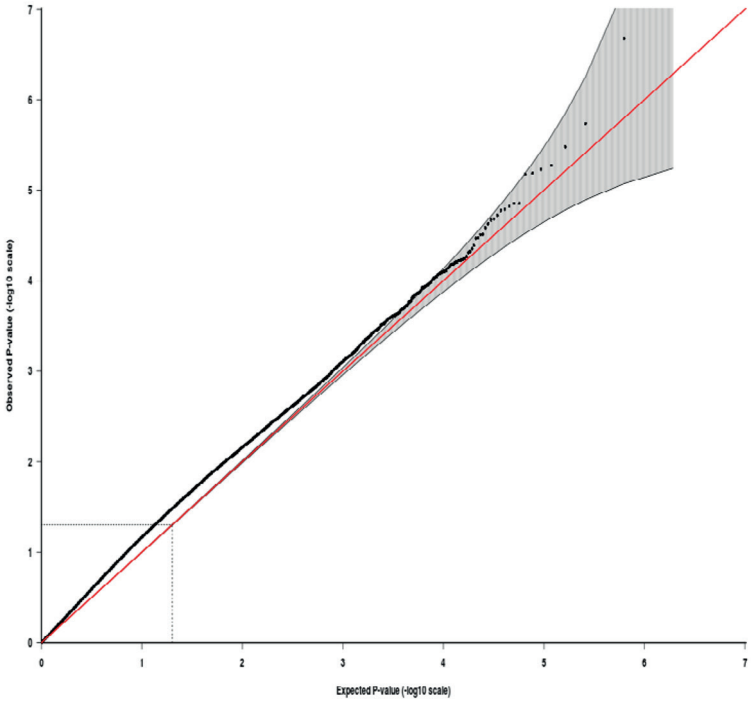

Figure 2 | Quantile-quantile (QQ) plot illustrating probability values from EWAS of PMSE score in cord blood, in the GEN-R sample (N = 912). The red line indicates the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

Epigenome-wide pathway analysis

GeneMANIA pathway analysis tool was used to test for over-representation of specific pathways within the suggestive (p < 1.00e-05) GpG probes identified by our EWAS analysis. We identified over-representation of specific pathways related to prenatal maternal stress exposure (e.g., the DNA damage response/ signal transduction by p53 class mediator pathway, p = 3.95e-13). The output of the pathway analysis tool is presented in more detail in Table 3.

Table 3 | Pathway analysis of suggestive CpG probes (p < 1.00e-05) derived from EWAS of PMSE score in neonates (N = 912).

Pathway	Coverage *	FDR corrected p $^{\$}$
DNA damage response/signal transduction by p53 class mediator	11/109	3.95e-13
Regulation of cellular amino acid metabolic process	9/57	1.60e-12
Lymphocyte regulated immunity	6/208	4.17e-04

* Number of genes in the network with a given function/ all genes in the genome with the same or similar function; $^{\$}$ FDR = false discovery rate

Differentially Methylated Regions (DMRs) analysis

We identified $N_{clusters}$ = 151,704 clusters of which $N_{>2}$ = 49,091 clusters contained more than 2 probes. We used the clusters containing more than 2 probes to test for an association with PMSE score in neonates. This analysis identified no Bonferroni- corrected (p = 0.05/49,091 = 1.00e-06) significant DMRS associated with PMSE score in the GEN-R sample. However, three DMRs, located in 20q13.33, 7q33, and 17q25.1, showed suggestive associations with the PMSE score in neonates. The results of the DMR analysis are presented in Table 4. A detailed annotation table of the DMRs can be found in the Supplementary Material (Table S3).

Table 4 | Top Differential methylated regions (DMRs) associated with the global prenatal maternal stress exposure (PMSE) score in cord blood (N =912).

Cluster Number	N CpGs	Chromosome	Start Position	End position	p	Nearest gene(s)
90946	3	20	62948037	62948235	3.75e-06	*MYT1; PCMTD2; CICP4*
135488	11	7	133811808	133812369	1.27e-05	*LRGUK*
66361	15	17	73974861	73976089	3.55e-04	*ACOX1; C17orf106*

Discussion

This study followed an hypothesis-free epigenome-wide (EWAS) approach to identify novel differentially methylated chromosomal sites in the cord blood of neonates exposed to prenatal maternal stress. Our EWAS analysis in a population- based cohort study, the Generation R Study (N = 912), did not provide evidence of Bonferroni-corrected significant DNA methylation differences in the cord blood of children exposed to prenatal maternal stress.

Assuming that the effects of prenatal maternal stress in DNA methylation in the neonates are mild to moderate, it is possible that among our suggestive CpG probes are some true positive findings, but the power to detect them in this study was insufficient. Of the suggestive CpG probes, the top hit (cg25937149, p = 2.10e-07) was located in chromosome 5q35.2, in the *KIAA1191* gene. The product of this gene has been characterized as an oxidoreductase, it is expressed in the brain, and might regulate dynamic processes related to neuronal growth [31]. The over-expression of this gene has also been implicated in Alzheimer's disease [32]. The exact contribution of this gene in conditions of maternal stress exposure is not straightforward and warrants further investigation.

Follow-up analysis of the CpG probes that showed suggestive (p < 1.00e-05) associations with prenatal maternal stress exposure indicated an over-representation of specific pathways, related to cell cycle control (i.e., the DNA damage response pathway, p = 3.95e-13), metabolic processes (i.e., the regulation of cellular amino acid metabolic process pathway, p = 1.60e-12), and immunity (i.e., the lymphocyte regulated immunity pathway, p = 4.17e-04). It is possible that fetal exposure to maternal stress activates the epigenetic machinery, regulating key cellular processes (e.g. cell division, metabolism), and preparing the child to better adapt to an adverse environment, which may continue after birth [33]. However, future molecular and animal studies are needed to investigate the strength of this hypothesis.

It has been shown that levels of DNA methylation are correlated in adjacent sites, although the patterns of this correlation are not yet clear [34]. In our sample, we searched for DNA methylation signatures over a window of 1kb, defined as differentially methylated regions (DMRs). Our analysis did not identify any Bonferroni-corrected significant DMR, although suggestive regions were identified. The top DMR, is located in chromosome 20q13.33 and spreads over several genes, such as the *MYT1* gene (a neural specific gene, playing a role in the developing nervous system [35]), the *PCMTD2* gene (associated with protein methylation [36]), and the *CICP4* gene (a pseudogene, action unknown). Although we can only speculate on the role of this DMR in relation to prenatal maternal stress exposure, we consider that DMR-identification methods could be helpful tools in the search of DNA signatures, providing maybe more generalizable results in comparison to single probe analyses [28].

The finding of no Bonferroni-corrected significant hit corroborates the previous EWAS by Schroeder et al. (2012), who reported no differential DNA methylation in neonates born to mothers with a lifetime history of mood disorder [37], but contrasts with the EWAS by Cao-Lei et al. (2014), who reported that prenatal exposure to a natural disaster was associated with offspring differential

methylation in several genes during childhood [16]. It is noteworthy that these studies differ in both the (i.e., depressive disorder versus participation in a natural disaster) and the timing (i.e., life- time versus acute) of stress exposure. What is more, according to a recent meta-analysis, the effect of prenatal maternal stress exposure on infant birth weight and gestational age may vary according to how stress is operationalized [38]. In the current study, we used a prenatal maternal stress exposure (PMSE) construct that incorporated a variety of stress domains, including life stress (e.g., death in family, illness, work problems), contextual stress (e.g., financial difficulties, housing problems), maternal stress (e.g., psychopathology, substance abuse), as well as interpersonal stress (e.g., family relationship difficulties, arguments with friends). It is possible that the effects of PMSE are more subtle than those of acute extremely stressful events (e.g., a natural disaster) and that larger sample sizes are needed to identify these small, but potentially biologically important DNA methylation differences in cord blood.

We argue that the stress domains used in the current study, more so than acute disaster-type stresses, are potentially generalizable to the broader population. For example, natural disasters are less common than financial and relationship difficulties. Using a more normative range of prenatal stresses, the current study promises to have wide-spread implications. The finding that there are no large effects of prenatal maternal stress exposure on neonatal DNA methylation but that more subtle effects cannot be ruled out may provide a springboard for future research identifying the targets and timing of intervention. A potential factor to be taken into account in future EWASs is the timing of the prenatal maternal stress exposure. In support of the fetal programming hypothesis, Tobi et al. (2015) showed in a recent EWAS that prenatal famine exposure in early gestation, but not in mid or late gestation, is associated with DNA methylation [39]. Findings of a recent EWAS by Richmond et al. (2015) suggest that, in contrast to the view that early pregnancy represents a critical time-window for influencing development, sustained exposure of the fetus to maternal smoking in utero is required to induce neonatal DNA methylation modifications [40]. The prenatal maternal stress measures used in the current study spanned 12 to 30 weeks of pregnancy and most indicators were assessed only once during pregnancy. There is a need for longitudinal EWASs investigating the relative roles of the early, mid, and late intrauterine environment in stress-induced DNA methylation changes.

The present results should be interpreted in the context of three main limitations. First, our analyses are limited to cord blood samples with heterogeneous cell types. Although it has become common practice to adjust for the proportion of cell types in the blood is EWASs [26], this approach has been validated for adult and not for cord blood samples. Alternative methods are needed to assess cell mixture distribution in cord blood. Second, although it has been shown that blood samples are adequate proxies of DNA methylation in other tissues [e.g. the brain, [41]], there is also accumulative evidence of tissue-specific DNA methylation [42]. Future research could investigate the effects of prenatal maternal stress exposure in specific tissues and cell types. Finally, an inherent characteristic of EWASs is that methylation at the majority of the CpG probes measured by the Illumina 450K array show low variation between individuals [43]. The pre-selection of CpG probes showing "meaningful" inter- individual variation, and/or the use of region discovery methods [28], may reduce the multiple

testing problem and increase the power to detect true associations. However, a consensus of how to deal with this issues has not been established yet.

The present study is the first, to our knowledge, to examine the association between prenatal maternal stress exposure and offspring genome-wide cord blood methylation. We used data from a population-based sample of 912 Caucasian mothers and neonates and performed an EWAS analysis, identifying several novel suggestive differentially methylated sites but no single CpG probe with strong associations with prenatal maternal stress exposure. The identification of differentially methylated regions across the genome was strengthened by follow-up analyses, including epigenome-wide pathway analysis, and identification of suggestive DMRs. The extent to which the severity, duration and timing of prenatal stress exposures associate with neonatal DNA methylation should be further investigated in large longitudinal studies with more extreme variations in relevant phenotypes.

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Supplementary Material

Table S1 | Prenatal maternal stress exposure (PMSE) items.

