Like mother, like child?

Intergenerational transmission of psychopathology;
a focus on genes and parenting

Rolieke A.M. Cents

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PhD thesis, Erasmus University Rotterdam, The Netherlands

ACKNOWLEDGEMENTS

The Generation R Study is conducted by the Erasmus Medical Center in close collaboration with the Erasmus University Rotterdam, School of Law and Faculty of Social Sciences, the Municipal Health Service Rotterdam area, Rotterdam, the Rotterdam Homecare Foundation, Rotterdam, and the Stichting Trombosedienst & Artsenlaboratorium Rijnmond (STAR), Rotterdam. We gratefully acknowledge the contribution of general practitioners, hospitals, midwives and pharmacies in Rotterdam. The first phase of the Generation R Study is made possible by financial support from: Erasmus Medical Center, Rotterdam, Erasmus University Rotterdam and the Netherlands Organization for Health Research and Development (ZonMw) (grant no.10.000.1003). The work presented in this thesis was conducted at the Department of Child and Adolescent Psychiatry/Psychology, Erasmus Medical Center, Rotterdam, the Netherlands. It was supported by additional grants from the Sophia Foundation for Scientific Research (SSWO; grant 547, 2008) and the Netherlands Organization for Scientific Research (NWO) (VIDI 017.106.370). Financial support for the publication of this thesis was provided by the 'Department of Child and Adolescent Psychiatry/Psychology', the 'Generation R Study', the 'Erasmus Medical Center', 'SBOH' employer of GP trainees, 'Chipsoft', and 'Cents Assurantiën' insurances and financial advice.

ISBN / EAN: 978-94-6233-348-2

Cover design, invitation design, and design chapter pages: Gea Zwart, kunstenaar & vormgever; www.geazwart.nl
Layout and printing: Gildeprint, Enschede

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Intergenerational transmission of psychopathology; a focus on genes and parenting

Zo moeder, zo kind?
Intergenerationele transmissie van psychopathologie;
een focus op genen en opvoeding

Proefschrift
ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam

op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 28 september 2016 om 15:30 uur

door
Rolieke Annedore Maria Cents
geboren te Wijhe

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. H.W. Tiemeier Prof.dr. F.C. Verhulst
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Ben ik een som van het verleden
Geschiedenis in elke gen?
Doe ik wat al degenen deden
Waaruit ik voortgekomen ben?
En als ik verander
Wat van wie komt er dan bij?
Wat van mij is van een ander
En wat van mij van mij?

(Uit: ‘Wat van mij ben ik?’ Jeroen van Merwijk, 1999)

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PREFACE

CHAPTER 1

The intergenerational transmission of psychopathology, an introduction

Mental health problems (e.g. psychopathology) affecting adults of parenting age are very common. According to a Dutch study, approximately four out of ten adults aged 18- 64 years have had a psychiatric disorder in their life, and almost one out of five adults experienced a psychiatric disorder in the last 12 months (de Graaf et al., 2010). Similar prevalence rates have been found in other Western countries (Kessler et al., 2005). These high prevalence rates are worrying because it is well known that parental psychopathology is one of the most important risk factors for child psychopathology and a wide range of other developmental problems in children (Goodman & Gotlib, 1999; Stein et al., 2014). In turn, psychopathology in young children is predictive of psychopathology in later childhood and even in adulthood (Egger & Angold, 2006; Feng et al., 2008; Goodman, 2007; Hofstra et al., 2002), thereby sustaining a familial cycle of psychopathology over the course of multiple successive generations.

The continuity of certain types of psychopathology across successive generations has been well described. Children of parents with a depressive disorder have a 3- to 4-fold increased risk of developing a depressive disorder compared to children of healthy parents (Beardslee et al., 1998; Weissman, 2006). Likewise, children of parents with an anxiety disorder have an increased risk of developing an anxiety disorder than children of parents without an anxiety disorder (Beesdo et al., 2009). Studies have also shown the transgenerational continuity of antisocial and disruptive behaviors (Bornovalova et al., 2010; Hicks et al., 2004). Importantly, there is emerging evidence that the risks for children extend beyond their parents' type of psychopathology (Dean et al., 2010; McLaughlin et al., 2012). For example, parental depression is also associated with child externalizing (i.e. behavioral) problems, and parental substance abuse and antisocial disorder are also associated with an increased risk of internalizing (i.e. emotional) problems in offspring (Hussong et al., 2008; Kerr et al., 2013).

Next to psychopathology, children of parents with mental health problems are at risk for developing cognitive problems (Sohr-Preston & Scaramella, 2006). Among others, parental psychopathology has been associated with impairments in early language development (Bjornebekk et al., 2015; Stein et al., 2014; Talge et al., 2007). Furthermore, associations between parental psychopathology and working memory problems have been described (Hughes et al., 2013; Jensen et al., 2014). Early identification of these cognitive problems is of concern because language and working memory are predictive of later academic functioning (Clark et al., 2010; Schoon et al., 2010; Young et al., 2002). Also, cognitive problems in young childhood may be an early manifestation of an increased vulnerability for later psychopathology (Goodman and Gottlib 1999, Petersen ea 2015, Schoon ea 2010, Brocki ea 2010).

Because psychopathology often recurs in the same families over the course of multiple generations, the intergenerational transmission of psychopathology remains a subject of great interest for researchers, policy makers, and the society. The main challenge is to interrupt the intergenerational cycle of psychopathology. Hence, a better understanding of the nature of continuities in psychopathology across multiple generations, and a better understanding of the mechanisms underlying the intergenerational transmission of psychopathology is needed. Ultimately this will help the designing of prevention and intervention programs aimed at children and their families at risk for psychopathology.

INTERGENERATIONAL TRANSMISSION OF PSYCHOPATHOLOGY

Typically, research on the intergenerational transmission of psychopathology has focused on documenting continuities in psychopathology across parents and their children. Much of this research focused on investigating associations between maternal depressive disorder and child psychopathology. This interest in maternal depressive disorder is not surprising given that depression is among the most prevalent and debilitating psychiatric disorders affecting women in their childbearing years (Goodman, 2007; World Health Organization, 2008). Also, subclinical depressive symptoms (i.e. symptoms that do not meet the criteria for a major depression) are highly prevalent and are shown to have clinical relevance and public health importance as well (Judd et al., 2002; Pietrzak et al., 2013).

Although there is considerable evidence that maternal depressive symptoms are associated with child psychopathology (Goodman & Tully, 2006), the evidence about the patterns and nature of the associations is less overwhelming. The gross of research has defined maternal depressive disorder as a unitary and static construct while there is considerable variation in severity of symptoms, duration of symptoms, and timing of symptoms (Goodman, 2014). Nowadays, various statistical techniques are available that allow researchers to model the heterogeneity of maternal depressive symptoms by estimating trajectories of depressive symptoms over time (Nagin, 2005; Nagin & Tremblay, 2001). Studying the associations between maternal depressive symptoms trajectories and child psychopathology would further enhance insights into the nature of the associations, i.e. for which mothers and children associations are strongest. Furthermore, studying trajectories of maternal depressive symptoms also allows us to study the context in which maternal depression occurs. For example, more severe and chronic maternal depressive symptoms may be a marker for higher genetic risk and / or for higher environmental risk including impairments in parenting, marital conflict and a lower socio-economic status (Serbin & Karp, 2004).

Expanding studies on intergenerational continuities of psychopathology to three generations would also provide us with better insights into the patterns of transmission of psychopathology. In contrast to the amount of two-generational studies, studies assessing transmission of psychopathology over the course of three successive generations are relatively sparse. Regarding the transmission of depressive disorders, results of these three generational studies are inconclusive (Olino et al., 2008; Pettit et al., 2008; Weissman et al., 2005) and need further study. Furthermore, three generational studies would give us the unique opportunity to study continuities in risk mechanisms, for example parenting behaviors and marital discord, that account for the intergenerational transmission of psychopathology (Serbin & Karp, 2004).

MECHANISMS OF INTERGENERATIONAL TRANSMISSION OF PSYCHOPATHOLOGY

Predominantly based on research assessing the transmission of depression from mother to child, multiple mechanisms that mediate the associations between parental psychopathology and child development have been proposed and described (Goodman, 2007; Goodman & Gotlib, 1999; Serbin & Karp, 2004). From that research, it follows that the transmission of psychopathology over successive generations is a complex process involving multiple mechanisms that exert uni-directional (i.e. from parent to child) and bi-directional effects, but are also often inter-correlated and interactive (See Elgar et al. (2004); Goodman and Gotlib (1999) for figures of theoretical models summarizing the possible mechanisms underlying the associations between parental and child psychopathology).

Not surprisingly, one mechanism proposed to be accounting for an important part of the association between parental psychopathology and child development is shared genetic factors, as each parent transmits approximately 50% of their genes to their biological child. However, mediating environmental mechanisms are also thought to explain important parts of the intergenerational transmission of psychopathology. Examples of environmental mechanisms are parent-child interactions including parenting behaviors, and exposure to family stressors such as interparental conflict (Goodman & Gotlib, 1999). Especially ineffective parenting is considered a key mediator in the transmission of psychopathology and an important predictor of early behavioral development and cognitive growth (Serbin & Karp, 2004).

A focus on genes

Evidence that genetic factors are indeed important determinants of various forms of psychopathology is provided by family, adoptee, and twin studies. For example, the heritability of depressive disorder in adults is estimated at approximately 40% (Sullivan et al., 2000), and the heritability for antisocial disorder is estimated at approximately 50% (Rhee & Waldman, 2002). Moderate to high heritability estimates are found for the more broadly defined childhood internalizing (60%-80%) (Bartels et al., 2004; Boomsma et al., 2005) and externalizing problems (40%-60%) (Arseneault et al., 2003; Haberstick et al., 2008). Cognitive problems are found to be highly heritable with estimates ranging from 60%-80% (Jansen et al., 2015).

The identification of susceptibility genes for child psychopathology and cognitive problems is important because this will enhance our insights into the biological mechanisms that are regulated by the genes identified. With regard to studies including samples of unrelated individuals, there are two main approaches to examine whether a gene is associated with a particular outcome. One approach is the hypothesis free approach where the researcher scans the whole genome (i.e. more than 500.000 genetic variants at once) for associations with the outcome of interest. This approach is named the genome wide association study (GWAS). To reach sufficient power to account for multiple testing, and for the generally small magnitude of genetic effects of mostly common genetic variants, study samples often have to include thousands individuals for which (inter)national collaboration is needed (Wang et al., 2005). Next, the interpretation of GWAS results is challenged by the fact that a lack of robust replication of the genetic variant – outcome association is often observed, and that identified genetic variants often include genetic variants that are located in genomic locations with no known genes, or the function of a gene is not known yet (Pearson & Manolio, 2008).

The other approach to investigate genetic association with an outcome of interest is the candidate gene approach (Lewis, 2002). Using this approach, which is hypothesis driven, the researcher has to have an understanding of the underlying pathophysiology of the outcome to be able to make an 'educated guess' about which genetic variant to investigate. One candidate gene that has gained particular interest in studies of depression and associated traits, is the serotonin transporter gene. In the promotor region of the serotonin transporter gene a genetic variant is located (5-HTTLPR), which influences the transcription efficiency of the gene. The short allele of 5-HTTLPR is found to be less active than the long allele, resulting in decreased transcription of the serotonin transporter and subsequent higher levels of serotonin in the synaptic cleft (Lesch et al., 1996; Murphy & Lesch, 2008). This gene is often selected as a candidate gene because serotonin is an important

neurotransmitter that modulates many brain functions including mood (Murphy & Lesch, 2008). Another frequently studied gene in candidate gene studies in relation to cognitive functioning and mental health disorders such as schizophrenia and ADHD, is the Catechol-O-MethylTransferase (COMT) gene. This candidate gene is chosen because prefrontal functioning, including cognition, is known to be dependent on dopaminergic neurotransmission. The COMT gene encodes an enzyme critical for prefrontal dopamine levels. This gene has been found to be associated with prefrontal activation during cognitive and emotional processing (Mier et al., 2010).

Although the amount of genetic molecular research has increased tremendously last years, only a relatively small proportion of genes responsible for the heritability rates mentioned above are identified. This phenomenon is likely explained by the fact that depressive disorder and depressive symptoms, but also language development, working memory, and parenting (i.e. the outcomes studied in this thesis) are so-called complex traits; traits that result from the combination of numerous genes with small to modest effects and multiple environmental factors, with the potential of interactions among them (Donnelly, 2008).

Candidate gene by environment interaction studies (cGxE studies) examine whether the effect of a candidate genetic variant depends on an environmental variable or vice versa, thereby taking into account that both nature and nurture explain why some individuals develop psychopathology and others do not. While it is widely accepted that both genetic factors and environmental factors influence an individual's development, and various researchers acknowledge the importance of gene-by-environment interactions in psychiatric research (Moffitt et al., 2005; van Winkel, 2015; Winham & Biernacka, 2013), there is also skepticism about the validity of GxE results mostly due to the frequent non-replication of results (Dick et al., 2015; Duncan et al., 2014). Factors contributing to the inconsistency in reported GxE results are the use of different definitions of phenotypes and environmental factors, small sample sizes, multiple testing, and publication bias (Dick et al., 2015; Duncan & Keller, 2011; Duncan et al., 2014).

Original cGxE findings were reported by Caspi et al. (2002); Caspi et al. (2003). They reported that a functional polymorphism in the promotor region of the gene encoding the enzyme monoamine oxidase A (MAO-A) moderated the effect of childhood maltreatment on antisocial personality and violent crime, and that the 5-HTTLPR polymorphism moderated the influence of stressful life events on depression. Following these findings, many studies assessed cGxE effects on psychopathology with psychosocial stress as the environmental risk factor reporting inconsistent findings. However, the challenge in framing cGxE hypothesis lays in developing hypotheses that are biologically plausible (Moffitt et al., 2005): the genetic

and environmental risk factors should affect the same neurobiological pathway to the outcome. Following this criterion, the selection of environmental factors should not be limited to stressful life events but future research can also include toxic pathogens such as cigarette smoke, and parenting behaviors as environmental risks in cGxE studies (Moffitt et al., 2005).

A focus on parenting behavior

Children, especially younger children and infants, are dependent on their parents for their physical and mental wellbeing. Therefore, parental behaviors such as warmth, support, guidance and structure during these developmental periods are important for the child to achieve developmental milestones and they contribute to a longterm healthy development (Sroufe et al., 2005). There is accumulating evidence that ineffective parenting behaviors, i.e. parenting behaviors that do not meet the child's need to sustain healthy development, are one of the primary mechanisms by which risk from a parent with psychopathology is transmitted to the child (Cummings & Davies, 1994; Goodman & Gotlib, 1999; Lovejoy et al., 2000).

The associations between parental psychopathology, including depressive disorder, anxiety disorder, antisocial personality disorder and substance abuse disorder, and ineffective parenting behaviors are well documented (Johnson et al., 2006; Johnson et al., 2001; McCabe, 2014). For example, mothers suffering from depressive symptoms use more harsh disciplining styles, and engage in more frequently in rejective and hostile parenting styles (Lovejoy et al., 2000). In turn, ineffective parenting behaviors are associated with an increased risk of psychopathology and cognitive problems in children (Bayer et al., 2008; Neppl et al., 2009). Moreover, long lasting consequences of exposure to ineffective parenting behaviors and abuse during childhood have been shown with an increased risk of adult psychopathology (Schilling et al., 2007; Schilling & Christian, 2014; Scott et al., 2010).

Accumulating evidence points out that parents display parenting behaviors similar to those they have experienced while growing up (Serbin & Karp, 2004). This holds especially true for harsh and aggressive parenting behaviors (Capaldi et al., 2003; Conger et al., 2003). Explanations for the intergenerational transmission of parenting behaviors include observational and experimental learning (Conger et al., 2009; van Ijzendoorn et al., 1992), and genetic factors. While research has indeed shown that substantial genetic influences are involved in parenting (Collins et al., 2000; Neiderhiser et al., 2004; Plomin et al., 1994), much less is known about the molecular genetic determinants of parenting (Swain et al., 2007).

Also, the simultaneously focus on parenting behaviors and interparental conflict or marital discord is a promising avenue for further research. Substantial research has shown that interparental conflict or marital discord is a robust predictor of child psychopathology, but may also indirectly affect child development through negatively impacting on parenting behaviors (Cummings & Davies, 2002). Studying continuities in marital discord and parenting behaviors is promising from a treatment and intervention standpoint, as that these family processes are more easily altered than, for example, genetic factors.

AIM AND OUTLINE OF THIS THESIS

The overall aim of this thesis is to further enhance our insights in the intergenerational transmission of psychopathology and its effects on the young child's psychopathology and cognitive development.

We therefore formulated two specific aims:
The first specific aim is to examine the nature of associations between parental and grandparental psychopathology and child psychopathology. In **Part I** of this thesis, two studies addressing this specific aim are included: We first examined whether the course of maternal depressive symptoms influenced the level of child psychopathology in chapter 2. That is, we modeled trajectories of maternal depressive symptoms with regard to severity and duration, and assessed how the different symptom-trajectories were related to child internalizing and externalizing problems. In chapter 3, we examined whether grandparental depressive and anxiety disorders were related to child psychopathology, independent of psychopathology of the parental generation.

The second specific aim of this thesis is to increase our understanding of the complex roles of genes and parenting behaviors in the transmission of psychopathology. The studies addressing this specific aim are presented in **Part II** of this thesis: In chapter 4 we performed a Genome Wide Association (GWA) study to identify new genes related to early spoken language. In chapter 5 and chapter 6, we included two candidate gene by environment interaction studies: In chapter 5, we assessed whether 5-HTTLPR interacts with maternal smoking during pregnancy to affect child emotional problems. In chapter 6, the influence of an interaction between genetic variation in COMT and harsh parenting on child working memory was assessed. Next, in chapter 7, we included a study that examined the effect of 5-HTTLPR on observed sensitive parenting. Last,

we included a study that examined the roles of grandparental divorce, parental marital discord and ineffective parenting behaviors over the course of three successive generations in relation to child psychopathology, see chapter 8.

All associations that were tested in the different chapters included in this thesis, are depicted in Figure 1.

SETTING

All studies included in this thesis were embedded in the Generation R Study, a prospective population-based cohort study from fetal life onwards in the city of Rotterdam, the Netherlands. The Generation R Study was designed to identify early biological, and environmental determinants of growth, development, and health in fetal life and childhood (Jaddoe et al., 2012; Tiemeier et al., 2012). In short, mothers resident in the study area at their delivery date and with an expected delivery date between April 2002 and January 2006 were eligible. While enrolment ideally took place during pregnancy, it was also possible after birth of the child. In total, 9.778 pregnant women were included, of whom 8.879 (91%) enrolled during pregnancy and 899 at birth of the child. During the two postnatal phases of the study (0-4 and 5 years), information was obtained in 7893 and 8305 children respectively.

Detailed assessments were conducted in a randomly assigned subgroup of Dutch children defined as having two parents and four grandparents born in the Netherlands. Assessments included prenatal psychiatric interviews, observations of maternal sensitivity until the child's age of four years, and a computerized working memory tasks at the child's age of four years, among others.

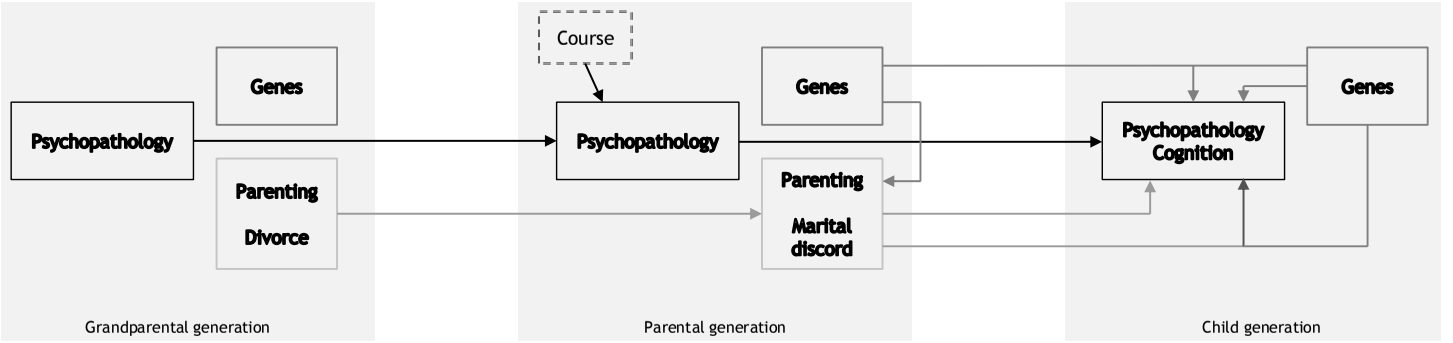

Figure 1. Overview of the hypotheses studied in this thesis. All solid lines represent associations that were investigated in this thesis.

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PART I

ASSOCIATIONS BETWEEN GRANDPARENTAL, PARENTAL, AND CHILD PSYCHOPATHOLOGY

CHAPTER 2

Trajectories of maternal depressive symptoms predict child problem behavior

ABSTRACT

Background: It is unclear how the course of maternal depressive symptoms affects child development. We modelled trajectories of maternal depressive symptoms from mid-pregnancy to three years after childbirth to better determine their associations with child problem behavior.

Methods: Mother-child dyads (n=4167) participated in a population-based prospective cohort in the Netherlands. Depressive symptoms were assessed with the Brief Symptom Inventory during pregnancy and at 2, 6 and 36 months postnatally. When children were three years old, problem behavior was assessed with the Child Behavior Checklist completed by each parent. A group-based modelling technique was used to model trajectories of maternal depressive symptoms and to examine their association with child problem behavior. The added value of trajectory modelling was determined with successive linear regressions.

Results: We identified four trajectories of maternal depressive symptoms; 'no' (34%), 'low' (54%), 'moderate' (11%) and 'high' (1.5%). Child problem behavior varied as a function of maternal trajectory membership. Whether rated by mother or father, children of mothers assigned to higher trajectories had significantly more problem behaviors than children of mothers assigned to lower trajectories. The model including trajectories had additive predictive value over a model relying only on a summed repeated measure of severity and a predefined chronicity variable.

Conclusions: Depending on their course, maternal depressive symptoms have different effects on child problem behavior. More information is gained by studying trajectories of symptoms, than only predefined measures of severity and chronicity. Also, trajectories can help identifying clinically depressed mothers who are possible candidates for early interventions.

INTRODUCTION

Depressive symptoms are very common, especially among women in their child-bearing years (Goodman, 2007; Judd et al., 1994; McLennan et al., 2001). Because women are usually the primary caregivers, children are substantially exposed to the mother's depressive symptoms during childhood. The effect of maternal depressive symptoms on child psychopathology has been the subject of a tremendous amount of research (Goodman & Tully, 2006). Better understanding is, however, complicated by the fact that maternal depressive symptoms are very heterogeneous as they can vary in severity of symptoms, and duration of symptoms.

Up to this date, few studies have examined the effects of chronicity of maternal depressive symptoms on child problem behavior. In general, these studies demonstrated that young children exposed to more severe and more chronic maternal depressive symptoms display more internalizing and externalizing behavior problems (Brennan et al., 2000; Early Child Care Research Network, 1999; Kim-Cohen et al., 2005). More severe and chronic depressive symptoms may indicate a higher genetic risk, have adverse effects on mother-child interactions, and tend to co-occur with various socio-emotional risk factors (Cummings & Davies, 1994) such as a lower educational level, a lower household income, and a non-Western ethnicity (McLennan et al., 2001; Pascoe et al., 2006). Also, mothers with depressive symptoms, compared to non-depressed mothers, are more likely to experience marital or family distress which also places the child at risk for behavioral problems (Rehman et al., 2008).

Although the longitudinal studies examining the effects of severity and chronicity of maternal depressive symptoms on child problem behavior (Brennan et al., 2000; Early Child Care Research Network, 1999; Kim-Cohen et al., 2005) have contributed significantly to the understanding of the effect of maternal depressive symptoms on child problem behavior, they are also subject to an important limitation. Severity and chronicity were modelled as pre-defined variables on the basis of subjective assignment rules such as cut-off scores to categorize individuals. As a consequence, it is difficult to disentangle the effects of severity and chronicity as these two dimensions are commonly confounded; more severe symptoms tend to last longer (Pettit et al., 2009).

Statistical methods are now available that allow modelling of the course and severity of maternal depressive symptoms simultaneously by identifying trajectories of mothers reporting similar patterns of depressive symptoms (Muthen, 2002; Nagin, 2005). So far, not many studies have addressed the heterogeneity of depressive symptoms by modelling trajectories (for a detailed overview see Nandi et al., 2009).

We located only three studies that modelled trajectories of maternal depressive symptoms to examine their relation with child problem behavior (Campbell et al., 2007; Campbell et al., 2009; Gross et al., 2009), of which only one examined child behavioral outcome during early childhood (Campbell et al., 2007). Exposure to maternal depressive symptoms during this developmental period may have particular adverse effects on child behavior. Campbell and colleagues (Campbell et al., 2007) identified six distinct trajectories of maternal depressive symptoms from one month to 56 months postpartum and found that children of mothers in the higher symptom trajectories displayed the most severe problem behaviors.

Although these findings provided insight in the course of maternal depressive symptoms measured during infancy and childhood, it is not yet clear whether other trajectories are identified if maternal depressive symptoms are also assessed during pregnancy. This may lead to the identification of a trajectory showing new-onset postpartum depressive symptoms. It is important to take any depressive symptomatology during pregnancy into account because of the possible adverse effects on the developing fetus (Deave et al., 2008; Luoma et al., 2001; Pawlby et al., 2009). Furthermore, it remains unclear whether the association between the trajectories and child problem behavior can also be explained by concurrent maternal depressive symptoms, the symptom level at the endpoint of the trajectories. Previous research has shown that concurrent symptoms have a particularly adverse effect on child problem behavior, possibly because they are the best indicator of ongoing depressive symptoms and interfere with mother-child interaction (Bayer et al., 2008; Brennan et al., 2000; Trapolini et al., 2007). Along the same line, the added value of modelling trajectories of maternal depressive symptoms to predict child problem behavior over more straightforward indicators of severity and chronicity of maternal depressive symptoms is unclear. Simple summary measures of severity and chronicity are generally reasonable. However, there is a risk of under- and over-fitting the data by creating pre-defined groups rather than identifying real trajectories. Also, the uncertainty of an individual's category membership cannot be accounted for (Nagin, 2005). While these limitations have been demonstrated (see Nagin 2005), to our knowledge, it has not yet been assessed whether formal modelling of trajectories is of significant added value.

We addressed these issues in the current study, conducted within a large population-based cohort including 4167 mother-child dyads. The study had three main aims; first, to identify trajectories of maternal depressive symptoms from mid-pregnancy until 36 months postnatally, using a group-based modelling approach (Nagin, 2005). Second, to assess the associations with child problem behavior as reported by each parent. Third, to examine the additive predictive value of trajectories

over indicators of severity or chronicity such as concurrent depressive symptoms, mean scores or predefined categories that summarize maternal depressive symptoms.

METHODS

Setting

The study was conducted within the Generation R Study, a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands; it has been described in detail elsewhere (Jaddoe et al., 2010). Mothers who were resident in the Rotterdam study area at their delivery date and had a delivery date from April 2002 until January 2006 were contacted. Midwives and obstetricians informed eligible mothers about the study at their first prenatal visit in routine care, and asked them to make an appointment at our research center. The study staff contacted these mothers by phone for additional information, and in person at the first appointment at the research center to obtain informed consent.

The study was conducted in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (MEC 198.782/2001/31 (prenatal) and MEC 217.595/2002/202 (postnatal)). Written informed consent was obtained from all participants.

Study population

In total, n = 9778 (61%) mothers contributed data to the Generation R Study of which n = 8880 were enrolled during pregnancy (69% in early pregnancy (< 18 wks), 19% in mid-pregnancy (18 – 25 wks), and 3% (> 25 wks) in late pregnancy). These mothers gave birth to n = 9745 known live born children. Of these, n = 1163 mother-child dyads were excluded because of birth outside the study area, and another n = 1287 because of no consent for postnatal follow-up. Therefore, a total of n = 7295 mother-child dyads were considered eligible.

Mothers without any information on depressive symptoms, or with information on only one episode of depressive symptoms were excluded (n = 1605). Mother-child dyads without information on child behavior (e.g. neither mother nor father-report, n = 1174) were also excluded. Of the remaining 4516 dyads, some mothers participated with more than one child in the cohort (e.g. older or younger siblings). To avoid paired data 349 siblings were randomly excluded, yielding a sample size of 4167 single mother-child dyads for the present study. Of these, the majority (n = 2252, 54%) had data on all four episodes of depressive symptoms, 33% had data

on three episodes of depressive symptoms, and 13% had data on two episodes of depressive symptoms.

For a detailed overview of our study population, including the attrition at different assessments, see Supplementary Material, Figure S1.

Measures

Maternal Depressive Symptoms

Information on maternal depressive symptoms was obtained by mailed questionnaires at 20 weeks of pregnancy (range 18 – 25 weeks), 2 months postnatally, 6 months postnatally, and 36 months postnatally. Maternal depressive symptoms were assessed using the Brief Symptom Inventory (BSI), a short version of the Symptom Checklist 90 (SCL-90) (de Beurs, 2004; Derogatis, 1993). The BSI is a self-report instrument with good reliability and validity. For the current study, the 6-item depression scale was used, which consists of the following items: 'feeling suicidal', 'feeling lonely', 'feeling down', 'having no interest in anything anymore', 'feelings of desperation about the future', 'feeling worthless'. Each item was rated on a 5-point scale ranging from '0' (not at all) to '4' (extremely). The depressive symptoms score was rated according to the BSI manual by summing the item scores and dividing the result by the number of endorsed symptoms; this resulted in a range of scores from 0.0-4.0 (de Beurs, 2004). Women with a score above 0.80 typically meet criteria for clinically significant depression (de Beurs, 2009). In the current study, internal consistencies (Cronbach's alpha) at the different time points ranged from 0.82 to 0.87.

Child problem behavior

When children were 3 years old, mothers and fathers each filled out the Child Behavior Checklist (CBCL/1,5-5), a parents' questionnaire that contains 99 problem items rated on a 3-point scale: 0 (not true), 1 (somewhat or sometimes true) and 2 (very true or often true). The Internalizing scale is the sum score of items in four syndrome scales: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn. The Externalizing scale is the sum score of Attention Problems and Aggressive Behavior. Higher scores represent higher severity. Good reliability and validity have been reported for the Child Behavior Checklist (Achenbach & Rescorla, 2000). The internal consistencies for the Internalizing problem scores (mother report: α=0.82, father report α=0.81) and Externalizing problem scores (mother report: α=0.89, father report α=0.89) were very good in the current study. Mother reports correlated moderately with father reports (Internalizing problem score: $r = 0.4$, $p < 0.001$; Externalizing problem score: $r = 0.5$, $p < 0.001$).

Covariates

Information on maternal age, educational level, family income, ethnicity, parity, marital status, and history of a clinically significant depressed mood were obtained at enrolment using self-report questionnaires. Educational level (highest education finished) was dichotomized into 'primary or secondary education' and 'higher education'. Monthly family income (based on the social security level for a 2-person household) was categorized into 'less than €1200' (below social security level), '€1200 to €2000' and 'more than € 2000' (above modal income). Ethnicity of the mother (based on the country of birth of the mother's parents) was categorized into 'Dutch', 'Other Western' and 'Non Western'. Parity was dichotomized into '0' and '1 or more'. Marital status was dichotomized into 'married or living together' and 'living alone'. History of a depressed mood was defined as 'present' as the mother reported to have experienced a period of depressed mood for which she received treatment from a general practitioner, a psychologist or a psychiatrist. Family stress was assessed during pregnancy by the seventh subscale (General Functioning) of the Family Assessment Device (FAD) which is a validated overall self-report measure of health or psychopathology of the family (Byles et al., 1988). A score > 2.17 on the General Functioning subscale denotes unhealthy family functioning. We used that score as a cut-off for 'family stress present'.

Statistical methods

To achieve the aims of the current study, data were analyzed in three steps.

In the first step, trajectories of mothers' depressive symptoms were modelled with a semi-parametric mixture model using the SAS procedure Proc Traj (Jones et al., 2001; Nagin, 2005; Nagin & Tremblay, 2001). This allows comparison with a previously published study on maternal depressive symptoms that also used this procedure (Campbell et al., 2007). Another approach is the growth mixture modelling approach (Muthen, 2002). Although both approaches share the common goal of identifying developmental trajectories, there is also an important technical assumption that distinguishes the two approaches: while growth mixture modelling allows for individual variation around the mean within trajectories relying on the normality assumption, group-based modelling assumes that there are groupings of distinct trajectories. Therefore, differences that may explain or predict individual-level heterogeneity can be expressed in terms of group differences. We choose the group-based modelling technique because we aimed to identify distinctive trajectories and relate those to a distal outcome and group-based modelling is ideally suited for this (see also Nagin, 2005).

Insofar a large proportion of the sample had a (near) 0 score on depressive symptoms, the developmental trajectories were modelled with the censored normal distribution. If data was missing, full information maximum likelihood estimates were computed. As previous research had led us to expect to find three to six trajectory groups, models with 3 to 6 trajectories were estimated (Campbell et al., 2007; Campbell et al., 2009; Gross et al., 2009; Mora et al., 2009; Nandi et al., 2009). Model selection was based on the Bayesian information criterion (BIC), the model with the largest BIC value (i.e. closest to 0) best fitting the data. Next, the model was refined by setting the orders of the trajectories (i.e. linear, quadratic or cubic). Multinomial logit models were estimated, relating maternal group membership to predictor variables including maternal age, educational level, family income, ethnicity, parity, marital status, history of depressed mood, and family stress. In this way, the parameters defining the trajectories and the probabilities of trajectory membership were estimated jointly (Nagin 2005). To define a good model, the average posterior probabilities of trajectory membership should be at least equal to 0.7 for all groups (Nagin, 2005).

In the second step of data analysis, the associations between maternal trajectory membership and child internalizing and externalizing problems were assessed with linear regression analyses. Mother-reported and father-reported data on child problem behavior were right-skewed. Square root transformations were applied to normalize the distribution. All models included the posterior probability of maternal trajectory membership as a covariate. Additionally, all models were adjusted for the predictor variables, excluding history of depressed mood, and for gender of the child.

In the third step, successive linear regression models were performed to examine the additive predictive value of trajectories of maternal depressive symptoms over concurrent depressive symptoms, and other indicators of severity and chronicity of maternal depressive symptoms. To this aim we defined severity of maternal depressive symptoms as the mean symptoms score across all four time points and chronicity as the number of assessments during which the mother experienced severe depressive symptoms above a certain cut-off, in line with previous research (Brennan et al., 2000; Early Child Care Research Network, 1999). Severe depressive symptoms were defined as a symptom score above 0.80 as measured by the BSI.

Percentage of missing data for predictor variables ranged from 0.9% to 15.3% (see footnote Table 1). Maternal reports of child behavior were missing for 159 (3.5%) children. Paternal reports of child behavior were missing for 24.2% of the children. To avoid the possible bias introduced by a complete case analysis, for missing data on categorical variables a 'missing' category was included. Missing data on continues variables (including if only one parent-report of child behavior was available) were imputed using expectation maximization (EM) in SPSS version 17.

Response analyses

The first group of mothers (n = 1605), who had participated in no or only one assessment of depressive symptoms, were lower educated (73.3% vs 46.1%, X^2 = 285.90, p < 0.001), had lower income (<1200; 36.3% vs 13.5%, X^2 = 322.98, p < 0.001), were more likely to be of non-Western ethnicity (57.0% vs 26.3%, X^2 = 441.06, p < 0.001), and reported more family stress (15.9% vs 8.2%, X^2 = 46.651, p < 0.001) than the mothers included in the study.

In the second group of mothers excluded from the study (n = 1174), no parent-report of child behavior was available. They were also lower educated (67.2% vs. 40.9%, X^2 = 239.42, p < 0.001), received lower income (<1200; 30.0% vs 19.7%, X^2 = 315.23, p < 0.001), and were more likely to be of non-Western ethnicity (45.8% vs 21.5%, X^2 = 275.99, p < 0.001) than mothers included in the analyses.

RESULTS

Descriptive statistics of the participating mother-child dyads are presented in Table 1.

Trajectories of maternal depressive symptoms

Models with 3 to 6 trajectories were estimated. The BIC score kept increasing as more groups were added. Trajectories in the four group model were conceptually interesting and average posterior probabilities, ranging from 0.70 to 0.92 (mean = 0.84), indicated a good to very good model fit. Because, in the five group model, the smallest trajectory estimated in the four group model (1.5%) was further divided into two even smaller groups (1.2% and 0.4%), we considered the four group model as the most optimal model. If the BIC criterium is not useful for model selection Nagin (Nagin, 2005) recommends selecting a model with no more groups than is necessary to describe the distinct features of the data. See Supplementary Material, Table S1 for the descriptives of the trajectories.