	Valid Range	M *(SD)*	Scale	Time Points	Measurement Unit	
Life Stress *n* = 886	0.00-5.37	1.47 (1.20)	Life events scale	20-25w gestation	Dichotomous	Your partner or child died
			Life events scale	20-25w gestation		A friend or relative died
			Life events scale	20-25w gestation		Child, partner or relative was seriously ill
			Quality of life scale	30w gestation		You were admitted to hospital (> 24 hours)
			Quality of life scale	20-25w gestation		You were ill (moderate or poor health)
			Life events scale	20-25w gestation		You lost your job
			Life events scale	20-25w gestation		You had problems at work or school
			Life events scale	20-25w gestation		You moved house
			Quality of life scale	20-25w gestation		You experienced vaginal bleeding
			Quality of life scale	30w gestation		You had a test to see if your baby was abnormal
			Life events scale	20-25w gestation		Your house or car was burgled
			Quality of life scale	12-20w gestation		This pregnancy was unplanned
			Pregnancy outcome questionnaire	12-20w gestation		You were often worried about the health of the baby
			Quality of life scale	30w gestation		You were unhappy about the obstetric care
			Pregnancy outcome questionnaire	12-20w gestation		You were often worried about the pregnancy

Table S1 | *(Continued)*

	Valid Range	M *(SD)*	Scale	Time Points	Measurement Unit	
Contextual Stress *n* = 839	0.00-3.61	0.58 (0.93)	Quality of life scale	30w gestation	Dichotomous	Housing basic living (indequate heating, no washing machine, or no refrigerator)
			Quality of life scale	30w gestation		Housing defects (cold or draught, windows are damp inside, or walls or furniture are damp)
			Long lasting difficulties questionnair	20-25w gestation		Housing adequacy (e.g., too small, lack of privacy)
			Long lasting difficulties questionnair	20-25w gestation		You had a major financial problem
			Quality of life scale	30w gestation		Financial difficulties
			Life events scale	20-25w gestation		Your income was reduced
Maternal Stress *n* = 902	0.00-1.41	0.16 (0.38)	Moral values questionnaire	20-25 gestation		You were in trouble with the law
			Family background question	Pregnancy composite		Early parenthood (age mother < 19 years)
			Family background question	Pregnancy composite		Maternal education
			Brief symptom inventory	20-25w gestation		Psychopathology of mother
			Lifestyle questionnaire	20-25w gestation		substance abuse
			Moral values questionnaire	20-25w gestation		Violence offence
			Moral values questionnaire			Public order offence

Table S1 | *(Continued)*

	Valid Range	M *(SD)*	Scale	Time Points	Measurement Unit	
Interpersonal Stress *n* = 884	0.00-6.36	1.04 (1.44)			Dichotomous	You argued with your partner
			Long lasting difficulties questionnai	20-25w gestation		You had arguments with your family or friends
			Family background question	Pregnancy composite		Partner status (single)
			Family assessment device	20-25w gestation		Family affection problems
			Social circumstances questionnaire	20-25w gestation		Family size (> 3)
			Life events scale	20-25w gestation		You were divorced
			Family assessment device	20-25w gestation		Family major problems: difficult making plans
			Family assessment device	20-25w gestation		Family major problems: do not accept each other
			Family assessment device	20-25w gestation		Family major problems: cannot talk about sadness
			Family assessment device	20-25w gestation		Family major problems: avoid talking about worries and problems
			Family assessment device	20-25w gestation		Family major problems: feel unaccepted
			Family assessment device	20-25w gestation		Family major problems: unpleasant and painful feelings
			Family assessment device	20-25w gestation		
			Family assessment device			Family major problems: cannot solve problems
			Family assessment device	20-25w gestation		Family major problems: decision-making is a problem
			Family assessment device	20-25w gestation		Family major problems: do not trust each other
			Family assessment device	20-25w gestation		Family major problems: conflict, argued
			Family assessment device	20-25w gestation		Family support problems
			Long lasting difficulties questionnair	20-25w gestation		Difficulties in contact with others

Confirmatory factor analysis model fit indices: (i) *life stress*: RMSEA = 0.039; CFI = 0.834; TLI = 0.806; (ii) *contextual stress:* RMSEA = 0.056; CFI = 0.982; TLI = 0.971; (iii) *maternal stress*: RMSEA = 0.026; CFI = 0.941; TLI = 0.911; (iv) interpersonal stress: RMSEA = 0.051; CFI = 0.922; TLI = 0.912.

Table S2 | Inter-correlations between the prenatal stress domains.

Life Stress		**Contextual Stress**	**Parental Stress**	**Interpersonal Stress**
Life Stress	–			
Contextual Stress	.22***	–		
Parental Stress	.16***	.26***	–	
Interpersonal Stress	.22***	.31***	.24***	–

*** $p < 0.001$

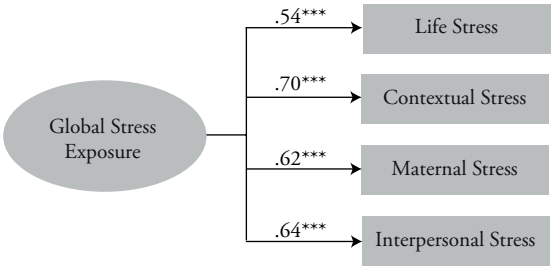

Figure S1 | Confirmatory factor analysis measurement model of global prenatal maternal stress exposure.

*** $p < 0.001$

Note: Values represent standardized factor loadings for the confirmatory factor analysis of global prenatal maternal stress exposure. CFA model fit indices $\chi^2(2) = 5.07$, $p = 0.079$; RMSEA = 0.014; CF I= 0.999; TLI = 0.997

Table S3 | Annotation of top DMRs associated with PMSE in cord blood (*N* = 912).

Cluster Name	CpG probes	Chromosome	Position	Nearest gene(s)
90946	cg09141953	20	62948235	*MYT1;PCMTD2; CICP4*
	cg19192585	20	62948037	*MYT1; PCMTD2; CICP4*
	cg24189721	20	62948134	*MYT1; PCMTD2; CICP4*
135488	cg07355590	7	133812072	*LRGUK*
	cg10839521	7	133811911	*LRGUK*
	cg12531972	7	133811940	*LRGUK*
	cg14455998	7	133811808	*LRGUK*
	cg14706178	7	133812217	*LRGUK*
	cg15280728	7	133812269	*LRGUK*
	cg15955731	7	133812369	*LRGUK*
	cg16776231	7	133812041	*LRGUK*
	cg17676607	7	133811837	*LRGUK*
	cg19967800	7	133811828	*LRGUK*
	cg22578125	7	133812031	*LRGUK*
66361	cg01906998	17	73974972	*ACOX1; C17orf106*
	cg02701084	17	73975226	*ACOX1;C17orf106*
	cg02760553	17	73975121	*ACOX1;C17orf106*
	cg10931252	17	73975914	*ACOX1; C17orf106*
	cg11507926	17	73975697	*ACOX1; C17orf106*
	cg12452675	17	73975215	*ACOX1*
	cg14770570	17	73975813	*ACOX1*
	cg15269548	17	73975372	*ACOX1*
	cg16419345	17	73976089	*ACOX1;C17orf106*
	cg19198791	17	73975107	*ACOX1;C17orf106*
	cg19652678	17	73975160	*ACOX1; C17orf106*
	cg21356179	17	73974861	*ACOX1; C17orf106*
	cg21697561	17	73974931	*ACOX1; C17orf106*
	cg24015057	17	73975129	*ACOX1; C17orf106*
	cg24357503	17	73975484	*ACOX1; C17orf106*

8

Early Pregnancy Maternal Thyroid Function and Genome-wide DNA Methylation in Neonates Evidence from the Generation R Study

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Manuscript in preparation

Abstract

Maternal thyroid hormones during early pregnancy are the only source of thyroid hormones for the foetus. The effects of prenatal maternal thyroid dysregulation in child development have repeatedly been investigated, but the exact mechanisms underlying the relation remain unclear. Epigenetic regulation is recognized as an important mediator of the effects of prenatal environment in child's development. In this study, we performed epigenome-wide association (EWA) analyses to investigate the effects of maternal thyroid hormones during early pregnancy and DNA methylation patterns in neonates. We measured both levels of free thyroxine (FT4) and thyroid stimulation hormone (TSH) to assess maternal thyroid function in a population-based sample of $N = 751$ ethnically homogenous mothers and their children. Second, we performed follow-up pathway analyses based on our EWAS results. We found no significant effects of FT4 levels in DNA methylation. Maternal TSH levels indicated significant differential methylation in the *RNF208* gene (cg14199276, $p = 3.17e\text{-}08$). Pathway analyses indicated over-representation of several pathways for both FT4 and TSH levels, related to development, immunity and metabolism. This is the first study to provide evidence of epigenetic regulation in neonates by levels of maternal thyroid hormones during early pregnancy. Future studies should replicate our findings in larger samples.

Introduction

Pregnancy is a period of particularly high demand on thyroid function, during which the maternal thyroid gland undergoes fundamental changes to adapt to the needs of both mother and child [1]. In the first trimester, the fetal thyroid gland is not yet mature, however maternal thyroid hormones [i.e. thyroid stimulating hormone, (TSH), thyroxine (T4), and triiodothyronine (T3)] cross the human placenta [2,3] and modulate fetal development, being the only source of thyroid hormones for the foetus [4]. Previous research indicated that prenatal maternal thyroid hormones are critical for normal brain development, and that even subclinical, mild cases of maternal hypothyroidism during early pregnancy may have long-lasting effects later in life [5]. Longitudinal studies have provided further evidence of the association between lower levels of thyroid hormones during early pregnancy and lower cognition [6,7], delayed neuropsychological development [8,9], increased attention deficits [10,11], autistic-like [12], and externalizing problems [13] in childhood.

The effects of thyroid hormones are exerted at the cellular level and can modulate gene expression. Although the role of thyroid hormones as activators and inhibitors of gene expression is well-established, the exact molecular mechanisms remain to a great extent unclear. One of the potential mechanisms by which thyroid hormones can exert their influence on gene expression is via epigenetic mechanisms, such as DNA methylation. Previous research in rats has indicated that perinatal hypothyroidism can alter expression of the reelin and *BDNF* genes in the hippocampus via increased DNA methylation of these genes [14]. In this study we investigate whether prenatal maternal thyroid function is associated with genome-wide methylation differences in a large (N = 751) population-based sample of neonates.

Previous studies have shown that the fetal period is critical for genome-wide methylation regulation [15]. Epigenome-wide associations studies (EWAS) provide a systematic approach which can uncover the molecular mechanisms underlying the association of prenatal maternal thyroid (dys)function with neurodevelopmental outcomes in the offspring. In this study, we measured both levels of free T4 (FT4) and TSH to assess maternal thyroid function in early pregnancy. Although there is a known inverse relation between levels of TSH and FT4, this normal relationship is altered during pregnancy [16]. Furthermore, it has been shown that TSH and FT4 may have independent physiological effects [17]. Thus, we used both maternal measurements (i.e. FT4 and TSH levels) to examine their association with DNA methylation in neonates.

The Illumina 450K Beadchip array [18] was used to assess genome-wide methylation in N = 751 ethnically homogenous (Caucasian) neonates. Our aim was to examine to what extent the range of prenatal maternal thyroid function is associated with epigenome-wide differential methylation in neonates. To this aim, hypothesis- free EWAS analyses on the continuum of FT4 and TSH levels were performed Follow-up pathway analyses based on our EWAS results aimed to integrate the results of our EWA analyses into gene networks, and help us speculate on their potential functionality.

Methods

Study Design and Participants

The Generation R (GEN-R) Focus Study is embedded in the Generation R Study, a prospective cohort in Rotterdam, the Netherlands [19]. For *N* = 969 ethnically homogenous, Dutch children participating in the GEN-R Focus Study, genome-wide methylation data were available. For *N* = 751 children with genome-wide DNA methylation data, maternal FT4 and TSH levels in early pregnancy were also available. All children participating were born between April 2002 and January 2006. The Generation R Study is designed to identify early environmental and genetic predictors of growth, development, and health from fetal life to young adulthood. The details of this study have been previously described [20]. All parents gave their informed consent for participation. Study protocols were approved by the local ethics committee.

Maternal thyroid function measurements

Blood samples were collected in early pregnancy (mean = 13.3 weeks, range 10.9-17.2 weeks). Free thyroxine (FT4) and thyroid stimulation hormone (TSH) were assessed in plasma using chemiluminescence assays, as previously reported [13,16]. The reference values of FT4 for non-pregnant women in our laboratory were 11-25 pmol/L [13]. The reference values for maternal TSH in pregnancy are 0.03-2.5 mIU/L, as recommended by the Endocrine Society Clinical Practice Guideline published in 2007 [21]. The assessment techniques for thyroid parameters are described in detail in previous publications of the GEN-R Study [10,13].

Genome-wide DNA methylation

Five hundred nanograms of DNA from cord-blood (birth, *N* = 979) underwent bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, USA). We used the Illumina Infinium HumanMethylation450 BeadChip Kit (Illumina Inc., San Diego, USA) to measure DNA methylation at 485,577 CpG probes. Following standardized criteria, quality control of data was performed by determining sample call rate, color balance, staining efficiency, extension efficiency, hybridization performance, stripping efficiency after extension, and bisulfite conversion. The final sample consisted of *N*= 969 neonates which passed DNA methylation quality control.

All 49,564 probes identified as having (i) a single nucleotide polymorphism in the single base extension site with a frequency of > 1% in the GoNLv4 reference panel [22] or (ii) a non-optimal binding (non-mapping or mapping multiple times to either the normal or the bisulfite-converted genome) were removed from the dataset, leaving a total of 436,013 CpG probes for analysis. Samples were normalized using the 'dasen' method described by Pidsley et al. [23] and dye bias corrected [24]. Normalized values were converted into β-values, which represent the methylation level at a CpG probe for each neonate. β-values were subsequently converted into M-values (log2 ratio of β-value) to account for heteroscedasticity and allow analysis assuming a normal distribution [25].

Covariates

We adjusted for technical covariates, including the sample's array number (range = 1 to 83) and position on the array (range = 1 to 12). We used the method developed by Houseman et al. [26], to estimate proportions of cells in whole blood [% of CD8 T-cells, CD4 T-cells, natural killer (NK) cells, B-cells, monocytes and granulocytes] and adjust for different cell type compositions, since DNA methylation across the genome can be cell-type specific [27,28].

Furthermore, we adjusted for sex of the child, and maternal smoking during pregnancy. Information on sex was obtained from midwife and hospital registries at birth. Recent research indicates sex-specific DNA methylation patterns in autosomal CpG sites [29]. The effect of smoking during pregnancy on DNA methylation of the offspring has also been extensively investigated [30]. Information on maternal tobacco smoking was obtained by postal questionnaires in early, mid-, and late pregnancy. Maternal smoking was categorized on the basis of all three questionnaires into "never smoked during pregnancy", "quit as soon as pregnancy was known", and "continued smoking during pregnancy".

Statistical analysis

For the N= 751 children participating in this study, the continuous range of FT4 levels was used for further analyses. We applied a natural log (ln) transformation to TSH levels to achieve a normal distribution. All analyses were conducted in SPSS 21.

Epigenome-wide association study (EWAS) analyses

First, single probe analysis was performed to investigate the association between maternal FT4 and neonatal DNA methylation for all available CpG probes that passed quality control (N = 436,013) using a linear mixed effects model and adjusting for fixed (i.e., sex, maternal smoking, and cell-type distribution) and random (i.e. batch effects) covariates, as described above. Second, a similar model was used to investigate the association of maternal TSH levels and genome-wide DNA methylation in neonates. All analyses were performed in the statistical framework R. The strict EWAS Bonferroni-corrected threshold for significance was estimated as p = 0.05 / 436,013 = 1.15e-07, while the level of suggestive significance was set to p < 1.00e-05.

To formally examine whether the direction of effects differs for the two maternal thyroid parameters (i.e., FT4 and TSH levels), a sign test was performed. The direction of the effect of the suggestive probes derived from the EWAS analysis on TSH levels was compared with the direction of the effect of the same probes derived from the EWAS analysis on FT4 levels. These analyses were conducted in SPSS 21.

Epigenome-wide pathway analysis

A pathway analysis was performed with our EWAS results, to investigate whether specific pathways are over-represented among the suggestive CpG probes. For the EWAS on maternal FT4 levels, N_{CpGs} = 18 with suggestive statistical significance (p < 1.00e-05) were selected and annotated to genes

(N_{genes} = 15), using the IlluminaHumanMethylation450kCHR36 file. Similarly, for the EWAS on maternal TSH levels, N_{CpGs} = 26 with p < 1.00e-05 were selected and annotated to genes (N_{genes} = 26). We used GeneMANIA pathway tool [31] to sort the suggestive genes into pathways, based on the Gene Ontology (GO)-Slim Biological Function annotation dataset. Since in pathway analyses, the same genes are allowed to participate in different pathways, resulting in not completely independent pathways, the false discovery rate (FDR) was used to adjust for multiple testing.

Results

Sample characteristics

The sample characteristics of 751 ethnically homogenous mothers and children participating in this study are presented in Table 1. FT4 and TSH levels were moderately negatively correlated in our sample (r = -.26, p = .01, N = 751). The mean methylation values (β-values) in the cord blood showed a bimodal distribution, with 42% of the CpG probes being hypomethylated (i.e. β-value < 0.3), 11% of the CpG probes showing intermediate levels of methylation (i.e. β-value ≥ 0.3-0.7), and 47% of the CpG probes being hypermethylated (i.e. β-value ≥ 0.7).

Table 1 | Sample characteristics of 751 pregnant women and children from the Generation R (GEN-R) Focus Study.

Child & mother characteristics (*N* = 751)	**Percentages or means (*SD*)**
Gender, % girls	48
Gestational age at delivery, weeks	40.2 (1.43)
Age mother at intake, years	31.60 (4.17)
Gestational age at maternal blood sampling, weeks	13.3 (1.7)
Maternal TSH, mU/l	1.69 (.04)*
range	0.00-10.70
Maternal FT4, pM	15.19 (.10)
range	8.06-26.13
Smoking during pregnancy, %	
Never	77
Until pregnancy was known	9
Continued during pregnancy	14

FT4 = free T4; TSH = thyroid stimulation hormone; * untransformed maternal TSH values.

Epigenome-wide pathway analysis

Single probe analysis of maternal FT4 levels and genome-wide methylation in neonates revealed no Bonferroni-corrected significant associations. However, several suggestive hits (p < 1.00e-05) with differential methylation in multiple genes across the genome were observed. The top hits are presented

in Table 2. The Manhattan and quantile-quantile plot are presented in Supplementary Material (Figures S1 and S2, respectively).

Table 2 | Top CpG sites (p < 1.00e-05) associated with maternal prenatal FT4 levels in neonates (N =751).

Probe name	Chromosome	Position	Effect (*SE*)	*P*	Nearest gene(s)
cg27588119	17	58042227	0.02 (.004)	3.03e-06	*RNFT1*
cg13336814	3	194775368	-0.02 (.004)	9.04e-06	*NA*
cg26445772	5	137090088	-0.04 (.009)	1.80e-05	*HNRNPA0*
cg18719157	X	133680925	-0.03 (.006)	2.15e-05	*MIR424;MIR503*
cg17794320	15	90190822	-0.02 (.005)	2.49e-05	*KIF7*
cg02332216	12	66563692	0.02 (.006)	3.39e-05	*TMBIM4*
cg15256944	17	79651359	-0.03 (.008)	4.05e-05	*ARL16;HGS*
cg19732469	14	96968481	0.01 (.003)	4.29e-05	*PAPOLA*
cg12285605	15	68785674	-0.02 (.004)	4.81e-05	*NA*
cg00629244	5	142783379	-0.02 (.005)	5.36e-05	*NR3C1*
cg01271423	10	100366981	-0.02 (.007)	5.62e-05	*HPSE2*
cg11983939	7	153574	0.03 (.007)	6.19e-05	*NA*
cg27327785	6	33176447	0.03 (.006)	7.03e-05	*RING1*
cg00296938	3	49044463	-0.03 (.007)	7.14e-05	*P4HTM;WDR6*
cg06259158	6	36846157	-0.02 (.004)	7.31e-05	*NA*
cg09131844	2	27651398	-0.04 (.009)	7.76e-05	*NRBP1*
cg17741689	1	4714574	0.02 (.004)	9.35e-05	*AJAP1*
cg10386483	15	35047189	0.02 (.006)	9.57e-05	*GJD2*

NA = not available

Single probe analysis of maternal prenatal TSH levels and genome-wide methylation in cord blood indicated a Bonferroni-corrected significant CpG probe in the transcription start site (TSS1500) of *RNF208* gene (cg14199276, chromosome 9q34.3, p = 3.17e-08). A detailed list of the top hits are presented in Table 3. The Manhattan and quantile-quantile plot are shown in Supplementary Material (Figures S3 and S4, respectively).

For the N_{CpGs} = 26 suggestive probes (p < 1.00e-05) derived from the EWAS analysis of maternal TSH levels, the direction of effect was also extracted for the results of the EWAS analysis on FT4 levels. We found no significant differences in the direction of the effect in DNA methylation for the two parameters of maternal thyroid function (sign test, p = .81, one-tailed).

Table 3 | Top CpG sites (p < 1.00e-05) associated with maternal prenatal TSH levels in neonates (N =751).