Figure 1 illustrates the four trajectory groups of maternal depressive symptoms. The first trajectory (N=1427, 34%) consisted of mothers who reported no or very few depressive symptoms throughout all four assessments (mean BSI score 0.00, 95% C.I. 0.00, 0.05). The second and largest trajectory (N=2221, 54%) constituted of mothers who reported low levels of depressive symptoms. However, mothers in this trajectory named 'low depressive symptoms' did report significantly higher depressive symptoms at 2 and 6 months postnatally (mean BSI scores 0.16 (95% C.I. 0.15, 0.17) and 0.17 (95% C.I. 0.16, 0.18) respectively) than in the prenatal and

third year assessment (mean BSI scores 0.13 (95% C.I. 0.13, 0.13) and 0.11 (95% C.I. 0.11, 0.11) respectively); as indicted by a significant quadratic term ($p < 0.001$). The third trajectory (N=457, 11%) was named 'moderate depressive symptoms. Mothers assigned to this trajectory reported levels of depressive symptoms just below the score of 0.80 which signals clinically significant symptoms. In these mothers, depressive symptoms were higher at the first two postnatal assessments (mean BSI scores 0.76 (95% C.I. 0.66, 0.86) and 0.76 (95% C.I. 0.65, 0.88) respectively) than at the prenatal assessment or the assessment 3 years after childbirth (mean BSI scores 0.71 (95% C.I. 0.59, 0.83) and 0.47 (95% C.I. 0.38, 0.56) respectively).

Again, the quadratic term was significant ($p < 0.001$). The last trajectory compromised only 62 mothers (1.5%). Mothers assigned to this trajectory reported consistently high levels of depressive symptoms that increased during the postnatal assessments at 2 and 6 months (quadratic term $p < 0.001$, mean BSI scores 1.47 (95% C.I. 1.27, 1.67), 2.31 (95% C.I. 2.16, 2.46), 2.61 (95% C.I. 2.40, 2.82), and 1.04 (95% C.I. 0.81, 1.27)). As the estimated mean depressive symptom scores were above 0.80 at each assessment, the 'high depressive symptoms' trajectory included mothers fulfilling the criteria of a clinically significant depression.

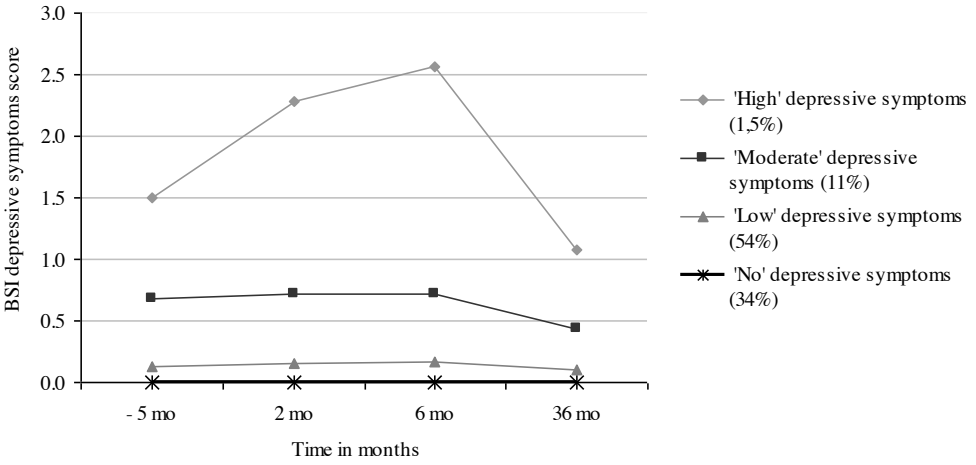

Figure 1. Trajectories of maternal depressive symptoms from pregnancy to 3 years postpartum

Table 1. Sample descriptives (N=4167)

Maternal characteristics	
Depressive symptoms [a]	
20 weeks of pregnancy	
% reporting no depressive symptoms	66.2
Median (interquartile range)	0.00 (0.00 – 0.17)
2 months postnatally	
% reporting no depressive symptoms	62.8
Median (interquartile range)	0.00 (0.00 – 0.17)
6 months postnatally	
% reporting no depressive symptoms	61.0
Median (interquartile range)	0.00 (0.00 – 0.17)
36 months postnatally	
% reporting no depressive symptoms	71.0
Median (interquartile range)	0.00 (0.00 – 0.17)
Age in years, mean (sd)	31.4 (4.6)
Educational level, % higher education	56.6
Family monthly income	
< €1200, %	9.4
€1200 - €2000, %	14.6
> € 2000, %	67.3
Ethnicity	
Dutch, %	63.1
Other Western, %	12.9
Non Western, %	22.2
Parity, % 1 or more	39.1
Marital status, % married or living together	88.9
History of depressed mood, % no depressed mood	71.3
Family stress, % no stress	82.3
Child characteristics	
Gender, % boy	49.6
Problem behavior (raw scores) [b]	
Internalizing problems, median (interquartile range)	4.0 (2.0 – 7.0)
% above clinical cut-off	3.3
Externalizing problems, median (interquartile range)	7.0 (4.0 – 12.0)
% above clinical cut-off	2.8

Note: Values were missing for educational level (0.9%), family income (8.7%), ethnicity (1.8%), parity (1.8%), marital status (3.0%), history of depressed mood (15.3%), and family stress (11.4%).

[a] Information on depressive symptoms were available for *n* = 3527 (85%) at 20 weeks of pregnancy, and *n* = 3436 (82%) at 2 months, *n* = 3187 (76%) at 6 months, and *n* = 4068 (98%) at 36 months postnatally.

[b] Children's problem behavior as rated by the mother. Clinical range based on clinical cut-off of Dutch normative sample (Tick et al., 2007).

Table 2 presents the results of the multinomial logit models that were performed to examine the contribution of predictor variables. Compared to the 'no depressive symptoms' trajectory, the probability of being assigned to the 'moderate' or 'high depressive symptoms' trajectories increased if mothers were lower educated (moderate; OR = 1.39, p = 0.06, high; OR = 3.03, p = 0.02) and reported an income less than €1200 per month (moderate; OR = 6.42, p < 0.001, high; 4.71, p = 0.01). Also, mothers assigned to the higher depressive symptoms trajectories were significantly more often of non-Dutch ethnicity than mothers assigned to the 'no depressive symptoms' trajectory. Being ethnically non-Western increased the risk by approximately 13-fold of being assigned to the highest depressive symptoms trajectory (OR = 13.2, p < 0.001). The probability of being assigned to the higher trajectories also significantly increased if mothers reported a history of depressed mood (moderate; OR = 11.5, p = < 0.001, high; OR = 23.8, p = < 0.001). Mothers assigned to the higher depressive symptoms trajectories also experienced significantly more family stress than mothers assigned to the 'no depressive symptoms' trajectory (moderate; OR = 9.58, p < 0.001, high; OR = 9.87, p < 0.001).

Child problem behavior as a function of maternal depressive symptoms trajectory

Child behavioral problems were examined as a function of trajectories of maternal depressive symptoms. Results are presented in Table 3. Overall, mothers assigned to any of the higher (i.e. low, moderate and high) trajectories of depressive symptoms rated their children as having significantly more internalizing problems than children of mothers assigned to the 'no depressive symptoms' trajectory, independent of socio-demographic variables and gender of the child (overall Beta = 0.25, p < 0.001). Likewise, children of mothers assigned to the 'low' trajectories had significantly more externalizing problems than children of mothers assigned to the 'no depressive symptoms' trajectory (overall Beta = 0.21, p < 0.001).

Results with father ratings of child problem behavior were very similar to the results with maternal ratings of child behavior (See Table 3). Fathers rated children of mothers assigned to the higher trajectories as having significantly more internalizing (overall Beta = 0.16, p<0.001) and externalizing problems (overall Beta = 0.13, p<0.001) than children of mothers assigned to the lower trajectories.

Table 2. Multivariate multinomial logistic regression of predictor variables on trajectories of maternal depressive symptoms

	No (N=1427)			Low (N=2221)			Moderate (N=457)			High (N=62)		
Determinants	OR	(95% CI)	p-value	OR	(95% CI)	p-value	OR	(95% CI)	p-value	OR	(95% CI)	p-value
Maternal age	1.00	(ref)	-	0.99	(0.97, 1.01)	0.5	0.98	(0.94, 1.02)	0.3	0.90	(0.83, 0.97)	0.002
Educational level; until secondary school	1.00	(ref)	-	1.28	(0.99, 1.66)	0.05	1.39	(0.98, 1.98)	0.06	3.03	(1.16, 7.97)	0.02
Family income												
% > 2000	1.00	(ref)	-	1.00	(ref)	-	1.00	(ref)	-	1.00	(ref)	-
% 1200 – 2000	1.00	(ref)	-	1.25	(0.86, 1.81)	0.2	2.56	(1.63, 4.03)	< 0.001	2.32	(0.90, 5.90)	0.08
% < 1200	1.00	(ref)	-	1.90	(0.85, 4.25)	0.1	6.42	(2.92, 14.1)	< 0.001	4.71	(1.44, 15.4)	0.01
Ethnicity												
Dutch	1.00	(ref)	-	1.00	(ref)	-	1.00	(ref)	-	1.00	(ref)	-
Other Western	1.00	(ref)	-	1.14	(0.83, 1.56)	0.4	2.12	(1.37, 3.27)	< 0.001	3.94	(1.23, 12.6)	0.02
Non Western	1.00	(ref)	-	2.24	(1.47, 3.50)	< 0.001	4.39	(2.74, 7.05)	< 0.001	13.2	(4.98, 34.3)	< 0.001
Parity; 1 or more	1.00	(ref)	-	0.76	(0.60, 0.94)	0.02	0.59	(0.43, 0.83)	0.002	0.94	(0.49, 1.80)	0.9
Marital status; living alone	1.00	(ref)	-	1.49	(0.75, 2.97)	0.2	1.79	(0.90, 3.56)	0.1	1.27	(0.47, 3.47)	0.6
History of depressed mood; present	1.00	(ref)	-	3.74	(2.11, 6.63)	< 0.001	11.5	(6.35, 20.7)	< 0.001	23.8	(9.62, 58.9)	< 0.001
Family stress; present	1.00	(ref)	-	2.29	(0.93, 5.68)	0.07	9.58	(4.10, 22.4)	< 0.001	9.87	(3.34, 29.2)	< 0.001

Note: Values were missing for Family income (N=363, 8.7%), Ethnicity (N=75, 1.8%), Parity (N=74, 1.8%), Marital status (N=127, 3.0%), History of depressed mood (N=636, 15.3%), and Family stress (N=475, 11.4%). Separate missing categories were run.

Table 3. Regression analyses of maternal depressive symptoms trajectories on child problem behavior as reported by each parent

		Internalizing				Externalizing			
	Trajectories	B	(95% CI)	Beta	p-value	B	(95% CI)	Beta	p-value
Mother report									
Model 1 [a]	No	0.00	(ref)	0.00	-	0.00	(ref)	0.00	-
	Low	0.52	(0.44, 0.61)	0.24	< 0.001	0.44	(0.34, 0.53)	0.19	< 0.001
	Moderate	1.05	(0.93, 1.16)	0.30	< 0.001	0.87	(0.74, 1.00)	0.23	< 0.001
	High	1.59	(1.32, 1.85)	0.18	< 0.001	1.18	(0.87, 1.48)	0.12	< 0.001
	Trend	*0.52*	*(0.47, 0.58)*	*0.33*	*< 0.001*	*0.43*	*(0.37, 0.48)*	*0.25*	*< 0.001*
Model 2 [b]	No	0.00	(ref)	0.00	-	0.00	(ref)	0.00	-
	Low	0.35	(0.26, 0.45)	0.16	< 0.001	0.35	(0.24, 0.54)	0.15	< 0.001
	Moderate	0.79	(0.66, 0.92)	0.23	< 0.001	0.73	(0.58, 0.88)	0.19	< 0.001
	High	1.21	(0.93, 1.49)	0.14	< 0.001	0.99	(0.68, 1.30)	0.10	< 0.001
	Trend	*0.40*	*(0.34, 0.46)*	*0.25*	*< 0.001*	*0.35*	*(0.29, 0.42)*	*0.21*	*< 0.001*
Father report									
Model 1 [a]	No	0.00	(ref)	0.00	-	0.00	(ref)	0.00	-
	Low	0.29	(0.21, 0.37)	0.15	< 0.001	0.21	(0.13, 0.30)	0.10	< 0.001
	Moderate	0.53	(0.42, 0.63)	0.17	< 0.001	0.40	(0.29, 0.52)	0.12	< 0.001
	High	0.82	(0.58, 1.07)	0.10	< 0.001	0.65	(0.38, 0.92)	0.08	< 0.001
	Trend	*0.27*	*(0.22, 0.32)*	*0.19*	*< 0.001*	*0.21*	*(0.16, 0.26)*	*0.14*	*< 0.001*
Model 2 [b]	No	0.00	(ref)	0.00	-	0.00	(ref)	0.00	-
	Low	0.21	(0.13, 0.30)	0.11	< 0.001	0.19	(0.10, 0.29)	0.09	< 0.001
	Moderate	0.44	(0.31, 0.56)	0.14	< 0.001	0.39	(0.25, 0.52)	0.12	< 0.001
	High	0.68	(0.42, 0.93)	0.09	< 0.001	0.61	(0.32, 0.89)	0.07	< 0.001
	Trend	*0.22*	*(0.16, 0.28)*	*0.16*	*< 0.001*	*0.20*	*(0.14, 0.26)*	*0.13*	*< 0.001*

[a] included covariates: posterior probability of maternal trajectory membership.
[b] included covariates: posterior probability of maternal trajectory membership, maternal age, educational level, family income, ethnicity, parity, marital status, family stress, gender of the child.

The added value of trajectory modelling

Successive linear regressions were performed to assess whether the course of the maternal depressive symptoms predicted child problem behavior above the concurrent depressive symptom score at 36 months. The results are presented in Table 4, model 1. In the first step, predictor variables and gender of the child were entered as a block. In the second and third step concurrent depressive symptoms and the trajectories of maternal depressive symptoms were entered. The trajectories were entered as a categorical variable, with the 'no depressive symptoms' trajectory as the reference group. Although concurrent depressive symptoms significantly predicted child problem behavior, the trajectories remained independent predictors of child internalizing and externalizing behavior.

Next, we assessed the added predictive value of trajectories of maternal depressive symptoms above a model including two predefined measures of severity and chronicity (Table 4, model 2). Variables representing severity and chronicity of maternal depressive symptoms were added to the basic model. In the third step, trajectories of maternal depressive symptoms (categorical variable) were added. Severity of maternal depressive symptoms, defined as the mean depressive symptoms score over all four assessments, independently predicted child internalizing (Beta = 0.23, $p < 0.001$) and externalizing problems (Beta = 0.20, $p < 0.001$). The trajectories also predicted child internalizing problems independently of severity and chronicity of maternal depressive symptoms (internalizing: overall Beta = 0.21, $p < 0.001$, externalizing: overall Beta = 0.23, $p < 0.001$). Moreover, the trajectories added to the predictive capability of the model; internalizing: $\Delta R^2 = 0.01$, DF = 3, 4144, F (ΔR^2) = 11.24, p = < 0.001, and externalizing: $\Delta R^2 = 0.01$, DF = 3, 4144, F (ΔR^2) = 9.56, p = < 0.001.

All analyses were repeated with father reports of child problem behavior as the outcome (see Supplementary Material, Table S2.). Results were very similar as compared to the analyses using mother reports.

To test consistency of the results, we also defined chronicity by a stricter cut-off and defined the top 15% of the depressive symptoms score as positive instead of a score above 0.80 on the BSI (cut-off scores now ranged between 0.34 and 0.51). The trajectories remained significant predictors of child internalizing and externalizing problems and had additional predictive capability (results not reported here).

Table 4. Successive linear regression models assessing the predictive value of trajectories on child problem behavior as reported by the mother

		Internalizing problems						Externalizing problems					
Model 1	Predictors	B (se)	Beta	R^2	ΔR^2	df	ΔF	B (se)	Beta	R^2	ΔR^2	df	ΔF
Block													
1	Covariates [a]			0.08	0.08	17, 4149	22.34***			0.05	0.05	17, 4149	12.84***
2	Concurrent symptoms	0.58 (0.06)***	0.17	0.13	0.04	1, 4148	202,85***	0.53 (0.07)***	0.14	0.08	0.03	1, 4148	126.41***
3	Trajectories			0.14	0.01	3, 4145	18,42***			0.09	0.01	3, 4145	13.66***
	No	0.00 (ref)	0.00 (ref)					0.00 (ref)	0.00 (ref)				
	Low	0.29 (0.05)***	0.05					0.29 (0.05)***	0.12				
	Moderate	0.51 (0.07)***	0.07					0.48 (0.08)***	0.13				
	High	0.56 (0.16)***	0.16					0.40 (0.18)*	0.04				
	Trend	*0.25 (0.03)*	*0.16*					*0.23 (0.04)*	*0.13*				
Model 2													
Block													
1	Covariates [a]			0.08	0.08	17, 4149	22.34***			0.05	0.05	17, 4149	12.84***
2	Predefined measures			0.12	0.04	2, 4147	87.05***			0.07	0.02	2, 4147	49.31***
	Severity	0.84 (0.16)***	0.23					0.77 (0.18)***	0.20***				
	Chronicity	-0.03 (0.05)	-0.02					-0.10 (0.06)	-0.06				
3	Trajectories			0.13	0.01	3, 4144	11.24***			0.08	0.01	3, 4144	9.56***
	No	0.00 (ref)	0.00 (ref)					0.00 (ref)	0.00 (ref)				
	Low	0.23 (0.05)***	0.11					0.24 (0.06)***	0.10***				
	Moderate	0.25 (0.11)*	0.07					0.34 (0.12)**	0.09**				
	High	-0.16 (0.26)	-0.02					-0.03 (0.29)	-0.00				
	Trend	*0.21 (0.05)****	*0.13*					*0.23 (0.05)****	*0.14*				

Note: Reported (standardized) regression coefficients are based on the full model.
[a] Included covariates: maternal age, educational level, family income, ethnicity, parity, marital status, family stress, gender of the child, and the posterior probability of maternal trajectory membership.
* p<0.05, ** p<0.01, ***p<0.001
The ΔR^2 depends on the order in which variables were entered into the model. For example, trajectories add an ΔR^2 of 0.01 if added to the predefined measures of depressive symptoms: This reflects the added explained variance due to modelling of trajectories of depressive symptoms. However, when trajectories are the only indicator of maternal depressive symptoms they would add an ΔR^2 of 0.04 to the model predicting internalizing problems, resulting in a total variance of 12% (data not shown).

DISCUSSION

In a large population-based sample of mother-child dyads, we identified four trajectories of maternal depressive symptoms from mid-pregnancy through the first three years of a child's life: a 'no' trajectory (34%), a 'low' trajectory (54%), a 'moderate' trajectory (11%), and a 'high' trajectory (1.5%). Children of mothers assigned to the higher trajectories had significantly more problem behavior than children of mothers assigned to the lower trajectories as reported by each parent. Also, trajectory modelling was of added value in predicting child problem behavior over classical approaches that define severity using symptoms-scores or cut-off scores to define chronicity. Further, father reports provided evidence that our findings were not due to a 'depressed mother reporter bias', i.e. sad mothers report more behavioral problems in their offspring than non-sad mothers.

Our results show that the vast majority of mothers (88%) report no or low depressive symptoms from the peripartum through the first years of their child's life. However, we also identified a group of 11% of the mothers who consistently reported symptoms around the level of clinical symptoms. Also, a small group of mothers of 1.5% consistently reported symptoms well above the level of clinical symptoms. Overall, these results are in line with a previous study that modelled trajectories of maternal depressive symptoms (Campbell et al., 2007) as assessed with the Centre for Epidemiologic Studies-Depression Scale (CES-D). In this study the vast majority of mothers also reported no symptoms or symptoms below the clinical cut-off on the CES-D. The remaining mothers reported symptoms around or above the clinical cut-off score on the CES-D, of which a small group of 2.5% of the mothers reported chronically high symptoms. Campbell and colleagues (2007) identified six trajectories. Possibly the three smaller trajectories of mothers with intermitted, moderate increasing, and high decreasing symptom levels, were merged into the 'moderate' trajectory identified in our study. However, studies must be compared cautiously, as the present sample is larger, of European descent and assessed with an instrument not including somatic symptoms or happiness as part of the depression scale.

A significant increase in symptom-severity was noted at two and six months postnatally in all but the 'no' symptoms group. Indeed, up to 80% of all woman experience some kind of emotional problems after childbirth (Henshaw, 2003). The increase in symptom-severity was most pronounced for the 'high' trajectory. This may be explained by the fact that adverse socio-economic characteristics predict postpartum depression (Buist et al., 2008) and mothers assigned to the 'high' trajectory had significantly more adverse socioeconomic and demographic

characteristics than mothers assigned to other trajectories. Moreover, elevated depressive symptoms during pregnancy are an important predictor for postpartum depressive symptoms (Gotlib et al. 1991). This suggests that increased levels of depressive symptomatology are already present prior to birth of the child in some of the mothers experiencing postpartum depressive symptoms. Symptoms may already be present before pregnancy, which is in line with the finding that mothers assigned to the higher trajectories more often reported a history of depressed mood than mothers assigned to the lower trajectories. This may then explain why we did not identify a trajectory of new-onset postpartum depressive symptoms. Overall, the prevalence of postpartum depressive symptoms is estimated at approximately 7% for diagnosed caseness (Gavin et al., 2005) and at 13% for self-reported symptoms of postpartum depression (O'Hara & Swain, 1996), which resembles the percentage of mothers assigned to the 'moderate' and 'high' trajectories (12.5%).

Children whose mothers were assigned to the higher depressive symptoms trajectories were more likely to have internalizing and externalizing problems than children whose mothers were assigned to the lower trajectories. Even children of mothers assigned to the 'low' trajectory already had significantly more problem behaviors. This suggests that chronic exposure to maternal depressive symptoms, even if symptom-severity is low, has an adverse effect on child problem behavior. Campbell and colleagues (Campbell et al., 2007) reported similar effects, but no significant difference in child problem behavior between children of mothers assigned to the two lowest trajectories. Using predefined variables representing severity and chronicity, Brennan and colleagues (Brennan et al., 2000) reported that children were at increased risk for behavioral problems if their mother's depressive symptoms were more severe and more chronic.

More severe and more chronic maternal depressive symptoms often occur within a context of environmental risk, which may also place children at increased risk for problem behavior (Elgar et al., 2004). Mothers assigned to the higher depressive symptoms trajectories were lower educated and had lower family income. Also, these mothers were more likely to be of a non-Western ethnicity and experienced more family stress than mothers assigned to the lower trajectory groups. However, maternal trajectory membership remained a significant predictor of child internalizing and externalizing problems after adjusting for these socio-economic and demographic variables. Although the present study was conducted in a longitudinal cohort, causality cannot be inferred. For example, the odds of experiencing family stress (marital discord) were much higher in mothers assigned to the 'moderate' and 'high' trajectories than in mothers in the lower trajectories. It is not clear what comes first: depression or family stress? Most likely, there are reciprocal influences

between depressive symptoms and family stress without causal primacy of one of the two (Rehman et al., 2008). Along the same lines, there are other possible influential factors that could have contributed to the association between trajectories of depressive symptoms and child problem behavior. For example, more severe or more chronic maternal symptoms may reflect a higher genetic loading, manifesting itself as behavioral problems in the children who inherit maternal genes. Also, intra-uterine factors may transmit the risk from mothers to their children, as may parenting styles (Goodman & Gotlib, 1999).

We found that trajectories of maternal depressive symptoms predicted child problem behavior independently of concurrent depressive symptoms. That finding implies that not only the level of concurrent symptoms (e.g. the end-point of the trajectories) is related to child problem behavior, but also the course of the preceding symptoms. Most importantly, trajectories were of additional predictive value for child problem behavior, over measures of severity, defined as the mean symptom score over all time-points, and chronicity of depressive symptoms, defined by cut-off scores. The additional explained variance of the model including the trajectories was small. However, this is only the explained variance added by modelling depressive symptoms differently with trajectories. Overall, trajectories of depressive symptoms accounted for 33% of the explained variance. Another advantage of trajectory modelling is that this approach gives insight into the distinct underlying patterns of depressive symptoms in a population (Nandi et al., 2009).

The trajectories of maternal depressive symptoms were modelled with a semi-parametric mixture model using the SAS procedure Proc Traj, i.e. group-based modelling (Nagin & Tremblay, 2001). When comparing this approach to growth mixture modelling, Campbell and colleagues (Campbell et al., 2009) identified similar trajectories of maternal depressive symptoms with the growth mixture procedure as with the Proc Traj procedure (Campbell et al., 2007). We identified three distinct trajectories showing an increase in symptom severity postnatally and a decrease towards 3 years postpartum (e.g. the 'low', 'moderate' and 'severe' trajectories). The increase and decrease were steepest for the 'high' trajectory, but similar for the 'low' and 'moderate' trajectories. Therefore, the latter two trajectories could have been merged into one trajectory using growth mixture modelling.

The current study has several strengths such as longitudinal and repeated assessments, a large community sample, and the use of both mother and father ratings. Some limitations also need to be considered. First, it is not yet clear to what extent current results are generalizable to the broader population. Trajectories may differ as different or more assessment points are included and as different measures are used to define depression. Second, non-respondents in the current study were

characterized by more adverse socioeconomic and demographic circumstances and this may preclude more differentiated high trajectories. Third, it remains unclear to what extent the associations are due to mother-child interactions or to genetic or intrauterine influences.

Implications of current findings are two-fold. First, future research would benefit from trajectory analyses. Not only would this provide further insight in maternal depressive symptoms, but it would also contribute to the understanding of the association with child development. Importantly, the risk of maternal depressive symptoms for child behavioral problems is not fully captured with a traditional approach. Second, current results seem to identify a clinically depressed group of mothers. More research is warranted to determine whether this group of mothers can be identified early based on certain characteristics. They would be a candidate group for early interventions to decrease or even prevent depressive symptoms and adverse child outcomes. Also, future research may benefit from designs that integrate information on depressive symptomatology and other possible causal factors in the association with child behavioral problems, such as family stress or genetic factors.

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Table S1. Descriptives and average posterior probabilities (APP) for the trajectories.

Trajectories of depressive symptoms	N (%)	APP (sd)	Parameters	Parameter Estimates		
				B	(se)	p-value
1 'No' depressive symptoms	1427 (34)	0.71 (0.08)	Intercept	0.00	(0.00)	1.00
2 'Low' depressive symptoms	2221 (54)	0.89 (0.15)	Intercept	-0.50	(0.05)	< 0.001
			Linear	3.25	(0.48)	< 0.001
			Quadratic	-6.86	(0.91)	< 0.001
3 'Moderate' depressive symptoms	457 (11)	0.84 (0.15)	Intercept	0.40	(0.11)	< 0.001
			Linear	2.90	(1.03)	0.005
			Quadratic	-7.73	(1.97)	< 0.001
4 'High depressive symptoms	62 (1.5)	0.92 (0.13)	Intercept	-0.70	(0.33)	0.03
			Linear	28.0	(3.15)	< 0.001
			Quadratic	-58.9	(5.98)	< 0.001

Table S2. Successive linear regression models assessing the predictive value of trajectories on child problem behavior as reported by the father

		Internalizing problems						Externalizing problems					
Model 1	Predictors	B (se)	Beta	R^2	ΔR^2	df	ΔF	B (se)	Beta	R^2	ΔR^2	df	ΔF
Block													
1	Covariates [a]			0.04	0.04	17, 4149	9.89***			0.03	0.03	17, 4149	6.34***
2	Concurrent symptoms	0.16 (0.06)**	0.05	0.05	0.01	1, 4148	36.00***	0.06 (0.06)	0.02	0.03	0.00	1, 4148	13.94***
3	Trajectories			0.05	0.01	3, 4145	10.53***			0.04	0.01	3, 4145	8.78***
	No	0.00 (ref)	0.00 (ref)					0.00 (ref)	0.00 (ref)				
	Low	0.20 (0.05)***	0.10					0.19 (0.05)***	0.09				
	Moderate	0.36 (0.07)***	0.12					0.36 (0.08)***	0.11				
	High	0.50 (0.15)***	0.06					0.54 (0.16)***	0.06				
	Trend	*0.18 (0.03)****	*0.13*					*0.18 (0.04)****	*0.12*				
Model 2													
Block													
1	Covariates [a]			0.04	0.04	17, 4149	9.89***			0.03	0.03	17, 4149	6.34***
2	Predefined measures			0.05	0.01	2, 4147	30.98***			0.03	0.01	2, 4147	18.10***
	Severity	0.50 (0.15)***	0.16					0.35 (0.17)*	0.10				
	Chronicity	-0.03 (0.05)	-0.02					-0.03 (0.05)	-0.02				
3	Trajectories			0.06	0.01	3, 4144	4.16**			0.04	0.00	3, 4144	3.05*
	No	0.00 (ref)	0.00 (ref)					0.00 (ref)	0.00 (ref)				
	Low	0.14 (0.05)**	0.07					0.14 (0.05)**	0.07				
	Moderate	0.13 (0.10)	0.04					0.18 (0.11)	0.05				
	High	-0.11 (0.24)	-0.01					0.08 (0.26)	0.01				
	Trend	*0.12 (0.04)***	*0.08*					*0.13 (0.05)***	*0.08*				

Note: Reported (standardized) regression coefficients are based on the full model.
[a] Included covariates: maternal age, educational level, family income, ethnicity, parity, marital status, family stress, gender of the child, and the posterior probability of maternal trajectory membership.
* p<0.05, ** p<0.01, ***p<0.001
The ΔR^2 depends on the order in which variables were entered into the model. For example, trajectories add an ΔR^2 of 0.01 if added to the predefined measures of depressive symptoms: This reflects the added explained variance due to modelling of trajectories of depressive symptoms. However, when trajectories are the only indicator of maternal depressive symptoms they would add an ΔR^2 of 0.02 to the model predicting internalizing problems, resulting in a total variance of 6% (data not shown).

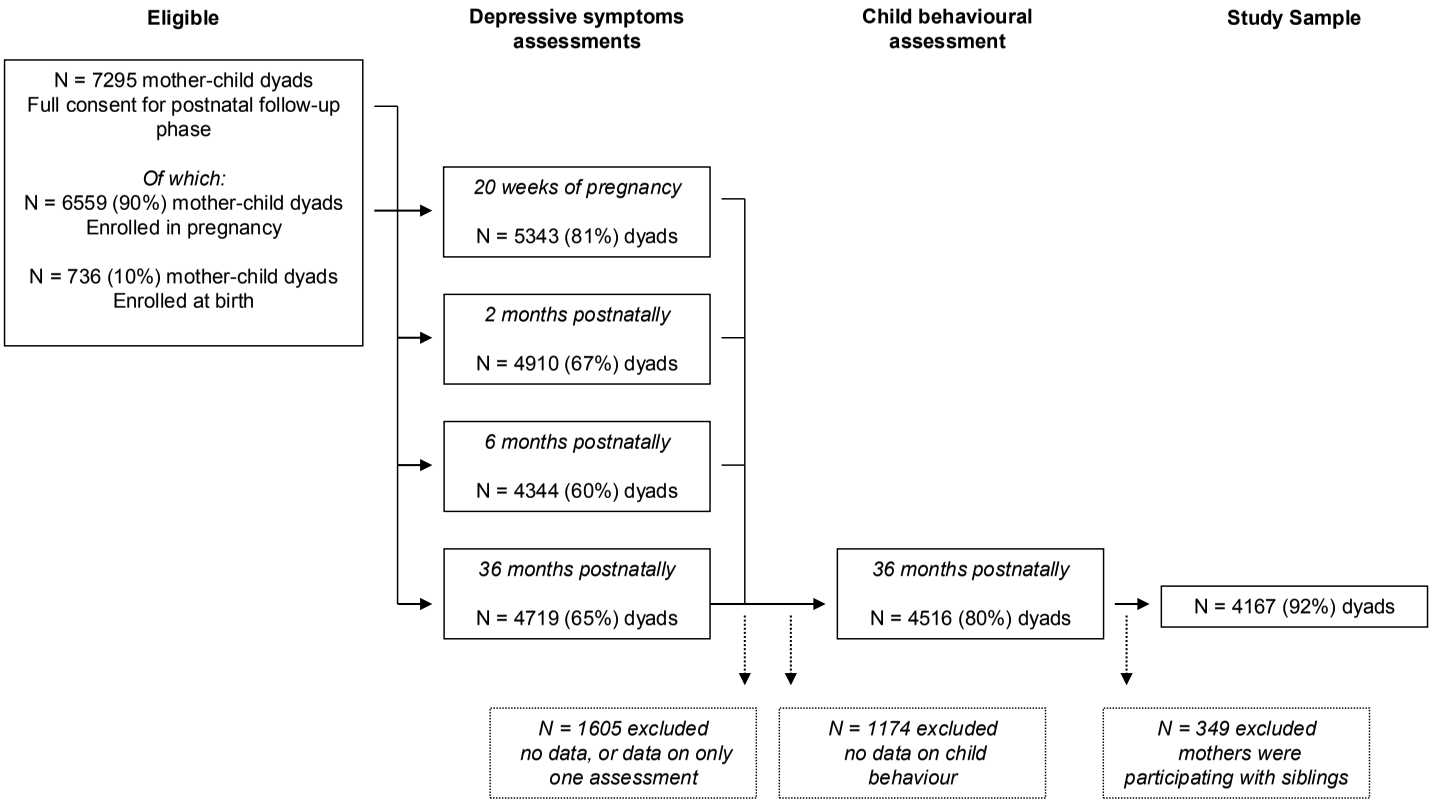

Figure S1. Flow chart on sample selection and participation
Note: Of the 4167 mother-child dyads included in the analyses, final response rates on depressive symptoms were the following: 85% at 20 weeks of pregnancy, and 82% at 2 months, 76% at 6 months, and 98% at 36 months postnatally.
For 72.3% of the 4167 children included in the analyses, information on child behavior was provided by both parents, for another 24.2% only mother-report on child behavior was available, and for 3.5% of the children only father-report was available.

CHAPTER 3

Grandparental anxiety and depression predict young children's internalizing and externalizing problems

ABSTRACT

Background: Family history is a major risk factor for child problem behavior, yet few studies have examined the association between grandparental psychiatric disorder and child problem behavior. Results are inconsistent as to whether the effect of grandparental depression on child problem behavior is independent of parental psychopathology.

Methods: Mothers and their children participated in an ethnically Dutch sub cohort of a population-based prospective cohort in the Netherlands. N=816 (66%) mothers and n=691 fathers participated in the prenatal interviews. N=687 (84%) mothers and children and n=565 (82%) fathers participated three years postpartum. (Grand)parental psychopathology was assessed during pregnancy of the mothers with the Family Informant Schedule and Criteria (FISC), the Composite International Diagnostic Interview (CIDI) and the Brief Symptom Inventory (BSI). Child behavior was assessed with the Child Behavior Checklist (CBCL) by mother and father when the child was three years old.

Results: Grandparental anxiety disorder predicted maternal reports of children's internalizing problems (OR=1.98, 95% C.I. (1.20, 3.28), p-value<0.01) and externalizing problems (OR=1.73, 95% C.I. (1.04, 2.87), p-value=0.03), independent of parental psychopathology. Results were similar for grandparental depression; internalizing OR=1.75, 95% C.I (1.11, 2.75), p-value=0.02 and externalizing OR=1.67, 95% C.I. (1.05, 2.64) p-value=0.03. However, grandparental psychopathology was not associated with children's problem behavior as reported by the father.

Conclusions: These results confirm the importance of a family history including not only the parental but also the grandparental generations.

INTRODUCTION

Family history is one of the most important risk factors for developing internalizing and externalizing problems at a young age and may help identification of young children at risk for problem behaviors (Bayer et al., 2008; Goodman & Gotlib, 1999). Studies of depressed or anxious children indicated that first degree relatives have higher prevalence rates of depressive disorders compared to the first degree relatives of controls (Birmaher et al., 1996; Klein et al., 2001; Kovacs et al., 1997). Overall, children with a depressed parent are three times more likely to develop a depressive disorder than children of healthy parents (Birmaher et al., 1996). However, children of depressed or anxious parents are not only at risk for internalizing behaviors. They are also at risk for externalizing behaviors (Beidel & Turner, 1997; Connell & Goodman, 2002; O'Connor et al., 2002).

Goodman and Gotlib (Goodman & Gotlib, 1999) provided an integrated model for the transmission of risk to children of depressed mothers. Their model posits that there are four possible mechanisms through which maternal depression can adversely affect child behavior. The first mechanism is through genetic factors as children inherit 50% of their DNA from their mother. The second mechanism proposes that maternal depression (during pregnancy) causes abnormal fetal development. These abnormalities may manifest after birth as, for example, behavioral inhibition. Third, the depressed mother may expose her child to negative cognitions, behaviors and affect which consequently place the child at risk for developing behavioral problems. Studies assessing the interaction between depressed mothers and their children documented numerous parenting difficulties among depressed mothers with these mothers displaying more hostile, irritable and intrusive behaviors towards their children (Lovejoy et al., 2000). The fourth mechanism refers to contextual stressors of maternal depression that mediate the association between maternal depression and child problem behavior. Examples of such contextual stressors are low social support, marital conflict and parenting stress.