Probe name	Chromosome	Position	Effect (*SE*)	*P*	Nearest gene(s)
cg14199276	9	140117005	0.21 (.04)	3.17e-08	*RNF208*
cg00461578	4	146819333	-0.11 (.03)	7.36e-06	*ZNF827*
cg24790764	12	104344310	0.10 (.02)	1.36e-05	*C12orf73*
cg25558406	X	133594204	-0.22 (.05)	2.05e-05	*HPRT1*
cg09673602	6	30042630	-0.15 (.04)	2.69e-05	*RNF39*
cg00012203	2	219082015	-0.18 (.04)	2.69e-05	*ARPC2*
cg14180747	9	37647717	0.13 (.03)	2.78e-05	*NA*
cg18868146	1	216939291	0.13 (.03)	3.13e-05	*ESRRG*
cg08524698	22	21213167	-0.10 (.02)	3.26e-05	*SNAP29;PI4KA*
cg09660043	10	118395444	0.11 (.03)	3.40e-05	*PNLIPRP2*
cg13388253	19	51505507	0.19 (.04)	3.51e-05	*KLK8*
cg19234089	11	65728168	0.12 (.03)	3.78e-05	*SART1*
cg17070935	8	51364244	0.11 (.03)	3.87e-05	*SNTG1*
cg17696847	20	57414217	-0.08 (.02)	3.99e-05	*GNASAS;GNAS*
cg07592828	X	70364353	0.16 (.04)	5.14e-05	*NLGN3*
cg00620552	2	48010097	0.10 (.02)	5.35e-05	*MSH6*
cg11199642	14	100405934	0.12 (.03)	5.63e-05	*EML1*
cg06069179	17	33288421	-0.13 (.03)	6.20e-05	*CCT6B;ZNF830*
cg09219182	22	37255542	-0.31 (.08)	6.68e-05	*NCF4*
cg14080254	11	46722302	-0.12 (.03)	6.95e-05	*ZNF408;ARHGAP1*
cg09678228	12	44230058	-0.13 (.03)	7.08e-05	*TMEM117*
cg16974075	10	13093279	0.12 (.03)	7.12e-05	*NA*
cg14511376	9	35607900	0.23 (.06)	7.21e-05	*TESK1*
cg21284880	13	40177740	-0.08 (.02)	7.47e-05	*LHFP*
cg04169369	16	4053199	-0.10 (.03)	8.53e-05	*ADCY9*
cg06393098	19	46235602	-0.12 (.03)	9.22e-05	*FBXO46*

NA = not available

Epigenome-wide pathway analysis

The pathway analysis based on the suggestive CpG probes identified by our EWAS on maternal FT4 levels indicated the over-representation of several biological pathways [e.g., the PRC1 (Polycomb repressive complex 1), FDR-corrected p = 2.00e-07]. The output of the pathway analysis for EWAS on maternal FT4 levels is presented in Table 4.

Similarly, pathway analysis on the suggestive CpG probes identified by our EWAS on maternal TSH levels indicated over-representation of specific pathways (e.g., immune response-regulating cell surface receptor signaling pathway involved in phagocytosis, FDR-corrected p = 8.71e-05). The results of the pathway analysis for EWAS on maternal TSH levels is presented in Table 5.

Table 4 | Pathway analysis of suggestive CpG probes (p < 1.00e-05) derived from EWAS on maternal FT4 levels in neonates (N = 751).

pathway	Coverage *	FDR corrected p $
PRC1 complex #	5/12	2.00e-07
nuclear ubiquitin ligase complex	5/37	5.14e-05
PcG protein complex +	5/39	5.14e-05
mRNA processing	6/288	3.78e-02

PRC1 = polycomb-group repressive complex 1; + PcG = polycomb gene complex number of genes in the network with a given function/ all genes in the genome with the same or similar function; $ FDR = false discovery rate

Table 5 | Pathway analysis of suggestive CpG probes (p < 1.00e-05) derived from EWAS on maternal TSH levels in neonates (N = 751).

Pathway	Coverage *	FDR corrected p $
immune response-regulating cell surface receptor signaling pathway involved in phagocytosis	6/70	8.71e-05
purine nucleotide biosynthetic process	7/147	1.15e-04
actin cytoskeleton	8/277	2.54e-04
positive regulation of cAMP metabolic process	5/60	2.79e-04
DNA secondary structure binding/ mismatch repair	3/13	1.63e-03
response to glycagon	3/33	2.22e-03

* number of genes in the network with a given function/ all genes in the genome with the function; $ FDR = false discovery rate

Discussion

In this study, we investigated whether maternal thyroid function parameters in early pregnancy are associated with differences in DNA methylation in the cord blood of children collected at birth. To this aim, we measured levels of free thyroxine (FT4) and thyroid stimulating hormone (TSH) during the first trimester of pregnancy, in a sample of N = 751 Dutch women and their children. Maternal FT4 levels were not significantly associated with epigenome-wide differences in methylation in children's cord blood. In contrast, maternal TSH levels were associated with epigenome-wide significant methylation levels of one CpG probe (cg14199276, p = 3.17e-08) at chromosome 9, in the regulatory area of *RNF208* (ring finger protein 208) gene. Pathway analyses on the suggestive CpG probes of both maternal FT4 and TSH levels indicated the over-representation of several pathways, related to development (e.g., the PRC1 complex pathway), immunity (e.g., the phagocytosis signaling pathway), and metabolism (e.g., the purine nucleotide biosynthesis pathway). To our knowledge, this is the first study in humans examining the hypothesis that maternal thyroid function during early pregnancy may affect growth and development in children, via epigenome-wide differential methylation. This mode of action of thyroid-related hormones has been so far only tested in animal models [14], and offers opportunities for further research in humans.

Further replication of our results with TSH is required, we can only speculate as to the biological importance of the differentially methylated locus on the *RNF208* gene. This gene (chromosome 9q34.3) codes for a ubiquitin E3 ligase, and previous research has linked *RNF208* to the regulation of apoptosis and neurodevelopmental diseases [32]. Furthermore, a microdeletion syndrome affecting the same area [del(9)(q34.3)], has been characterized by severe developmental delay, mental retardation, and characteristic craniofacial features [33], indicating that although the exact mechanism remains to be explored, the fine-tuned regulation of this chromosomal region may be of great importance for normal development.

Pathway analysis using the suggestive CpG probes that were identified by the EWAS on maternal FT4 levels, indicated significant over-representation of the PRC1 (Polycomb repressive) complex pathway. Interestingly, PRC1 complex is important for normal development in mammals, regulating downstream genes at the epigenetic, transcriptional and post-transcriptional level [34]. This suggests that the effects of maternal FT4 levels in neonates could be amplified or attenuated by epigenetically activating a cascade of key regulators, ultimately influencing expression of target genes. This hypothesis is strengthened by the assumed biological function of the other pathways that were over-represented in our top EWAS results (e.g. the mRNA processing pathway).

Similar pathway analysis using the top results of the EWAS on maternal TSH levels indicated significant over-representation of immune related pathways (i.e. the immune response-regulating cell surface receptor signaling pathway involved in phagocytosis). Previous research has shown that TSH levels in adults do not only influence thyroid hormone production, but they are also involved in immune regulation [35]. Our research indicates that this role may be mediated by DNA methylation differences in cord blood.

In this study, both EWAS single probe and EWAS pathway analyses indicated distinctive patterns of the associations of maternal FT4 and TSH levels during early pregnancy. Although there is a negative relationship between FT4 and TSH levels, the complex relationship between the two has been previously reported [36]. Furthermore, we found random direction of effects of maternal FT4 and TSH levels on DNA methylation in neonates (sign test, $p = .81$). Thus, we suggest to use both measurements as parameters of maternal thyroid function in epigenetic analyses, as it is possible to exert their action through distinct pathways.

In summary, this study provided the first evidence of complex, fine-tuned regulation of the epigenome in neonates by maternal levels of FT4 and TSH during the 1st trimester of pregnancy. The results of this study remain to be replicated in future collaborative efforts, in larger samples. An increase in sample size would provide additional statistical power to detect even smaller effects on the epigenome and it would test the validity of our findings in independent samples.

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Supplementary Material

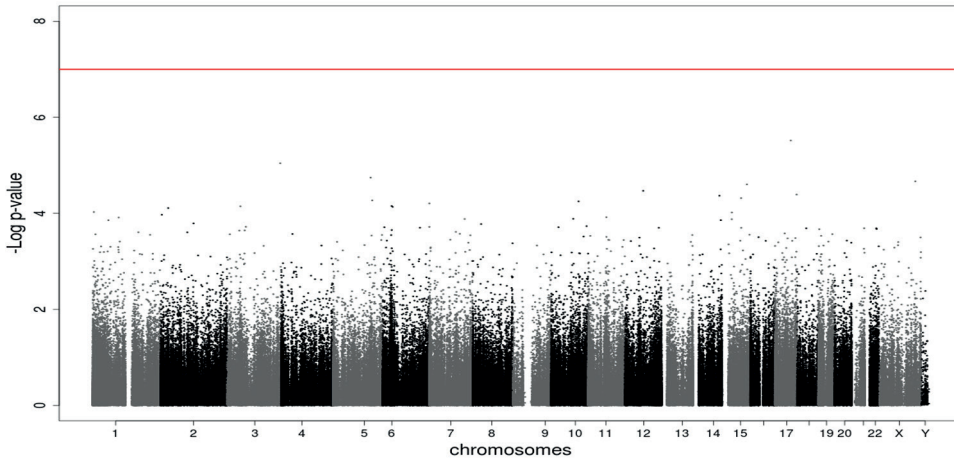

Figure S1 | Manhattan plot of the EWAS of maternal prenatal FT4 levels in cord blood (N = 751). The x-axis represents the autosomal (1-22) and sex (X,Y) chromosomes and the y-axis shows the -$\log_{10}(p)$. The red line indicates the Bonferroni-corrected epigenome-wide significance level (p = 1.00e-07)