While there is an extensive amount of research assessing the association between parental psychopathology and child mental health, to our knowledge, only four studies have gone beyond the assessment of two successive generations (Hammen et al., 2004; Olino et al., 2008; Pettit et al., 2008; Weissman et al., 2005). Yet, the familial risk is insufficiently evaluated when only assessing parental history. Knowledge of family history including the grandparents will help identifying children in need for prevention and treatment programs. The four existing studies reported an increased risk for internalizing and externalizing problems in children and adolescents in the presence of grandparental depression. However, results are

inconsistent as to whether the effect of grandparental depression on child problem behavior is independent of (e.g. mediated by) psychopathology of the parental generation. This inconsistency may be due to some limitations of the existing studies. First, most of the studies did not assess psychopathology of all four grandparents and parents (Hammen et al., 2004; Pettit et al., 2008; Weissman et al., 2005). Second, most importantly, none of the studies assessed global psychiatric symptoms next to lifetime psychiatric diagnoses to better assess psychopathology of the parental generation with a continuous measure. Third, most studies only took depression of the parental generation into account (Hammen et al., 2004; Pettit et al., 2008; Weissman et al., 2005). As a result it is not clear whether the risk of grandparental depression is transmitted specifically through parental depression or may also be transmitted through other parental psychopathology. For example, research demonstrated substantial sharing of genetic and, to a lesser extent, environmental factors across depression and anxiety indicating a common underlying vulnerability (Kendler et al., 2007; Kendler et al., 2003). Also, it was found that relatives of persons with depression had higher rates of dysthymia but also substance abuse disorders (Goldstein et al., 1994). By disregarding the three issues raised above, studies may not have well captured the genetic and environmental mechanisms underlying the risk transmitted. Also, except for one study sample sizes were relatively small (Hammen et al., 2004). This may have reduced power to detect significant associations between grandparental psychopathology and grandchild problem behaviors.

To address the issues raised, we examined whether grandparental anxiety and depressive disorder predicted internalizing and externalizing problems in a large community sample of preschoolers. Next we assessed whether this association was independent of psychopathology of the parental generation. Diagnostic information on the (biological) grandparental and parental generations was complete and included lifetime psychiatric diagnoses and psychiatric symptoms assessed with a continuous measure at two time points. In this way we wanted to account for as much genetic and environmental variation as possible. To provide further insights in the association between grandparental and child psychopathology, we additionally assessed whether the association was further mediated by maternal sensitivity (available for a subgroup of participating mothers and children) and maternal parenting stress. Mother reports of child behavior as well as father reports of child behavior were available and provided data from two informants.

METHODS

Setting

The study was conducted within Generation R, a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail (Jaddoe et al., 2008).

In a randomly assigned subgroup of Dutch pregnant women and their children, the Focus Cohort, detailed assessments were conducted including prenatal psychiatric interviews. This subgroup is ethnically homogeneous to exclude confounding or effect modification by ethnicity. All children were born between February 2003 and August 2005 and form a prenatally enrolled birth-cohort. The study was conducted in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (numbers: prenatal, MEC 198.782/2001/31 and postnatal, MEC 217.595/2002/202). Written informed consent was obtained from all participants.

Study population

A flowchart illustrating sample selection and participation is given in Figure 1. Of the n=1232 mothers who constituted the Focus Cohort, diagnostic information on the occurrence of a lifetime psychiatric disorder was available for n=972 mothers. For a total of n=816 of these mothers, diagnostic information on both her biological parents (i.e. the child's maternal grandparents) was obtained. Diagnostic information from the biological father and both of his parents (i.e. the child's paternal grandparents) was available for n=691 fathers. Mother reports of child behavior were obtained for a total of n=687 children and included in analyses. For a total of n=630 children, father reports of child behavior were available and included in analyses.

Measures

Grandparental psychopathology

Grandparental lifetime anxiety and depression were assessed at 30 weeks of pregnancy using the Family Informant Schedule and Criteria updated for DSM-IV (FISC) (Mannuzza et al., 1985; Schleyer B, 1995); an interview derived from the Family History-Research Diagnostic Criteria (FH-RDC) (Andreasen et al., 1977; Endicott et al., 1978). The FH-RDC showed good inter-rater reliabilities ranging from kappa = 0.72 for depression to kappa = 0.75 for anxiety (Ptok et al., 2001). The FISC is used to assess lifetime psychiatric disorders of relatives. It screens for mood disorders, substance use disorders, psychotic disorders, anxiety disorders,

antisocial personality disorder and dementia. Three levels of confidence for the diagnoses are generated; 'definite', 'probable' or 'absent'. For the current study, we considered only 'definite' diagnoses as positive diagnoses. Mothers and fathers were interviewed separately about their parents, with the partner not present in the same room at the time of the interview. Groups of diagnoses included in the present study consisted of; 1) any lifetime history of an anxiety disorder, consisting of generalized anxiety disorder, obsessive-compulsive disorder, panic disorder, agoraphobia, social phobia, specific phobia or posttraumatic stress disorder and 2) any lifetime history of a (unipolar) depressive disorder, consisting of a major depressive episode or dysthymia.

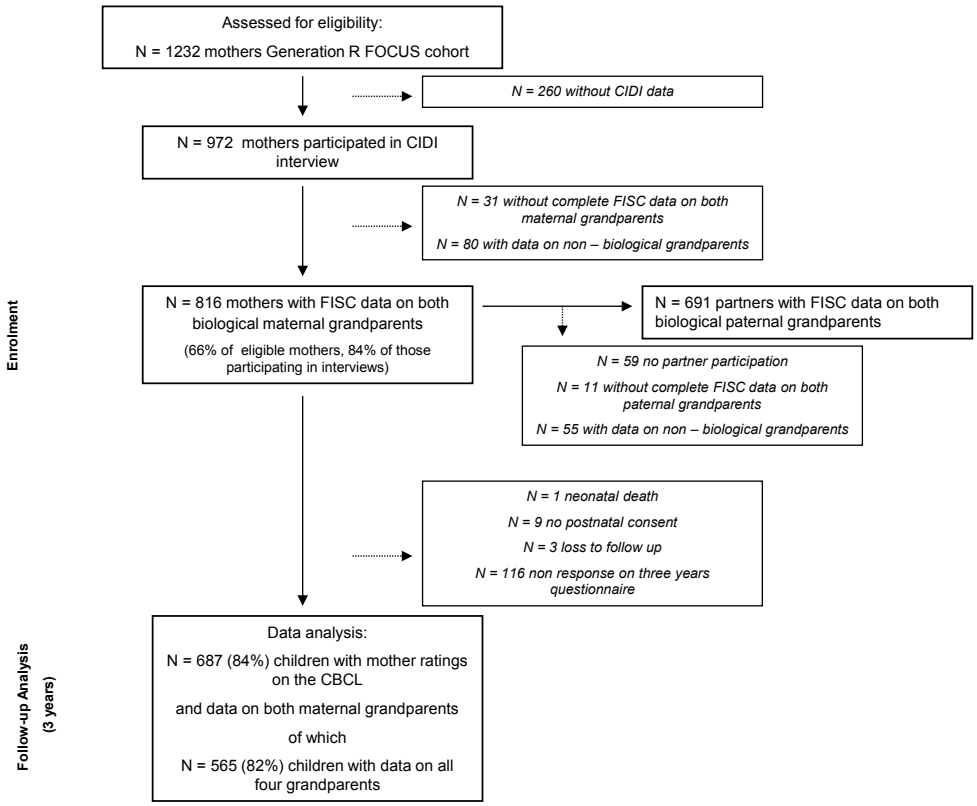

Figure 1. Sample selection and participation
Abbreviations: CIDI = Composite International Diagnostic Interview; FISC = Family Informant Schedule and Criteria; CBCL = Child Behavior Checklist

Parental psychopathology

Parental psychopathology was assessed in three different ways to rigorously test the independent effect of grandparental psychopathology.

First, lifetime psychopathology of both mother and father was assessed by means of the Composite International Diagnostic Interview (CIDI) Version 2.1. The CIDI is a structured interview based on DSM-IV criteria. Good reliability (Kappa's for inter-rater reliability for 17 / 20 diagnoses higher than 0.90) and validity (Kappa's 0.66 and 0.77 in non-clinical samples) have been reported (Andrews & Peters, 1998). A home interview was conducted 30 weeks during pregnancy by research assistants trained in an official training center. Mother and father were interviewed separately, with the partner not present in the same room at the time of the interview. For the purpose of the current study, lifetime diagnoses were divided into three categories; 1) any anxiety disorder, consisting of generalized anxiety disorder, obsessive-compulsive disorder, panic disorder, agoraphobia, social phobia, specific phobia or posttraumatic stress disorder; 2) any (unipolar) depressive disorder, consisting of a mild to severe depressive episode or dysthymia and 3) any substance abuse disorder.

Second, mother and father each completed the Brief Symptom Inventory (BSI) at 20 weeks of pregnancy. The BSI is a well validated self-report questionnaire with 53 items to be answered on a 5-point scale, ranging from 0 (not at all) to 4 (extremely) (de Beurs, 2004; Derogatis, 1993). The BSI is a short version of the Symptom Checklist 90 (SCL-90) (Derogatis & Melisatores, 1983). The items of the BSI define a broad spectrum of psychiatric symptoms in the preceding seven days. Next to anxious and depressive symptoms, symptoms of hostility and interpersonal sensitivity are covered among other dimensions. For the purpose of this study, we used the Global Severity Index (GSI). The GSI is generated by summing all item scores (range 0-4) of all subscales and then dividing the sum by the number of endorsed symptoms. The internal consistency for the GSI was excellent ($\alpha = 0.94$) in this study. This continuous symptom score allows us to control for global psychiatric symptoms including anxious and depressive symptoms among others.

Third, mother and father each completed the anxious and depressive subscales of the BSI when the child was 3 years old, i.e. at the time they completed the child behavior reports. The total scores for the anxious and depressed subscales were calculated by first summing the item scores (range 0-4) and then dividing by the number of endorsed symptoms. The internal consistencies in the study were $\alpha = 0.68$ for the anxious subscale and $\alpha = 0.75$ for the depressive subscale. Including these subscales in the analyses allowed us to further control for concurrent parental anxious and depressive psychopathology. This is important as parental mood may influence ratings of their child's behavior.

Child problem behavior

When the child was 3 years old, mothers and fathers each filled out the Child Behavior Checklist (CBCL/1,5-5), a self-administered parents-report questionnaire that contains 99 problem items rated on a 3-point scale: 0 (not true), 1 (somewhat or sometimes true) and 2 (very true or often true). By summing the raw scores, seven syndromes (Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems and Aggressive Behavior) can be computed. Moreover, two broadband scales (Internalizing and Externalizing problems) can be derived that were used in this study. The Internalizing problem score is a summary score for the items on the first four syndrome scales and the Externalizing problem score is a summary score for attention problems and aggressive behavior. A higher score represents a higher severity. Good reliability (mean test-retest Pearson's r = 0.85, interparental agreement r = 0.61) and validity have been reported for the Child Behavior Checklist (Achenbach & Rescorla, 2000). Respective raw item scores were summed to derive the two raw broadband scales.

Other covariates

Parental educational levels, family income, marital status and parental age at intake were determined at enrolment using questionnaires. Educational level was dichotomized into less or equal and higher than secondary education. Family income was dichotomized into less or equal and more than € 2000. Marital status was dichotomized into married or living together and living alone. The level of maternal stress in the parent-child dyad was measured by the Nijmeegse Ouderlijke Stress Index-Kort (NOSIK) (De Brock et al., 1992), the Dutch version of the Parenting Stress Index-Short Form (Abidin, 1983). Mothers completed this scale at the child's age of 18 months. The NOSIK has good reliability (Cronbach's alpha = .95) and validity (De Brock et al., 1992). Maternal sensitivity was observed during free play in the 14-month lab visit with Ainsworth's rating scales for sensitivity (Ainsworth et al., 1974). The mean duration of the play session was 5 minutes (SD = 2.0). Sensitivity scores were based on the subscale scores for sensitivity and cooperation (r = .84), both scored on 9-point rating scales with higher scores indicating more sensitivity. The intraclass correlation was .79 for sensitivity and .69 for cooperation (n = 24).

Statistical analyses

Multiple logistic regression analyses were run to examine the associations of grandparental psychiatric disorder with child internalizing and externalizing problems. CBCL internalizing and externalizing raw scores were the dependent variables. These scores were right-skewed and could not be transformed to satisfy

the assumption of normality. Therefore we analyzed these scores as dichotomous variables. To this aim, we calculated separate 80th percentiles of mother and father ratings as a cut-off point for internalizing and externalizing problems. To test consistency we also examined the 75th and 85th percentiles as cut-offs. Grandparental anxiety and depressive disorders were coded as disorder present in one or more grandparents and as number of grandparents per disorder.

We analyzed the data in several steps. Per step, three logistic regressions were conducted; for maternal grandparents, paternal grandparents and all grandparents. This way, reporting bias was addressed as psychopathology of maternal and paternal parents were also studied in relation to child problem behavior as reported by the other parent. In the first step, we performed logistic regressions to assess if grandparental anxiety and depression were associated with child internalizing and externalizing problems. In the next step, we tested the independency of the effect of grandparental psychiatric disorder on child problem behavior by additionally adjusting for parental psychopathology; i.e. lifetime anxiety and depressive disorders, prenatal psychopathology and concurrent anxious-depressive symptoms. Lifetime anxiety and depressive disorders were entered as dichotomous variables (disorder present yes or no). Prenatal psychopathology was entered as a continuous variable as was concurrent anxious-depressive symptoms. In the third and final step, we tested whether maternal sensitivity and maternal parenting stress mediated the effect of grandparental psychiatric disorder on child problem behavior by additionally adjusting the full models (see step 2) with these variables. All models were tested with mother reports of child behavior (unless otherwise specified) and with father reports of child behavior as the outcome.

All models were adjusted for gender of the grandchild, family income and parental educational level. The consideration of potential confounders was determined a priori. To test confounding we added the considered variables to the unadjusted models. Covariates were selected and included in the models if they changed the effect estimates meaningfully, defined as more than 5%. As a result, child age at assessment of the outcome, maternal and paternal age at intake, marital status and parental substance abuse disorder were tested as possible confounding variables but not included in the present analyses.

Percentages of missing values on covariates ranged from 0.3% to 12.5%. For missing values on continuous variables, the mean value was imputed. For missing values on a categorical variable, a separate 'missing' category was included in the regression analyses.

Measures of association are presented with 95% confidence intervals. Statistical analyses were carried out using the Statistical Package for the Social Sciences, version 15.0 for Windows (SPSS, Inc. Chicago, Illinois).

Response analyses

Respondents are mothers who filled out the Child Behavior Checklist (CBCL) when the child was three years old. Non-respondents are mothers who did not fill out the CBCL. The latter and their children where therefore excluded from the current study. Response analyses showed that the families of respondents and non-respondents did not differ on prevalences of grandparental lifetime anxiety and depression. They also did not differ on lifetime prevalences of anxiety and depression. However, the non-respondents had a higher psychiatric symptoms score (0.14 vs. 0.10, 95% range 0.14-0.16, p=0.001) than the respondents. Partners of non-respondents (fathers) had similar levels of psychopathology as partners of respondents. Both non–respondents and their partners were lower educated (54.3% vs. 28.6%, X^2= 54.1, p<0.001 (mothers) and 48.2% vs. 32.8%, X^2= 11.8, p=0.001 (fathers)) and reported lower income (less than 2000 euro's 29.6% vs. 16.2%, X^2=13.9, p<0.001) than the respondents and their partners.

RESULTS

Results are presented using the 80th percentile as a cut-off point for child problem behavior. Using the 75th and 85th percentiles as cut-off points yielded similar results. Descriptive statistics for the total sample (N=687) are summarized in Table 1. Univariate associations between (grand)parental characteristics and child problem behavior are summarized in Table 2. Parental prenatal psychiatric symptoms were significantly associated with child internalizing problems (OR=8.76, se=0.42, p-value<0.01 for mothers, OR=9.40, se=0.62, p-value<0.01 for fathers). Likewise, parental psychiatric symptoms were significantly associated with child externalizing problems. Also, concurrent anxious – depressive symptoms were significantly associated with child problem behavior. Maternal parenting stress was significantly associated with child internalizing problems (OR=1.16, se=0.03, p-value<0.01) and externalizing problems (OR=1.12, se=0.03, p-value<0.01). However, maternal insensitivity was not associated with internalizing (OR=0.92, se=0.12, p-value=0.5) or externalizing (OR=1.03, se=0.12, p-value=0.8) behavioral problems.

Table 3 shows the univariate associations between grandparental and parental psychopathology. In these analyses the highly skewed parental prenatal and concurrent psychiatric symptoms scores were dichotomized using a cut-off for psychiatric symptoms of the top 15% in line with a previous study (O'Connor et al., 2002). Overall, grandparental lifetime disorder was significantly associated with parental psychopathology.

Table 1. Descriptive statistics (N=687)

	Total sample descriptives Percentage / mean (sd) / median (interquartile range)	
Children		
boys	48.6	
age at CBCL rating, months	36.3	(0.7)
parenting stress	2.00	(1.00–4.00)
maternal sensitivity[$]	6.00	(5.50-6.75)
Mothers		
lifetime anxiety disorder	14.0	
lifetime depressive disorder	12.7	
lifetime substance abuse disorder	4.7	
prenatal global psychiatric symptoms	0.10	(0.06 – 0.23)
concurrent anxious – depressive symptoms	0.00	(0.00 – 0.33)
education (< higher education)	28.6	
income (< 2000 euro's)	16.2	
age at intake, years	32.0	(3.7)
marital status (living alone)	3.4	
Fathers		
lifetime anxiety disorder	6.7	
lifetime depressive disorder	7.3	
lifetime substance abuse disorder	19.8	
prenatal global psychiatric symptoms	0.04	(0.00 – 0.13)
concurrent anxious – depressive symptoms	0.00	(0.00 – 0.17)
education (< higher education)	32.8	
age at intake, years	34.0	(4.7)
Grandparents mother's side		
lifetime anxiety disorder	11.1	
lifetime depressive disorder	23.3	
Grandparents father's side		
lifetime anxiety disorder	10.1	
lifetime depressive disorder	17.3	

[$] Data were available for a subgroup of n=320 children.
Values were missing for parenting stress (n=37), maternal prenatal psychiatric symptoms (n=40), maternal concurrent anxious – depressive symptoms (n=3), maternal educational level (n=2), family income (n=26), marital status (n=14), paternal lifetime disorders (n=71), paternal prenatal psychiatric symptoms (n=86), paternal concurrent anxious – depressive symptoms (n=79), paternal educational level (n=72), paternal age at intake (n=4) and lifetime disorders grandparents father's side (n=122).
Abbreviations: CBCL = Child Behavior Checklist

Table 2. Univariate association between descriptive variables and CBCL scores (N=687)

	Associations with internalizing problems OR (se)		Associations with externalizing problems OR (se)	
Children				
boys	1.01	(0.19)	1.53	(0.20)*
age at CBCL rating, months	1.04	(0.13)	0.77	(0.15)
parenting stress	1.16	(0.03)**	1.12	(0.03)**
maternal sensitivity[$]	0.92	(0.12)	1.03	(0.12)
Mothers				
lifetime anxiety disorder	1.41	(0.26)	2.03	(0.25)**
lifetime depressive disorder	1.53	(0.26)	1.49	(0.27)
lifetime substance abuse disorder	2.20	(0.39)*	2.29	(0.39)*
prenatal global psychiatric symptoms	8.76	(0.42)**	3.87	(0.47)**
concurrent anxious – depressive symptoms	3.00	(0.21)**	2.22	(0.20)**
education (< higher education)	1.00	(0.003)	0.99	(0.02)
income (< 2000 euro's)	1.00	(0.001)	1.00	(0.001)
age at intake, years	0.95	(0.03)	0.99	(0.03)
marital status (living alone)	1.70	(0.24)*	1.64	(0.25)*
Fathers				
lifetime anxiety disorder	1.52	(0.36)	1.62	(0.37)
lifetime depressive disorder	0.60	(0.45)	1.42	(0.36)
lifetime substance abuse disorder	0.92	(0.26)	1.36	(0.25)
prenatal global psychiatric symptoms	9.40	(0.62)**	5.97	(0.61)**
concurrent anxious – depressive symptoms	0.98	(0.26)	1.58	(0.24)*
education (< higher education)	1.00	(0.001)	1.00	(0.001)
age at intake, years	0.96	(0.02)*	1.00	(0.02)
Grandparents mother's side				
lifetime anxiety disorder	1.76	(0.27)*	2.13	(0.27)**
lifetime depressive disorder	1.45	(0.21)*	1.71	(0.21)*
Grandparents father's side				
lifetime anxiety disorder	2.60	(0.30)**	1.62	(0.32)**
lifetime depressive disorder	2.14	(0.42)**	2.10	(0.24)**

Ratings of internalizing and externalizing problems are based on mother reports.
Internalizing problems were reported for N=137 children and externalizing problems for N=133 children.
[$] Data were available for a subgroup of N=320 children.
Values were missing for parenting stress (n=37), maternal prenatal psychiatric symptoms (n=40), maternal concurrent anxious – depressive symptoms (n=3), maternal educational level (n=2), family income (n=26), marital status (n=14), paternal lifetime disorders (n=71), paternal prenatal psychiatric symptoms (n=86), paternal concurrent anxious – depressive symptoms (n=79), paternal educational level (n=72), paternal age at intake (n=4) and lifetime disorders grandparents father's side (n=122).
* $p < 0.05$
** $p < 0.01$
Abbreviations: CBCL = Child Behavior Checklist

Table 3. Univariate associations between grandparental and parental psychopathology

Mothers				
Grandparental psychiatric disorder	lifetime anxiety disorder	lifetime depressive disorder	prenatal global psychiatric symptoms (top 15%)	concurrent anxious – depressive symptoms (top 15%)
Grandparents mother's side	crude OR (se)	crude OR (se)	crude B (se)	crude B (se)
lifetime anxiety disorder	1.61 (0.31)	1.66 (0.32)	1.97 (0.32)*	1.66 (0.32)
lifetime depressive disorder	3.27 (0.23)**	3.60 (0.24)**	1.35 (0.26)	1.90 (0.45)**
Fathers				
	lifetime anxiety disorder	lifetime depressive disorder	prenatal global psychiatric symptoms (top 15%)	concurrent anxious – depressive symptoms (top 15%)
Grandparents father's side	crude OR (se)	crude OR (se)	crude B (se)	crude B (se)
lifetime anxiety disorder	2.43 (0.43)*	0.67 (0.62)	2.65 (0.33)**	1.19 (0.33)
lifetime depressive disorder	1.91 (0.37)	1.33 (0.39)	1.74 (0.29)	2.13 (0.24)*

Note: OR=odds ratio, se=standard error
* p <0.05, ** p < 0.01

Table 4 shows the associations between grandparental anxiety and depressive disorder and child problem behavior. These analyses were adjusted for gender of the child, educational level of the parents and family income. Both grandparental anxiety disorder and grandparental depressive disorder were significantly associated with internalizing problems and externalizing problems as reported by the mother (internalizing problems; OR=2.21, 95% C.I. (1.38, 3.54), p-value=0.001 and OR=1.92, 95% C.I. (1.26, 2.92), p-value=0.002, externalizing problems; OR=1.97, 95% C.I. (1.21, 3.19), p-value=0.006 and OR=2.02, 95% C.I. (1.31, 3.11), p-value=0.001). There were no substantial differences between the effect estimates of maternal and paternal grandparents and confidence intervals substantially overlapped. The effect estimates remained essentially the same when we restricted analyses of maternal grandparents to those children with complete paternal information on grandparents present (data not shown). The analyses of grandparental psychopathology and child behavior as reported by the father are also presented in Table 4. Only grandparental anxiety disorder was associated with the Internalizing broadband scale of the CBCL.

Next, we tested whether the observed associations between grandparental psychopathology and child internalizing and externalizing problems were independent of psychopathology of the parental generation. Analyses were additionally adjusted for parental lifetime psychiatric disorders, prenatal psychiatric symptoms and current anxious and depressive symptoms at the time of child behavior report. Results are summarized in Table 5. Grandparental lifetime anxiety disorder and grandparental lifetime depression remained significantly associated with a higher likelihood of grandchild internalizing and externalizing problems as reported by the mother. Moreover, with each grandparent with a lifetime history of psychiatric disorder, the risk of child internalizing problems increased (OR=1.65, 95% C.I. (1.09, 2.48) *p*-value=0.02 for grandparental anxiety, OR=1.67, 95% C.I. (1.23, 2.27) *p*-value<0.001 for grandparental depression) and similarly for externalizing problems. A notable finding was that the maternal reports of child behavior were not only predicted by maternal grandparental psychopathology, but by paternal grandparental psychopathology as well (see Table 5).

Results did not substantially change using other cut-offs of the CBCL. Next, we assessed whether child internalizing problems as reported by the father was associated with grandparental anxiety disorder adjusted for parental psychopathology; the odds ratio was attenuated and became non-significant. No other analysis was performed as initial analyses were not significant (see Table 4).

Table 4. Associations between grandparental anxiety and depression and child internalizing and externalizing problems not adjusted for parental psychopathology

Grandparental psychiatric disorder	Maternal report						Paternal report					
	Internalizing			Externalizing			Internalizing			Externalizing		
Maternal grandparents	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
Anxiety disorder, any	1.74	1.02, 2.98	0.04	2.05	1.21, 3.50	0.008	1.06	0.55, 2.07	0.9	1.23	0.66, 2.28	0.5
Depressive disorder, any	1.51	0.99, 2.31	0.06	1.72	1.12, 2.63	0.01	1.15	0.70, 1.89	0.6	1.21	0.75, 1.95	0.4
Paternal grandparents												
Anxiety disorder, any	2.65	1.47, 4.78	0.001	1.59	0.84, 3.02	0.2	2.01	1.02, 3.93	0.04	0.73	0.32, 1.63	0.4
Depressive disorder, any	2.16	1.32, 3.53	0.002	2.19	1.32, 3.64	0.002	1.03	0.55, 1.91	0.9	1.14	0.62, 2.07	0.7
All four grandparents												
Anxiety disorder, any	2.21	1.38, 3.54	0.001	1.97	1.21, 3.19	0.006	1.31	0.75, 2.27	0.3	0.87	0.49, 1.55	0.6
Depressive disorder, any	1.92	1.26, 2.92	0.002	2.02	1.31, 3.11	0.001	1.17	0.73, 1.88	0.5	1.00	0.62, 1.60	1.0
Anxiety disorder, per[$]	1.78	1.21, 2.63	0.003	1.76	1.19, 2.62	0.005	1.24	0.79, 1.94	0.4	0.93	0.58, 1.50	0.8
Depressive disorder, per	1.76	1.33, 2.33	< 0.001	1.83	1.38, 2.43	< 0.001	1.14	0.82, 1.57	0.4	1.08	0.78, 1.49	0.6

Values are odds ratios (95% confidence intervals) from logistic regression models, adjusted for gender, family income, maternal educational level and paternal educational level.
Models are based on n=687 sets of maternal grandparents and n=565 sets of paternal and all four grandparents for maternal report.
Models are based on n=630 sets of maternal grandparents and n=527 sets of paternal and all four grandparents for paternal report.
[$] per: defined as per grandparent. The risk of child problem behavior increases as more grandparents are affected (dose response relationship).
Abbreviations: OR = Odds ratio, CI = Confidence interval

Table 5. Associations between grandparental anxiety and depression and child internalizing and externalizing problems, independent of parental psychopathology

Grandparental psychiatric disorder	Maternal report						Paternal report					
	Internalizing			Externalizing			Internalizing			Externalizing		
Maternal grandparents	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
Anxiety disorder, any	1.57	0.88, 2.80	0.1	1.81	1.04, 3.15	0.03	-	-	-	-	-	-
Depressive disorder, any	-	-	-	1.59	1.00, 2.51	0.05	-	-	-	-	-	-
Paternal grandparents												
Anxiety disorder, any	2.10	1.11, 3.98	0.02	-	-	-	1.71	0.85, 3.46	0.1	-	-	-
Depressive disorder, any	1.90	1.11, 3.25	0.02	1.67	0.97, 2.87	0.06	-	-	-	-	-	-
All four grandparents												
Anxiety disorder, any	1.98	1.20, 3.28	0.008	1.73	1.04, 2.87	0.03	-	-	-	-	-	-
Depressive disorder, any	1.75	1.11, 2.75	0.02	1.67	1.05, 2.64	0.03	-	-	-	-	-	-
Anxiety disorder, per[$]	1.65	1.09, 2.48	0.02	1.65	1.09, 2.49	0.02	-	-	-	-	-	-
Depressive disorder, per	1.67	1.23, 2.27	0.001	1.60	1.18, 2.18	0.003	-	-	-	-	-	-

Values are odds ratios (95% confidence intervals) from logistic regression models adjusted for gender, family income, maternal educational level, paternal educational level, maternal lifetime anxiety and depressive disorders, maternal prenatal psychiatric symptoms, maternal concurrent anxious - depressive symptoms, paternal lifetime anxiety and depressive disorders, paternal prenatal psychiatric symptoms and paternal concurrent anxious - depressive symptoms.
"-" = not calculated as the primary analyses (Table 4) were not significant.
Models are based on n=687 sets of maternal grandparents and n=565 sets of paternal and all four grandparents for maternal report.
Model is based on n=527 sets of paternal grandparents for paternal report.
per: defined as per grandparent. The risk of child problem behavior increases as more grandparents are affected (dose response relationship).
Abbreviations: OR = Odds ratio, CI = Confidence interval

Finally, we tested whether perceived maternal parenting stress was a mediating factor in the association between grandparental psychiatric disorder and child problem behavior. Maternal parenting stress significantly predicted child problem behavior (see Table 2; all *p*-values<0.01). But, the associations between grandparental anxiety and depressive disorders and child behavior remained unchanged. The maximum change of effect estimates was 4% (data not presented). As maternal insensitivity did not predict child internalizing and externalizing problems (see Table 2), we did not further assess this variable as a mediating factor.

DISCUSSION

The main finding of this study was that anxiety and depressive disorders of the grandparental generation predicted child problem behavior independent of psychopathology of the parental generation. Children with one or more grandparent with a lifetime history of an anxiety or depressive disorder had an increased risk of internalizing and externalizing problems at the age of three years.

To the best of our knowledge, an independent effect of grandparental psychopathology has only reported once (Olino et al., 2008). Other three-generational studies also concluded that grandparental psychopathology may increase the likelihood of problem behavior, but did not find an independent effect (Hammen et al., 2004; Pettit et al., 2008; Weissman et al., 2005). The present study had sufficient power to detect an independent effect and diagnostic information on (grand)parents was complete. Moreover, in contrast to previous studies, we included two continuous measures of parental psychiatric symptoms next to a measure of lifetime psychiatric disorders. Our aim was to assess as best possible the independent effect of grandparental psychopathology on child problem behavior. We further extend previous findings by demonstrating that grandparental anxiety disorder similarly increases the risk of child internalizing and externalizing problems as depression. This may indicate anxiety and depression have a common underlying vulnerability. This hypothesis is supported by several findings of previous research such as co-aggregation of anxiety and depressive disorders in families, evidence for a common underlying genetic factor provided by twin studies and possible common vulnerability genes for example the serotonine gene (Gorwood, 2004).

The findings can be best explained in terms of the four proposed mechanisms underlying transmission of psychopathology of Goodman & Gotlib (Goodman & Gotlib, 1999). The first mechanism proposed that genetic factors underlie the transmission of risk. The importance of genetic factors, even at the age of three, is

documented by several studies with heritability rates ranging from 24% till 56% for the common behavioral problems (Derks et al., 2004; Van Hulle et al., 2007). Bartels et al state that genetic factors are the most important cause of individual differences in internalizing and externalizing behaviors between the ages three and twelve (Bartels et al., 2007). Neither in the current study nor in the other three-generational studies, may the genetic risk of the parental generation not fully be accounted for. Firstly, genetic variants associated with psychiatric disorders most likely have reduced penetrance. In other words, participants carrying the high risk variant of a gene may not be affected. The genetic variants may have late penetrance; subjects carrying the genetic variant may still develop the psychiatric disorder at a later age. Secondly, psychiatric disorders are complex disorders, i.e. depending on multiple genetic variants which converge to increase susceptibility for a disorder. Thus, the susceptibility for a psychiatric disorder depends on the combination of genetic variants inherited. Therefore, the parents may have had a combination of genetic variants which did not make them more susceptible for developing a psychiatric disorder, while the children could well have inherited a combination of these genetic variants which did increase their likelihood of developing problem behavior. Also, there is the issue of uncertain paternity. A recent meta-analysis showed that non-paternity rates are approximately 3.1% (Voracek et al., 2008). Consequently, we might have included a small number of non-biological (grand)fathers and not adequately have taken all genetic factors into account. Next to genetic factors, intra-uterine factors may underlie the transmission of intergenerational risk. All analyses were adjusted for prenatal global psychiatric symptoms, including symptoms of depression and anxiety, but a residual effect of maternal stress during pregnancy cannot be ruled out. The third mechanism described by Goodman and Gotlib proposes that depressed parents expose their children to negative cognitions, behaviors and affect which also extend to their parenting styles (Goodman & Gotlib, 1999). We examined maternal sensitivity as a parenting factor, but no main effects of maternal sensitivity were found. This is in line with earlier studies. These studies found that maternal sensitivity was associated with externalizing behaviors only in children with difficult temperament (Mesman et al., 2009) and that maternal sensitivity in middle childhood rather than in early childhood is associated with child problem behavior (Bradley & Corwyn, 2007). It could be that young children's behavioral problems relate more to negative aspects of parenting than to variation in positive aspects of parenting. For example, harsh discipline is found to be a consistent and cumulative predictor of internalizing and externalizing behavior in a population based sample of three year olds (Bayer et al., 2008). Fourth, contextual factors may contribute to the transmission of transgenerational risk. We examined maternal parenting stress.

The extent to which this measure influenced the association between grandparental psychopathology and children's behavior was however limited. Other contextual risk factors such as low social support and marital conflict may still mediate part of the association between grandparental and child psychopathology. Earlier studies have found strong links between parental psychopathology, marital functioning and child problem behavior (Cummings & Davies, 2002; Cummings et al., 2005).

Grandparental psychopathology predicted child problem behavior as reported by the mother, whereas grandparental psychopathology did not predict child behavior as reported by the father. An explanation is that fathers observe and interpret behaviors of their preschoolers not as accurately as mothers. Behaviors of young children are not as distinct as behaviors of older children and adolescents. This may especially be true if fathers are not the primary caretakers. Moreover, Hay et al state that father ratings on the CBCL were primarily associated with behavioral problems due to cognitive ability of the child while mother ratings of behavioral problems were associated with the family climate. This suggests that fathers rate different behaviors than mothers (Hay et al., 1999). Alternatively, maternal reports of child behavior may be particularly influenced by the mother's mental state. However, we adjusted for prenatal psychiatric symptoms and for psychiatric symptoms at the moment of child assessment as well. It is also known that women are more prone to develop and subsequently report anxious and depressive symptoms. In the current study, mothers reported higher rates of psychopathology than fathers did. Mechanisms underlying these gender differences are not well understood (Nolen-Hoeksema, 1987), although it has been said that they are moderated by socialization processes that prescribe gender specific expectations regarding the expression of anxious and depressive behaviors (McLean & Anderson, 2009). Also, it was found that differential reporting did not account for the gender differences (Bogner & Gallo, 2004). However, there is evidence that subjects who experience higher rates of psychopathology, are more sensitive for reporting psychopathology in family members (Chapman et al., 1994). In the current study, mothers reported substantially higher depression rates for her parents than fathers did for their parents. However, we analyzed the data for maternal and paternal grandparents separately, with the other parent reporting child behavior.

This study has several strengths. We assessed the effect of grandparental anxiety and depression on children's problem behavior in a large population based study. We examined whether the effect of grandparental psychopathology on child behavior was independent of psychopathology of the parental generation. To this aim, psychopathology of the parental generation was assessed as carefully as possible. Diagnostic information on the parental generation was complete including lifetime

psychiatric disorders, psychiatric prenatal symptoms and concurrent psychiatric symptoms.