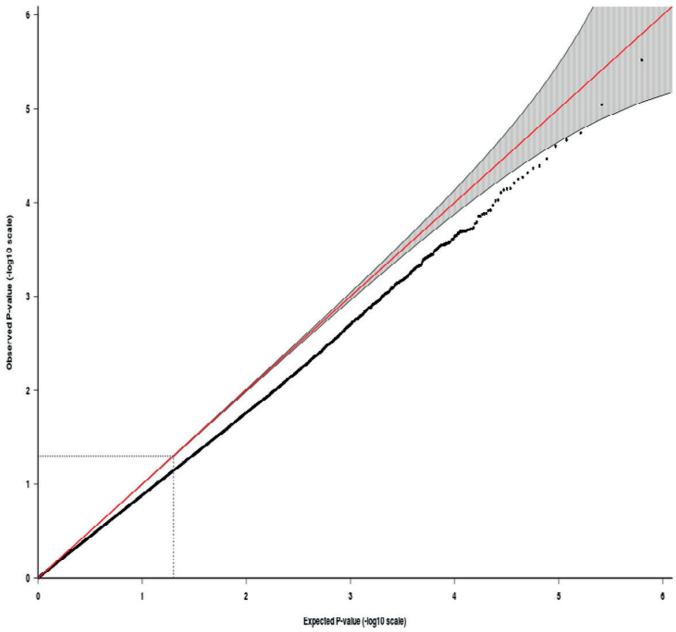

Figure S2 | Quantile-quantile (QQ) plot illustrating probability values from EWAS of maternal FT4 levels in cord blood (N = 751). The red line indicates the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

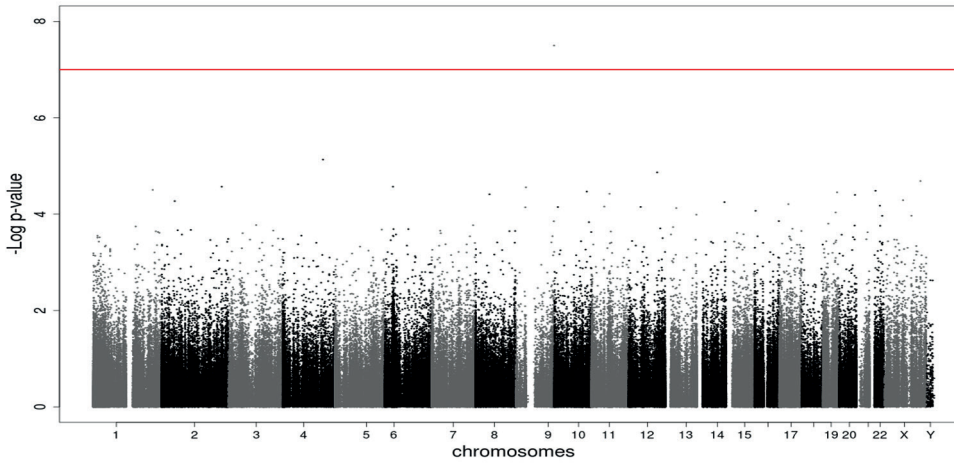

Figure S3 | Manhattan plot of the EWAS of maternal prenatal TSH levels in cord blood (N = 751). The x-axis represents the autosomal (1-22) and sex (X,Y) chromosomes and the y-axis shows the -$\log_{10}(p)$. The red line indicates the Bonferroni-corrected epigenome-wide significance level (p = 1.00e-07).

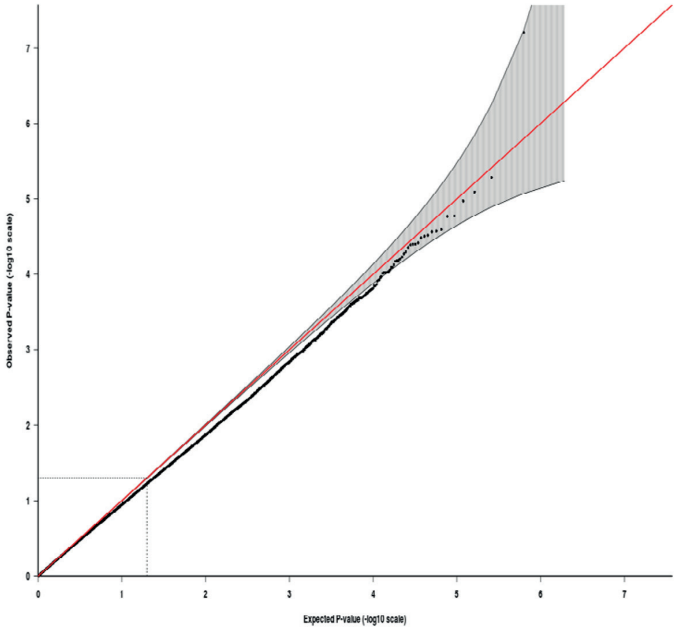

Figure S4 | Quantile-quantile (QQ) plot illustrating probability values from EWAS of maternal TSH levels in cord blood (N = 751). The red line indicates the distribution under the null hypothesis and the shaded area indicates the 95% confidence band.

General discussion

9

Rationale

Since 1960s, we have become increasingly aware of the importance of genetic factors in the etiology of behavior problems in children. In the course of time, many complementary approaches have been developed (i.e. the *candidate gene*, the *genome- wide*, and the *epigenome* approach) offering us a tremendous potential to advance our understanding of the etiology of behavior problems in children. In this set of studies, we used multiple cutting-edge approaches with the aim of estimating the proportion of heritability attributed to common genetic polymorphisms, and identifying the specific genetic and epigenetic determinants that influence children's behavior problems. In this chapter, we first provide an overview of the main findings of this thesis. Second, we consider some methodological issues and the practical implications of our research. Finally, we provide recommendations for future research.

Main findings

The candidate gene approach

In **section A**, we focused on candidate genes. Candidate gene studies allow us to investigate genes previously selected based on *a priori* hypotheses about their functional role in biological pathways leading to a disease or disorder [1]. In **chapter 2**, we investigated the widely-studied variable number tandem repeat (VNTR) in the dopamine receptor D4 gene (*DRD4*). Long *DRD4* VNTRs have been previously associated with increased risk of attention deficit/ hyperactivity disorder (ADHD) in children [2,3]. In this study, we replicated this association and expanded it in a typically developing, non-clinical sample of 6-year old children. Secondly, we focused on the behavioral patterns of children with ADHD symptoms. These children often show lower levels of behavioral inhibition, higher impulsivity, and lower levels of stranger fear compared with healthy children. The phenotypic overlap of ADHD symptoms and lower stranger fear implies a biological link, which has not been previously established. We measured stranger fear with an observational task modelled after real-life events [4]. Our study demonstrated that less fearful behavior towards strangers at 3 years could be an early indicator of ADHD symptoms at 6 years, and that this relationship was partially explained by variation in the *DRD4* gene.

An additional contribution of this study was the cataloging of up to 5 different genotypic classifications of *DRD4* VNTRs used in the literature. We then showcased that although the direction of the effect sizes is mostly consistent across studies, the interpretation of the significance tests depends on the choice of the classification. In **chapter 3**, we systematically reviewed all studies exploring the biological differences between *DRD4* VNTRs. We concluded that although there is some evidence of functional differences between the most common alleles (i.e. the 2-, 4-, and 7-repeat), there is uncertainty about the functionality of rare alleles. In addition, we systematically reviewed the literature for association studies of *DRD4* VNTRs and behavior outcomes in non-clinical samples of children

and adolescents. We identified five classification systems (the 7-repeat carriers vs. non-carriers was the most commonly used classification), but there was no evidence supporting the use of one classification over the others. Our meta-analytical efforts did not support the association of *DRD4* VNTRs with normative differences in attention problems, externalizing problems, and reactive temperament in children and adolescents. However, we identified that longer *DRD4* VNTRs were associated with less optimal social and emotional development, and executive function during childhood and adolescence. These associations were attenuated when we used the genotypic classification system as a moderator, indicating that the use of alternative genotypic classifications introduces heterogeneity in the outcomes of the studies. The problems of data dredging and the winner's curse in candidate gene studies make the need for full transparency and replication of the initial findings in independent samples prominent, before we can draw safe conclusions.

The genome-wide approach

In **section B** we focused on the hypothesis-free genome-wide approach [5]. In **chapter 4**, we used a novel approach developed by Yang et al.,(Genome-wide Complex Trait Analysis, GCTA) to estimate genetic variance explained by common, genome-wide single nucleotide polymorphisms (SNPs) [6]. The goal of GCTA is not to identify the individual polymorphisms that are common among those who share a given trait, but to determine whether this trait can be associated with a (potentially large) number of unidentified polymorphisms. Thus, the heritability estimates from GCTA (SNP h^2) are commonly considered as the upper limit of the variance that can be explained by GWAS efforts. To estimate SNP h^2, large sample sizes are required. In this study, we combined genotype and phenotype data from two independent, population-based Dutch cohorts, focusing on four domains of children's behavior problems, rated by the mother or teacher: attention deficit problems, externalizing, internalizing, and pervasive developmental problems. We also estimated SNP h^2 of observational ratings of non-verbal cognition in children. Our study provided evidence of substantial SNP h^2 for all four domains of children's behavior problems, measured at ages 3, 7 and 9 years. As expected, SNP h^2 estimates were lower but consistent with previously reported twin heritabilities of the same phenotypes. Our study was in contrast with a recent study in 12-year old children [7], indicating that SNP h^2 is a population-based property and as such, it can differ under different environments, developmental stages, and study design.

Our findings of quantifiable SNP h^2 of common behavior problems in children supported further research aiming to identify the specific genetic variants and pathways associated with these complex traits. Since we expect that behavior in children is shaped by many genes, which could work together under an additive model [8], a GWAS approach in large samples is needed to detect these common genetic variants. The Early Genetics and Lifecourse Epidemiology (EAGLE) consortium of birth cohorts aims to investigate the genetic basis of behavior in childhood. Within the framework of the EAGLE consortium we performed a GWAS meta-analysis to identify genetic associations with children's aggressive behavior (**chapter 5**). We combined data from nine population-based studies, using parental reports of children's aggressive behavior, generating the largest sample size to date

(*N* = 18,988). Our study confirmed that common genetic variation explains 10% to 54% of the phenotypic variance of aggressive behavior in children. What is more, we identified a near genome-wide significant locus, in *LRRTM4* gene (rs11126630), known to regulate excitatory synapse development [9]. Finally, our gene-based analysis indicated that functional variation in the *AVRP1A* gene is linked to higher levels of aggressive behavior in children, in line with previous research conducted in rodents [10]. The results of this study, although requiring replication in independent samples, demonstrate that many SNPs, not individually significant in GWAS, are responsible for the genetic variance of aggressive behavior in children. Finally, our study offers opportunities for further research in both novel and candidate genes.

In **chapter 6**, we turned our attention to infant attachment. Although attachment quality (e.g. secure or disorganized attachment) is strongly shaped by environmental factors [11], the contribution of genetic factors may be larger than previously expected [12]. To explore this hypothesis, we used a multidimensional approach in the largest ethnically homogeneous sample available (*N* = 657 14-month old children). Although no SNP reached the genome-wide level of significance, we were able to identify three novel genes possibly associated with attachment disorganization (i.e., *HDAC1*, *ZBF675*, and *BSDC1*) and one novel gene possibly associated with attachment security (i.e., *BECN1*). The function of these genes remains largely unknown, but offers new opportunities for research, potentially in animal models [13]. Finally, pathway analyses indicated overrepresentation of synaptic and neuronal pathways associated with attachment disorganization. No pathway was significantly associated with attachment security. This confirms the hypothesis that disrupted, atypical phenotypes, such as attachment disorganization, are under genetic influence more than normative phenotypes, such as attachment security [14].