However, there are some limitations that need to be considered. First, information on grandparental lifetime psychiatric information was assessed through a parental interview which may have compromised the validity of the reported diagnoses as prevalence rates may have been underestimated. However, external validity increases as a direct grandparental interview is often not feasible in clinical settings. Second, these informants also reported about their child's problem behavior. Information bias, however, was addressed in several ways; a) the maternal and paternal grandparents were assessed separately. b) child outcome was reported by both parents. c) Information on grandparental and parental psychopathology was assessed prior to the birth of the child and d) we included measures of parental psychopathology at the moment of child assessment. A third limitation to be mentioned was the lack of observational measures or semi-structural interviews of child behavior. On the other hand, we had two informants reporting child problem behavior. Fourth, we lost those mothers (and their families) with higher prevalence rates of psychopathology and higher social stressors. This may have reduced power to detect relationships due to these characteristics. Last, because we selected an ethnically Dutch sub cohort caution should be taken with generalizing these results to the broader (multi-ethnic) population. However, this approach most likely makes the present results more valid.

In conclusion, lifetime anxiety and depressive disorders of the grandparental generation predict child problem behavior at the age of three independent of parental psychopathology. This finding confirms the importance of a family history including not only the parental but also the grandparental generations, at least for children aged three. Future studies will investigate whether the observed associations between grandparental and child psychopathology change with age. Results also showed an increased risk of grandparental anxiety next to depression, thereby supporting the hypothesis of a common underlying vulnerability for problem behavior.

Future research should try to further disentangle the genetic and environmental factors underlying the transmission of intergenerational risk. These issues can be addressed by assessing whether subjects carry a functional polymorphism. Subsequently, studies should address gene x environment interactions in the pathogenesis of anxiety and depression (Caspi et al., 2003; Fox et al., 2005; Kaufman et al., 2006). Also, research must investigate the different environmental risks underlying the transmission of anxiety and depression. It is not yet clear to what extend specific (negative) parenting practices such as harsh discipline and contextual factors such as social support and marital conflict mediate the association

between grandparental and child psychopathology. Last, it would be interesting to investigate whether these genetic and environmental factors follow distinct patterns based on gender, or that they moderate gender differences.

The current findings have practical implications as well for developing primary prevention at the population level. Obtaining a family history including the grandparents, for which relatively easy family screens are available (Weissman et al., 2000), can help identify children at risk for developing problem behaviors at a young age. There is some evidence that group-based parenting programs improve children's problem behaviors (Barlow et al., 2010).

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PART II

THE ROLES OF GENES AND PARENTING IN THE TRANSMISSION OF PSYCHOPATHOLOGY

CHAPTER 4

Common variation near ROBO2 is associated with expressive vocabulary in infancy

ABSTRACT

Background: Twin studies suggest that expressive vocabulary at ~24 months is modestly heritable. However, the genes influencing this early linguistic phenotype are unknown.

Methods: Here we conduct a genome-wide screen and follow-up study of expressive vocabulary in toddlers of European descent from up to four studies, analyzing an early (15-18 months, 'one-word stage', N_{Total}=8,889) and a later (24-30 months, 'two-word stage', N_{Total}=10,819) phase of language acquisition.

Results: For the early phase, one SNP (rs7642482) at 3p12.3 near *ROBO2,* encoding a conserved axon binding receptor, reaches the genome-wide significance level (p=1.3x10^{-8}) in the combined sample. This association links language-related common genetic variation in the general population to a potential autism susceptibility locus and a linkage region for dyslexia, speech-sound disorder and reading.

Conclusions: The contribution of common genetic influences is, although modest, supported by Genome-wide Complex Trait Analysis (meta-GCTA $h^2_{15\text{-}18\text{-months}}$=0.13, meta-GCTA $h^2_{24\text{-}30\text{-months}}$=0.14) and in concordance with additional twin analysis (5,733 pairs of European descent, $h^2_{24\text{-months}}$=0.20).

INTRODUCTION

The number of distinct spoken words is a widely used measure of early language abilities, which manifests during infancy[1]. Word comprehension (known as receptive language) in typically developing children starts at the age of about 6 to 9 months[2] and the spontaneous production of words (known as expressive language) emerges at about 10 to 15 months[1,3]. During the next months the accumulation of words is typically slow, but then followed by an increase in rate, often quite sharp, around 14 to 22 months ('vocabulary spurt')[1,4]. As development progresses, linguistic proficiency becomes more advanced, with 2-word combinations (18-24 months of age)[1,3] and more complex grammatical structures (24-36 months of age)[1,3] arising, accompanied by the steady increase in vocabulary size. Expressive vocabulary is therefore considered to be a rapidly changing phenotype, especially between 12 and 24 months[5], with zero size at birth, ~50 words at 15 to 18 months[1,3], ~200 words at 18 to 30 months[1,3] and ~14.000 words at six years of age[3,4], and ≥50.000 words in high school graduates[6,7].

Twin analyses of cross-sectional data suggest that expressive vocabulary at ~24 months is modestly heritable (h^2=0.16-0.38)[8,9] and longitudinal twin analyses have reported an increase in heritability of language-related factors during development (h^2=0.47-0.63, ≥7 years of age)[10]. Large-scale investigations of common genetic variation underlying growth in language skills however are challenging due to the complexity and varying nature of the phenotype. This is coupled with a change in psychological instruments, which are used to assess these abilities with progressing age. Current genome-wide association analysis studies (GWAS) using cross-sectional data on language abilities in childhood and adolescence have failed to identify robust signals of genome-wide association[11,12] and genes influencing earlier, less complex linguistic phenotypes are currently unknown.

To attempt to understand genetic factors involved in language development during infancy and early childhood, we perform a GWAS and follow-up study of expressive vocabulary scores in independent children of European descent from the general population and analyze an early ('one-word stage') and a later ('two-word stage') phase of language acquisition. We report a novel locus near *ROBO2,* encoding a conserved axon binding receptor, as associated with expressive vocabulary during the early 'one-word' phase at the genome-wide significance level, and provide heritability estimates for expressive vocabulary during infancy and early childhood.

METHODS

Phenotype selection and study design

Consistent with the developmental pattern of language acquisition, the analysis of children's expressive vocabulary in infancy was divided between an early phase (15-18 months of age, Fig. 1) and a later phase (24-30 months of age, Fig. 2) and conducted using independent individuals of up to four population-based European studies with both quantitative expressive vocabulary scores and genotypes available (early phase: total N=8,889; later phase: total N=10,819).

Expressive vocabulary scores were measured with age-specific defined word lists and either ascertained with adaptations of the MacArthur Communicative Development Inventories (CDI)[13–17] or the Language Development Survey (LDS)[18] and based on parent-report. The CDIs were developed to assess the typical course and variability in communicative development in children of the normal population (8-30 months of age)[13]. The LDS was designed as a screening tool for the identification of language delay in 2-year old children[18]. Both measures have sufficient internal consistency, test-retest reliability and validity[18,53,54].

Expressive vocabulary during the early phase was captured by an abbreviated version of the MacArthur CDI (Infant Version[13], 8-16 months of age, Supplementary Data 1) within the discovery cohort (ALSPAC, N=6,851, Supplementary Fig. 1a). Note, the Infant CDI has recently become also known as CDI Words&Gestures[55]. A Dutch adaptation of the short-form version of the MacArthur CDI (N-CDI 2A)[14,16] was used within the follow-up cohort (GenR, N=2,038). Scores in both cohorts comprised both expressive and receptive language aspects ('says and understands') and showed a positively skewed data distribution ($1.95 < \text{skewness} \leq 2.39$; Supplementary Data 1, published online).

Vocabulary production during the later phase was measured with an abbreviated version of the MacArthur CDI (Toddler version, 16-30 months of age)[13,15] in the discovery cohort (ALSPAC, N=6,299, Supplementary Fig. 1b). Note, the Toddler CDI has recently become also known as CDI Words&Sentences[55]. Within the follow-up cohorts, expressive vocabulary was either assessed with the LDS (GenR N=1,812; the Raine study N=981) or a short form of the MacArthur CDI (MCDI)[13] (Twins Early Development Study, TEDS, N=1,727, one twin per pair). Later phase expressive vocabulary scores measured expressive language only ('says') and were either symmetrically distributed or negatively skewed ($-1.68 < \text{skewness} \leq 0.24$; Supplementary Data 1, published online).

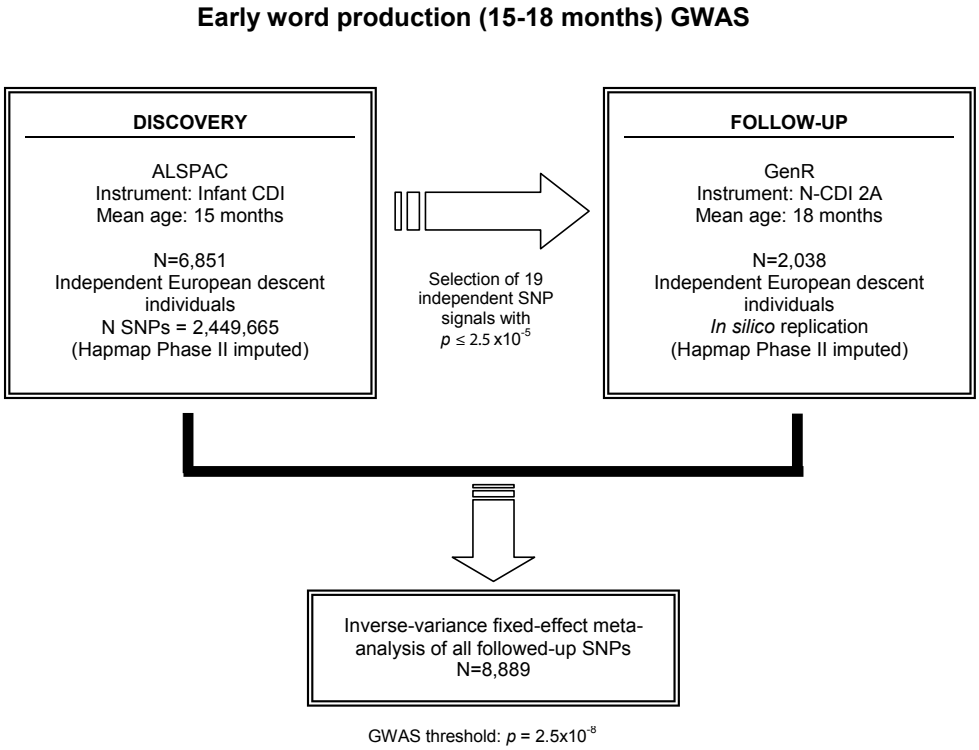

Figure 1. Study design for the genome-wide screen of early expressive vocabulary between 15 and 18 months of age.
Expressive vocabulary was assessed with different forms of the MacArthur Communicative Development Inventories (CDI). Detailed phenotype descriptions are given in Supplementary Data 1, published online.

In total, three different languages were included in our analyses: English (three samples: ALSPAC; TEDS; Raine), Dutch (one sample: GenR), and Finnish (sensitivity analysis: NFBC1966). The cross-cultural comparability of the CDI has been explored, and the measures in many languages, including Dutch and English, show minimal differences in vocabulary production scores in the early years[56]. In addition, the standardization within each sample (see below) would have removed any minor differences between instruments.

Basic study characteristics, details on phenotype acquisition and psychological instruments as well as summary phenotype characteristics (including mean, standard deviation, kurtosis, skewness and age at measurement) are presented for each cohort and developmental phase in Supplementary Data 1, published online.

For each participating study, ethical approval of the study was obtained by the local research ethics committee, and written informed consent was provided by

all parents and legal guardians. Detailed information on sample-specific ethical approval and participant recruitment is provided in Supplementary Note 1.

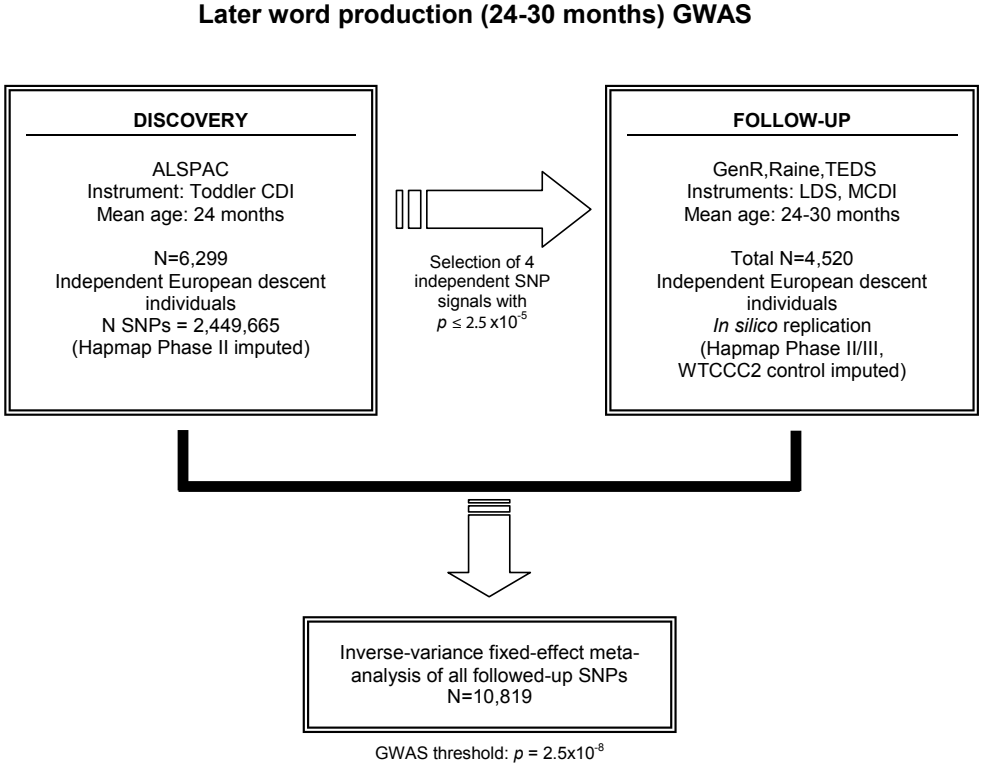

Figure 2. Study design for the genome-wide screen of later expressive vocabulary between 24 and 30 months of age.
Expressive vocabulary was assessed with different forms of the MacArthur Communicative Development Inventories (CDI) and the Language Development Survey (LDS). Detailed phenotype descriptions are given in Supplementary Data 1, published online.

Genotyping and imputation

Genotypes within each cohort were obtained using high-density SNP arrays (Supplementary Data 1). Cohort-specific genotyping information including genotyping platform, quality control (QC) for individuals and SNPs, the final sample size, the number of SNPs before and after imputation as well as the imputation procedures are detailed in Supplementary Data 1. Briefly, for individual sample QC, this included filtering according to call rate, heterozygosity and ethnic/other outliers, and for SNP QC (prior to imputation) filtering according to MAF, call rate, and SNPs with deviations from Hardy-Weinberg equilibrium (detailed exclusion criteria are listed in Supplementary Data 1). Genotypes were subsequently imputed to HapMap CEU (Phase II and/or III) and/or Wellcome Trust Controls (Supplementary Data 1).

Single variant association analysis

Within each cohort expressive vocabulary scores were adjusted for age, age squared, sex and the most significant ancestry-informative principal components[57] and subsequently rank-transformed to normality to facilitate comparison of the data across studies and instruments. The association between SNP and expressive vocabulary score was assessed within each cohort using linear regression of the rank-transformed expressive vocabulary score against allele dosage, assuming an additive genetic model.

In the discovery cohort, the genome-wide association analysis for each phase was carried out using MACH2QTL[58] using 2,449,665 imputed or genotyped SNPs. SNPs with a minor allele frequency (MAF) of <0.01 and SNPs with poor imputation accuracy (MACH $R^2 \leq 0.3$) were excluded prior to the analysis and all statistics were subjected to genomic control correction[59] (Supplementary Data 1). All independent SNPs from the early and later phase GWAS below the threshold of $p<10^{-4}$ (85 and 50 SNPs respectively) were selected for subsequent follow-up analysis in additional cohorts. Independent SNPs were identified by linkage disequilibrium (LD)-based clumping using PLINK[60] (±500kb, $r^2>0.3$, Hapmap II CEU, Rel 22). All analyses within the follow-up samples were carried out *in silico* using MACH2QTL or SNPTEST[61] software (Supplementary Data 1). For the selected SNPs, estimates from the discovery (genomic-control corrected) and follow-up cohort(s) were combined using fixed-effects inverse-variance meta-analysis (R 'rmeta' package), while testing for overall heterogeneity using Cochran's Q-test. Signals below a genome-wide significance threshold of $p <2.5x10^{-8}$ (accounting for two GWAS) were considered to represent robust evidence for association.

An empirical approach (Bootstrapping with 10,000 replicates) was selected to obtain meaningful genetic effects (Basic 95% bootstrap confidence interval) of the reported SNPs in the discovery cohort. For this, we utilized a linear model of z-standardized expressive vocabulary scores against allele dosage, adjusted for age, age squared, sex and the most significant ancestry-informative principal components. The local departmental server of the School of Social and Community Medicine at the University of Bristol was used for data exchange and storage.

Direct genotyping of reported SNPs

Reported SNPs with a medium imputation accuracy (MACH $R^2< 0.8$) were re-genotyped in the discovery cohort (ALSPAC) to confirm the validity of the observed association signal (rs10734234, MACH $R^2 = 0.76$). Genotyping was undertaken by LGC Genomic Ltd (http://www.lgcgenomics.com/) using a form of competitive allele-specific polymerase chain reaction system (KASPar) for SNP analysis.

Variance explained

To estimate the variation in expressive vocabulary scores explained by each reported SNP and jointly by all reported SNPs together, we calculated the adjusted regression R^2 values from i) univariate linear regression of the rank-transformed expressive vocabulary score (see above) against allele dosage and ii) multivariate linear regression of the rank-transformed expressive vocabulary score (see above) against the allele dosage from all reported SNPs. All analyses were performed using R/STATA software.

Phenotypic characterization of reported SNP association signals

First single-word expressive vocabulary: To investigate whether there is association between the first single-word utterances at ~12 months of age and the reported SNPs, we conducted an association analysis in the Northern Finnish Birth Cohort 1966 (NFBC 1966). The number of spoken words in the NFBC 1966 (word-list free assessment, 'words' are undefined) were based on parental response to a questionnaire administered at 12 months of age (Supplementary Data 1). Given the scarcity of categories referring to 3 or more spoken words, word numbers were dichotomized into '1+ words' (one or more words, 1) versus 'no words' (0). The association between early word-production scores and allele dosage of the reported SNPs was studied using logistic regression models, adjusted for sex and the most significant principal components (as exact age at measurement was not available) using SNPTEST.

Pre-school language deficits have been repeatedly associated with later problems in language development especially reading skills[62]. To assess whether genetic effects affecting expressive language skills early in life also influence language competencies during later development, we investigated the association between reported SNP signals and a series of language-related cognitive measurements in the ALSPAC cohort (Supplementary Table 9). All outcomes were Z-standardized prior to analysis. The association between the transformed outcome and SNP allele dosage was investigated using linear regression adjusted for sex, the most significant principal components and age (except for age-normalized scores, Supplementary Table 10).

To assess whether gestational age and maternal education influence the association between the reported signals and early expressive vocabulary scores, we i) investigated the association between these potential covariates and the SNPs directly and ii) adjusted the association between genotypes and language measures for potential covariate effects. Gestational age in the relevant cohorts was either estimated from medical records or obtained from midwife and hospital registries at birth (Supplementary Data 1), and measured in completed weeks of gestation.

Information on maternal education was obtained from antenatal questionnaire data, and dichotomized into lower (1) and higher (0) maternal education (Supplementary Data 1). The association between gestational age and allele dosage for reported SNPs was investigated with linear regression models and adjusted for sex and the most significant principal components in each cohort. The link between maternal education and these SNPs was studied using logistic regression models adjusted for the most significant principal components in each cohort.

We furthermore created new transformations of expressive vocabulary scores, i.e. the reported number of words were in addition to the previously described variables (see above) adjusted for gestational age and maternal education respectively, before they were rank-transformed. Association analysis for reported SNPs was then carried out as described for discovery, follow-up and combined analysis before. All analyses were carried out using R, SPSS and STATA software.

Genome-wide Complex Trait Analysis

The proportion of additive phenotypic variation jointly explained by all genome-wide SNPs together (narrow-sense heritability) was estimated for all cohorts and analyses windows using GCTA[32]. In brief, using a sample of independent individuals the method is based on the comparison of a matrix of pairwise genomic similarity with a matrix of pairwise phenotypic similarity using a random-effects mixed linear model[32]. Pertinent to this study, GCTA (Supplementary Data 1) was carried out using rank-transformed expressive vocabulary scores (previously adjusted for age, sex and the most significant ancestry-informative principal components in each cohort, see above) and directly genotyped SNPs (ALSPAC, GenR, Raine), or most likely imputed genotypes (TEDS). GCTA estimates from different cohorts were combined using fixed-effects inverse-variance meta-analysis assuming symmetrically distributed standard errors (SE), while testing for overall heterogeneity using Cochran's Q-test.

The extent to which the same genes contribute to the observed phenotypic correlation between two variables can be furthermore estimated through genetic correlations[63]. For all cohorts with expressive vocabulary measures at two time-points (ALSPAC, GenR), the genetic correlation (r_g) between the rank-transformed scores was estimated using bivariate GCTA analysis[33] (based on the genetic covariance between two traits).

Twin analysis

Twin analyses allow the estimation of the relative contributions of genes and environments to individual differences in measured traits. Twin intraclass correlations were calculated[64], providing an initial indication of the relative contributions of

additive genetic (A), shared environmental (C), and non-shared environmental (E) factors. Additive genetic influence, also commonly known as heritability, is estimated as twice the difference between the identical and fraternal twin correlations. The contribution of the shared environment, which makes members of a family similar, is estimated as the difference between the identical twin correlation and heritability. Non-shared environments, i.e. environments specific to individuals, are estimated by the difference between the identical twin correlation and 1 because they are the only source of variance making identical twins different. Estimates of the non-shared environment also include measurement error.

Maximum likelihood structural equation model-fitting analyses allow more complex analyses and formal tests of significance[65]. Standard twin model-fitting analyses were conducted using Mx[66]. The model fit is summarized by minus two times the log likelihood (-2LL). Differences in -2LL between models distributes as chi-square, which provides a goodness of fit statistic. A change in chi-square of 3.84 is significant for a 1 degree of freedom test. Model fit was compared between the full ACE model and the saturated model (where variances are not decomposed into genetic and environmental sources). Reduced models testing CE, AE, and E models were compared to the full ACE model and the saturated model. A significant p-value indicates a significantly worse fit.

Twin analysis was carried out on rank-transformed expressive vocabulary scores at 24 months (adjusted for age, age squared and sex), which were assessed in 5,733 twin pairs (Monozygotic twins N=1,969; Dizygotic twins (male, female, opposite sex) N=3,764) from the Twins Early Development Study[67].

The URLs for all utilized web pages are given in Supplementary Note 2.

RESULTS

Genome-wide association analyses

We conducted two cross-sectional genome-wide screens corresponding to an early (15-18 months, N_{Total}=8,889) and a later (24-30 months, N_{Total}=10,819) phase of language acquisition respectively, each adopting a two-stage design (Fig. 1-2, Supplementary Data 1). During these developmental phases expressive vocabulary was captured with age-specific wordlists (adaptations of the MacArthur Communicative Development Inventories (CDI)[13–17] and the Language Development Survey (LDS)[18], Methods). However, measures of expressive vocabulary were not normally distributed and differed in their symmetry (Supplementary Data 1, Supplementary Fig. 1), and association analysis was therefore carried out

using rank-transformed scores (Methods). Within the discovery cohort, a total of 2,449,665 autosomal genotyped or imputed SNPs were studied in 6,851 15-month-old and 6,299 24-month-old English-speaking toddlers respectively. Genome-wide plots of the association signals are provided in Supplementary Fig. 2-3. For the early phase, the strongest association signal was observed at rs7642482 on chromosome 3p12.3 near *ROBO2* (p=9.5x10^{-7}, Supplementary Table 1) and for the late phase at rs11742977 on chromosome 5q22.1 within *CAMK4* (p=3.5x10^{-7}, Supplementary Table 2). All independent variants from the discovery analysis (associated $p \leq 10^{-4}$, Supplementary Table 1-2), including these SNPs, were taken forward to a follow-up study (Methods). This included 2,038 18-month-old Dutch-speaking children for the early phase and 4,520 24 to 30-month-old Dutch or English-speaking children for the later phase (Supplementary Data 1).

For four independent loci from the early phase GWAS (rs7642482, rs10734234 and rs11176749, rs1654584), but none for the later phase analysis, we found evidence for association within the follow-up cohort (p<0.05), assuming the same direction of effect as in the discovery sample (Table 1, Supplementary Table 1-4). In the combined analysis of all available samples (Table 1, Fig. 3a-d) rs7642482 on chromosome 3p12.3 near *ROBO2* (the strongest signal in the discovery cohort) reached the genome-wide significance level (p=1.3x10^{-8}), and the three other signals approached the suggestive level (rs10734234 on chromosome 11p15.2 near *INSC*, p=1.9x10^{-7}; rs11176749 on chromosome 12q15 near *CAND1*; p=7.2x10^{-7}, rs1654584 on chromosome 19p13.3 within *DAPK3*; p=3.4x10^{-7}).

Each of these four polymorphisms explained only a small proportion of the phenotypic variance (Adjusted-regression-R^2: rs7642482=0.34-0.35%; rs10734234=0.27-0.35%, rs11176749=0.25-0.27%, rs1654584=0.22-0.49%) in both the discovery and the follow-up cohort, but together the four SNPs accounted for more than 1% of the variation in early expressive vocabulary scores (Joint adjusted-regression-R^2=1.10-1.45%). For the SNP reaching genome-wide significance, rs7642482, each increase in the minor G-allele was associated with lower expressive vocabulary, although, due to the rank-transformation, an interpretation of the magnitude of the genetic effect is not informative. An empirical estimate of the genetic effect in the discovery sample, suggested a decrease of 0.098 standard deviations in expressive vocabulary scores (95%-CI: 0.058;0.14) per increase in G-allele. We are aware however that this signal might be prone to the 'winner's curse' (i.e. an overestimation of the effect) and requires further replication within independent samples.

Table 1: Lead association signals for early expressive vocabulary (15-18 months of age)

					Discovery (N=6851)			Follow-up (N=2038)			Meta-analysis (N=8889)			
SNP	E/A	Chr	Pos[a]	Gene[b]	EAF	Beta(SE)[c]	*p*[c]	EAF	Beta(SE)	*p*	EAF	Beta(SE)	*p*	p_{het}
rs7642482	G/A	3p12.3	77,800,446	*ROBO2*	0.18	-0.11(0.022)	$9.5x10^{-7}$	0.19	-0.12(0.040)	$4.4x10^{-3}$	0.19	-0.11(0.019)	$1.3x10^{-8}$	0.90
rs10734234	T/C	11p15.2	15,422,436	*INSC*	0.90	-0.14(0.032)	$1.1x10^{-5}$	0.90	-0.17(0.059)	$4.5x10^{-3}$	0.90	-0.15(0.028)	$1.9x10^{-7}$	0.72
rs11176749	T/A	12q15	66,139,051	*CAND1*	0.11	-0.12(0.027)	$2.1x10^{-5}$	0.11	-0.13(0.050)	$1.0x10^{-2}$	0.11	-0.12(0.024)	$7.2x10^{-7}$	0.83
rs1654584	G/T	19p13.3	3,921,683	*DAPK3*	0.23	-0.081(0.020)	$6.2 x10^{-5}$	0.23	-0.13(0.038)	$9.2x10^{-4}$	0.23	-0.091(0.018)	$3.4 x10^{-7}$	0.30

Genome-wide screen of rank-transformed expressive vocabulary scores between 15-18 months of age in children of European ancestry. Discovery analysis was conducted in ALSPAC (Infant CDI[13]) and independent signals were followed up in GenR (N-CDI-2A[14,16]). Combined results are from inverse-variance fixed effect meta-analysis. Beta coefficients represent the change in rank-transformed score (adjusted for sex, age, age squared and the most significant principal components in each cohort) per effect allele from weighted linear regression of the score on allele dosage. The imputation accuracy (Supplementary Table 3) for rs7642482, rs11176749 and rs1654584 was high (MACH $R^2 \geq 0.95$), and for rs10734234 moderate (MACH $R^2 = 0.75$-0.76). Thus, the signal at rs10734234 in the discovery cohort was confirmed by direct genotyping (Supplementary Table 4)

E – Effect allele, A – Alternative allele, Chr – Chromosome, Pos – Position, EAF – Effect allele frequency, p_{het} – Heterogeneity *p*-value based on Cochran's Q-test; a – hg18, b – Nearest known gene within ±500kb; c - Genomic-control corrected

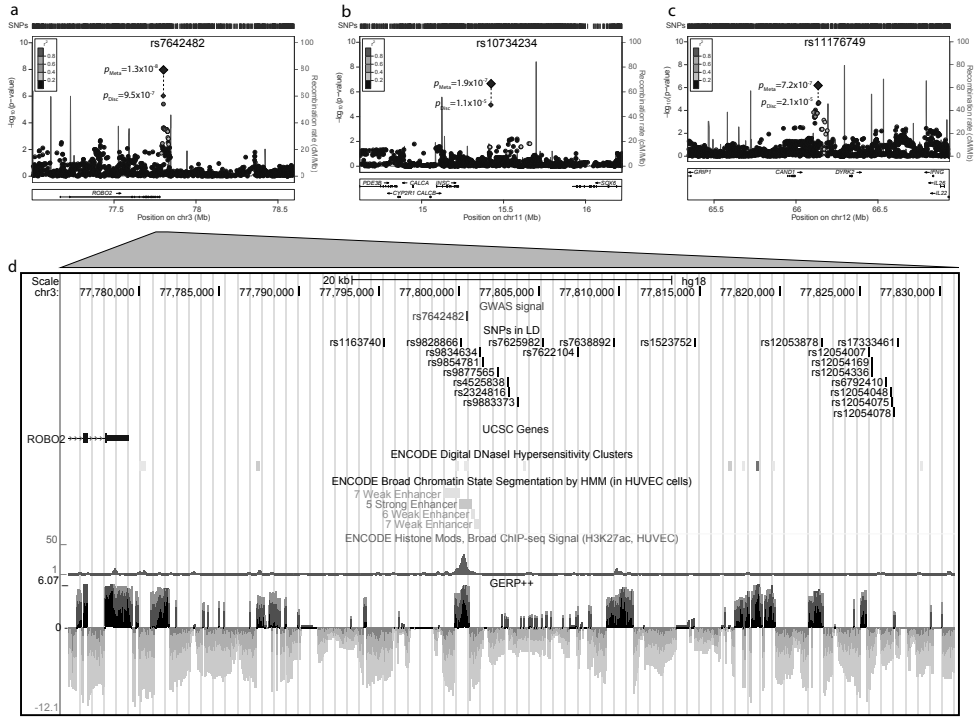

Figure 3: Association plots for early expressive vocabulary signals (15-18 months) (a-d). For the 3p12.3 (a), the 11p15.2 (b), 12q15 (c) and 19p13.3 (d) region, SNPs are plotted with their discovery -log10 p-value as a function of the genomic position (hg18). *P*-values of discovery SNPs taken forward to the follow-up analysis are denoted by a small purple diamond (p_{Disc}) and their combined meta-analysis p-value (p_{Meta}) is represented by a large purple diamond. The local linkage disequilibrium (LD) structure near the associated region is reflected by recombination rates estimated from Hapmap CEU (Phase II). SNPs were colored on the basis of their correlation with the lead signal (based on pair-wise r^2 values). (e) Detailed annotation of the genomic region at 3p12.3 using the UCSC Genome Browser (hg18) including rs7642482 and SNPs in LD (±500kb, r^2>0.3, Hapmap). Tracks for ENCODE digital DNaseI hypersensitivity clusters, ENCODE histone modifications and chromatin state segmentation in umbilical vein endothelial cells (HUVEC), as well as Genomic Evolutionary Rate Profiling (GERP++) scores (lifted from hg19) are included.

Characterization of the lead association signals

rs7642482 is located ~19 kb 3' of *ROBO2* (OMIM: 602431), which encodes the human roundabout axon guidance receptor homolog 2 (Drosophila) gene. An *in silico* search for potentially functional effects using the University of California Santa Cruz (UCSC) Genome Browser[19] provided no evidence that rs7642482 or proxy SNPs (r^2>0.3) relate to protein-coding variation within *ROBO2*. For this, we also confirmed the observed linkage disequilibrium structure within the discovery cohort through local imputation of chromosome 3 using the 1000 Genomes reference

panel (v3.20101123, data not shown).The sequence at rs7642482 and the flanking genomic interval are however highly conserved (rs7642482 Genomic Evolutionary Rate Profiling (GERP)[20] score = 3.49; regional average GERP score near rs7642482 (derived from 100 bases surrounding rs7642482, GWAVA[21])=3.06; average GERP score for coding sequences[20] >2). Encyclopedia of DNA elements (ENCODE)[22] data indicate that in umbilical vein endothelial cells (HUVEC) rs7642482 overlaps with regulatory chromatin states, such as H3K27ac[23,24], which are predicted to be a strong enhancer[25] (Fig. 3e). Additional searches using HaploReg v2[26] identified overlaps with further regulatory DNA features such as DNase I hypersensitive sites and binding sites for transcription factors (lrx, Pou3f2_1). This suggests that variation at rs7642482 might be implicated within regulatory mechanisms in embryonic cell types, consistent with a peak of *ROBO2* expression in the human brain during the first trimester (Supplementary Fig. 4). There was no evidence for *cis* expression quantitative trait loci (eQTL) within ±1 Mb of rs7642482 in postnatally derived cell-types such as lymphoblastoid cell lines, which were available for children of the discovery cohort (ALSPAC, data not shown), or adult brain tissue, based on searches of public eQTL databases (seeQTL)[27,28].

Since little is known about the genetic factors affecting language acquisition, the 'suggestive' signals at 11p15.2, 12q15 and 19p13.3 may also stimulate future research. rs10734234 resides within the vicinity of *INSC* (197 kb 3' of the gene), encoding an adaptor protein for cell polarity proteins (OMIM: 10668). rs11176749 is located near *CAND1* (144 kb 3' of the gene) encoding a F-box protein-exchange factor (OMIM: 607727), which regulates the ubiquitination of target proteins, and rs1654584 is an intronic SNP within *DAPK3* encoding the Death-associated protein kinase 3, which plays a key role in apoptosis (OMIM: 603289).

Within a further step we investigated whether the reported association signals are influenced by potential covariates, such as gestational age[29] and maternal education[30]. These have been previously linked to late language emergence in infancy[29] and the total number of spoken words in early childhood[30] respectively. Studying up to 8,889 15 to 18-month-old children from the discovery and follow-up cohort, the association signal at rs7642482 increased when gestational age was adjusted for (adjusted p_{meta} =4.0x10^{-9}, 0.36-0.38% explained variance), while adjustment for maternal education did not affect the association(Supplementary Table 5-6). For the remaining SNPs, there was little or no effect on the strength of the genetic association when these covariates were controlled for.

To explore whether the reported association signals influence linguistic skills other than early phase expressive vocabulary, we also investigated a series of language-related measures during development. We observed no evidence for association

between the four SNPs and first single-word utterances in 4,969 12-month-old Finnish children (Supplementary Data 1, Supplementary Table 7). However, this age pertains to a developmental stage where expressive vocabulary is very low, i.e. the majority of children speak about one or two words, and pre-linguistic communication skills are still developing[31]. All early phase signals were furthermore attenuated or even abolished when investigated for association with word-production scores during the later phase of language acquisition (24-30 months, Supplementary Fig. 5). This age band spans a phase where growth in linguistic proficiency may relate more to early grammar development including two-word combinations[1], than a vocabulary of single words. Overall, the phenotypic correlations between early and later expressive vocabulary scores were moderate within cohorts with multiple linguistic measures ($0.48<\rho\leq0.57$, Supplementary Data 1), and evidence for genetic correlations, based on Genome-wide Complex Trait Analysis (GCTA)[32,33], was mixed (Avon Longitudinal Study of Parents and Children (ALSPAC): r_g(SE)=0.69(0.20), p=0.02), Generation R Study (GenR): r_g(SE)=-0.32(0.97), p=0.18). There was also no association between the four reported SNPs and other language-related cognitive outcomes, including verbal intelligence scores, in middle childhood (8-10 years of age) when studying up to 5,540 children from the discovery cohort, apart from nominal associations with reading speed (rs7642482 p=0.009; rs1654584 p=0.0035; Supplementary Table 8-9). Thus, the observed genetic associations, especially at rs7642482, are likely to be time-sensitive and specific to the early phase of language acquisition.

Twin analysis and GCTA

A twin study of 5,733 twin pairs of European descent, including a subset of children from the follow-up cohorts, supported the (modest) influence of additive genetic effects on variability in expressive vocabulary at ~24 months (a^2(SE)=0.20(0.008); Table 2, Supplementary Table 10-11, Methods) and was consistent with previous reports on a smaller sample[9]. Estimates from twin analysis and GCTA[32], performed on the discovery sample, were furthermore in close concordance (ALSPAC GCTA-$h^2(SE)_{15\text{-months}}$=0.13(0.05); GCTA-$h^2(SE)_{24\text{-months}}$=0.17(0.06); Table 2). However, in the smaller-sized follow-up samples, GCTA heritability, especially for the later phase, was close to zero (Table 2), and is likely to reflect impaired power during the follow-up. Combining GCTA heritability estimates using meta-analysis techniques (Methods), provided similar estimates as observed for the discovery cohort alone (meta-GCTA $h^2(SE)_{15\text{-18-months}}$=0.13(0.05), meta-GCTA $h^2(SE)_{24\text{-30-months}}$=0.14(0.05)).

Table 2: Heritability of expressive vocabulary (15-30 months of age)

GCTA: Early expressive vocabulary (15-18 months)						
Sample	Age (m)	Measure	h^2(SE)[a]	LRT(df)	*p*	N[b]
ALSPAC	15	Infant CDI	0.13(0.05)	5.66(1)	0.009	6194
GenR	18	N-CDI-2A	0.19(0.17)	1.23(1)	0.10	1828
Total[c]			0.13(0.05)			8022
GCTA: Later expressive vocabulary (24-30 months)						
Sample	Age (m)	Measure	h^2(SE)[a]	LRT(df)	*p*	N[b]
ALSPAC	24	Toddler CDI	0.17(0.06)	8.09(1)	0.002	5739
Raine	24	LDS	<0.01(0.34)	<0.01(1)	0.50	866
TEDS	24	MCDI	<0.01(0.15)	<0.01(1)	0.50	1720
GenR	30	LDS	0.11(0.19)	0.33(1)	0.30	1641
Total[c]			0.14(0.05)			9966
Twin analysis: Later expressive vocabulary (24 months)						
Sample	Age (m)	Measure	a^2(SE)[d]			N[e]
TEDS	24	MCDI	0.20(0.008)			5733

Expressive vocabulary was captured with different forms of the MacArthur Communicative Development Inventories (CDI: Infant CDI, Toddler CDI, N-CDI-2A and MCDI) [13-17] and the Language development Survey (LDS)[18] (Supplementary Data 1). GCTA – Genome-wide Complex Trait Analysis; m - Months

a – Narrow-sense GCTA heritability based on rank-transformed expressive vocabulary scores adjusted for age, age squared, sex and the most significant ancestry-informative principal components in each cohort

b – Sample number after exclusion of individuals with a relatedness of ≥2.5%

c – Estimates were combined with fixed effects inverse-variance meta-analysis (Heterogeneity *p*-value based on Cochran's Q-test based $p_{het} \geq 0.72$)

d – Additive genetic influence using rank-transformed expressive vocabulary scores adjusted for age, age squared and sex, based on an ACE model (Supplementary Table 10-11)

e – Number of twin pairs

DISCUSSION

This study reports a genome-wide screen and follow-up study of expressive vocabulary scores in up to 10,819 toddlers of European origin investigating an early phase (15-18 months) and a later phase (24-30 months) of language acquisition. Based on the combined analysis of all available samples, our study identifies a novel locus near *ROBO2* as associated with expressive vocabulary during the early phase of language acquisition.

Robo receptors and their Slit ligands (secreted chemorepellent proteins) are highly conserved from fly to human[34,35] and play a key role in axon guidance and cell migration. In vertebrates, Robo2 is involved in midline commissural axon guidance[36], the proliferation of central nervous system progenitors[37], the spatial positioning of

spiral ganglion neurons[38] and the assembly of the trigeminal ganglion[39], which is the sensory ganglion of the trigeminal nerve. The latter is particularly important for speech production in humans[40] as the trigeminal nerve provides motor supply to the muscles of mastication, which control the movement of the mandibles, and in addition the nerve transmits sensory information from the face. Thus, genetic variation at *ROBO2* may be linked to both speech production abilities and expressive vocabulary size within children of the general population. This is not dissimilar to genetic defects implicated in both speech- and language-related symptoms in familiar studies, such as the famous KE family[41]. There, affected individuals present an autosomal dominant form of speech and language disorder including both a deficiency in the use of grammatical suffixation rules and severe orofacial dyspraxia, which are linked to mutations in *FOXP2*[41]. However, the underlying genetic mechanisms affecting subtle population variation in language acquisition, compared with severe disturbances of speech and language, will most certainly fundamentally vary.

Rare recurrent *ROBO2* deletions have been discovered in patients with autism spectrum disorder[42], a severe childhood neuro-developmental condition where core symptoms include deficits in social communication[43], and decreased *ROBO2* expression has been observed in the anterior cingulate cortex[44], and in lymphocytes of individuals with autism[45]. Indeed, the 3p12-p13 region has been linked to dyslexia[46], and quantitative dyslexia traits[47], as well as quantitative speech-sound disorder traits and reading[48]. The dyslexia linkage findings[46] have been recently related to a specific SNP haplotype within *ROBO1*[49], a neighboring gene of *ROBO2*. In animal models, *Robo1* and *Robo2* are mostly co-expressed and it has been shown that both receptors function cooperatively, for example with respect to the guidance of most forebrain projections[50]. Thus, it is possible that variation within both *ROBO1* and *ROBO2* might also contribute to the linkage signals within the reported regions, and our findings highlight *ROBO2* as a novel, not yet investigated candidate locus.

Common polymorphisms within *ROBO1* have also been associated with reading disability[51] and with performance on tasks of non-word repetition[52], which is related to phonological short-term memory deficits. However, none of these previously reported *ROBO1* variants (rs6803202, rs4535189, rs331142 and rs12495133)[51,52] was associated with early word production scores within our study (data not shown). Vice versa, we also found no association between rs7642482 (*ROBO2*) and language-related measures including phonological memory and verbal intelligence in middle childhood, nor was there any association with expressive vocabulary during the later phase of language acquisition (24-30 months of age) or with very first single-word utterances at about 12 months of age. Instead, our findings suggest that the identified

ROBO2 signal is specific for an early developmental stage of language acquisition (15-18 months of age), which is characterized by a slow accumulation of single words, followed by an increase in rate that is sometimes related to a 'vocabulary spurt'[1,4]. Both *in silico* analyses and the increase in signal after adjustment for gestational age support the hypothesis that expressive vocabulary during this phase may be affected by perinatal or early postnatal gene regulatory mechanisms. It is furthermore possible that the enhancer effect predicted within HUVEC also relates to a yet uncharacterized embryonic cell type, where expression changes are only detectable on the single cell level. For example, during the trigeminal ganglion formation placode/neural crest cells travel as individual cells to the site of ganglion formation, and *Robo2* appears to be expressed in discrete, dispersed regions in the surface ectoderm[39]. This is characteristic of cells, which are about to detach and migrate[39]. Thus, it will require further molecular studies to characterize the biological mechanisms underlying the observed *ROBO2* association in more detail.

In line with previous findings[8,9], estimates from twin analysis and GCTA (based on large samples) suggest that the proportion of phenotypic variation in early expressive vocabulary, which is attributable to genetic factors, is modest. The concordance of twin and large-sample GCTA heritability estimates indicates however that most of this genetic variation is common and that there is little 'missing heritability'. Thus, a large proportion of common genetic variation influencing early expressive vocabulary might be captured by current GWAS designs, given sufficient power.

To conclude, this study describes genome-wide association between rs7642482 near *ROBO2* and expressive vocabulary during an early phase of language acquisition where children typically communicate with single words only. The signal is specific to this developmental stage, strengthened after adjustment for gestational age and links overall language-related common genetic variation in the general population to a potential autism susceptibility locus as well as a linkage region for dyslexia, speech-sound disorder and reading on chromosome 3p12-p13.

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SUPPLEMENTARY MATERIAL

Table S1. Association signals for early expressive vocabulary (15-18 months, $p \leq 10^{-4}$)

				Discovery(N=6851)			Meta-analysis (N=8889)			
SNP	E/A	Chr	Pos[a]	EAF	Beta(SE)[b]	*p*[b]	EAF	Beta(SE)	*p*	Dir
rs11804375	T/C	1	16,001,561	0.11	0.12(0.028)	$4.2x10^{-5}$	0.11	0.099(0.025)	$6.0x10^{-5}$	++
rs11208754	A/G	1	40,948,896	0.89	0.11(0.028)	$5.6x10^{-5}$	0.88	0.09(0.024)	$2.1x10^{-4}$	++
rs11810174	T/C	1	98,907,589	0.94	0.16(0.038)	$3.9x10^{-5}$	0.94	0.1(0.034)	$3.2x10^{-3}$	+-
rs1234318	A/G	1	171,364,058	0.65	-0.082(0.02)	$5.0x10^{-5}$	0.65	-0.056(0.018)	$1.7x10^{-3}$	-+
rs1498029	A/C	1	217,150,795	0.98	0.29(0.067)	$1.7x10^{-5}$	0.98	0.25(0.06)	$3.6x10^{-5}$	++
rs4669286	A/G	2	8,612,388	0.10	-0.16(0.039)	$5.5x10^{-5}$	0.09	-0.12(0.034)	$7.0x10^{-4}$	-+
rs4952624	A/C	2	40,506,606	0.97	0.22(0.055)	$6.0x10^{-5}$	0.97	0.17(0.048)	$5.4x10^{-4}$	+-
rs13420384	A/G	2	59,642,081	0.08	-0.14(0.033)	$5.2x10^{-5}$	0.08	-0.12(0.029)	$3.8x10^{-5}$	--
rs16826639	C/G	2	230,704,789	0.74	0.077(0.019)	$6.1x10^{-5}$	0.74	0.049(0.017)	$3.9x10^{-3}$	+-
rs4684234	A/G	3	14,596,299	0.37	0.081(0.02)	$6.2x10^{-5}$	0.38	0.073(0.018)	$4.3x10^{-5}$	++
rs12487696	A/G	3	59,538,614	0.78	-0.1(0.021)	$2.0x10^{-6}$	0.79	-0.078(0.019)	$2.8x10^{-5}$	-0
rs17061008	A/G	3	59,618,671	0.54	0.074(0.018)	$4.8x10^{-5}$	0.53	0.058(0.016)	$2.9x10^{-4}$	++
s7642482	**A/Gr**	**3**	**77,800,446**	**0.82**	**0.11(0.022)**	$\mathbf{9.5x10^{-7}}$	**0.81**	**0.11(0.019)**	$\mathbf{1.3x10^{-8}}$	**++**
rs1874655	A/C	3	99,552,155	0.75	0.08(0.02)	$7.6x10^{-5}$	0.75	0.056(0.018)	$1.5x10^{-3}$	+-
rs4234700	A/G	3	130,989,172	0.90	0.12(0.028)	$3.1x10^{-5}$	0.90	0.08(0.025)	$1.5x10^{-3}$	+-
rs1006742	A/G	3	152,108,938	0.84	-0.16(0.041)	$8.4x10^{-5}$	0.84	-0.084(0.036)	$1.9x10^{-2}$	-+
rs9857706	A/G	3	152,129,866	0.29	-0.081(0.019)	$2.5x10^{-5}$	0.29	-0.061(0.017)	$2.8x10^{-4}$	-+
rs13073941	T/C	3	160,680,164	0.27	-0.085(0.019)	$9.6x10^{-6}$	0.27	-0.062(0.017)	$2.3x10^{-4}$	-+
rs792354	T/C	3	174,456,839	0.34	-0.072(0.018)	$7.6x10^{-5}$	0.34	-0.059(0.016)	$2.0x10^{-4}$	--
rs2537921	T/C	4	18,588,912	0.27	-0.075(0.019)	$9.4x10^{-5}$	0.27	-0.071(0.017)	$2.9x10^{-5}$	--
rs6814920	A/G	4	58,173,417	0.23	0.082(0.02)	$5.0x10^{-5}$	0.23	0.056(0.018)	$1.6x10^{-3}$	+-
rs342435	A/G	4	88,262,230	0.41	0.068(0.017)	$7.6x10^{-5}$	0.41	0.06(0.015)	$8.1x10^{-5}$	++
rs7694786	A/C	4	187,916,334	0.67	-0.076(0.019)	$7.6x10^{-5}$	0.67	-0.054(0.017)	$1.3x10^{-3}$	-+
rs2652492	T/C	5	5,967,817	0.37	0.075(0.019)	$9.4x10^{-5}$	0.37	0.06(0.017)	$4.6x10^{-4}$	++
rs13164951	T/G	5	50,015,814	0.9	-0.13(0.03)	$2.4x10^{-5}$	0.9	-0.11(0.027)	$2.2x10^{-5}$	--
rs16750	A/G	5	159,062,555	0.87	0.1(0.025)	$5.4x10^{-5}$	0.87	0.085(0.022)	$1.4x10^{-4}$	++
rs6894268	A/G	5	178,965,094	0.32	-0.075(0.018)	$3.8x10^{-5}$	0.32	-0.048(0.016)	$2.9x10^{-3}$	-+

rs4715888	A/G	6	14,462,126	0.31	0.073(0.018)	$6.0x10^{-5}$	0.31	0.051(0.016)	$1.7x10^{-3}$	+-
rs3799344	T/C	6	25,894,972	0.44	-0.076(0.017)	$9.8x10^{-6}$	0.44	-0.048(0.015)	$1.6x10^{-3}$	-+
rs314214	A/G	6	69,935,117	0.82	0.087(0.022)	$9.2x10^{-5}$	0.82	0.079(0.02)	$5.7x10^{-5}$	++
rs1340080	A/G	6	164,754,183	0.13	-0.1(0.026)	$8.9x10^{-5}$	0.13	-0.082(0.023)	$3.7x10^{-4}$	--
rs4720231	A/C	7	36,955,623	0.27	-0.08(0.019)	$3.1x10^{-5}$	0.28	-0.062(0.017)	$2.0x10^{-4}$	--
rs921908	T/C	7	50,212,082	0.76	0.092(0.022)	$3.5x10^{-5}$	0.76	0.047(0.019)	$1.6x10^{-2}$	+-
rs7005662	T/C	8	6,464,034	0.29	-0.08(0.019)	$3.1x10^{-5}$	0.29	-0.061(0.017)	$2.7x10^{-4}$	-+
rs2201476	A/G	8	15,302,254	0.39	-0.073(0.018)	$6.0x10^{-5}$	0.39	-0.058(0.016)	$2.4x10^{-4}$	--
rs7015219	A/G	8	38,939,594	0.55	-0.07(0.017)	$4.6x10^{-5}$	0.54	-0.059(0.015)	$8.3x10^{-5}$	--
rs1897446	A/C	8	130,530,423	0.35	-0.071(0.018)	$9.5x10^{-5}$	0.34	-0.047(0.016)	$3.1x10^{-3}$	-+
rs7861219	T/C	9	28,199,655	0.17	0.098(0.023)	$2.5x10^{-5}$	0.17	0.078(0.02)	$1.2x10^{-4}$	++
rs7851060	A/T	9	75,612,036	0.82	-0.093(0.023)	$6.3x10^{-5}$	0.82	-0.076(0.02)	$1.9x10^{-4}$	--
rs10820378	T/C	9	98,132,570	0.84	-0.092(0.023)	$7.6x10^{-5}$	0.84	-0.07(0.02)	$5.6x10^{-4}$	-0
rs10759964	A/G	9	120,090,898	0.36	0.071(0.018)	$9.5x10^{-5}$	0.36	0.054(0.016)	$7.1x10^{-4}$	+-
rs12359510	T/C	10	9,446,646	0.29	-0.076(0.019)	$7.6x10^{-5}$	0.29	-0.051(0.017)	$2.6x10^{-3}$	-+
rs10734234	**T/C**	**11**	**15,422,436**	**0.9**	**-0.14(0.032)**	**$1.1x10^{-5}$**	**0.9**	**-0.15(0.028)**	**$1.9x10^{-7}$**	**--**
rs198750	T/C	11	61,144,666	0.11	-0.12(0.027)	$6.6x10^{-6}$	0.11	-0.1(0.024)	$2.7x10^{-5}$	--
rs2726888	T/G	11	108,142,998	0.13	-0.099(0.025)	$9.0x10^{-5}$	0.13	-0.086(0.022)	$1.2x10^{-4}$	--
rs7113211	A/G	11	112,147,842	0.5	-0.078(0.019)	$4.9x10^{-5}$	0.5	-0.073(0.017)	$8.7x10^{-6}$	--
rs11221096	T/C	11	127,254,104	0.08	-0.19(0.046)	$3.9x10^{-5}$	0.08	-0.14(0.04)	$5.3x10^{-4}$	-+
rs318961	T/C	11	130,869,705	0.78	0.083(0.021)	$9.2x10^{-5}$	0.78	0.061(0.019)	$1.0x10^{-3}$	+-
rs893951	T/G	11	133,763,626	0.32	-0.074(0.018)	$4.8x10^{-5}$	0.33	-0.054(0.016)	$7.3x10^{-4}$	-+
rs10845602	A/G	12	12,716,504	0.59	0.072(0.017)	$2.8x10^{-5}$	0.59	0.064(0.015)	$2.1x10^{-5}$	++
rs11608516	T/C	12	47,074,366	0.05	0.23(0.054)	$1.8x10^{-5}$	0.05	0.2(0.046)	$1.9x10^{-5}$	++
rs10878615	C/G	12	66,118,859	0.16	-0.091(0.023)	$9.1x10^{-5}$	0.16	-0.078(0.02)	$1.3x10^{-4}$	--
rs11176749	**A/T**	**12**	**66,139,051**	**0.89**	**0.12(0.027)**	**$2.1x10^{-5}$**	**0.89**	**0.12(0.024)**	**$7.2x10^{-7}$**	**++**
rs7306190	A/G	12	69,455,688	0.83	0.1(0.023)	$7.7x10^{-6}$	0.83	0.089(0.021)	$1.6x10^{-5}$	++
rs11106179	A/G	12	90,420,543	0.20	-0.094(0.021)	$9.5x10^{-6}$	0.20	-0.087(0.019)	$3.4x10^{-6}$	--
rs2453165	T/C	12	103,737,294	0.97	-0.25(0.065)	$8.6x10^{-5}$	0.97	-0.22(0.057)	$1.0x10^{-4}$	--
rs4772487	T/G	13	85,903,906	0.34	-0.072(0.018)	$7.6x10^{-5}$	0.34	-0.05(0.016)	$1.8x10^{-3}$	-+
rs17096641	A/C	14	29,926,756	0.92	-0.14(0.031)	$4.3x10^{-6}$	0.92	-0.12(0.028)	$1.9x10^{-5}$	--
rs11632744	T/C	15	59,701,327	0.72	0.087(0.022)	$9.2x10^{-5}$	0.72	0.055(0.019)	$4.1x10^{-3}$	+-
rs12148373	A/G	15	63,321,342	0.52	0.084(0.017)	$1.0x10^{-6}$	0.52	0.065(0.015)	$2.1x10^{-5}$	+-

rs8025474	T/C	15	66,173,577	0.31	0.08(0.018)	$1.1x10^{-5}$	0.3	0.059(0.016)	$2.8x10^{-4}$	+-
rs12920537	A/G	16	20,329,057	0.72	0.078(0.019)	$4.9x10^{-5}$	0.72	0.046(0.017)	$6.2x10^{-3}$	+-
rs187680	A/C	16	50,981,658	0.6	0.068(0.017)	$7.6x10^{-5}$	0.6	0.064(0.015)	$2.4x10^{-5}$	++
rs12445038	T/C	16	56,817,301	0.04	-0.21(0.053)	$9.6x10^{-5}$	0.04	-0.13(0.044)	$3.9x10^{-3}$	-+
rs7209108	T/C	17	44,218,897	0.26	0.076(0.019)	$7.6x10^{-5}$	0.26	0.069(0.017)	$5.4x10^{-5}$	++
rs2465411	A/G	17	58,063,009	0.41	0.072(0.017)	$2.8x10^{-5}$	0.4	0.053(0.015)	$4.2x10^{-4}$	+-
rs6501538	T/C	17	67,885,084	0.5	0.072(0.017)	$2.8x10^{-5}$	0.5	0.057(0.015)	$1.7x10^{-4}$	++
rs11657164	T/C	17	67,885,977	0.83	0.092(0.023)	$7.6x10^{-5}$	0.83	0.079(0.02)	$1.2x10^{-4}$	++
rs9907431	A/G	17	74,071,124	0.34	-0.084(0.02)	$3.3x10^{-5}$	0.34	-0.054(0.018)	$2.5x10^{-3}$	-+
rs4395181	T/C	18	62,952,025	0.38	0.075(0.018)	$3.8x10^{-5}$	0.38	0.065(0.016)	$4.6x10^{-5}$	++
rs10514093	A/G	18	69,667,638	0.98	0.37(0.093)	$5.8x10^{-5}$	0.98	0.28(0.08)	$5.8x10^{-4}$	+-
rs1654584	**T/G**	**19**	**3,921,683**	**0.77**	**0.081(0.02)**	$\mathbf{6.2x10^{-5}}$	**0.77**	**0.091(0.018)**	$\mathbf{3.4x10^{-7}}$	**++**
rs3865452	T/C	19	45,902,896	0.5	0.07(0.017)	$4.6x10^{-5}$	0.5	0.052(0.015)	$6.0x10^{-4}$	+-
rs12463085	A/G	19	51,161,251	0.69	-0.079(0.019)	$3.9x10^{-5}$	0.69	-0.053(0.017)	$1.5x10^{-3}$	-+
rs11697413	A/G	20	1,213,152	0.06	-0.14(0.036)	$9.5x10^{-5}$	0.06	-0.12(0.032)	$1.7x10^{-4}$	--
rs2273189	C/G	20	8,613,751	0.95	0.2(0.048)	$2.8x10^{-5}$	0.95	0.15(0.042)	$4.5x10^{-4}$	+-
rs6032829	T/C	20	10,178,291	0.16	0.098(0.024)	$5.4x10^{-5}$	0.16	0.07(0.021)	$9.7x10^{-4}$	+-
rs11699690	A/G	20	33,735,188	0.09	0.13(0.031)	$4.4x10^{-5}$	0.09	0.095(0.028)	$5.4x10^{-4}$	+-
rs11700299	T/C	20	34,012,279	0.11	0.12(0.028)	$4.2x10^{-5}$	0.11	0.077(0.025)	$1.9x10^{-3}$	+-
rs6066696	A/T	20	46,386,093	0.33	-0.072(0.018)	$7.6x10^{-5}$	0.33	-0.063(0.016)	$8.4x10^{-5}$	--
rs802952	T/C	20	47,519,676	0.56	-0.071(0.017)	$3.6x10^{-5}$	0.56	-0.046(0.015)	$2.3x10^{-3}$	-+
rs8130870	T/C	21	23,803,965	0.75	0.083(0.02)	$4.0x10^{-5}$	0.75	0.074(0.018)	$2.6x10^{-5}$	++
rs4818184	A/G	21	41,124,301	0.13	-0.1(0.025)	$3.9x10^{-5}$	0.13	-0.1(0.022)	$7.0x10^{-6}$	--
rs132503	T/C	22	37,616,345	0.8	0.09(0.022)	$5.2x10^{-5}$	0.8	0.068(0.02)	$5.2x10^{-4}$	+-
rs713628	C/G	22	42,012,214	0.7	0.084(0.019)	$1.2x10^{-5}$	0.71	0.061(0.017)	$2.8x10^{-4}$	+-

Genome-wide screen of expressive vocabulary scores between 15-18 months of age. Discovery analysis was conducted in ALSPAC and independent signals ($p \leq 1x10^{-4}$) were followed up in GenR (N=2038; Supplementary Data 1). Combined results are from inverse-variance fixed effect meta-analysis. Beta coefficients represent the change in rank-transformed score (adjusted for sex, age, age squared and the most significant principal components in each cohort) per effect allele from weighted linear regression of the score on allele dosage. E – Effect allele, A – Alternative allele, Chr – Chromosome, Pos – Position, EAF – Effect allele frequency, Dir – Direction of the genetic effect in the discovery and follow-up cohort; a - hg18, b - Genomic-control corrected

Table S2. Association signals for later expressive vocabulary (24-30 months, $p \leq 10^{-4}$)

				Discovery(N=6299)			Meta-analysis(N=10819)			
SNP	E/A	Chr	Pos[a]	EAF	Beta(SE)[b]	p[b]	EAF	Beta(SE)	p	Dir
rs12410765	A/T	1	206,254,554	0.87	-0.12(0.03)	$4.6x10^{-5}$	0.87	-0.086(0.025)	$7.7x10^{-4}$	-+-?[c]
rs16850132	A/G	1	227,902,789	0.7	0.077(0.019)	$6.5x10^{-5}$	0.7	0.052(0.015)	$4.3x10^{-4}$	+++-
rs449993	A/G	2	14,095,240	0.96	0.23(0.059)	$8.0x10^{-5}$	0.96	0.16(0.044)	$3.8x10^{-4}$	+-++
rs10496465	A/G	2	114,974,533	0.85	0.099(0.024)	$4.8x10^{-5}$	0.85	0.054(0.019)	$4.6x10^{-3}$	+-+-
rs1561444	T/G	2	139,745,456	0.25	-0.085(0.021)	$6.6x10^{-5}$	0.25	-0.029(0.016)	$7.0x10^{-2}$	++-+
rs17207382	A/G	2	159,666,010	0.63	0.073(0.018)	$6.4x10^{-5}$	0.63	0.041(0.014)	$3.1x10^{-3}$	+++-
rs16825679	T/C	2	229,661,770	0.16	0.097(0.024)	$6.8x10^{-5}$	0.17	0.060(0.018)	$1.1x10^{-3}$	++++
rs6791159	C/G	3	2,905,924	0.11	-0.14(0.034)	$5.5x10^{-5}$	0.11	-0.11(0.027)	$1.2x10^{-4}$	---?[c]
rs7629002	A/C	3	14,580,991	0.31	-0.076(0.019)	$8.0x10^{-5}$	0.31	-0.038(0.015)	$9.9x10^{-3}$	++--
rs9837325	A/C	3	130,798,521	0.2	-0.088(0.022)	$8.0x10^{-5}$	0.2	-0.040(0.017)	$1.9x10^{-2}$	++--
rs16889	A/C	3	131,196,174	0.49	-0.077(0.018)	$2.5x10^{-5}$	0.48	-0.041(0.014)	$3.1x10^{-3}$	+---
rs1512077	A/G	3	147,575,442	0.3	0.075(0.019)	$1.0x10^{-4}$	0.3	0.051(0.015)	$6.4x10^{-4}$	-+++
rs10937169	T/C	3	185,613,725	0.42	-0.074(0.018)	$5.1x10^{-5}$	0.42	-0.045(0.014)	$1.2x10^{-3}$	+---
rs7650510	A/G	3	191,845,810	0.76	0.087(0.022)	$9.7x10^{-5}$	0.76	0.051(0.017)	$2.1x10^{-3}$	-+++
rs4305462	A/T	3	192,013,125	0.19	-0.1(0.025)	$6.8x10^{-5}$	0.18	-0.052(0.019)	$6.7x10^{-3}$	++--
rs1395821	T/C	4	148,267,000	0.82	0.093(0.023)	$6.7x10^{-5}$	0.82	0.070(0.018)	$8.9x10^{-5}$	+-++
rs9312489	A/C	4	171,874,489	0.23	0.085(0.021)	$6.6x10^{-5}$	0.23	0.049(0.016)	$2.5x10^{-3}$	+-+-
rs11742977	C/G	5	110,624,391	0.59	0.093(0.018)	$3.5x10^{-7}$	0.59	0.065(0.014)	$3.5x10^{-6}$	++++
rs13182561	A/G	5	140,624,668	0.80	0.096(0.023)	$3.9x10^{-5}$	0.8	0.055(0.018)	$1.8x10^{-3}$	--++
rs9371371	A/G	6	155,666,796	0.4	-0.078(0.019)	$5.2x10^{-5}$	0.41	-0.033(0.014)	$2.0x10^{-2}$	++-+
rs10155941	T/C	7	134,073,272	0.54	-0.076(0.018)	$3.2x10^{-5}$	0.54	-0.046(0.014)	$9.8x10^{-4}$	-+-+
rs6981525	A/G	8	57,772,551	0.46	-0.077(0.019)	$6.5x10^{-5}$	0.47	-0.036(0.015)	$1.4x10^{-2}$	+--+
rs2182746	T/C	9	37,521,193	0.05	0.18(0.044)	$4.5x10^{-5}$	0.05	0.11(0.033)	$1.1x10^{-3}$	+++-
rs1863898	A/G	10	79,449,156	0.48	0.075(0.018)	$4.0x10^{-5}$	0.48	0.054(0.014)	$1.0x10^{-4}$	++++
rs10748710	A/G	10	83,084,355	0.29	0.083(0.021)	$9.8x10^{-5}$	0.29	0.058(0.016)	$2.9x10^{-4}$	++++
rs2421140	A/T	10	124,777,360	0.03	-0.23(0.057)	$6.0x10^{-5}$	0.03	-0.088(0.042)	$3.8x10^{-2}$	++--
rs7929580	A/G	11	36,415,193	0.88	-0.11(0.027)	$3.2x10^{-5}$	0.88	-0.081(0.021)	$1.1x10^{-4}$	---+

rs2510894	T/G	11	74,739,826	0.47	0.073(0.018)	$6.4x10^{-5}$	0.47	0.03(0.014)	$3.2x10^{-2}$	+-+-
rs12796629	T/G	11	98,205,037	0.01	-0.38(0.091)	$2.7x10^{-5}$	0.01	-0.25(0.07)	$3.5x10^{-4}$	---+
rs17532216	T/G	13	66,968,886	0.9	-0.12(0.03)	$5.3x10^{-5}$	0.9	-0.065(0.023)	$5.1x10^{-3}$	-+-+
rs9600102	T/C	13	72,709,016	0.06	0.16(0.04)	$5.2x10^{-5}$	0.06	0.086(0.03)	$4.0x10^{-3}$	-+++
rs924895	T/C	13	75,804,599	0.55	-0.076(0.018)	$3.2x10^{-5}$	0.56	-0.041(0.014)	$3.4x10^{-3}$	-+-+
rs7320524	T/G	13	87,184,488	0.19	0.099(0.024)	$4.8x10^{-5}$	0.18	0.079(0.019)	$2.9x10^{-5}$	-+++
rs11617458	A/G	13	97,665,446	0.74	0.08(0.02)	$8.0x10^{-5}$	0.74	0.04(0.016)	$1.1x10^{-2}$	--++
rs8020079	A/G	14	89,980,698	0.95	-0.17(0.043)	$8.0x10^{-5}$	0.95	-0.13(0.036)	$2.9x10^{-4}$	+--?[c]
rs8034690	A/G	15	51,350,929	0.83	0.1(0.025)	$6.8x10^{-5}$	0.83	0.042(0.019)	$2.7x10^{-2}$	--+-
rs1280396	A/G	15	55,519,020	0.19	0.094(0.023)	$5.6x10^{-5}$	0.19	0.044(0.018)	$1.3x10^{-2}$	--+-
rs16946198	A/C	15	61,055,589	0.92	-0.14(0.035)	$7.1x10^{-5}$	0.92	-0.089(0.026)	$5.8x10^{-4}$	+--+
rs8031639	A/C	15	64,763,977	0.57	-0.074(0.018)	$5.1x10^{-5}$	0.57	-0.051(0.014)	$2.8x10^{-4}$	----
rs12600092	A/G	16	20,057,104	0.77	0.087(0.021)	$4.4x10^{-5}$	0.78	0.067(0.017)	$4.9x10^{-5}$	++++
rs2447098	A/C	17	2,224,470	0.52	0.08(0.02)	$8.0x10^{-5}$	0.51	0.059(0.015)	$1.1x10^{-4}$	-+++
rs3816577	T/C	17	9,289,321	0.23	0.085(0.021)	$6.6x10^{-5}$	0.23	0.036(0.016)	$2.8x10^{-2}$	-++-
rs4594325	A/G	18	2,451,449	0.29	-0.079(0.02)	$9.9x10^{-5}$	0.29	-0.062(0.015)	$5.1x10^{-5}$	+---
rs11151979	T/C	18	52,558,537	0.23	0.11(0.022)	$8.3x10^{-7}$	0.22	0.045(0.017)	$7.4x10^{-3}$	--++
rs2430894	A/G	18	52,568,589	0.7	-0.089(0.02)	$1.2x10^{-5}$	0.7	-0.035(0.015)	$1.9x10^{-2}$	++-+
rs9962179	T/C	18	54,221,584	0.68	-0.077(0.019)	$6.5x10^{-5}$	0.68	-0.059(0.015)	$5.8x10^{-5}$	-+--
rs344575	T/C	19	6,607,497	0.88	0.16(0.039)	$5.2x10^{-5}$	0.89	0.11(0.029)	$8.4x10^{-5}$	+++-
rs4816169	A/C	20	972,652	0.68	0.12(0.029)	$5.2x10^{-5}$	0.67	0.089(0.025)	$2.9x10^{-4}$	-++?[c]
rs6030120	T/C	20	40,367,121	0.77	-0.083(0.021)	$9.8x10^{-5}$	0.77	-0.051(0.016)	$1.7x10^{-3}$	+---
rs11907293	T/G	20	54,867,252	0.02	-0.45(0.1)	$1.3x10^{-5}$	0.02	-0.29(0.088)	$1.1x10^{-3}$	+--?[c]

Genome-wide screen of expressive vocabulary scores between 24-30 months of age. Discovery analysis was conducted in ALSPAC and independent signals ($p \leq 1x10^{-4}$) were followed up in Raine (N=981), TEDS (N=1727) and GenR (N=1812; Supplementary Data 1). Combined results are from inverse-variance fixed effect meta-analysis. Beta coefficients represent the change in rank-transformed score (adjusted for sex, age, age squared and the most significant principal components in each cohort) per effect allele from weighted linear regression of the score on allele dosage. Signals based on more than one missing cohort were excluded. E – Effect allele, A – Alternative allele, Chr – Chromosome, Pos – Position, EAF – Effect allele frequency, Dir – Direction of the genetic effect in the discovery and follow-up cohort; a - hg18, b - Genomic-control corrected, c - Available in ALSPAC, Raine and GenR only (Total N=9092)

Table S3. Genotyping characteristics for lead association signals (15-18 months)

SNP		ALSPAC (Discovery)	GenR (Follow-up)	Raine (Follow-up)	TEDS (Follow-up)	NFBC1966 (Sensitivity)
rs7642482 (G,A)	EAF (G)	0.18	0.19	0.18	0.17	0.15
	Imputation quality (MACH R^2 / PROPERINFO)	R^2 = 0.95	R^2= 0.96	R^2=0.97	PROPERINFO=0.93	PROPERINFO=0.97
rs10734234 (C,T)	EAF (T)	0.90	0.90	0.90	0.88	0.91
	Imputation quality (MACH R^2 / PROPERINFO)	R^2= 0.76	R^2= 0.75	R^2= 0.72	PROPERINFO=0.77	PROPERINFO=0.53
rs11176749 (T,A)	EAF (T)	0.11	0.11	0.12	0.11	0.13
	Imputation quality (MACH R^2 / PROPERINFO)	R^2= 1.00	R^2= 1.00	R^2=1.00	PROPERINFO=0.91	PROPERINFO=0.99
rs1654584(G,T)[a]	EAF (G)	0.23	0.23	0.22	-	0.22
	Imputation quality (MACH R^2 / PROPERINFO)	R^2= 1.00	R^2= 1.00	R^2=1.00	-	PROPERINFO=0.98

EAF – Effect allele frequency; All SNPs were imputed, a - Available in ALSPAC, Raine and GenR only

Table S4. Association analysis at rs10734234

Phase	Age (m)	I/G	E/A	EAF	Beta(SE)	*P*
Early	15	0	T/C	0.88	-0.11(0.027)	$1.8x10^{-5}$
	15	1	T/C	0.90	-0.14(0.032)	$5.7x10^{-6}$
Late	24	0	T/C	0.88	-0.09(0.027)	$1.0x10^{-3}$
	24	1	T/C	0.90	-0.11(0.034)	$5.0x10^{-4}$

Association analysis comparing directly genotyped versus imputed SNP data (N=8,058) in the discovery cohort (ALSPAC). Beta coefficients represent the change in rank-transformed score (adjusted for sex, age, age squared and the most significant principal components in ALSPAC) per effect allele from linear regression of the score on allele dosage. I/G – Imputed (1)/ directly genotyped (0)SNP, E – Effect allele, A – Alternative allele, EAF – Effect allele frequency, m - months

Table S5. Adjustment of lead association signals (15-18 months) for potential covariates

		Discovery						Follow-up					Meta-analysis			
SNP	E/A	Model	N	EAF	Beta(SE)	*p*	R^2 (%)	N	EAF	Beta(SE)	*p*	R^2(%)	N	Beta(SE)	*p*	p_{het}
Covariate: Gestational age																
rs7642482	G,A	baseline	6851	0.18	-0.11(0.022)	$9.6x10^{-7}$	0.34	2038	0.19	-0.11(0.040)	0.0044	0.35	8889	-0.11(0.019)	$1.4x10^{-8}$	0.91
rs7642482	G,A	adj		0.18	-0.11(0.022)	$3.6x10^{-7}$	0.36		0.19	-0.12(0.040)	0.0032	0.38		-0.11(0.019)	$4.0x10^{-9}$	0.91
rs10734234	T,C	baseline		0.90	-0.14(0.032)	$9.7x10^{-6}$	0.27		0.90	-0.17(0.058)	0.0045	0.35		-0.15(0.028)	$1.5x10^{-7}$	0.71
rs10734234	T,C	adj		0.90	-0.14(0.032)	$9.0x10^{-6}$	0.27		0.90	-0.17(0.059)	0.0046	0.35		-0.15(0.028)	$1.4x10^{-7}$	0.72
rs11176749	T,A	baseline		0.11	-0.12(0.027)	$2.0x10^{-5}$	0.25		0.11	-0.13(0.050)	0.010	0.27		-0.12(0.024)	$6.6x10^{-7}$	0.83
rs11176749	T,A	adj		0.11	-0.12(0.027)	$8.0x10^{-6}$	0.28		0.11	-0.13(0.050)	0.0081	0.29		-0.12(0.024)	$2.1x10^{-7}$	0.85
rs1654584	G,T	baseline		0.23	-0.081(0.02)	$6.8x10^{-5}$	0.22		0.23	-0.13(0.038)	$9.1x10^{-4}$	0.49		-0.091(0.018)	$3.6x10^{-7}$	0.30
rs1654584	G,T	adj		0.23	-0.083(0.02)	$5.1x10^{-5}$	0.22		0.23	-0.13(0.038)	$6.1x10^{-4}$	0.53		-0.093(0.018)	$2.0x10^{-7}$	0.28
Covariate: Maternal education																
rs7642482	G,A	baseline	6681	0.18	-0.11(0.022)	$5.8x10^{-7}$	0.36	2015	0.19	-0.11(0.040)	0.0058	0.33	8696	-0.11(0.020)	$1.1x10^{-8}$	0.99
rs7642482	G,A	adj		0.18	-0.11(0.023)	$4.7x10^{-7}$	0.36		0.19	-0.11(0.040)	0.0077	0.30		-0.11(0.020)	$1.2x10^{-8}$	0.90
rs10734234	T,C	baseline		0.90	-0.14(0.032)	$1.2x10^{-5}$	0.27		0.90	-0.17(0.059)	0.0048	0.35		-0.15(0.028)	$2.0x10^{-7}$	0.71
rs10734234	T,C	adj		0.90	-0.14(0.032)	$1.2x10^{-5}$	0.27		0.90	-0.17(0.059)	0.0039	0.36		-0.15(0.028)	$1.6x10^{-7}$	0.68
rs11176749	T,A	baseline		0.11	-0.12(0.027)	$1.6x10^{-5}$	0.26		0.11	-0.13(0.050)	0.012	0.27		-0.12(0.024)	$5.6x10^{-7}$	0.88
rs11176749	T,A	adj		0.11	-0.12(0.027)	$1.6x10^{-5}$	0.26		0.11	-0.12(0.050)	0.012	0.26		-0.12(0.024)	$6.2x10^{-7}$	0.91
rs1654584	G,T	baseline		0.23	-0.08(0.021)	0.00011	0.21		0.23	-0.12(0.038)	0.0022	0.42		-0.089(0.018)	$8.8x10^{-7}$	0.36
rs1654584	G,T	adj		0.23	-0.08(0.021)	0.00011	0.21		0.23	-0.12(0.038)	0.0022	0.42		-0.089(0.018)	$8.9x10^{-7}$	0.36

Adjustment of lead signals for potential covariates are shown for the discovery (ALSPAC), follow-up (GenR) and inverse-variance fixed effect meta-analysis. Beta coefficients represent the change in rank-transformed score per effect allele from linear regression of the score on allele dosage (R/Stata software). Covariate details are described in Supplementary Data 1. An increase in signal after adjustment for covariates is indicated in bold.