The epigenome-wide approach

In **section C**, we performed epigenome-wide association studies (EWAS) to identify whether early environmental influences can affect DNA methylation in cord blood. In **chapter 7**, we constructed a score measuring maternal stress during pregnancy. Previous literature indicated that prenatal maternal stress is associated with higher methylation of the oxytocin receptor (*OXTR*) gene, in a small sample of children (*N* = 87) [15]. In this study, we followed an hypothesis-free EWAS approach using data from the Generation R Study (*N* = 912). Our study identified no large epigenome-wide effects of prenatal exposure to maternal stress in neonates. Follow-up pathway analysis identified over-representation of specific pathways, related to cell cycle regulation, metabolic processes, and immunity. Our study suggests that even larger samples may be required to identify modest effects of prenatal maternal stress. Follow-up studies are also needed to assess whether maternal stress *in utero* has long- lasting effects on DNA methylation of the offspring.

In **chapter 8**, we studied whether maternal thyroid function during early pregnancy is associated with epigenome-wide alterations in neonates. Previous research linked early pregnancy maternal hypothyroidism to delayed neuropsychological developments in childhood [16], and animal studies identified that this effect may be mediated by altered DNA methylation in specific genes (i.e. *reelin*

and *BDNF* genes [17]). In our study, we assessed maternal thyroid function by measuring both levels of free thyroxine (FT4) and thyroid stimulating hormone (TSH) in a population-based sample of N = 751 Caucasian mothers and their neonates. We identified no EWAS significant CpG probe associated with levels of maternal FT4. However, we found that increased differential methylation in the *RNF208* gene (cg14199276, p = 3.17e-08) in the neonates was associated with increased levels of maternal TSH. Follow-up pathway analysis identified the over-representation of several pathways related to development, immunity, and metabolism. These first results give opportunities for further research, and await replication in future collaborative efforts, within the PACE (Pregnancy And Child Epigenetics) consortium.

Methodological considerations

Candidate genes vs. Hypothesis-free approaches

After decades of research with candidate genes, very few results show consistency and reproducibility [18]. The incomplete understanding of their functionality, the fact that most of the studies are underpowered, the differences in allele frequencies among different populations, and the heterogeneity of (empirical) approaches used are the main problems in the design and interpretation of candidate gene association studies.

More recently, GWAS has provided a new conceptual framework to uncover the genetic architecture of psychopathology, by genomic screening, without prior hypotheses for specific genetic regions [19]. Nevertheless, very large sample sizes are needed to find common variants with small effects on the traits of interest and replication of the initial findings in independent samples is not always possible. What is more, with the current design of GWAS other sources of variation, such as structural variation (copy-number variants, CNVs, inversions, microdeletions and – duplications) [20], rare, and de novo mutations [21], and epigenetic variation [22] cannot be identified.

Since all approaches have strengths and limitations, to better understand how genetic variation affects common behavior problems in children, complementary techniques are needed. These techniques should combine previous knowledge from candidate gene studies (e.g., polygenic risk scores of genes participating in the same functional pathway) with identification of novel genetic variants (e.g., GWAS, next- generation sequencing, mitochondrial DNA analysis) and inter-individual variation of DNA methylation levels. The integration of genetic and epigenetic analyses should reveal the biological mechanisms underlying child behavior in health and disease.

Association vs. causation

The research presented in this thesis is based on the principles of genetic epidemiology. Epidemiologic and genetic methods can help us identify genetic and epigenetic determinants associated with common behavior problems in children and adults. However, the statistical models used to identify association cannot establish causal relations between genetic/ epigenetic variants and the traits of interest [23].

Our genome- and epigenome-wide association studies have focused on common variants, often in linkage disequilibrium (LD) with the actual causal variants, with small effects on the trait of interest [24]. Although these genetic and epigenetic variants may explain a fraction of the phenotypic variance appearing in the population, expression of genes at specific chromosomal loci may be modified by other genes and/ or the environment. Thus, functional studies (e.g., identification of functional elements in DNA using bioinformatics tools, gene expression analysis, protein-protein interaction, RNAi methods etc.) should follow the initial findings of candidate genes, GWA and EWA studies, aiming to identify the possible mechanisms that guide translational progress. Animal models [25] and human induced pluripotent stem cells (iPSCs) [26] could also prove useful towards this goal, although it would be extremely difficult to prove individual differences in emergent phenomena (such as thought disorders, and empathy) at the neuronal level.

Many genes of small effect

One of the major criticisms of GWAS is that it reveals many genes of small effect on the trait of interest. However, the small effects of common genetic variants do not come as a surprise. Under the additive genetic model, we expect that psychopathology occurs only when an individual accumulates a large number of genetic variants, each one of which has only a small effect. The fact that common environmental factors (e.g. socio-economic status, SES) explain a larger proportion of the variance in children's behavior problems than any single genetic variant does, may be inflated by genetic confounding, which has not been properly controlled for. For example, a recent publication used the GCTA method and identified a significant genetic overlap between family SES and children's IQ [27]. This implies that the same group of genetic variants may influence both an typical "environmental" factor (i.e., SES) and the phenotype of interest (i.e., IQ). Other common environmental factors, such as parental education [28] and parenting practices [29], also have a genetic component. Moreover, many children's (genetically-driven) behaviors in turn have evocative effects by eliciting certain behaviors from parents, teachers or friends. This phenomenon can be seen as reflecting reverse causality and could also lead to over-estimation of the environmental influences.

It is maybe time that the debate between *"nature and nurture"* comes to a conclusion. Genetic and environmental factors are intertwined with each other, in ways not yet clear to us, but which are under thorough investigation. Integrated studies on genetics and epigenetics of children's behavior could contribute to these efforts.

The Missing Environment problem

In this thesis, we focused exclusively on main genetic effects on children's behavior problems. Although we recognize the importance of environmental (e.g., social) influences in developmental psychopathology, and we ultimately aim for an interactionistic view of both health and psychopathology, we decided to deal with the complexity of our phenotypes by focusing on one aspect of their underpinnings: the genetic part.

In the literature, a large body of research has investigated the role of environmental influences (e.g. parenting sensitivity [30], socio-economic status [31], and peer relationships [32]) on behavior problems in childhood and adolescence. More recently, the role of gene-environment interaction (GxE) has been recognized as a prominent mechanism influencing children's psychopathology [33,34]. The differential susceptibility theory has been developed, which proposes that some individuals who carry specific genetic variants are more susceptible to environmental influences, for better and for worse [35]. A genome-wide GxE study could help us identify novel gene- environment interactions influencing common behavior in childhood, when large samples with well-assessed environmental factors become available [36]. One of the most challenging tasks would be to adequately measure environmental factors, which usually cannot be observed directly, in large population-based studies. Relying only on general questionnaires can be problematic, as reporting bias is a major issue and cultural differences cannot be interpreted easily. New user-friendly software tools (mobile apps) became recently available in behavioral research, and promise to accurately measure behavior and environmental factors in real-time [37]. These mobile apps can also be implemented in population-based studies.

An additional level of complexity is added when considering that in reality the role of specific environmental effects may be mediated by epigenetic changes in specific genes, and moderated by the underlying genetic variation of the individual, influencing normal and disruptive development in children [38].

Population-based vs. clinical case/ control samples

The research reported in this thesis was performed in population-based studies. Although it can be argued that clinical cases represent the extreme end of the continuum of psychopathology, it is also possible that the genetic architecture of children diagnosed with behavior problems differs from that of children derived from population-based samples [39]. In this thesis, we were particularly interested in normative differences of common behavior problems, in non-clinical samples of children and adolescents. So far, little was known about the genetic basis of normative development of behavioral and emotional problems in the general population. While clinical cases are critically important for research, it is also very important to identify the general norms of various aspects of behavior, cognition, social, and emotional development, from infancy through adolescence. The genetic and epigenetic determinants of normal variation in population-based samples will help us understand how these variations shape the developmental outcomes and the healthy function of the children in a society. It remains to be assessed whether the findings of our studies also hold true in more extreme, clinical cases of children.

Multi-informants assessment of child behavior problems

In this thesis, children's behavior problems were assessed by parents, teachers or by direct observations. The use of multiple informants could help us assess behavior problems in different settings and gain a more comprehensive understanding of children's psychopathology. Often informant discrepancies

arise and could be interpreted as measurement error, informant's bias or true contextual differences of child's behavior [40]. We propose child psychiatric genetic research can only benefit from multi-informant assessments, by avoiding informant effects which could noise and/or bias the estimates of genetic contribution to common behavior problems in children. Additionally, peer [41] and self-reported [42] assessments could be used, when available, to improve phenotypic identification of children with behavior problems in different settings.

Ethical considerations

Ethical challenges are inherent to child psychiatric genetic research. The knowledge gained by our research should be interpreted carefully and it must be decided beforehand which types of results can/ should be communicated to the participants. Given the risk of chance findings, or true findings with small effects, revealing information about a child's genome carries the actual risk to both the individual and the family of stigmatization and unpredictable discrimination [43].

In addition, the use of new genetic and epigenetic technologies requires large cohorts, raising the issue of data sharing. There is a current trend to open access data sharing (e.g. as in the case of the Psychiatric Genomics Consortium, PGC), leading to international collaboration of experts. This policy is also accompanied by the development of biobanks, to store the ever-increasing, anonymized biosamples [44]. The ethical issues accompanying biobanking (e.g. how human samples are processed, is it possible to give an informed consent when donating a sample etc.) require constant reappraisal and discussion [45].

Practical implications

The ultimate goal of child psychiatric genetics research is to improve current predictive and diagnostic models, and therapeutic practices. In this thesis, we provide evidence of common genetic factors influencing ADHD symptoms and levels of stranger fear in children. Our study indicates that atypically low levels of fear in a social context could be an early indicator of later ADHD symptoms. ADHD is typically diagnosed in school-age children, and only when the symptoms cause impairment in two or more settings (e.g. home and school) [46]. Early childhood identification and intervention could effectively reduce the severity of the symptoms and improve later academic performance [47].

Moreover, we provide new insights of the genetic architecture of childhood aggressive behavior and infant attachment security and disorganization. The novel genes and pathways that were identified and presented in this thesis could indicate susceptibility to adverse outcomes (i.e. aggressive behavior, and attachment disorganization) and potentially indicate groups of individuals that benefit more from certain preventive or therapeutic interventions [48]. Although such reliable genetic markers have not been identified yet, genetic variants could ultimately be used in clinical tests with the potential to improve diagnosis, classification, and therapeutic response rates of children with behavior problems [49].Finally, this thesis provides evidence of early pregnancy influences of the DNA methylation

in the neonates. The differentially methylated CpG probes and pathways that were significantly associated with prenatal maternal thyroid levels may help us understand how prenatal environment affects normal development in children, provide guidelines for optimal *in utero* development [50], and provide us novel epigenetic targets with therapeutic potential [51].