Baseline - Baseline models: Expressive vocabulary scores were adjusted for sex, age, age squared and the most significant principal components in each cohort before rank-transformation, Adj - Adjusted models: As baseline models with additional adjustment for the covariate;. E – Effect allele, A – Alternative allele, EAF – Effect allele frequency, p_{het} – Heterogeneity *p*-value based on Cochran's Q-test, R^2 – Adjusted regression R^2 in %

Table S6. Association between lead association signals (15-18 months) and potential covariates

		Discovery			Follow-up			Meta-analysis			
Covariate: Gestational age											
SNP	E/A	N	Beta(SE)	*p*	N	Beta(SE)	*p*	N	Beta(SE)	*p*	p_{het}
rs7642482	G,A	7877	0.082(0.038)	0.028	2649	0.074(0.054)	0.17	10526	0.078(0.031)	0.0096	0.90
rs10734234	T,C		-0.0046(0.054)	0.93		0.0065(0.079)	0.93		-0.0011(0.045)	0.98	0.91
rs11176749	T,A		0.042(0.046)	0.36		0.068(0.067)	0.31		0.050(0.038)	0.18	0.74
rs1654584	G,T		0.012(0.034)	0.73		0.050(0.05)	0.32		0.024(0.028)	0.40	0.52
Covariate: Maternal education											
SNP	E/A	N	OR(SE)	*p*	N	OR(SE)	*p*	N	OR(SE)	*p*	p_{het}
rs7642482	G,A	7407	0.98(0.049)	0.75	2596	1.01(0.017)	0.49	10003	1.01(0.016)	0.59	0.60
rs10734234	T,C		0.96(0.068)	0.55		0.98(0.025)	0.32		0.97(0.023)	0.25	0.82
rs11176749	T,A		1.03(0.061)	0.63		1.01(0.021)	0.75		1.01(0.02)	0.64	0.73
rs1654584	G,T		0.98(0.044)	0.60		1.01(0.016)	0.36		1.01(0.015)	0.49	0.42

Association between potential covariates and lead signals for rank-transformed CDI expressive vocabulary scores between 15-18 months of age. Results are shown for the discovery (ALSPAC), follow-up (GenR) and inverse-variance fixed effect meta-analysis. Beta coefficients represent the change in gestational age (weeks) per effect allele from linear regression of gestational age on allele dosage, adjusted for sex and the most significant principal components in each cohort. Odds ratios (OR) represent the odds of having lower compared to higher maternal education per effect allele from logistic regression of maternal education (low=1, high=0) on allele dosage, adjusted for the most significant principal components in each cohort. Covariate details are described in Supplementary Data 1. E – Effect allele, A – Alternative allele, EAF – Effect allele frequency, p_{het} – Heterogeneity *p*-value based on Cochran's Q-test

Table S7. Association between lead association signals (15-18 months) and first single-word utterances (12 months)

SNP	E/A	EAF	OR(SE)	*p*
rs7642482	G/A	0.15	1.03(0.07)	0.68
rs10734234	T/C	0.91	1.08(0.13)	0.51
rs11176749	T/A	0.13	0.98(0.07)	0.76
rs1654584	G/T	0.22	0.96(0.06)	0.47

Expressive vocabulary was assessed as the number of words spoken at the age of 12 months In the NFBC1966. Odds ratios (OR) represent the odds of speaking one or more words compared with speaking no words per effect allele and were obtained from logistic regression of expressive vocabulary (1+ words (1) = 3856 children, 0 words (0) = 1113 children, N=4969) on allele dosage, adjusted for sex and the most significant principal components. E – Effect allele, A – Alternative allele, EAF – Effect allele frequency

Table S8. Language-related cognitive outcomes in later childhood

SNP	Mean score(SD) untransformed	Mean age at measurement (SD) in years
Phonological memory	7.26(2.51)	8.63(0.3)
Verbal intelligence	107.81(16.75)	8.64(0.31)
Reading speed (Words read per minute)	105.51(12.47)	9.89(0.32)
Reading comprehension	100.4(11.83)	9.89(0.32)

Sample descriptives for language-related cognitive outcomes in later childhood measured in ALSPAC. Phonological memory was assessed with 'The Children's Test of Nonword Repetition'[3], verbal intelligence quotient scores (Verbal IQ) with the' Wechsler Intelligence Scale for Children'[4,5], and reading speed ('words read per minute') as well as reading comprehension with the Neale analysis of reading ability test[6]. SD – Standard deviation

Table S9. Association between lead association signals (15-18 months) and language-related cognitive outcomes in later childhood

SNP	E/A	N	Beta(SE)	*p*
Phonological memory (8 years)				
rs7642482	G/A	5552	0.021(0.025)	0.39
rs10734234	T/C		0.011(0.036)	0.77
rs11176749	T/A		-0.034(0.030)	0.27
rs1654584	G/T		-0.018(0.023)	0.43
Verbal IQ (9 years)				
rs7642482	G/A	5540	-0.032(0.025)	0.20
rs10734234	T/C		-0.040(0.036)	0.26
rs11176749	T/A		-0.022(0.030)	0.48
rs1654584	G/T		-0.035(0.023)	0.12
Reading speed (Words read per min, 10 years)				
rs7642482	G/A	5275	-0.067(0.026)	0.0093
rs10734234	T/C		0.052(0.037)	0.16
rs11176749	T/A		-0.011(0.031)	0.72
rs1654584	G/T		-0.068(0.023)	0.0035
Reading comprehension (10 years)				
rs7642482	G/A	5287	-0.042(0.026)	0.10
rs10734234	T/C		0.004(0.042)	0.93
rs11176749	T/A		-0.012(0.031)	0.70
rs1654584	G/T		-0.051(0.023)	0.028

Sample descriptives for all cognitive measures are given in Supplementary Table 8. Beta coefficients represent the change in cognitive outcome (Z-standardised) per effect allele from linear regression of the cognitive outcome on allele dosage, adjusted for sex, the most significant principal components and age (except for age-normalised Verbal IQ scores). E – Effect allele, A – Alternative allele

Table S10. Twin analysis of expressive vocabulary scores (24 months)

Twin intra-class correlations		ACE model Variance components (95% CI)		
		A	C	E
MZ	0.94; N = 1969	0.20	0.73	0.07
DZ all	0.84; N = 3764	(0.19;0.22)	(0.72;0.75)	(0.06;0.07)

Expressive vocabulary was assessed with the MCDI at 24 months of age within TEDS. Twin analysis was conducted on 5,733 twin pairs using rank-transformed expressive vocabulary scores adjusted for age, age squared and sex. The best-fitting model (ACE) was chosen in comparison to a CE and AE model on the basis of model fit parameters (Supplementary Table 11).

MZ – Monozygotic twins, DZall – Dizygotic twins (male, female, opposite sex), N – Complete twin pairs, A – Additive genetic influence, C – Shared environmental influence, E – Non-shared environmental influence

Table S11. Model fit parameters for twin analysis

Model	-2LL	df	AIC	*p*(1)	*p*(2)
Saturated	24279.678	11504	1271.678	-	-
ACE*	24286.858	11507	1272.858	.07	-
CE	24749.167	11508	1733.167	<0.001	<0.001
AE	25972.380	11508	2956.380	<0.001	<0.001
E	32631.156	11509	9613.156	<0.001	<0.001

-2LL - Minus twice the log likelihood; df - Degrees of freedom; AIC - Akaike Information Criterion; p(1) - *P*-value of model fit compared to the saturated model; p(2) - *P*-value of model fit compared to the ACE model; * - Best-fitting model; A-Additive genetic influence; C-Shared environmental influence; E-Non-shared environmental influence.

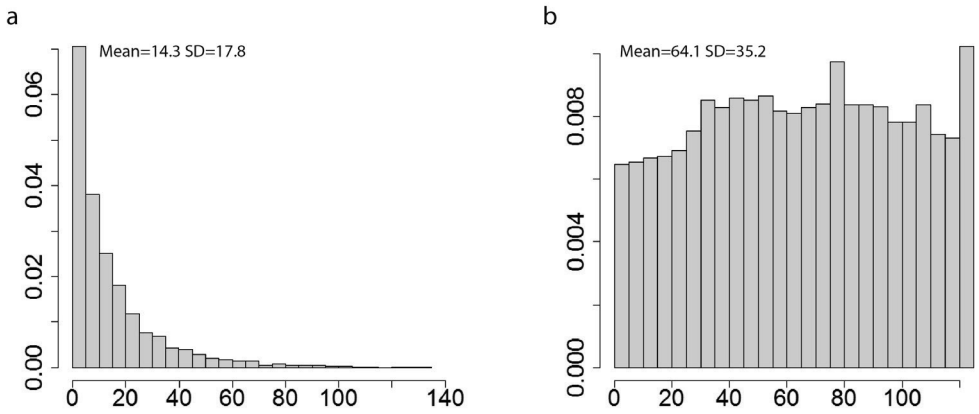

Figure S1. Phenotype distribution of expressive vocabulary in the discovery cohort. Measures were ascertained within ALSPAC at 15 months (a) and 24 months of age (b). Expressive vocabulary during the early phase was captured by an abbreviated version of the MacArthur CDI (Infant Version[1], 8-16 months of age), and vocabulary production during the later phase was measured with an abbreviated version of the MacArthur CDI (Toddler version, 16 to 30 months of age)[1,2]. Detailed phenotype descriptions are given in Supplementary Data 1.

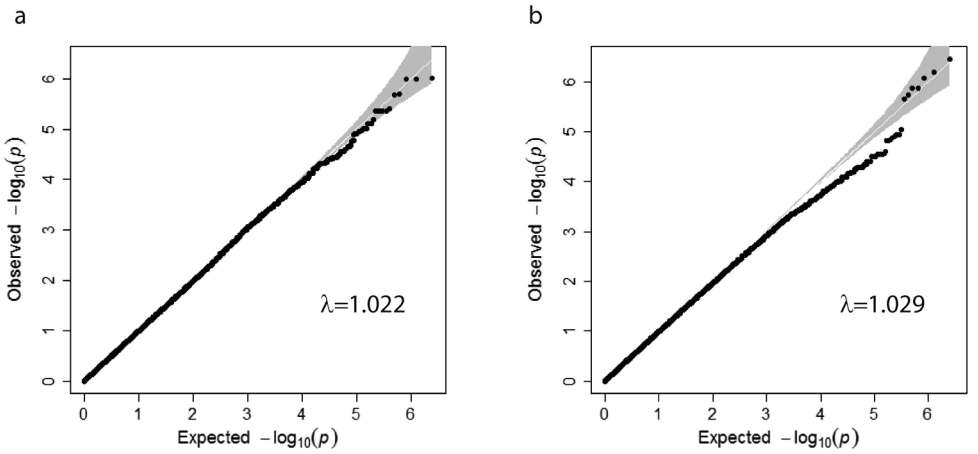

Figure S2. Quantile-quantile plot of genome-wide signals in the discovery cohort.
Genome-wide analysis (2,449,665 SNPs) within ALSPAC was carried out for an early (a, 15-18 months, N=6,851) and a later (b, 24-30 months, N=6,299) phase of language acquisition. Black circles depict the observed association signals (p-values), the white diagonal line represents the distribution of signals under the null hypothesis and the shaded area corresponds to the 95% confidence interval.
λ – Genomic-control factor

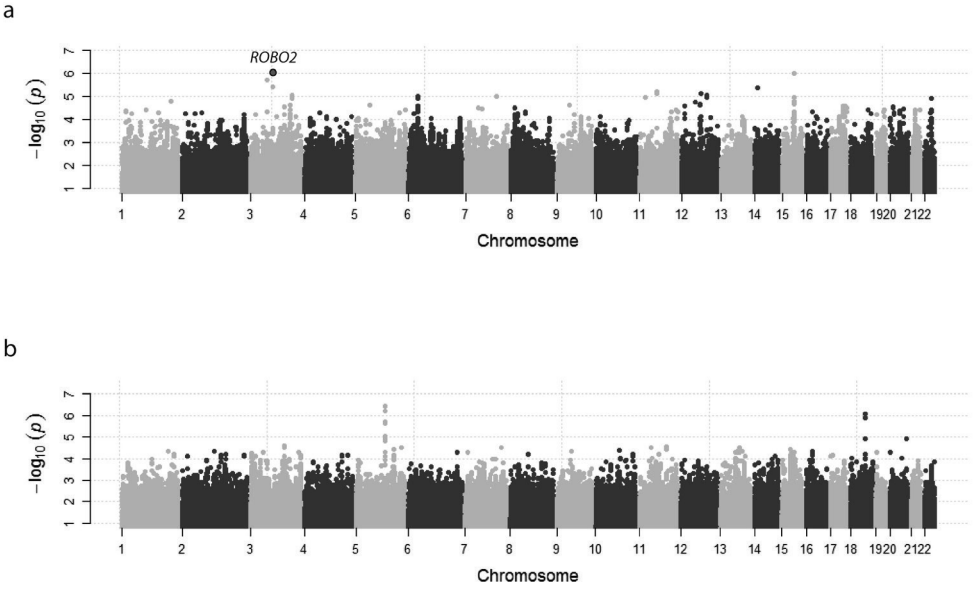

Figure S3. Manhattan plot of genome-wide signals in the discovery cohort.
Genome-wide analysis (2,449,665 SNPs) within ALSPAC was carried out for an early (a, 15-18 months, N=6,851) and a later (b, 24-30 months, N=6,299) phase of language acquisition. -log10 p-values are plotted against genomic position (hg18). Association signals with genome-significance in the meta-analysis of discovery and follow-up cohorts are shown in red.

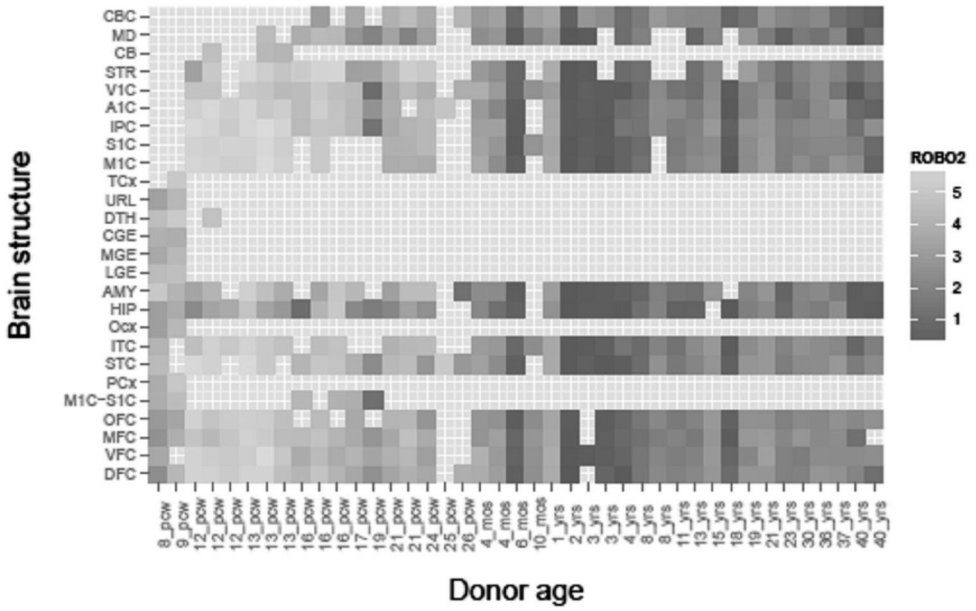

Figure S4. Developmental expression profile of *ROBO2* in brain.
The *ROBO2* mRNA expression profile (Brainspan, http://www.brainspan.org/) is reported as log2 RPKM (reads per kilobase per million) according to donor tissue and donor age (8 weeks of gestation – 41 years). DFC - dorsolateral prefrontal cortex, VFC - ventrolateral prefrontal cortex, MFC - anterior (rostral) cingulate (medial prefrontal) cortex, OFC - orbital frontal cortex, M1C-S1C - primary motor-sensory cortex (samples), PCx - parietal neocortex, STC - posterior (caudal) superior temporal cortex, ITC - inferolateral temporal cortex, Ocx - occipital neocortex, HIP - hippocampus (hippocampal formation), AMY - amygdaloid complex, LGE - lateral ganglionic eminence, MGE - medial ganglionic eminence, CGE- caudal ganglionic eminence, DTH - dorsal thalamus, URL - upper (rostral) rhombic lip, TCx - temporal neocortex, Ocx - occipital neocortex, M1C - primary motor cortex, S1C - primary somatosensory cortex, IPC - posteroventral (inferior) parietal cortex, A1C - primary auditory cortex, V1C - primary visual cortex, STR – striatum, CB – Cerebellum, MD - mediodorsal nucleus of thalamus, CBC - cerebellar cortex, pcw – week gestation, mos – months, yrs - years

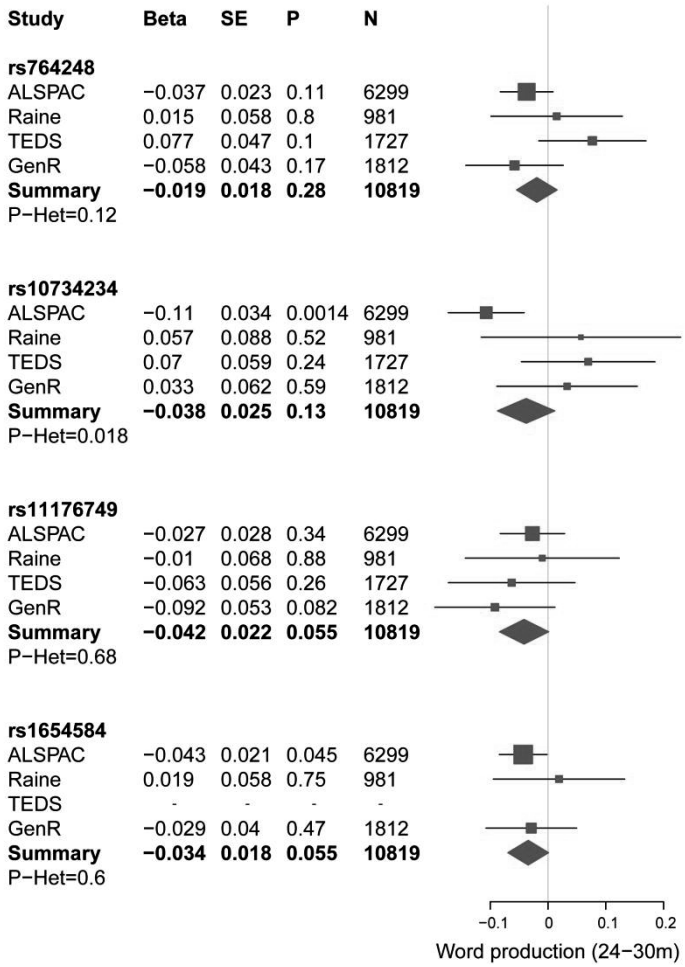

Figure S5. Association between lead association signals for early expressive vocabulary (15-18 months) and later expressive vocabulary scores (24-30 months).
Forest plots include results from ALSPAC (Genomic-control corrected), Raine, TEDS and GenR and an inverse-variance fixed effect meta-analysis of all cohorts. Beta coefficients represent the change in rank-transformed expressive vocabulary score (adjusted for sex, age, age squared and the most significant principal components in each cohort) per effect allele from weighted linear regression of the score on allele dosage. Effects are given with respect to the following effect alleles: rs7642482 (G), rs10734234 (T), rs11176749 (T) and rs1654584 (G).

SUPPLEMENTARY NOTES

Supplementary Note 1. Cohort description and study-specific ethical approval

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. 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 [7,8]. 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 (http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/). 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.

Generation R (GenR)

The Generation R Study is a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail elsewhere[9]. Typically, enrolment took place in early pregnancy. All children were born between April 2002 and January 2006, forming a prenatally enrolled birth-cohort that is now followed. The study was conducted in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (numbers: MEC 198.782/2001/31 (prenatal) and MEC 217.595/2002/202 (postnatal)). Written informed consent was obtained from all participants.

Northern Finnish Birth Cohort 1966 (NFBC 1966)

The Northern Finland Birth Cohort 1966 (NFBC1966)[10] were recruited through maternity health centres and data were collected from women living in Finland's two northernmost provinces, Oulu and Lapland, with expected deliveries between 1st January to 31st December 1966 (n=12,055 mothers). A total of 12,231 babies were born from the pregnancies, of which 12,058 were live births babies and 173 were

stillborn babies. Individuals born in NFBC1966 were found to be representative of all births in the area. All cohort members are Finns (white Caucasians), and less than 1% of these are Gypsies or Lapps. Birth outcomes were collected at delivery by trained medical staff and input into the medical records. The individuals were then followed-up with questionnaires from birth to ages at 1, 14 and clinical examination at 31 years, covering information on health, lifestyle and socio-economic indicators. Each participant or their parents gave written informed consent for the use of the data (Protocols approved by the Ethical Committee of the Northern Ostrobothnia Hospital District).

The Twins Early Development Study (TEDS)

The Twins Early Development Study (TEDS) is a large longitudinal sample of twins born in England and Wales between 1994 and 1996[11]. The focus of TEDS has been on cognitive and behavioral development, including difficulties in the context of normal development. TEDS began when multiple births were identified from birth records and the families were invited to take part in the study; 16,810 pairs of twins were originally enrolled in TEDS. More than 10,000 of these twin pairs remain enrolled in the study to date. DNA has been collected for more than 7,000 pairs, and genome-wide genotyping data for two million DNA markers are available for 3,500 individuals. The TEDS families have taken part in studies when the twins were aged 2, 3, 4, 7, 8, 9, 10, 12, 14, 16 and currently at 18 years of age. Ethical approval for each stage of TEDS has been obtained from the Institute of Psychiatry Ethics Committee (REC approval 05/Q0706/228), and informed consent was collected from the parents for each assessment.

Western Australian Pregnancy Cohort study (Raine)

The Western Australian Pregnancy Cohort study (Raine)[12] was started as a randomized controlled trial to evaluate the effects of repeated ultrasound in pregnant women in Perth, Western Australia. In total, 2,900 pregnant women were recruited between 1989 and 1991 prior to 18 weeks gestation at the King Edward Memorial Hospital (Perth, Western Australia). Women were randomized to repeated ultrasound measurements at 18, 24, 28, 34 and 38 weeks gestation or to a single ultrasound assessment at 18 weeks. Children have been assessed at average ages of 1, 2, 3, 5, 8, 10, 14 and 17 and both height and weight were collected at each assessment. The study was conducted with appropriate institutional ethics approval (Ethics approval number for DNA collection and storage: EC03-14.7 and EC06-29), and written informed consent was obtained from mothers at all follow-ups and participants at the year 17 follow-up.

Supplementary Note 2. URLs

ALSPAC, http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary
Brainspan, http://www.brainspan.org
EAGLE, http://research.lunenfeld.ca/eagle
Golden Path Genome Browser, http://genome.ucsc.edu
GWAVA http://www.sanger.ac.uk/sanger/StatGen_Gwava
HaploReg, v2 http://www.broadinstitute.org/mammals/haploreg/haploreg.php
HapMap, http://hapmap.ncbi.nlm.nih.gov
LocusZoom, http://csg.sph.umich.edu/locuszoom
PLINK, http://pngu.mgh.harvard.edu/~purcell/plink
OMIM, http://www.ncbi.nlm.nih.gov/omim
seeQTL, http://www.bios.unc.edu/research/genomic_software/seeQTL

Supplementary Note 3. Consortium membership

Wellcome Trust Case Control Consortium 2 (WTCCC2)

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A detailed overview of the participating cohorts, the working groups and the analytic committee of the EAGLE consortium can be found online (http://research.lunenfeld.ca/eagle/).

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CHAPTER 5

Maternal smoking during pregnancy and child emotional problems: the relevance of maternal and child 5-HTTLPR genotype

ABSTRACT

Background: Serotonin is involved in the development of neural circuits modulating emotional behavior. The short allele (s) of a polymorphism (5-HTTLPR) of the serotonin transporter gene is a risk factor for psychopathology in the presence of environmental stressors. Maternal smoking is associated with growth restriction of the human fetal brain and adverse effects of nicotine on the developing serotonin system have been documented. We hypothesized that maternal smoking interacts with both child and mother 5-HTTLPR genotype as a risk factor for later child emotional problems.

Methods: In a sample of n=1529 mother-child dyads, smoking habits were assessed by questionnaires during pregnancy. Child emotional problems were measured by the Child Behavior Checklist at the child's age of 3 years.

Results: Maternal smoking during pregnancy significantly increased the risk for emotional problems in children carrying the s-allele; ß=0.24, p=0.03 (mother-report) and ß=0.46, p=.001 (father-report). In children heterozygous at 5-HTTLPR and exposed to maternal prenatal smoking (n=79) risk of emotional problems increased with each additional s-allele the mother carried. The associations between 5-HTTLPR and child emotional problems were not moderated by paternal prenatal smoking.

Conclusions: These findings imply that the vulnerability for emotional problems in s-allele carriers may already originate in fetal life.

INTRODUCTION

Serotonin is an important neurotransmitter that modulates many brain functions including anxiety and mood (Murphy et al., 2008a). In addition to its role as a neurotransmitter, serotonin plays an important role in brain development, modulating processes such as neurogenesis and cell proliferation, migration and differentiation and synaptogenesis (Gaspar et al., 2003; Whitaker-Azmitia, 2001). As such, serotonin is critically involved in the development of brain circuits modulating emotional behavior (Gaspar et al., 2003; Gross & Hen, 2004). Rodent studies have shown that high levels of serotonin during early development disrupt the architecture and function of these circuits, having lasting negative effects on adult emotional behavior (Ansorge et al., 2008; Salichon et al., 2001).

The serotonin transporter is a key receptor protein involved in regulating synaptic serotonin levels. The gene encoding the serotonin transporter has been the focus of many candidate gene and gene by environment association studies. A functional polymorphism in the promoter region of this gene (5-HTTLPR) is known to influence the transcription efficiency of the gene; the short (s) allele of the 5-HTTLPR is found to be less active than the long (l) allele, resulting in decreased transcription of the serotonin transporter and subsequent higher levels of serotonin in the synaptic cleft (Lesch et al., 1996; Murphy & Lesch, 2008b).

Although there has been significant debate on the role of the 5-HTTLPR polymorphism (Risch et al., 2009) a recent meta-analyses by Karg et al provides relatively convincing evidence supporting the role of the s-allele of the 5-HTTLPR polymorphism in the association with higher levels of trait anxiety and with an increased risk for depressive disorders in interaction with environmental stress exposure (Karg et al., 2011).

In many of the prior papers, environmental exposure is broadly defined, including various stressors across the lifespan such as childhood maltreatment, life events, cardiovascular disease and Parkinson's disease. Recently, Pluess and colleagues (Pluess et al., 2010) extended current literature by providing the first evidence that the s-allele of the 5-HTTLPR also increases the vulnerability to stressful environments during fetal life. Within the Generation R Study, the authors assessed the relationship between the child's 5-HTTLPR status, prenatal maternal anxiety and child negative emotionality. They found that infants carrying the s-allele were more negatively emotional at the age of six months when their mothers reported anxiety during pregnancy. This finding could be explained by the fact that the effects of variation of the 5-HTTLPR system are active during fetal life and play an important role during brain development.

In the present study, also embedded in the Generation R sample, we tested the moderating effect of a classical environmental risk factor, maternal smoking during pregnancy, on the association between 5-HTTLPR and child emotional problems. We included maternal 5-HTTLPR next to the child's 5-HTTLPR to better understand whether maternal or child genes underlie the vulnerability of the child's brain to maternal smoking during pregnancy. Mice studies have indicated that serotonin present during early gestation is of maternal origin and determines a normal development of the offspring (Cote et al., 2007; Fligny et al., 2009). Therefore, emotional problems in the presence of maternal prenatal smoking could in theory also be explained by the maternal genotype rather than the child's 5-HTTLPR. The concept of a maternal (genetic) effect on child neurodevelopment is not without precedent: Variations of the maternal MTHFR gene have been associated with spina bifida of the child due to low folate levels (van der Put et al., 1997; van der Put et al., 1995).

Prenatal smoking exposure is an important risk factor for fetal growth restriction, including reduced growth of the fetal head and brain (Jaddoe et al., 2007; Roza et al., 2007; Vardavas et al., 2010). The few human studies that assessed the direct effect of prenatal smoking on the fetal brain reported an adverse effect of nicotine by affecting the expression of receptors involved in neurogenesis and synaptic functioning (Falk et al., 2005; Hellstrom-Lindahl et al., 2001). These findings are consistent with evidence from a large body of animal literature that reported that nicotine is a neuroteratogen causing cell damage, cell loss and impaired synaptic functioning. More specifically, animal studies have reported that nicotine exposure negatively affects the developing serotonin system throughout the brain, showing damage to serotonin projections to the cerebral cortex and the brainstem and by showing impairments in synaptic functioning of the serotonergic system (Muneoka et al., 1997; Slotkin et al., 2006a; Slotkin et al., 2006b; Xu et al., 2001).

Previous research assessing the moderating effect of maternal smoking on the association between genetic factors and child problem behavior mainly focused on behavioral problems such as aggression and ADHD-related behaviors, rather than emotional problems. Although findings from these studies are not entirely consistent, they support maternal smoking as a possible environmental factor in gene-environment interactions (GxE) explaining variation in child problem behavior. For example, Kahn and colleagues (2003) found that children carrying the 480 allele of DAT1 were at increased risk of oppositional and hyperactive symptoms in the presence of maternal prenatal smoking. While another study (Neuman et al., 2007) also reported a significant association between the child's DAT1 genotype and ADHD, they observed the findings for the other DAT1 allele (i.e. the 440 allele). Also,

the COMT genotype was found to interact with maternal smoking to predict youth aggressive behavior (Brennan et al., 2011).

Here we report on our examination of the moderating effect of maternal smoking during pregnancy on the association between 5-HTTLPR and emotional problems in a large population-based cohort of European descent, the Generation R Study. Data on 5-HTTLPR were available for both child and mother. Maternal smoking during pregnancy was prospectively assessed. To rule out that the effect of maternal smoking is due to confounding factors rather than an intra-uterine effect, we also included data on paternal smoking during pregnancy. Child emotional problems were reported by each parent using the Child Behavior Checklist allowing us to exclude possible maternal reporting bias. Child behavioral problems were also included as an outcome, to test for the specificity of any findings for 'emotional problems'.

METHODS

Setting

The study was conducted within the Generation R Study, a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail elsewhere (Jaddoe et al., 2010).Typically, enrolment took place in early pregnancy. All children were born between April 2002 and January 2006, forming a prenatally enrolled birth-cohort that is now followed. The study was conducted in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (numbers: MEC 198.782/2001/31 (prenatal) and MEC 217.595/2002/202 (postnatal)). Written informed consent was obtained from all participants.

Study population

Children were considered eligible for the current study if they were of genetically European descent (based on GWA data), if their mothers were enrolled during pregnancy, and if full consent for the postnatal phase of Generation R was obtained. A total of n=2307 children qualified. Of these, data on child and maternal 5-HTTLPR genotype was obtained for n=1857 children. Seven children were subsequently excluded because of missing data on maternal smoking habits during pregnancy. Of the remaining n=1850 children, maternal reports on child behavior at the child's age of three were obtained for n=1529 children. Of these, n=74 (5%) were siblings, but results did not meaningfully change when they were excluded.

Measures

5-HTTLPR genotyping

Both maternal and child 5-HTTLPR were included in the analyses.

Maternal DNA was derived from blood samples and child DNA was derived from cord blood samples at birth. The 43-base pair insertion/deletion in the promoter region of the 5HTT gene was genotyped using Taqman allelic discrimination. Upstream of the 5-HTTLPR promoter region, a single nucleotide polymorphism (rs25531) results in two functional variants of the L-allele: L_A and L_G. This polymorphism was genotyped only in the children. Primer sequences were taken from (Hu et al., 2006). Reactions were performed in a 384-wells format in a total volume of 5 ul containing 2 ng DNA, 120 nM FAM-probe, 80 nM VIC-probe, PCR primers (100 nM each), dimethyl sulfoxide (DMSO) (4% by volume), and 1 x genotyping master mix (Applied Biosystems Inc.). PCR cycling consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 96° C and annealing and extension for 90 seconds at 62.5° C. Signals were read with the Taqman 7900HT (Applied Biosystems Inc.) and analyzed using the sequence detection system 2.3 software (Applied Biosystems Inc.). To evaluate genotyping accuracy, 225 random child samples were genotyped a second time. No discrepancies were found.

Maternal smoking habits

Information on maternal smoking during pregnancy was prospectively obtained by three postal questionnaires during the first, second and third trimester of pregnancy. In each questionnaire was asked whether or not the mother smoked during the preceding three months. In the first questionnaire was specifically asked whether she quit smoking as soon as pregnancy was known. Maternal smoking was categorized into 'never smoked during pregnancy', 'quit smoking as soon as pregnancy was known', and 'smoked during pregnancy'.

Information on paternal smoking was obtained by the maternal questionnaire during the first trimester and by a partner-questionnaire during the second trimester of pregnancy. When partners did not complete the smoking question on the partner-questionnaire, we used maternal information on paternal smoking (13%).

Maternal and paternal smoking habits were significantly associated; of the mothers who smoked during pregnancy, 68% of the fathers smoked as well. Of the mothers who never smoked, only 32% of the fathers smoked (X^2=99.94, p<0.001).

Child emotional problems

Child emotional problems were assessed with the Child Behavior Checklist (CBCL/1,5-5) when the child was 3 years old. Mothers and fathers each filled out the parents-report questionnaire that contains 99 problem items rated on a 3-point

scale: 0 (not true), 1 (somewhat or sometimes true) and 2 (very true or often true). By summing the raw scores, seven syndrome scales (Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems and Aggressive Behavior) can be computed. Moreover, two broadband scales (Internalizing and Externalizing problems) can be derived. The Internalizing problem score, used in the current study to define emotional problems, is a summary score for the items on the first four syndrome scales. The Externalizing problem score, used in the current study to define behavioral problems, is a summary score for the items on the last two syndrome scales. Higher scores represent higher severity. Good reliability and validity have been reported for the Child Behavior Checklist (Achenbach & Rescorla, 2000).

Other covariates

Maternal age, maternal educational level and family income were determined at enrolment using a postal questionnaire. Educational level (highest education finished) was dichotomized into 'primary or secondary education' and 'higher education'. Family income was dichotomized into 'less than €2000' and 'more than € 2000'. Maternal alcohol use during pregnancy was obtained by three postal questionnaires during the first, second and third trimester of pregnancy. Alcohol use was categorized into 'never drank alcohol during pregnancy', 'quit as soon as pregnancy was known', and 'drank alcohol during pregnancy'. Maternal prenatal psychopathology was assessed during the second trimester of pregnancy with the Brief Symptoms Inventory (BSI), which is a self-report instrument. The BSI is a short version of the Symptom Checklist 90 (SCL-90) that covers a broad spectrum of psychiatric symptoms (Derogatis, 1993). Next to depressive and anxious symptoms, symptoms of interpersonal sensitivity and hostility are covered among other dimensions. Good validity and reliability are reported (de Beurs, 2004, 2009). For the purpose of this study the overall summary score, the Global Severity Index score (GSI score) was used. Child age was determined at the same time parents reported child behavior, using a postal questionnaire.

Statistical analyses

The 5-HTTLPR genotype was assessed as an additive trait; *ll*=0, *ls*=1 and *ss*=2. For the child 5-HTTLPR genotype, a further functional categorization based on the rs25531 polymorphism was possible: It has been shown that the L_G-allele is functionally different from the L_A-allele and S-allele (Hu et al., 2006). Therefore the L_A-allele was re-categorized as an S-allele. Again, the genotypes (5-HTTLPR + rs25531) were assessed as an additive trait; *l'l'*=0, *l's*=1 and *ss*=2, to test consistency of the findings including only 5-HTTLPR.

Because the data on child emotional problems were right skewed, we applied a square root transformation to fulfill the criterion of a normal distribution. As the data on maternal psychopathology were right-skewed as well, a natural log transformation was conducted to fulfill the criterion of a normal distribution.

Data were analyzed in several steps. Using linear regression models, we first assessed the main effects of maternal smoking and 5-HTTLPR on child emotional problems as reported by the mother. Second, we assessed whether the interactions between 5-HTTLPR and maternal smoking predicted child emotional problems as reported by the mother. To test consistency of the results and to guard against the possibility of a maternal rater bias, all models were repeated with father reports of child emotional problems as the outcome. To test the specificity of the results for 'emotional problems', all analyses were repeated including 'behavioral problems' as the outcome.

To assess whether maternal smoking has a causal, intra-uterine effect on child emotional problems and not a non-causal effect through many confounding factors such as socio-economic or behavior factors related both to maternal smoking and to child emotional problems, we repeated all analyses with paternal smoking as the dependent variable (Smith, 2008).

To exclude gene-environment correlations, we assessed whether there was an association between maternal 5-HTTLPR and maternal smoking using a multinomial logistic regression model, and whether there was an association between maternal 5-HTTLPR and maternal prenatal psychopathology using a linear regression model.

All analyses were adjusted for maternal educational level, maternal prenatal psychopathology, gender of the child, and child age at behavioral assessment which were determined a priori. Maternal age, family income and maternal alcohol use during pregnancy were also tested as possible confounding variables, but they did not change effect sizes meaningfully after inclusion of the other covariates (defined as more than 5%). Therefore, they were not included in the analyses.

There were missing values on maternal educational level (n=11, 0.7%), maternal psychopathology (n=135, 8.8%), family income (n=74, 4.8%), and maternal alcohol use (n=74, 4.8%). We used Multiple Imputation in SPSS 17 to impute the missing data. All test statistics and regression coefficients were averaged over 5 imputed datasets. We used an alpha of 0.05 to indicate statistical significance. All reported p – values are based on two-sided hypotheses. All statistical analyses were carried out using the Statistical Package for the Social Sciences, version 17.0 for Windows (SPSS, Inc. Chicago, Illinois).

Response analyses

The first group of non-respondents (n=450) consisted of children without data on the child's or maternal 5-HTTLPR genotype. Their mothers did not differ on smoking habits, symptoms-score of psychopathology and educational level compared to mothers of children included in the study. The children did not differ on emotional problems compared to the children included in the study.

The second group of non-respondents (n=321) consisted of children who were excluded because no data on emotional problems were available. Their mothers were more likely to smoke during pregnancy (24.6% vs 10.7%, X^2=47.61, p<0.001), reported more symptoms of psychopathology (0.18 vs 0.15, t=2.84, p=0.005) and were lower educated (51.8% vs 28.9%, X^2=60.89, p<0.001) than the mothers of children included in the study.

RESULTS

Descriptive statistics of the children and their mothers are presented in Table 1. Child and maternal 5-HTTLPR genotype distribution were both in Hardy Weinberg equilibrium (p=0.93, p=0.95 respectively). The vast majority of mothers (n=1236, 80.8%) never smoked during pregnancy. Approximately 8% (n=130) quit smoking as soon as pregnancy was known, and 11% (n=163) of the mothers continued to smoke during pregnancy.

The effects of maternal smoking and 5-HTTLPR on child emotional problems, as reported by the mother, are summarized in Table 2. Maternal smoking did not predict child emotional problems, neither did 5-HTTLPR. However, the interaction between maternal smoking and the child's 5-HTTLPR was significant in predicting child emotional problems; ß=0.24, 95% C.I. (0.02, 0.47), p=.03. The interaction between maternal smoking and maternal 5-HTTLPR was also significant in predicting child emotional problems; ß=0.25, 95% C.I. (0.03, 0.47), p=.03. We repeated the analyses additionally controlling for paternal smoking; the results remained the same (data not shown).

Table 1. Sample descriptives (n = 1,529)

	Percentage /mean (sd)
Mothers	
5-HTTLPR	
LL	32.3
LS	48.7
SS	19.0
Smoking	
Never smoked	80.8
Quit when pregnancy was known [a]	8.5
Smoked during pregnancy	10.7
Alcohol use	
Never drank alcohol	24.4
Quit when pregnancy was known [b]	15.4
Drank during pregnancy	60.2
Educational level	
No until secondary school	28.9
Higher	71.1
Income	
0 – 2000	12.0
>2000	88.0
Prenatal psychopathology [c]	0.15 (0.14)
% in clinical range [d]	2.2
Age, years	32.1 (3.76)
Children	
5-HTTLPR	
LL	33.5
LS	48.5
SS	18.0
Emotional problems [e]	1.80 (0.99)
% in borderline or clinical range [f]	3.7
Age at behavioral assessment, months	36.5 (1.1)
Gender	
Boy	50.2
Girl	49.8

[a] Mean (sd) weeks of gestation when pregnancy was known: 4.8 (1.5), range 1-10
[b] Mean (sd) weeks of gestation when pregnancy was known: 5.0 (1.4), range 1-10
[c] Range: 0 – 0.98
[d] Clinical range based on cut-off score of 0.71 based on the untransformed scores (de Beurs 2009)
[e] Range: 0 – 5.74
[f] Borderline / clinical range based on borderline cut-off of Dutch normative sample for Internalizing broadband scale (Tick et al., 2007).

Table 2. Main effects and interaction effects of maternal smoking and 5-HTTLPR on emotional problems

	Emotional problems					
	Maternal report (n=1529)			Father report (n=1348)		
	ß	95% C.I.	p	ß	95% C.I.	p
Main effects						
Smoking						
Never (reference)	0.00 (ref)	-	-	0.00 (ref)	-	-
Quit when pregnancy was known	-0.05	-0.22, 0.12	0.6	0.04	-0.16, 0.23	0.7
Smoking during pregnancy	0.01	-0.15, 0.17	0.9	-0.10	-0.29, 0.09	0.3
Child's 5-HTTLPR	-0.01	-0.07, 0.06	0.9	-0.01	-0.09, 0.07	0.9
Maternal 5-HTTLPR	-0.01	-0.07, 0.06	0.9	-0.02	-0.09, 0.06	0.7
Interaction effects						
Child's 5-HTTLPR * quit smoking	0.06	-0.19, 0.30	0.7	-0.14	-0.42, 0.14	0.3
Child's 5-HTTLPR * smoking during pregnancy	0.24	0.02, 0.47	0.03	0.46	0.18, 0.74	0.001
Maternal 5-HTTLPR * quit smoking	0.06	-0.19, 0.31	0.6	-0.25	-0.52, 0.02	0.07
Maternal 5-HTTLPR * smoking during pregnancy	0.25	0.03, 0.47	0.03	0.32	0.07, 0.57	0.01

Note: Values are Beta's (with 95% confidence intervals) from linear regression models, adjusted for maternal educational level, maternal psychopathology, child age at behavioral assessment and child gender. Maternal age, family income and maternal alcohol use during pregnancy were dropped as they did not change effect estimates meaningfully (>5%)

To test the consistency of the results, analyses were repeated with father reports of child emotional problems. Results are also summarized in Table 2. Again, there was no significant main effect of maternal smoking or of the 5-HTTLPR genotype on child emotional problems. Also consistent with the results when assessing mother reports of child emotional problems, there was a significant interaction between maternal smoking and 5-HTTLPR; ß=0.46, 95% C.I. (0.18, 0.74), p=.001 (child's genotype) and ß=0.34, 95% C.I. (0.07, 0.57), p=.01 (maternal genotype). The illustration of the interaction between maternal smoking and the child's 5-HTTLPR shows that there is a dose-response relationship; the more s-alleles the child carried, the higher the emotional problems score, see Figure 1.

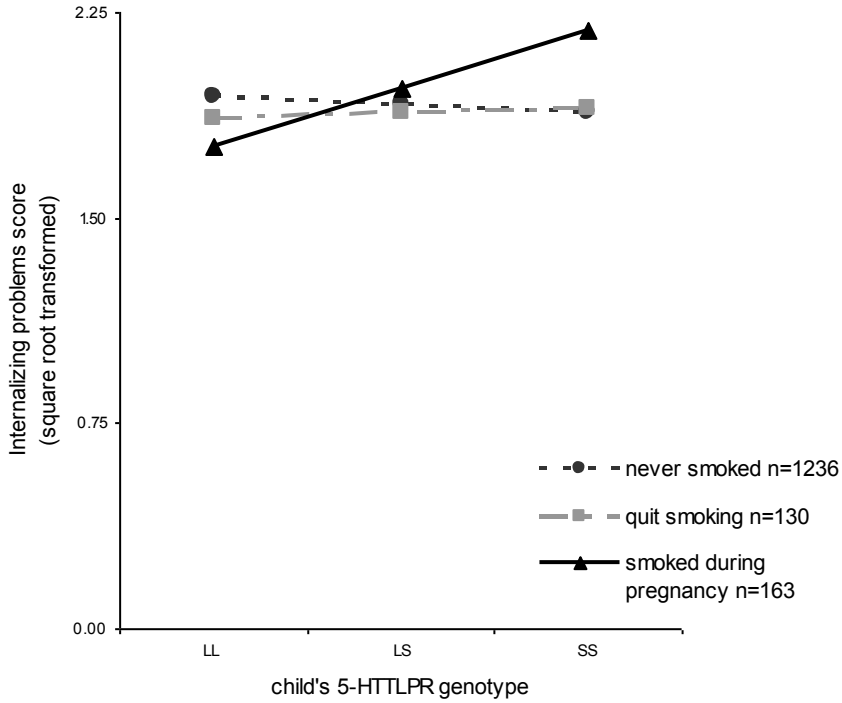

Figure 1. The effect of the child's 5-HTTLPR genotype by maternal smoking during pregnancy on child emotional problems.

To test the specificity of the results for emotional problems, analyses were repeated with behavioral problems as the outcome. There were no significant main effects of maternal smoking or 5-HTTLPR on child behavioral problems. Neither was the interaction between 5-HTTLPR and maternal smoking significant in predicting child behavioral problems (Supplementary Table S1.).

As maternal smoking during pregnancy moderated the effect of both the child's 5-HTTLPR and maternal 5-HTTLPR, the question remained to what extent the effect was influenced by the maternal genotype. Therefore, an additional linear regression analyses in the subgroup of children heterozygous at 5-HTTLPR and whose mothers continued to smoke during pregnancy (n=79) was performed. In this group of children, mothers could have the ll-genotype, as well as the ls-genotype or the ss-genotype which provided power-variation to detect any independent effect of the maternal genotype. Results showed that there was a significant effect of maternal genotype on child emotional problems; with each s-allele the mother carried, the risk of emotional problems increased with 0.30 points (ß=0.30, 95% C.I. (0.04, 0.56), p=.02). Results are presented in Figure 2.

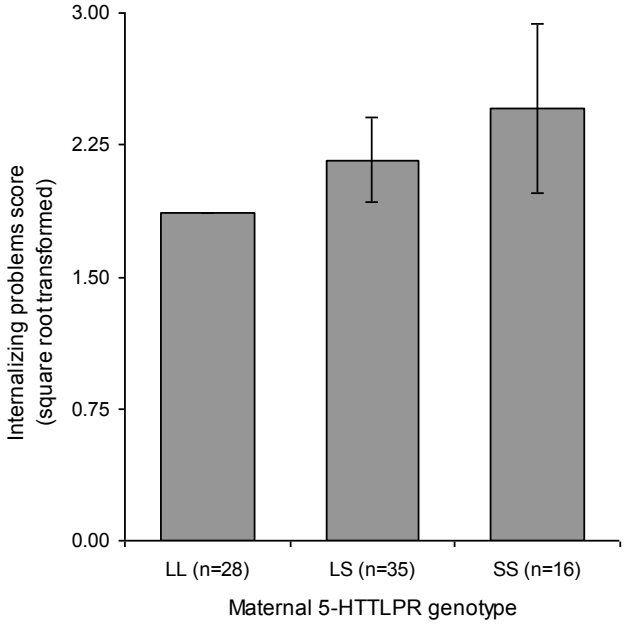

Figure 2. The additive effect of maternal 5-HTTLPR genotype on child emotional problems (n=79).
The linear regression analysis was restricted to the group of children with the ls-genotype whose mothers smoked during pregnancy (n=79). The model was adjusted for maternal educational level, maternal psychopathology, child age at behavioral assessment, and child gender. Maternal age, family income, and maternal alcohol use during pregnancy were dropped as they did not change effect estimates meaningfully (>5%).

To test the consistency of the findings including the 5-HTTLPR genotype, all analyses including the child's 5-HTTLPR were repeated with the 5-HTTLPR + rs25531 categorization. No discrepancies were found; results were essentially the same (see Supplementary Table S2.).

To confirm that the effects reported above are due to a causal, intra-uterine effect of maternal smoking and not to a non-causal effect through socio-economic, behavioral and genetic factors related to maternal smoking and child emotional problems, we reran all analyses using paternal smoking during pregnancy. Mothers who continued to smoke during pregnancy were excluded. If the effects reported are due to an intra-uterine effect, no moderating effect of paternal smoking during pregnancy on the association between 5-HTTLPR and child emotional problems should exist. Indeed, no moderating effect of paternal smoking was found (paternal smoking*child's 5-HTTLPR: ß=0.06, 95% C.I. (-0.09, 0.21), p=.4). Results are illustrated in Figure 3. We repeated the analyses with paternal smoking not excluding the mothers who smoked, but statistically controlling for maternal smoking during pregnancy. Results were essentially the same.

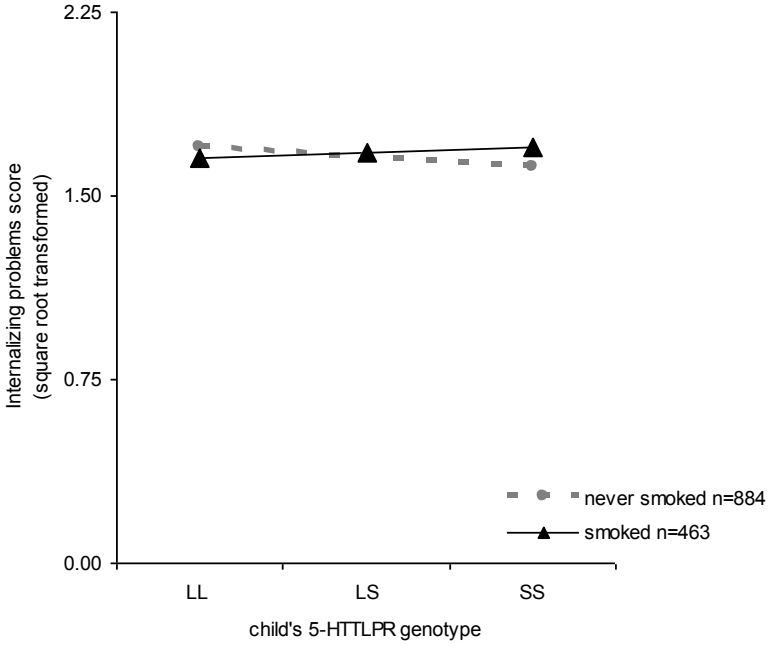

Figure 3. The effect of the child's 5-HTTLPR genotype by paternal smoking on child emotional problems (n=1,347).
Regression models with paternal smoking as a dependent variable were run to rule out that the effect of maternal smoking is due to confounding factors rather than an intra-uterine effect. Mothers who continued to smoking were excluded.

To exclude the possibility that the reported results are gene-environment correlations misinterpreted as a gene-environment interaction (Rutter et al., 2006), we assessed whether maternal 5-HTTLPR genotype predicted maternal smoking. This was not the case (data not shown). We also assessed whether maternal 5-HTTLPR genotype predicted maternal psychopathology, which was also not the case (data not shown).

DISCUSSION

The present study investigated the moderating effect of maternal smoking during pregnancy on the association between the 5-HTTLPR polymorphism and child emotional problems. As hypothesized, children carrying the 5-HTTLPR s-allele and whose mothers continued to smoke during pregnancy were at increased risk for emotional problems at the age of three years. The risk was greatest for children carrying two copies of the *s*-allele and intermediate for children carrying one copy of the s-allele. Similar results were found when we assessed maternal 5-HTTLPR instead of the child's 5-HTTLPR. Additional analyses in the group of children carrying the l/s-genotype and whose mothers smoked during pregnancy showed that the risk of emotional problems significantly increased with each s-allele the mother carried.

These findings shed new light on the understanding of why some children may have more emotional problems than others. Risk of emotional problems is elevated based on genetic vulnerability of both mother and child in interaction with maternal smoking during pregnancy. These data support the basic science findings that the effect of the 5-HTTLPR s-allele originates in neurodevelopment (Gaspar et al., 2003). These data are also consistent with previous research that implicated the s-allele as the risk allele for anxious and depressive behaviors in the presence of environmental stressors (Caspi et al., 2010; Karg et al., 2011; Uher & McGuffin, 2008).

By adjusting all analyses for maternal educational level, maternal psychopathology, maternal age, family income, and maternal drinking during pregnancy, our findings help reconcile the fact that the literature to date presents a mixed picture of the relations between maternal smoking and subsequent child problem behavior. Others have argued that it is not possible to directly relate maternal smoking during pregnancy to later child problem behavior due to the claim that there is evidence that the association is fully accounted for by socio-economical and behavioral factors such as maternal age, education and depression (Brion et al., 2010; Ernst et al., 2001). We have controlled for some of these potential factors in our work. Moreover, we repeated all our analyses with paternal smoking during pregnancy. Our results

indicate that there was not a moderating effect of paternal smoking during pregnancy on the association between 5-HTTLPR and emotional problems. In sum, our data support the hypothesis that the moderating effect of maternal smoking during pregnancy on the association between 5-HTTLPR and child emotional problems is an intra-uterine effect.

We also tested the confound that it was maternal genotype that led to the increased risk of child emotional problems. The finding that the interaction between the child's 5-HTTLPR and maternal smoking during pregnancy remained significant after adjusting for maternal psychopathology also suggests that the effect of the interaction is not likely due to shared environmental or genetic factors (i.e. heritability) in which mothers would pass on genes or environments that increase both the likelihood that she experiences emotional problems during pregnancy and that her child experiences emotional problems (Van den Bergh et al., 2005).

Perhaps most importantly, we controlled for maternal rating bias by also obtaining and analyzing father reports of child emotional problems. Our results clearly show that similar effects were found when child emotional problems were reported by fathers, demonstrating that maternal reporting bias is not a likely explanation for the reported findings.

Our data also are useful in consideration of the importance of 'quitting smoking'. No moderating effect on the association between 5-HTTLPR and emotional problems was found for mothers who quit smoking as soon as pregnancy was known. There are three sources of fetal smoking exposure: fetal skin absorption, fetal gastrointestinal reabsorption of urine, and maternal circulation. Skin absorption is possible from around 8 weeks of gestation and the latter two sources from around 10 weeks of gestation (Huppertz & Peeters, 2005; Jauniaux et al., 1999). The mean week that pregnancy was known in the mothers who quit smoking was 4.8 weeks of gestation, providing a possible explanation why no moderating effect in this group was found.

We observed that the maternal 5-HTTLPR has an independent, additional effect on child emotional problems. This was hypothesized as mice studies have indicated that serotonin present during early gestation is of maternal origin and needed for a normal development of the offspring's brain and other organs (Cote et al., 2007; Fligny et al., 2009). Moreover, a recent mice study showed that anxiety related behaviors in the offspring can be caused by a serotonin-receptor deficit in the mothers, independent of the offspring's own genotype (Gleason et al., 2010). Also, Halmøy and colleagues (2010) assessed human adult offspring of mothers with TPH1 deficiencies, leading to impaired serotonin production. They found that the offspring reported higher symptom scores of bipolar and attention-deficit/hyperactivity disorder as compared to controls. These seemingly inconsistent findings about the role of serotonin (i.e.

increased versus very low levels of serotonin) in early neurodevelopment are, however, in line with animal literature: Both mice with increased and depleted levels of serotonin during development show increased anxiety and aggression. This underscores the complexity of the serotonin system. The effects of the system on early brain development differ according to the developmental stage, the types of receptors assessed, and the localization of the receptors (Gaspar et al., 2003). Yet, the findings suggest that maternal serotonin, in interplay with other factors, moderates the intra-uterine environment in which the fetus develops. Our finding of an independent, additional effect of the maternal 5-HTTLPR on child emotional problems is compatible with a prenatal effect.

It should be noted that emotional and behavioral problems were rated using broadband scales. Furthermore problem behavior was assessed at the child's age of three years. Associations with other psychiatric disorders such as attention-deficit/hyperactivity disorder, bipolar disorder, and schizophrenia, cannot be established at this young age.

Although our study has notable strengths, including the large sample size and prospective data assessment, there are some limitations that also need to be considered. First as with many general population studies it is important to note that our findings are on mothers and children who were generally in the non-clinical range rather than on children who have severe psychopathology typically seen in a clinic. Second, there was selective attrition of children whose mothers were more likely to have smoked during pregnancy and had higher levels of psychopathology. This may have reduced our power to detect relationships due to these characteristics.

In conclusion, we showed that maternal smoking during pregnancy moderates the association between the 5-HTTLPR and emotional problems in three year old children; children carrying the s-allele and whose mothers smoked during pregnancy seem to be at increased risk for emotional problems. Also, the maternal 5-HTTLPR genotype had an additional, independent effect on child emotional problems. These findings imply that the vulnerability for emotional problems in s-allele carriers may already originate in fetal life. However, replication of our findings is warranted to substantiate the reported associations.

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SUPPLEMENTARY MATERIAL

Table S1. Main effects and interaction effects of maternal smoking and 5-HTTLPR on behavioral problems

	Behavioral problems					
	Maternal report (n=1529)			Father report (n=1348)		
	ß	95% C.I.	p	ß	95% C.I.	p
Main effects						
Smoking						
Never (reference)	0.00 (ref)	-	-	0.00 (ref)	-	-
Quit when pregnancy was known	-0.02	-0.28, 0.20	0.8	0.00	-0.22, 0.23	1.0
Smoking during pregnancy	0.15	-0.05, 0.34	0.1	0.80	-0.14, 0.30	0.5
Child's 5-HTTLPR	-0.02	-0.11, 0.06	0.6	-0.01	-0.10, 0.08	0.8
Maternal 5-HTTLPR	-0.04	-0.12, 0.05	0.4	-0.03	-0.12, 0.06	0.5
Interaction effects						
Child's 5-HTTLPR * quit smoking	-0.00	-0.30, 0.30	1.0	-0.08	-0.41, 0.24	0.6
Child's 5-HTTLPR * smoking during pregnancy	0.19	-0.08, 0.46	0.2	0.32	-0.00, 0.65	0.06
Maternal 5-HTTLPR * quit smoking	-0.03	-0.34, 0.27	0.8	-0.15	-0.46, 0.17	0.4
Maternal 5-HTTLPR * smoking during pregnancy	0.08	-0.19, 0.35	0.6	0.10	-0.19, 0.39	0.5

Note: Values are Beta's (with 95% confidence intervals) from linear regression models, adjusted for maternal educational level, maternal psychopathology, child age at behavioral assessment and child gender. Maternal age, family income and maternal alcohol use during pregnancy were dropped as they did not change effect estimates meaningfully (>5%)

Table S2. Main effects and interaction effects of maternal smoking and the child's 5-HTTLPR + rs25531 on emotional problems

	Emotional problems					
	Maternal report (n=1454)			Father report (n=1285)		
	ß	95% C.I.	p	ß	95% C.I.	p
Main effects						
Child's 5-HTTLPR	0.01	-0.07, 0.08	0.9	0.01	-0.07, 0.09	0.8
Child's 5-HTTLPR + rs25531	-0.02	-0.09, 0.06	0.7	0.03	-0.06, 0.11	0.5
Interaction effects						
Child's 5-HTTLPR * quit smoking	0.07	-0.19, 0.33	0.6	-0.15	-0.43, 0.14	0.3
Child's 5-HTTLPR + rs25531 * quit smoking	0.04	-0.21, 0.30	0.7	-0.08	-0.37, 0.20	0.6
Child's 5-HTTLPR * smoking during pregnancy	0.23	-0.00, 0.46	0.05	0.51	0.22, 0.81	0.001
Child's 5-HTTLPR + rs25531 * smoking during pregnancy	0.23	0.00, 0.46	0.05	0.54	0.26, 0.82	<0.001

Note: Values are Beta's (with 95% confidence intervals) from linear regression models, adjusted for maternal educational level, maternal psychopathology, child age at behavioral assessment and child gender. Maternal age, family income and maternal alcohol use during pregnancy were dropped as they did not change effect estimates meaningfully (>5%). Sample was restricted to children with available data on rs25531

CHAPTER 6

The COMT Val158Met polymorphism interacts with maternal harsh parenting to impact on child working memory

ABSTRACT

Background: The Met-allele of the COMT Val[158]Met polymorphism is associated with increased dopamine availability and better performance on cognitive tasks in contrast to the Val-allele. However, there are indications that under circumstances of a further rise in dopamine levels Met/Met carriers may perform less, and Val/Val carriers may improve on cognitive performance. A rise in dopamine levels may be induced by pharmacological agents, but also by exposure to stressful circumstances. Therefore, we hypothesized that COMT genotype interacts with exposure to harsh parenting to impact on working memory (WM) performance in children.

Methods: The study sample consisted of 1856 children of genetically Caucasian descent. For 882 of these, informative data on parental COMT genotypes was available. Mothers reported their level of harsh parenting on an adapted version of the Parent-Child Conflict Tactics Scale at the child's age of 3 years. Child WM was reported by the mother using the Behavior Rating Inventory of Executive Functioning for Preschoolers (BRIEF-P) at the child's age of 4 years. For a total of 499 children WM was observed using a computerized task. We first examined the interaction-effect of COMT and harsh parenting on WM using gene-association regression analyses. Next we tested consistency of the results using family-based association testing (FBAT) in the subsample with parental genotype data.

Results: Children with the Met/Met genotype consistently showed superior reported WM performance in the presence of low levels of maternal harsh parenting relative to Val/Val carriers, and less WM performance in the presence of high levels of harsh parenting.

Conclusions: Maternal harsh parenting may differentially impact on the association between COMT genotype and child WM as reported by the mother.

INTRODUCTION

Working memory (WM) is typically defined as the ability to maintain and manipulate information necessary for guiding goal-directed behaviors over short periods of time (Baddeley, 1992) and plays an essential role in psychosocial and academic functioning. WM is commonly ascribed to the prefrontal cortex (PFC) and frontostriatal networks. The functioning of these circuits critically depends on modulation of dopamine (Goldman-Rakic, 1996; Puig et al., 2014). The relationship between prefrontal dopamine levels and PFC function seems to be best described by an inverted U-shape, with intermediate levels of dopamine appearing to be optimal for PFC function, whereas both deficient and excessive levels of dopamine are related to impairments in PFC function, including WM performance (Arnsten, 2009; Goldman-Rakic et al., 2000).

Dopamine levels in the PFC are mainly regulated by the enzyme Catechol-O-methyltransferase (COMT) (Tunbridge et al., 2006). The COMT gene, encoding the enzyme COMT, contains a single nucleotide functional polymorphism that impacts on COMT's enzyme activity: The Met-allele is associated with a three to four times reduced COMT enzyme activity compared to the Val-allele (Lachman et al., 1996). The lower enzyme activity results in increased synaptic dopamine availability. It is thought that dopamine levels in individuals homozygous for the Met-allele are closest to the peak of the previously mentioned inverted U-curve, and thus correspond with optimal PFC function. Dopamine levels of Val/Val carriers are found on the up slope of the normal range, corresponding to sub-optimal PFC function (Mattay et al., 2003).

It has been demonstrated that through the administration of amphetamine, which increases synaptic dopamine, Met/Met carriers may exceed the peak of the inverted U-curve with their dopamine levels, and show stable or even decreased performance on WM or attention tasks. In contrast, Val/Val carriers show improved performance, presumably because they have a beneficial increase in dopamine levels due to amphetamine, thus moving closer towards the peak of the inverted U-curve (Hamidovic et al., 2010; Mattay et al., 2003). Next to pharmacologic agents, psychosocial stress is also known to increase prefrontal dopamine levels (Finlay et al., 1995; Lataster et al., 2011) and negatively influence PFC function (Arnsten, 2009). Against this background, it has been hypothesized that psychosocial stress differentially impacts on the association between COMT and cognitive function; Met/Met carriers may show no or worse performance and Val/Val carriers may show improved performance (Stein et al., 2006; Tunbridge et al., 2006). In line with that hypothesis, Buckert et al. (2012) found that after the induction of acute stress, by

simulating a job interview, adult Met homozygotes performed worse on a WM task than Val homozygotes.

An important example of psychosocial stress that may negatively impact on PFC function is harsh or abusive parenting (Cicchetti & Toth, 2005; McCrory et al., 2010). We located two studies that examined the moderating effect of childhood maltreatment on the association between COMT and PFC function in healthy adults (Goldberg et al., 2013; Green et al., 2014): In contrast to the aforementioned hypothesis, Goldberg et al. (2013) found that Val/Val carriers performed worse on a task of cognitive flexibility with increasing number of events of childhood maltreatment, whereas Met/Met carriers remained stable in task performance. However, Green et al. (2014) found that, in line with the above hypothesis, a history of childhood physical abuse interacted with COMT genotype such that schizophrenic Val-allele carriers showed improved executive functioning in the presence of abuse. They also found a main effect of Met-allele carriers on attention and immediate memory, with superior performance of Met-allele carriers relative to Val/Val homozygotes.

In the present study, we investigate the association between variation in COMT Val158Met and WM in the presence of harsh parenting, in a large sample of healthy four year olds. We hypothesized that children homozygous for the Met-allele would show better WM function in the presence of low levels of maternal harsh parenting compared to Val/Val carriers, but would show less WM function in the presence of high levels of harsh parenting. We tested the interaction between COMT genotype and harsh parenting on WM using two different statistical approaches: First, we examined the interaction effect of COMT and harsh parenting on WM in a sample of unrelated children of Caucasian descent using gene-association regression analyses. Next, we examined the consistency of the interaction-effect in a subsample of children and their parents using family-based association testing (FBAT). Information on reported harsh parenting was assessed approximately one year prior to the assessments of the outcome. WM was assessed by parent report and by a computerized task to test consistency of the results and to diminish reporter bias.

METHODS

Setting

The study was conducted within the Generation R Study, a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail elsewhere (Jaddoe et al., 2010). All children were born

between April 2002 and January 2006, forming a prenatally enrolled birth-cohort that is now followed. The study was conducted in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (numbers: MEC 198.782/2001/31 (prenatal) and MEC 217.595/2002/202 (postnatal)). Written informed consent was obtained from all participants.

Study population

Children were considered eligible for the current study if they were singletons of genetically Caucasian descent (based on GWA data) and if full consent for the postnatal phase of Generation R was obtained. A total of n = 2420 children qualified. Of these, data on maternal harsh parenting was obtained for 1987 (82%) children. A total of 131 (7%) children were subsequently excluded because of missing data on reported working memory (WM). Thus, the sample for gene-association analyses was compromised of a total of 1856 children. For 499 of these children, data on observed WM was available.

For the family based association tests (FBAT), 706 (38%) children were subsequently excluded from the sample for gene-association analyses because of missing data on parental COMT genotype. Of the remaining 1150 parent-child trio's, 268 (23%) trio's were excluded because of homozygosity of both parents for the COMT genotype, leaving a sample of n = 882 genetically Caucasian children and their parents for FBAT. For n = 276 trio's, data on observed WM was available.

Measures

COMT genotyping

Data on parental and child COMT (rs4680, A/G) were included in the analyses. Parental DNA was derived from blood samples, child DNA was derived from cord blood samples at birth.

Genotyping of the COMT polymorphism in the parents was performed using Taqman allelic discrimination assay (Life Technologies). Signals were measured with the Taqman 7900HT Sequence Detection System (Life Technologies).

Data on the child's COMT genotype was extracted from a genome wide association dataset. Genome wide data were available for n = 5.809 samples. Of those, 314 samples were genotyped using the Human 660 Quad Arrays of Illumina, and 5495 samples were genotyped using the Human 610 Quad Arrays of Illumina. Samples were checked for duplicates, call rate (> 97.5%), genotyping of SNPs (> 0.05), and minor allele frequency (MAF) (> 0.001). After merging of the two datasets, SNPs were tested for HWE (p <= 1e-07), missingness, sample call rate (>95%), heterozygosity

and homozygosity. Also, a gender check was performed. The GWA dataset was imputed using MACH2QTL software. The imputation accuracy for the SNP used in the current study was excellent: MACH R^2 (rs4680) = 0.997. The data on rs4680 was extracted from the GWA dataset using Plink Best Guess.

Harsh parenting

Harsh parenting was assessed using an adapted version of the Parent-Child Conflict Tactics Scale (CTS-PC) (Straus et al., 1998) when the child was three years old. Mothers rated their use of disciplining practices during the past two weeks on a 6-point scale ranging from 'never' (0) to 'five times or more' (5). Higher scores reflect a higher incidence of harsh parenting practices. Factor analysis of this adapted CTS-PC has yielded a harsh discipline construct consisting of six items showing good psychometric properties (Jansen et al., 2012).

Child working memory

Reported working memory (WM) was assessed using the Behavior Rating Inventory of Executive Function for Preschoolers (BRIEF-P) when the child was four years old (Gioia et al., 2003). The inventory is a caregiver – reported questionnaire that assesses problems in executive functioning (EF) behaviors in young children (2 to 5 years). The BRIEF-P consists of 63 items in five related, but non-overlapping scales that measure children's ability in five different aspects of EF including working memory. Parents were asked to rate how often a particular behavior of the child was problematic in the preceding month on a three-point scale (never, sometimes, often). A score is derived by adding the scores of the respective scale. The raw scores yield T-scores based on age and gender. Higher scores indicate more problems. The content validity, and internal consistency of the BRIEF-P are adequate, and the scales show adequate to high test–retest reliability (Sherman & Brooks, 2010). For the purpose of the current study, the score on the WM scale was used that assesses the ability to hold information in mind for the purpose of completing a task. As the distribution were skewed, scores were transformed with the natural logarithm to approach normality.