Future research directions

The findings of our systematic review and meta-analyses of studies on *DRD4* VNTRs and behavior outcomes in children provide implications for further research. We propose that researchers analyze and present all available genotypic classifications, before more functional knowledge on *DRD4* VNTRs become available.

In this thesis, we also provide evidence of additive genetic influences on common behavior problems in children. The results of our study support the continuation of GWAS efforts in large samples investigating the genetic architecture of psychopathology (e.g., attention problems) in children. Future collaborative efforts on large samples of children, with well-measured phenotype and environmental data, will facilitate the continuous progress of child psychiatric genetics. In addition, advances in next generation sequencing could have a great impact in child psychiatry and developmental psychopathology [52].

Finally, DNA methylation provides a crucial regulatory mechanism of normal human development and psychopathology. The field of epigenome-wide association studies is new and promising. Future studies should focus on how genetic variation (i.e. methylation quantitative trait loci, meQTLs) [53] interacts with environmental hues to shape human behavior. The cis- and trans-acting regulation of gene expression [54], the stability and change of methylation patterns across different cell tissues [55] and time [56], and the use of epigenomic biomarkers in drug therapy [57] are also hot and promising topics for further research.

The progress in child psychiatric genetics has accelerated over the past few years, and although we become increasingly aware of the complexity accompanying child psychiatry and developmental psychopathology, we also begin to see the path from genetic discovery, to circuits, interactions, and epigenetic differences. Capitalizing the new tools and advances (e.g. induced pluripotent stem cells, gene editing, next generation sequencing, and open access genomic databases) will assure further development and progress of the field.

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10

Summary/Samenvatting

Summary

The aim of this thesis was to identify genetic and epigenetic determinants of children's behavior problems. It has been estimated that 7% of preschoolers and 13-20% of school-aged children and adolescents suffer from internalizing and/or externalizing problems. To investigate the role of genetic variation in children's behavior problems and search for specific epigenetic patterns related to the prenatal environment, we used a quantitative model of psychopathology, and multiple, cutting-edge approaches (i.e., the candidate gene, the genome-wide, and epigenome-wide approach).

In **section A**, we focused on candidate genes. As outlined in **chapter 2**, long variable number tandem repeats (VNTRs) in the dopamine receptor D4 (*DRD4*) gene were associated with increased risk of attention deficit/ hyperactivity symptoms in a population-based sample of N = 589 6-year old children. This relationship was partially mediated by lower levels of stranger fear at age 3 years, indicating a common biological link between attention deficit/ hyperactivity symptoms and fear in social context.

In **chapter 3**, we presented the results of a systematic review and meta-analysis of *DRD4* VNTRs and behavior outcomes in non-clinical samples of children and adolescents. We identified five classification systems of *DRD4* VNTRs, with the 7-repeat carriers vs. non-carriers being the most commonly used classification. We found inconclusive evidence of functional differences between the rare *DRD4* VNTRs. Our meta-analyses provided evidence of association between longer *DRD4* VNTRs and less optimal social and emotional development, and executive function in children and adolescence, but these associations were moderated by the choice of genotypic classification system.

In **section B**, we focused on the genome-wide approach. In **chapter 4**, we performed Genome-wide Complex Trait Analysis (GCTA) to estimate heritability based on common, genome-wide single nucleotide polymorphisms (SNPs). To this aim, we combined genotype and phenotype data from two independent, population-based Dutch studies. We provided evidence of substantial additive genetic heritability (SNP h^2) for four domains of children's behavior problems (i.e., attention deficit problems, internalizing, externalizing, and pervasive developmental problems) in N = 1,495 to 3,175 of 3,7, and 9 year old children.

Chapter 5 describes a genome-wide association (GWA) meta-analysis on children's aggressive behavior. We meta-analyzed data from 8 population-based samples (N = 18,988) and identified a near genome-wide significant locus in *LRRTM4* gene (rs11126630, p = 5.30e-08). Gene-based analyses indicated that genetic variation in *AVRP1A* gene is associated with higher levels of aggressive behavior in children.

Chapter 6 describes a GWA study on attachment disorganization and security. In this exploratory study of the largest sample available (N = 657 of 14-month old Dutch children), we identified suggestive genetic variants and novel genes associated with attachment disorganization (i.e., *HDAC1*, *ZBF675*, *BSDC1*) and security (i.e., *BECN1*). Follow-up pathway analyses indicated over-representation of synaptic and neuronal pathways associated with attachment disorganization, but no pathway associated with attachment security.

In **section C**, we focused on the epigenome-wide approach. **Chapter** 7 outlines the results of our epigenome-wide association study (EWAS) on prenatal exposure to maternal stress in neonates. A prenatal maternal stress exposure (PMSE) score was constructed in a sample of $N = 912$ Caucasian mothers and associated with DNA methylation of the neonates. Our single probe analysis did not identify any epigenome- wide significant result. Follow-up pathway analyses identified over-representation of pathways related to cell cycle regulation, metabolism and immunity. Our differentially methylated regions (DMRs) analysis identified only suggestive regions of differential methylation in children.

In **chapter 8**, we performed a EWA study on maternal thyroid function during early pregnancy, in a sample of $N = 751$ Caucasian mothers and their neonates. We assessed thyroid function during pregnancy by measuring both levels of free thyroxine (FT4) and thyroid stimulating hormone (TSH). Our EWA analysis identified no epigenome-wide significant CpG probe in cord blood associated with levels of FT4 during early pregnancy. However, we found that increased differential methylation in *RNF208* gene in cord blood was associated with increased levels of maternal TSH. Follow-up pathway analyses identified over-representation of specific pathways related to development, immunity and metabolism for maternal FT4 and TSH levels.

In **chapter 9** we discussed the results of the studies mentioned in this thesis in a broader perspective, and also the relevant methodological considerations, practical implications and future research directions.

Samenvatting

Het doel van dit proefschrift was om de genetische en epigenetische determinanten van gedragsproblemen bij kinderen te identificeren. 7% van de jonge kinderen en 13-20% van de oudere kinderen en adolescenten hebben internaliserende en/of externaliserende gedragsproblemen. Om de rol van genetische variatie in deze gedragsproblemen te onderzoeken en de specifieke epigenetische patronen met betrekking tot de prenatale omgeving te bestuderen, hebben we gebruik gemaakt van een dimensioneel model van psychopathologie, en van meerdere, innovatieve benaderingen (d.w.z. de kandidaat gen, GWAS, en EWAS methoden).

In **deel A**, hebben we ons op kandidaat-genen gericht. Zoals beschreven in **hoofdstuk 2**, werden lange VNTRs in de dopamine receptor D4 (*DRD4*) gen geassocieerd met een verhoogd risico op ADHD klachten in een cohort steekproef van N = 589 6-jarige kinderen. Deze relatie werd deels veroorzaakt door lagere niveaus van sociale angst.

In **hoofdstuk 3** hebben we een systematische review en meta-analyse van *DRD4* VNTRs en gedragsproblemen in niet-klinische steekproeven van kinderen en jongeren gepresenteerd. We identificeerden vijf classificatiesystemen voor *DRD4* VNTRs (het 7-repeat versus de rest werd meestal gebruikt). We hebben geen overtuigend bewijs van functionele verschillen tussen de zeldzame varianten van *DRD4* VNTRs. Onze meta-analyses lieten de associatie zien tussen langere *DRD4* VNTRs en minder optimale sociale en emotionele ontwikkeling, en executieve functies bij kinderen en adolescenten. Deze associaties werden door de keuze van genotypische classificatiesysteem gemodereerd.

In **deel B**, hebben we ons op GWA studies gericht. In **hoofdstuk 4** hebben we Genome-wide Complex Trait Analysis (GCTA) uitgevoerd om de erfelijkheid te schatten op basis van gemeenschappelijke, 'genome-wide single nucleotide polymorphisms' (SNPs). We hebben genotype en fenotype gegevens uit twee onafhankelijke, Nederlandse populatie-studies gecombineerd. Wij toonden substantiële additieve genetische erfelijkheid (SNP h^2) aan voor vier domeinen van gedragsproblemen (aandachtsproblemen, internaliserende- en externaliserende gedragsproblemen en pervasieve ontwikkelingsstoornis) in N = 1,495 tot 3,175 van 3,7 en 9 jaar kinderen.

Hoofdstuk 5 beschrijft een GWA meta-analyse van agressief gedrag bij kinderen. We hebben data van 8 populatiestudies geanalyseerd (N = 18.988) en vonden een bijna genome-wide significante locus in *LRRTM4* gen (rs11126630, p = 5.30e-08). Gene-based analyses lieten zien dat genetische variatie in *AVRP1A* gen met hogere niveaus van agressief gedrag bij kinderen geassocieerd is.

Hoofdstuk 6 beschrijft een GWA studie over attachment desorganisatie en veilige gehechtheid. In de grootste beschikbaar sample van N = 657 14-maanden Nederlandse kinderen), hebben we mogelijke genetische varianten geïdentificeerd en nieuwe genen geassocieerd met attachment desorganisatie (d.w.z. *HDAC1*, *ZBF675*, *BSDC1*) en veilige gehechtheid (d.w.z. *BECN1*) . Follow-up pathway analyses hebben synaptische en neuronale paden met attachment desorganisatie geassocieerd, maar geen pad was met veilige gehechtheid geassocieerd.

In **deel C**, hebben we ons op EWAS studies gericht. **Hoofdstuk** 7 beschrijft de resultaten van onze EWAS analyses op prenatale maternale stress bij pasgeborenen. Een prenatale maternale stress score werd gebouwd in een sample van *N* = 912 moeders. DNA methylatie werd in pasgeborenen gemeten. Onze single probe analyse liet geen significant EWAS resultaat zien. Follow-up pathway-analyses hebben verschillende paden geïdentificeerd met betrekking tot regulatie van de celcyclus, metabolisme, en immuniteit. Onze DMRs analyse heeft alleen suggestieve regio's van differentiële methylatie bij kinderen geïdentificeerd.

In **hoofdstuk 8**, hebben we een EWA studie over maternale schildklierfunctie tijdens de vroege zwangerschap uitgevoerd, in een steekproef van *N* = 751 moeders en hun pasgeborenen kinderen. We hebben de schildklierfunctie tijdens de zwangerschap beoordeeld door het meten van vrije thyroxine (FT4) en thyroid stimulating hormone (TSH). Onze EWA analyse heeft geen significante EWAS CpG probe geïdentificeerd in navelstrengbloed in verband met niveaus van FT4 tijdens vroege zwangerschap. Daarentegen hebben we gevonden dat verhoogde differentiële methylatie in het *RNF208* gen in navelstrengbloed met verhoogde niveaus van maternale TSH geassocieerd is. Follow-up pathway analyses hebben specifieke paden met betrekking tot ontwikkeling, immuniteit, en metabolisme geïdentificeerd voor FT4 en TSH niveaus van de moeder.

Tot slot werden in **hoofdstuk 9** de resultaten van dit proefschrift in een breder perspectief besproken. De methodologische overwegingen, de praktische implicaties en ideeën voor toekomstig onderzoek werden uitgewerkt.

Addendum 11

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Akpudo H, Aleksic B, Alkelai A, Burton C, Roa TC, Chen DTW, Cheng MC, Cocchi E, Davis LK, Giori IG, Hubbard LM, Merikangas A, Moily NS, Okewole A, Olfson E, **Pappa I**, Reitt M, Singh AB, Steinber Jm Strohmaier J, Ting T, van Hulzen KJE, O'Shea A, DeLisi LE. Summaries of oral sessions at the XXI World Congress if Psychiatric Genetics, Boston, Massachusetts, 17-21 October 2013: state of the field. *Psychiatric Genetics 2014, 24:125-150.*

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Eirini (Irene) Pappa was born on March 23rd 1985, in Athens, Greece. She completed her studies in Biology (2002-2006) and Medicine (2006-2011) with cum laude, in the National and Kapodistrian University of Athens, Greece. Part of her undergraduate studies was conducted in Pauls Stradins Clinical University Hospital, in Riga, Latvia (Department of Orthopedics) and in the Massachusetts General Hospital, Boston, USA (Department of Trauma, Emergency Surgency and Surgical Critical Care). In 2012, Irene moved to Rotterdam, the Netherlands, to work on the PhD program which resulted in this thesis. During the first 2 years of her PhD, Irene also completed the NIHES Master program in Genetic Epidemiology. Irene has presented her research in international scientific meetings and she won the Young Investigator Travel Grant Award in the World Congress of Psychiatric Genetics 2013, in Boston, USA. Irene's scientific interests are focused on genetics, epigenetics, child and adolescent psychiatry/ psychology. In the future, Irene would like to pursue a specialization in psychiatry.

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MSc-program Genetic Epidemiology, NIHES:	**Year**	**ECTS**
– Principles of Research in Medicine	2012	0.7
– Clinical Decision Analysis	2013	0.7
– Methods of Clinical Research	2013	1.4
– Genome-Wide Association Analysis	2012	0.4
– Conceptual Foundation of Epidemiologic Study Design	2013	1.4
– Principles of Genetic Epidemiology	2012	0.7
– Genomics in Molecular Medicine	2013	1.4
– Social Epidemiology	2013	0.7
– Markers and Prognostic Research	2012	0.7
– Advances in Genomics Research	2013	0.4
– Study Design	2013	4.3
– Biostatistical Methods I: Basic Principles	2012	5.7
– Biostatistical Methods II: Classical Regression Models	2012	4.3
– Genetic-Epidemiologic Research Methods	2013	5.1
– Linux for Scientists	2013	0.6
– Repeated Measurements in Clinical Studies	2013	1.4
– Psychiatric Epidemiology	2013	1.1
– Courses for the Quantitative Researcher	2012	1.4

MSc-program Genetic Epidemiology, NIHES:	Year	ECTS
In depth courses:		
– Research Integrity	2014	2.0
– SNP's and Human Diseases, MolMed	2014	1.4
– Advances in Genome-Wide Association Studies, NIHES	2013	1.4
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– Introduction to Genetic Epidemiology, Boerhaave Nascholing	2012	1.4
– Applied Multivariate Data Analysis, Leiden FSW	2012-2013	10
Symposia, Conferences & Workshops		
– EAGLE symposium, Imperial College, London, UK	2012	1
– International Expert Meeting: "Novel Directions in research on GxE interplay", Utrecht University, The Netherlands	2012	0.5
– Development of Executive Function Workshop Utrecht University, The Netherlands	2012	0.5
– Abuse & Neglect Conference, The Netherlands	2012	1
– European Association of Developmental Psychology (EADP) winter school, Leuven, Belgium	2013	1.4
– XXI World Congress of Psychiatric Genetics, Boston, USA Oral presentation: "A genome-wide approach to childhood aggression", Young Investigator Travel Award	2013	1.4
– EMBO workshop on Epigenetics of Neural (Dys)function, Braga, Portugal Oral presentation: "Implications of prenatal maternal psychopathology in the epigenome of neonates"	2014	1.4
Teaching/ educational activities		
– Lecturing & supervising students "De Gekte Voorbij: over de Neurobiologie van (Kinder)Psychiatrische Stoornissen" Department of Psychiatry, Erasmus MC	2012-2013	4
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Final words

René Descartes (1596- 1650) wrote: "*Archimedes used to demand just one firm and immovable point, in order to shift the entire earth, so I too can hope for great things if I manage to find just one thing, however slight, that is certain and unshakable*." With somehow similar thoughts, I began the research leading to this PhD thesis, considering that genes could be this "*firm and immovable point*" on which we can base our understanding of both normal behavior and psychopathology. Now that my PhD thesis is completed, I clearly see that indubitable knowledge does not exist, or has not been found yet. Genes are indeed a starting point, but not enough to explain complex behaviors. Environmental variables should be taken into account, the interactions of genotype- environment should be captured in proper mathematical models, while chance effects cannot be ruled out. This is why I dedicate this thesis to what I consider the cornerstones of life: *genes* (mine, and others'), *environment* (natural, and social) and *chance* (unpredictable patterns we are -yet- unable to model).

This PhD thesis has been a team work, and the contribution of many people is here acknowledged. Dear members of the Generation R management team *(professors Hofman, V. Jaddoe, E. Steegers, C. Klaver, E. Wolvius, H. Tiemeier, F. Verhulst, M. van IJzendoorn, J. de Jongste, H. Moll, O. Franco, C. van Duijn, A. Uitterlinden, F. Rivadeneira, A. van der Lugt, J. Mackenbach, and H. Raat)*, dear data managers *(M. Kooijman, and C. Kruithof)*, dear support team *(H. Gerritse-Kattouw, A. Koedoot, R. van den Nieuwenhof, E. Peters, K. Toebes, P. van Sichem-Maeijer, R. Slotema, L. Paling- Stout, and E. Kroos)*, dear FOCUS team, your hard work is sincerely appreciated. Dear parents and children, your decision to participate in the Generation R study has offered us tremendous possibilities for research. You have filled in questionnaires, accepted us in home visits, participated in observational episodes, and gave us biological samples to store and analyze. You have been more than an ID number to me. Without you, we wouldn't be able to gather such variety of measurements on different aspects of growth and development, and our knowledge would almost certainly be poorer than it is today. Dear colleagues *(H. Bakker, N. Bergen, Z. Brown, R. Gaillard, O. Gishti, M. Guxens, D. Heppe, G. van Ingen, T. Korevaar, L. Kragt, K. Miliku, B. Dhamo, C. Monnereau, G. Noppe, W. Tideman, L. Toemen, A. Vidakovic, S. Vucic, L. Blanken, K. Bolhuis, R. Cents, I. Derks, A. Hammerschlag, Pauline Jansen, Philip Jansen, D. Kocevska, N. van Mil, S. Mous, R. Mulder, A. Neumann, A. Ringoot, F. Serdarevic, J. Steenweg-de Graaf, S. Thijssen, E. Verhoeff, A. Wildeboer, M. den Dekker, N. Elbert, T. Gazibara, M. Basten, J. Rijlaarsdam, A. Ghassabian, H. El Marroun, M. Verlinden-Bondaruk, S. Beth, M. Jansen, G. Bai, A. Wijtzes, C. Ars, L. de Barse, E. van den Hooven, L. Leermakers, M. Tielemans, T. Voortman, R. van der Valk, R. Taal, E. Székely, N. Lucassen, R. Kok, L. Zuurbier, M. Verkerk, L. Broer, P. de Vries, A. Vitezova, D. Vojinovic, A. Tharner, A. van der Spek, A. Sajjad, I. Nedeljkovic, T. Muka, S. Mirza, J. Milic, A. Maksimovic, S. Ligthart, S. van der Lee, O. Jovanova, L. Jaspers, A. Iglesias Gonzalez, R. Freak-Poli, S. Bouthoorn, H. Adams, N. Amin, F. Velders, E. Motazedi, S. Voorthuis, and R. Huffmeijer)*, each and every one of you has contributed to the Generation R, Rotterdam Study, Erasmus Epidemiology and/ or Leiden FSW team and the collaboration, discussion, and brainstorming with you have substantial improved my

research. Dear colleagues of the School of Pedagogical & Educational Sciences of Erasmus University Rotterdam, thank you for hosting my research and for your endless support. Special thanks to *Trude Michels-Dalm* for her hard working. Finally, special appreciation to *Dafna Windhorst*- my PhD-buddy all this time, and *Ryan Muetzel*- for his continuous support and encouragement, who agreed to be my paranymphs.

This thesis was conducted under the auspices of: *Marinus H. van IJzendoorn*, *Marian J. Bakermans-Kranenburg*, and *Henning Tiemeier*. My gratitude also to: *Frank Verhulst*, for his valuable support in this research and beyond, *Viara Mileva-Seitz*, for her great supervision and for being such an inspiring human, *Ralph Rippe*, for his statistical expertize and smooth collaboration, *Janine Felix*, for being such a giving researcher, sharing scripts and knowledge, *Carolina Medina Gomez*, for being such a brilliant and hard-working biostatistician, *Maartje Luijk*, for her broad knowledge in parenting and attachment, and *Stefan Walter*, for his valuable help in biostatistics. Dear co-authors of the studies mentioned in this thesis, your contribution has already been acknowledged in several occasions. Although you are way too many to mention here by name, I have benefited greatly from our interaction and I cherish our collaboration.

"I am a part of all that I have a met; Yet all experience is an arch where through; Gleams that untraveled world; Whose margin fades; Forever and forever when I move." (Ulysses, by Alfred Tennyson). If all people I have met and influenced my life have also contributed to what I am today, how my genes are expressed and how I conduct and interpret research, then all these people are indirectly contributors of this PhD thesis. I should go deep in time, to my first teacher, my first friend, and all others I have met ever since and express them my gratitude. But this will still not be enough, there are lonely travelers I briefly spoke to, random acquaintances and other substantial interactions I should bring to memory. This is not necessary here. As I stride deeper and deeper into the world, I am determined to do the only think I can do – determined to love you with all my heart, and all my soul.