When the children were approximately 52 months old we evaluated their WM during a home visit by assessing the ability to remember colors in order of presentation on a computer screen. Children were first tested to ensure they could recognize and name the task colors. The backward memory test had a demonstration trial and eight test trials (four sections, each section repeated twice). In the demonstration trial, the child was first shown two circles of different colors, and then asked to identify these two colors on a five-color wheel. If the child passed the demonstration trial

twice, the test trials were started. The total test trial consisted of four memory trials of increasing difficulty, each repeated twice. In the first trial, the child was shown two circles of different colors, and then asked to identify the colors he or she saw, in order from last to first. In the second trial, the child was shown three circles of different colors, and so on. Thus, in the fourth trial, the child was shown five circles of different colors and asked to name the colors he or she saw, in order from last to first. If the child failed to answer on a trial, they were given an extra trial of the same number of colors. The test was stopped if the child failed a trial twice. Therefore, the child could get a maximum of two errors per trial. The total backward memory error-score of a child could vary between 0 (if the child succeeded on all four trials either with or without extra trials) and 8 (if the child failed the first test trial of two colors twice).

Other covariates

Maternal age at intake, maternal educational level, family income, marital status, and parity were determined at enrolment using postal questionnaires. Educational level was dichotomized into 'primary or secondary education' and 'higher education'. Family income was dichotomized into 'less than €2000' and 'more than € 2000'. Marital status was dichotomized into 'living alone' and 'living together or married'. Parity was dichotomized into 'one child' and 'two children or more'.

Child's gestational age at birth and gender were obtained from community midwife and hospital registries at birth.

Maternal psychopathology and child problem behavior were assessed at the child's age of three years, using postal questionnaires. Maternal psychopathology was assessed with the Brief Symptoms Inventory (BSI), a short version of the Symptom Checklist 90 (SCL-90) (Derogatis, 1993). In this questionnaire, the depressive, anxious, interpersonal sensitivity, and hostility scales of the BSI were covered. Good validity and reliability are reported (de Beurs, 2004). The scores on the subscales were summed to derive a total problems score. Next, the scores were square-root transformed to approach a normal distribution. Child problem behavior was assessed with the Child Behavior Checklist (CBCL/1,5-5). Parents each filled out the questionnaire containing 99 problem items rated on a 3-point scale: 0 (not true), 1 (somewhat or sometimes true) and 2 (very true or often true). By summing the raw scores a total problems score can be derived. Higher scores represent greater severity. Good reliability and validity have been reported for the Child Behavior Checklist (Achenbach & Rescorla, 2000). We calculated the mean total problem scores of mothers and fathers. Next, the mean scores were square root transformed to approach a normal distribution.

Child age was determined using the postal questionnaire assessing child executive functioning and assessed during the home-visit when working memory was observed.

Statistical analyses

First, linear regression analyses were performed to assess the main and interacting effects of COMT and parental harsh parenting on mother reports of child working memory (WM), using the population-based sample of unrelated children. The COMT genotype was analyzed as an additive trait; MetMet (AA) = 0, ValMet (AG) = 1, and ValVal (GG) = 2. Using this model an r-fold increased effect was assumed for ValMet, and a 2r-increased effect for ValVal. To test consistency of the results, all analyses were repeated using child WM as assessed with a computerized task. All regression analyses were adjusted for maternal educational level, family income, maternal age at intake, marital status, parity, maternal psychopathology, child's gestational age, child age at assessment of outcome, child gender, and child problem behavior. Prior to inclusion in the regression equations, all continues covariates were mean centered. All regression equations including interaction effects also included the main effects of the respective variables. The analyses were carried out using SPSS, version 21.0 for Windows (SPSS, Inc. Chicago, Illinois). We used an alpha of 0.05 to indicate statistical significance.

To test consistency of the results, all analyses were repeated using family based association tests (FBAT). For this purpose, the subsample of parent-child trio's was used. The FBAT approach extents the traditional transmission disequilibrium test (TDT) with incorporating the ability to handle continuous outcomes and gene – environment interactions (Laird & Lange, 2006; Vansteelandt et al., 2008). The FBAT is based on the principal that if a specific allele is associated with a trait of interest, that allele is expected to be more often transmitted from parents to children with high levels of the trait compared to children with low levels of the trait. As the non-transmitted alleles of parents are the control alleles, FBAT is robust to underlying population stratification. Assuming an additive model, counting the number of Val (G) alleles, we first tested the main effect of COMT on child WM as reported by the mother. Second, we tested the interaction effect between COMT and harsh parenting on child WM. Again, analyses were repeated using observed child WM as the dependent variable. All FBAT were adjusted for the same covariates included in the linear regression analyses as only the genetic variant is robust to possible confounders and not the interacting environmental variable. We used an alpha of 0.05 to reject the null-hypothesis of no linkage and no association. The FBAT were conducted using the PBAT statistical package (version 3.61 Harvard School of Public Health, Departments of Biostatistics and Environmental Health,

Program for Population Genetics, Boston, MA, USA), which allows for analyzing continuous traits (Lange et al., 2004).

For significant interactions, the results were displayed in a figure. Regions of significance and simple slopes were estimated to provide further insight into the nature of the interaction effects. For estimating the regions of significance, the SPSS Macro 'PROCESS' was used (Hayes & Matthes, 2009).

To exclude gene-environment correlation, we assessed whether COMT genotype predicted maternal harsh parenting using a linear regression model.

We used Multiple Imputation in the Statistical Package for the Social Sciences (SPSS), version 21.0 for Windows (SPSS, Inc. Chicago, Illinois) to generate one imputed dataset of the total sample of n = 1856 children because of missing data on covariates. Next, the sample for FBAT analyses was created. We created one imputed dataset as both FBAT and PROCESS can't handle multiple imputed datasets. When re-analyzing the data using gene-association linear regression with 10 imputed sets, results did not change meaningfully (data not shown).

Response analyses

Non-respondents (i.e. children with missing data on reported working memory, n = 131) did not differ on level of harsh maternal parenting or on COMT genotype distribution compared to children included in the study. Neither did non-respondents differ from respondents on any of the other covariates included in the analyses, with the exception of maternal age at intake (39.74 vs 40.08, t=-8.04, p<0.001).

RESULTS

Descriptive statistics of the sample used for gene-association regression analyses, are presented in Table 1. Child and parental COMT genotype distributions were in Hardy Weinberg equilibrium. The vast majority of mothers was higher educated and only a small proportion received a lower family income.

We first report the results obtained using multivariate linear regressions in the population-based sample. The effects of COMT and maternal harsh parenting on child working memory (WM), as reported by the mother, are summarized in Table 2. The child's COMT genotype did not predict child working memory. Maternal harsh parenting predicted child WM; ß=0.01, 95% C.I. (0.00, 0.02), Beta=0.06, p=0.01. The interaction between the child's COMT genotype and maternal harsh parenting was significant in predicting reported child WM; ß=-0.02, 95% C.I. (-0.03,-0.00), Beta=-0.08, p=0.02.

Table 1. Sample descriptives (n = 1856)

	Mean (SD) / %
Parental characteristics	
Maternal COMT genotype, ValVal / ValMet / MetMet[$]	13.6 / 66.4 / 20.0
Paternal COMT genotype, ValVal / ValMet / MetMet[$]	13.3 / 66.9 / 19.8
Maternal harsh parenting	1.19 (0.73)
Maternal psychopathology	0.54 (0.49)
Maternal educational level, % lower	28.2
Family income, % lower	12.1
Maternal age at intake, in years	32.22 (3.78)
Maternal marital status, % living alone	3.5
Parity, % multiple children	39.7
Children's characteristics	
COMT genotype, ValVal/ ValMet / MetMet	20.7 / 49.5 / 29.8
Child's gestational age, in weeks	40.15 (1.50)
Child's gender, % boys	51.4
Child's problem behavior	4.13 (1.36)
Working memory problem score, questionnaire	3.82 (0.18)
Child's age at questionnaire assessment, in months	48.47 (0.98)
Backward error score, observed^	6.72 (1.30)
Child's age at observational assessment, in months^	51.35 (1.26)

[$] n = 822 (trio-subsample)
^ n = 499

To test consistency of the results, analyses were repeated using a measure of observed WM. Results are also summarized in Table 2. Effect sizes were consistently in the same direction as compared to the results for reported child WM, but far from significant in this small sample.

Next, we used FBAT to assess the effects of COMT and maternal harsh parenting on child WM in the trio-subsample. Results are presented in Table 3. Again, we found no main effect of COMT on child reported WM, or on WM assessed with a computerized task. However, consistent with the results using linear regression, we observed an interaction effect of COMT and maternal harsh parenting on reported child working memory (β_{inter} (se) = -0.03, se (0.01), FBAT-I p=0.01).

Table 2. Effects of COMT genotype and harsh parenting on working memory, results from gene - association regression analyses

	Working memory							
	Reported (n = 1856)				Observed (n = 499)			
	ß	(95% C.I.)	Beta	p-value	ß	(95% C.I.)	Beta	p-value
Main effects								
Child's COMT	0.00	(-0.01, 0.01)	0.004	0.8	-0.01	(-0.17, 0.15)	-0.01	0.9
Maternal harsh parenting	0.01	(0.00, 0.02)	0.06	0.01	0.16	(-0.01, 0.32)	0.09	0.07
Interaction effects								
Child's COMT x Maternal harsh parenting	-0.02	(-0.03, -0.00)	-0.08	0.02	-0.18	(-0.40, 0.05)	-0.12	0.1

Note: Values are Beta's (with 95% confidence intervals) from linear regression models.
An additive genotype model was used with number of Val (G) alleles coded.
All models were adjusted for maternal educational level, family income, maternal age at intake, marital status, parity, maternal psychopathology, child's gestational age, child age at assessment of outcome, child's gender, and child problem behavior.

Table 3. Effects of COMT genotype and harsh parenting on working memory, results from family based association tests (FBAT)

	Working memory			
	Reported (n = 882)		Observed (n = 276)	
	$ß_{inter}$ (se)	FBAT(-Interaction) p-value	$ß_{inter}$ (se)	FBAT(-Interaction) p-value
Main effects				
Child's COMT	n.a.	0.6	n.a.	0.7
Interaction effects				
Child's COMT x Maternal harsh parenting	-0.03 (0.01)	0.01	-0.16 (0.16)	0.3

Note: Values are Beta's (with standard errors) derived from FBAT statistics. Estimates are reported for the Val (G)-allele.
All models were adjusted for maternal educational level, family income, maternal age at intake, marital status, parity, maternal psychopathology, child's gestational age, child age at assessment of outcome, child's gender, and child problem behavior.
The p-value is based on the score test for a main effect or a gene-environment interaction

The illustration of the interaction between the child's COMT genotype and maternal harsh parenting on WM (see Figure 1) shows that there is a differential effect of maternal harsh parenting on the association between COMT and the child's WM problem score; Compared to Val homozygotes, Met homozygotes perform better on WM in the presence of low maternal harsh parenting, but perform worse on WM in the presence of high maternal harsh parenting. The lower and upper bounds of the regions of significance were -1.10 and 1.58 respectively (range harsh parenting current sample: -1.19 to 2.28). The simple slopes for the MetMet and ValMet genotypes were significant ($p<0.001$ and $p=0.02$), while the simple slope for the ValVal genotype was not ($p=0.6$).

Multivariate linear regressions showed that COMT genotype did not predict maternal harsh parenting ($p=0.8$).

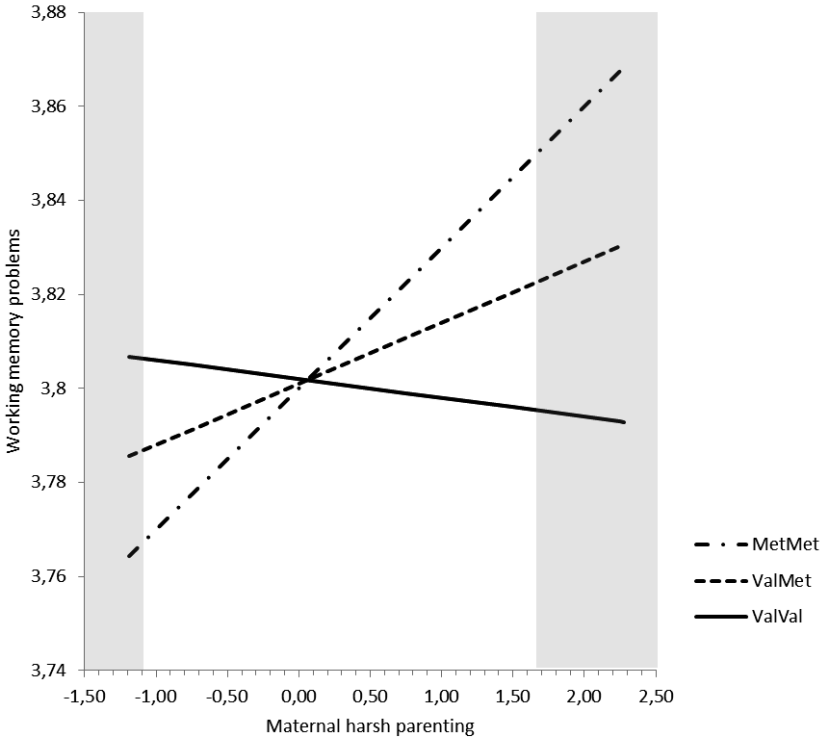

Figure 1. The associations between the child's COMT genotype, maternal harsh parenting, and working memory as reported by the mother.
Results based on multivariate gene – association regression analysis (n = 1856). The upper and lower boundaries of the regions of significance are indicated by the grey bars. The slopes for MetMet and ValMet were statistically significant at $p<0.001$ and $p=0.02$ respectively. The slope for ValVal was not significant ($p=0.6$).

DISCUSSION

The present study investigated the interacting effect of genetic variation in COMT Val158Met and maternal harsh parenting on child working memory (WM). Using population-based gene- association regression analyses and family based association testing (FBAT), we consistently found that four-year-old Caucasian children carrying the Met/Met genotype performed better on WM in the presence of low maternal harsh parenting, but performed worse on WM in the presence of high maternal harsh parenting in contrast to Val/Val carriers.

These findings are in part consistent with the hypothesis that psychosocial stress differentially impacts on the association between COMT and cognitive function, with Met/Met carriers showing worse performance on cognitive tasks in the presence of high stress and showing improved performance in the presence of low stress (Stein et al., 2006; Tunbridge et al., 2006). However, we did not find a significant improvement on WM performance for Val/Val carriers in the presence of high stress. Findings may be explained by a rise in dopamine levels as a result of stress exposure. Met/Met carriers exposed to stress may subsequently show a decline in WM because their dopamine levels exceed the optimum level for working memory performance, whereas Val/Val carriers may benefit from the increase in dopamine and show stable or improved WM performance (Hamidovic et al., 2010; Mattay et al., 2003).

The finding that Met-homozygotes may show better WM performance in circumstances of low harsh parenting, but may deteriorate on WM performance in the presence of high levels of maternal harsh parenting compared to Val homozygotes, is also in support of the genetic differential susceptibility hypothesis (Bakermans-Kranenburg & van IJzendoorn, 2015). This hypothesis proposes that children carrying a 'susceptible' genetic variant may disproportionally benefit from supportive environments and may deteriorate in adverse environments compared to children not carrying the susceptible variant. Dopamine-related genetic variants have been previously implicated as clear genetic susceptibility markers (Bakermans-Kranenburg & van Ijzendoorn, 2011). Also, other research contributes to the notion that the Met-allele may be a susceptibility marker for the effects of parenting practices on child cognitive and behavioral performance (Kok et al., 2013; Sulik et al., 2015).

Met-allele carriers have an advantage in cognitive paradigms relative to Val-allele carriers, but fMRI studies have also found that Met-allele carriers have a disadvantage in emotional paradigms (Mier et al., 2010). In line with the findings from fMRI studies, the Met-allele has also been associated with increased negative emotionality (Stein et al., 2005) and behavior problems (Albaugh et al., 2010). Conceivably, harsh parenting practices could be evoked by the problem behavior

associated with Met-allele status. This would imply that current findings are due to an evocative gene-environment correlation rather than an interaction. However, in the current analyses, we adjusted all analyses for problem behaviors as reported by each of the parents.

Although effect sizes were in the same direction, results were only found using parent reports of WM and not using a measure of WM obtained by a computerized task. Correlation between the scales of the BRIEF-P and performance based measures are positive, but very modest (Mahone & Hoffman, 2007). The BRIEF-P measures EF in a naturalistic setting and does not have the limitations of performance based tests and environmental effects during administration.

A major concern relating to the interpretation of the results of population-based gene – association studies is the possibility of false-positive findings due to underlying population stratification. Population stratification occurs when diversity in background subpopulations exist, resulting in different allele frequencies and different distributions of the trait under investigation. This may lead to spurious associations between the (candidate) gene and the trait (Cardon & Palmer, 2003). In our study we aimed at diminishing the risk of population stratification by using a stringent criterion for sample selection. We only considered children of genetically Caucasian descent as eligible. Moreover, we repeated all analyses using a family-based approach (FBAT). While less powerful than population-based approaches, family-based association testing (FBAT) is robust against confounding by population stratification because the non-transmitted parental allele is used as the control (Cardon & Palmer, 2003; Sillanpaa, 2011). Using FBAT, we found similar results compared to the results of the population-based gene-association tests, making underlying population stratification accounting for our results less likely. However, there are indications that when subpopulation specific exposure distribution (in our study maternal harsh parenting) is correlated with subpopulation specific allele frequencies, also FBAT analyses of gene by environment interactions remain vulnerable for Type I errors (Shi et al., 2011). Thus, replication of our results remains warranted before any firm conclusions can be drawn.

Our study has notable strengths, including prospective data assessment and the use of two different statistical approaches. However, there are some limitations that also need to be considered. First, it is important to note that our findings on behavioral and cognitive functioning (i.e. WM, harsh parenting) of mothers and children were generally in the non-clinical range and therefore lower than on children and mothers typically seen in a clinic. Second, harsh parenting is undoubtedly influenced by child behavior. Although we adjusted all analyses for child problem behavior, we cannot rule out that associations found in the current study are partly due to evocative gene-environment correlations rather than GxE.

In conclusion, these findings imply that the effects of COMT genetic variation on WM performance may be differentially impacted by maternal harsh parenting. Although replication is certainly warranted, these findings shed more light on why some children are disproportionally affected by harsh parenting and others are not as it comes to cognitive performance.

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CHAPTER 7

Variations in maternal 5-HTTLPR affect observed sensitive parenting

ABSTRACT

Background: Little is known about the genetic determinants of sensitive parenting. Two earlier studies examined the effect of the serotonin transporter polymorphism (5-HTTLPR) on sensitive parenting, but reported opposite results. In a large cohort we further examined whether 5-HTTLPR is a predictor of observed maternal sensitivity and whether observed child social fearfulness moderates the effect of 5-HTTLPR on maternal sensitivity.

Methods: The population-based cohort consisted of 767 mother-child dyads. Maternal sensitivity was repeatedly observed at the child's age of 14 months, 36 months and 48 months. Sensitivity was coded using the Ainsworth's rating scales for sensitivity and cooperation and the revised Erickson rating scales for Supportive presence and Intrusiveness. Child social fearfulness was observed using the Stranger Approach episode of the Laboratory Temperament Assessment Battery at 36 months.

Results: Repeated measurement analyses showed a consistent main effect of maternal 5-HTTLPR on sensitivity; mothers carrying the *S*-allele were more sensitive towards their children ($p = .005$). This effect was not explained by the child's 5-HTTLPR genotype. We found no evidence that child social fearfulness moderated the effect of 5-HTTLPR on sensitivity.

Conclusions: This study suggests that variations in maternal 5-HTTLPR genotype appear to be involved in the etiology of parenting behavior. The observed effects of this genetic variation are in line with the notion that parenting may have a genetic component, but large studies are needed to find the specific small molecular effects.

INTRODUCTION

Sensitive parenting is predictive of children's attachment security (Bakermans-Kranenburg et al., 2003), social problem solving (Raikes & Thompson, 2008), executive functioning (Bernier et al., 2010), and relationships with siblings and peers (McFarlane et al., 2010; Volling & Belsky, 1992).

Given the critical role of sensitive parenting in children's healthy development, a vast body of research has investigated the determinants of parenting. Belsky's (1984) widely cited process model of parenting distinguishes three main groups of determinants: parental characteristics, such as affective disorders and agreeableness (Bornstein et al., 2011; Clark et al., 2000), child characteristics such as negativity and difficult temperament (Mills-Koonce et al., 2007; Van den Boom, 1994; Vaughn et al., 2008), and contextual sources of stress and support in which the parent-child relationship is embedded.

While substantial genetic influences may also be involved in parenting (Collins et al., 2000; Neiderhiser et al., 2004; Plomin et al., 1994), molecular genetic determinants have been studied to a far lesser extent (Swain et al., 2007). In terms of Belsky's process model (1984), genetic factors may impact on parenting by their effects on parental and child characteristics.

In the current study we focus on the serotonin transporter polymorphism (5-HTTLPR), a polymorphic region in the promoter region of the serotonin transporter gene. In humans, 5-HTTLPR has two functional alleles, long (*L*) and short (*S*). The *S*-allele results in a decreased transcription of the serotonin transporter gene, and consequently in increased levels of serotonin in the synaptic cleft (Murphy & Lesch, 2008). Evidence shows that the S-allele is associated with higher levels of trait anxiety (Schinka et al., 2004; Sen et al., 2004), with selective attention to negative, threat-related stimuli (Pergamin-Hight et al., 2012), and with an increased risk of depressive disorders in the presence of environmental stress (Karg et al., 2011). Against this background, an association of the *S*-allele with less sensitive parenting may be hypothesized. However, the increased vulnerability of *S*-allele carriers for depressive symptomatology in the presence of stress also supports the increasing notion that the *S*-allele acts as a plasticity allele (Caspi et al., 2010). That is, the *S*-allele confers vulnerability to psychopathology in stressful environments, but confers an advantage in low-risk environments (Belsky & Beaver, 2011). Based on the enhanced sensitivity to the social environment of *S*-allele carriers, we hypothesize that mothers carrying the *S*-allele may be more able of providing sensitive parenting. Furthermore, there is accumulating evidence showing that the S-allele is related to improved decision making and cognitive flexibility (Borg et al., 2009; Homberg &

Lesch, 2011), and to social cognition (Canli & Lesch, 2007), which are fundamental components of parenting (Atkinson et al., 2009; Barrett & Fleming, 2011). This also provides initial support for the hypothesis that the S-allele may be positively associated with sensitive parenting. Because the S-allele was maintained throughout evolution in humans and rhesus macaques, it might be that positive effects of the allele offset negative ones (Homberg & Lesch, 2011).

Two previous studies focused on 5-HTTLPR and both found a direct effect of the polymorphism on observed sensitive parenting (Bakermans-Kranenburg & Van IJzendoorn, 2008; Mileva-Seitz et al., 2011). However, they reported opposite effects: In a sample of mothers with toddlers at high risk for behavioral problems, mothers carrying the *S*-allele had lower levels of sensitive parenting towards their toddlers (Bakermans-Kranenburg & Van IJzendoorn, 2008). In contrast, a general population-based study reported that mothers carrying the *S*-allele had higher levels of sensitive parenting (Mileva-Seitz et al., 2011). Moreover, Mileva-Seitz and colleagues (2011) found that mothers carrying the *S*-allele and reporting higher levels of early care quality, oriented away from the baby less frequently, which was positively associated with sensitivity.

In the current study we further examined the association between 5-HTTLPR and observed sensitive parenting. We used a four times larger sample than previous studies to increase power to detect any effect of 5-HTTLPR. Precision of the findings was improved by assessing maternal sensitivity repeatedly at three different time-points. We also assessed whether child social fearfulness moderated the effect of 5-HTTLPR on maternal sensitivity. It has been proposed that shy children are cognitively more challenged in new situations, eliciting maternal over involvement (Bates & Pettit, 2007). Also, previous research demonstrated that child characteristics such as shyness and approach withdrawal are associated with maternal intrusiveness and less warmth (Bates & Pettit, 2007; Brunk & Henggeler, 1984). Because social fear was previously associated with parenting, it is a good candidate factor (Moffitt et al., 2005). Additionally, we examined whether any associations between maternal 5-HTTLPR and sensitivity could be explained by the child's 5-HTTLPR genotype as maternal sensitivity includes reciprocal interactions between mother and child (Shin et al., 2008). Last, to test the specificity of any association between 5-HTTLPR and maternal sensitivity, we repeated all analyses with two other polymorphisms that have previously been examined in relation to sensitivity: the Val158Met polymorphism in the Catechol-O-Methyltransferase gene (COMT) and rs53576, a polymorphism in the oxytocin-receptor gene (OXTR).

METHODS

Setting

The study was embedded within the Generation R Study, a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail elsewhere (Jaddoe et al., 2010).

In a randomly assigned subgroup of Dutch pregnant women and their children, detailed assessments were conducted including observations of maternal sensitivity and child temperament. This subgroup is ethnically homogeneous to exclude confounding or effect modification by ethnicity. All children were born between February 2003 and August 2005 and form a prenatally enrolled birth-cohort. The study was conducted in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (numbers: prenatal, MEC 198.782/2001/31 and postnatal, MEC 217.595/2002/202). Written informed consent was obtained from all participants.

Study population

Mothers were considered eligible for the current study if they had singleton pregnancies and gave full consent for postnatal follow-up (n = 1079). Of these, data on 5-HTTLPR genotype was available for n = 919 mothers. Within this group, information on observed maternal sensitivity was available for n = 780 (85%) mothers. A total of n = *13* mothers participated with two siblings. In these cases, data from one of the siblings were randomly excluded so that each mother was included with only one child. Thus, the cohort for analysis compromised n = 767 mothers. Of these mothers, the majority (n = 584, 76%) participated in 2 or 3 assessments of sensitivity.

To study the main effect, information on all 767 mother-child dyads were included in the analyses. As for the 5-HTTLPR x child fearful temperament interaction-effect, data on 604 mother-child dyads with assessments of child fearful temperament was available.

Measures

5-HTTLPR genotyping

Maternal DNA was derived from blood samples at enrolment and child DNA was derived from cord blood samples at birth. The 43-base pair insertion/deletion in the promoter region of the 5-HTT gene was genotyped using Taqman allelic discrimination. Primer sequences were taken from Hu and colleagues (Hu et al.,

2006). Reactions were performed in a 384-wells format in a total volume of 5 ul containing 2 ng DNA, 120 nM FAM-probe, 80 nM VIC-probe, PCR primers (100 nM each), dimethyl sulfoxide (DMSO) (4% by volume), and 1 x genotyping master mix (Applied Biosystems Inc.). PCR cycling consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 96° C and annealing and extension for 90 seconds at 62.5° C. Signals were read with the Taqman 7900HT (Applied Biosystems Inc.) and analyzed using the sequence detection system 2.3 software (Applied Biosystems Inc.). To evaluate genotyping accuracy of 5-HTTLPR, 225 random child samples were genotyped a second time. No discrepancies were found.

Maternal sensitivity

During the lab visit at the child's age of 14 months, maternal sensitivity was observed during 5 minutes free play (SD=2.0). Maternal sensitivity was coded from DVD recordings with the Ainsworth's 9-point rating scales for sensitivity and cooperation (Ainsworth et al., 1974). The intraclass correlation (ICC) for intercoder agreement was .79 for sensitivity and .69 for cooperation (n = 24). Sensitivity and cooperation correlated strongly (r = .84). An overall 14-month sensitivity score was created by standardizing the two scores and computing the average.

During the lab visit at the child's age of 3 years and the home visit at age 4 years, maternal sensitivity was observed during two tasks that were too difficult for the child, considering his or her age: building a tower and etch-a-sketch. Mothers were instructed to help their child as usual. Maternal sensitivity was coded from DVD recordings with the revised Erickson 7-point rating scales for Supportive presence and Intrusiveness (Egeland et al., 1990). An overall sensitivity score was created by reversing the Intrusiveness scale, standardizing the scores, and computing the average across both scales and both tasks. The two tasks were independently coded by 13 and 10 extensively trained coders, respectively. At 3 years, average ICC's for the subscales were .75 for the tower task (n = 53) and .79 for the etch-a-sketch task (n = 55). At 4 years, average ICC's for the subscales were .85 for the tower task (n = 40) and .79 for the etch-a-sketch task (n = 40).

Overall, coders were trained in approximately 7 sessions and regularly supervised during the coding process; interreliability between coders was not only assessed directly after the training, but also monitored during the coding process to avoid rater drift. Coders were unaware which of their DVDs would be assigned to a second coder.

Child social fearfulness

Child social fearfulness was measured using the Stranger Approach (SA) episode of the Laboratory Temperament Assessment Battery Preschool Version (Lab-TAB) during the lab visit at 3 years of age (Goldsmith et al., 1999). The Lab-TAB is a widely used, standardized instrument for observational assessment of early temperament. During the SA episode the child has to deal with social fear when a novel, slightly threatening stranger approaches. The episode was modeled after real-life events: The child was left alone in a room. After 10 seconds a stranger entered the room and asked the child standard questions in a neutral tone of voice.

Episodes were coded from DVD recordings according to the coding system described in the Lab-TAB manual. Coders were blind to all other measures. Each episode was divided into nine epochs. Eight parameters were scored in each epoch: Intensity of fear expressions, distress vocalizations, activity decrease, approach, avoidance, gaze aversion, verbal hesitancy, and nervous fidgeting. For each parameter, average scores were calculated by dividing the child's overall score for that parameter across the 9 epochs. The mean intercoder agreement ICC for these average scores was .84 (n = 25). An overall 'fearfulness' score was created by taking the mean of the standardized average scores of the different parameters. This fearfulness score ranged from 0 to 1 with higher scores indicating a more social fearfulness.

Other covariates

Maternal age, educational level, marital status and parity were assessed using questionnaires at enrolment. Mothers were asked what their highest completed level of education was. Educational level was dichotomized into 'lower education' (primary school, lower or intermediate vocational education) and 'higher education' (higher vocational education or university).

At 20 weeks of pregnancy, family stress was assessed by a subscale, General Functioning, of the Family Assessment Device (FAD), which is a validated self-report measure of health or psychopathology of the family (Byles et al., 1988). The scores were square root transformed to approach a normal distribution. Maternal symptoms of psychopathology were assessed with the Brief Symptoms Inventory (BSI), a self-report instrument. The BSI is a short version of the Symptom Checklist 90 (SCL-90) (Derogatis, 1993). Good validity and reliability are reported (de Beurs, 2004, 2009). For the purpose of this study the overall summary score, the Global Severity Index score was used. This score covers a broad range of symptoms of psychopathology: Next to depressive and anxious symptoms, symptoms of interpersonal sensitivity and hostility are covered among other dimensions. The score was square root transformed to approach a normal distribution.

Amount of non-parental care was assessed using a questionnaire at the child's age of one year. Mothers were asked 'for how many hours per week is your child been taken care of by 1) a babysitter, 2) an au-pair, 3) a host-parent, 4) neighbors or family members, 5) daycare, or 6) some-one else ?'. The total hours of non-parental care per week was computed by summing the answers to the different items.

Statistical analyses

An additive model was used in the analyses with the 5-HTTLPR genotype, with LL=0, LS=1, and SS=2. Using this model an r-fold increased effect was assumed for LS, and a 2r-increased effect for SS. The genotypes were analyzed by the Armitage's test for trend (1 *DF*). The 5-HTTLPR genotype was also analyzed by a general genetic model. Using this model 5-HTTLPR was analyzed per genotype using dummy coding with the LL genotype as the reference group (2 *DF*).

Data were analyzed in three steps. We first assessed the main effect of maternal 5-HTTLPR on maternal sensitivity. To analyze the associations between the repeatedly measured sensitivity scores and 5-HTTLPR we used unbalanced repeated-measurements regression analysis. These regression models enable studies of repeatedly measured outcomes taking into account the correlation between measurements, and allowing for incomplete outcome data (Twisk, 2003). The covariance parameters were estimated using Restricted Maximum Likelihood (REML). We used unstructured covariance structures.

We also tested whether 5-HTTLPR interacts with child age, i.e. whether the development of maternal sensitivity over time differs between mothers with different alleles of 5-HTTLPR. However, as this term was not significant ($p = 0.5$) it was not further included in the models.

To test whether any effect of 5-HTTLPR on maternal sensitivity was driven by a specific time point, we examined the per time-point associations between 5-HTTLPR and maternal sensitivity using multivariate linear regression analyses.

Second, we tested whether the interaction between child social fearfulness and maternal 5-HTTLPR predicted maternal sensitivity. To this end, the fearfulness score was standardized. Again, unbalanced repeated-measurement regression analysis was used to test the repeated associations and multivariate linear regression analyses were performed to examine the per time-point associations.

Third, because maternal and child genotype are highly correlated, we choose a two-step approach to examine the role of the child's genotype in the association between 5-HTTLPR and sensitivity: We first reran all analyses using only the child's 5-HTTLPR genotype. This enabled us to test whether any effect of maternal 5-HTTLPR on sensitivity could not be explained by an effect of the child's genotype.

Second, we reran all analyses of the maternal 5-HTTLPR genotype, now additionally adjusted for the child's genotype if available (n = 624 out of n = 767). This allowed us to test whether the reported results for maternal 5-HTTLPR were independent of the child's genotype.

Bivariate correlations between the determinants, outcome and possible confounding covariates were assessed using Pearson correlations for continuous variables and Spearman's rho for categorical variables (see supplementary Table S1). All analyses were additionally adjusted for the hypothesized covariates.

To exclude gene-environment correlations, we assessed whether maternal or child 5-HTTLPR were correlated with child social fearfulness.

To test the specificity of our findings for 5-HTTLPR, the analyses testing the main effect of 5-HTTLPR and the interaction effect with social fearfulness were repeated using COMT and OXTR.

To exclude possible false-positive findings due to population heterogeneity, analyses were reran in a sample of n = 607 mother-child dyads of which the children were of genetically Caucasian descent based on GWA data.

We used Multiple Imputation in SPSS 17 to impute the missing data on covariates (family stress 6.9%, educational level 0.8%, parity 0.1%, psychopathology symptoms 5.6%). All test statistics and regression coefficients were averaged over 5 imputed datasets. We used an alpha of .05 to indicate statistical significance. All repeated measurements analyses were carried out using the Statistical Analysis System version 9.2 (SAS, Institute Inc.Gary NC, USA), including the PROC MIXED procedure for unbalanced repeated measurements. All per time-point analyses and correlations were carried out using the Statistical Package for the Social Sciences, version 17.0 for Windows (SPSS, Inc. Chicago, Illinois).

Response analyses

Non-respondents (i.e. mothers without any data on maternal sensitivity, n=139) did not differ on the distributions of 5-HTTLPR genotypes, parity, or family stress compared to mothers included in the study. Non-respondents were, however, lower educated than mothers included (43.6% vs 34.4%, X^2 = 4.22, p = .04). The children of non-respondents did not differ on social fearfulness compared to children of mothers included in the study.

Mothers included in the study (i.e. mothers participating in a subgroup of the Generation R Study) reported slightly less symptoms of psychopathology (0.36 (0.21) vs 0.38 (0.24), t = 2.41, p < .001) and less family stress (1.18 (0.16) vs 1.20 (0.17), t=3.68, p = .002) compared to Dutch mothers participating in the total sample of the Generation R Study. Also, mothers included in the study were higher educated

(65.5% vs 56.3%, $X^2 = 21.9$, $p < .001$) than Dutch mothers participating in the total study group of Generation R.

RESULTS

Descriptive statistics of the mothers and children are presented in Table 1. Maternal and child 5-HTTLPR genotype distribution were both in Hardy Weinberg equilibrium (p = .6 and p = .6, respectively). Correlations between predictor variables, maternal sensitivity, and covariates are presented in Supplementary material, Table S1.

The repeated measurement analyses showed that, overall, with each additional *S*-allele of the mother she was more sensitive towards her child (B = 0.11 (95% C.I. = 0.03, 0.18), p = .005) taking into account all covariates (see Table 2). Using a general genetic model we found that mothers carrying the *SS* and *SL* genotypes were more sensitive towards their children than mothers with the *LL* genotype.

The results of the individual per time-point analyses are summarized in Table 2. Maternal 5-HTTLPR was associated with maternal sensitivity at 14 months and with maternal sensitivity at 4 years. These associations remained significant after adjusting for all covariates. Although 5-HTTLPR did not predict maternal sensitivity at 3 years, the association was in the same direction as the associations observed at 14 months and 4 years, and was not significantly different from those associations.

The repeated measurements analysis showed no evidence for an interaction between 5-HTTLPR and child temperament in predicting maternal sensitivity; B = -0.08 (95% C.I. = -0.17, 0.01), p = .08 (see Table 3). Also, the per time-point analyses showed no evidence for a specific age-driven interaction effect between 5-HTTLPR and child temperament on sensitivity (see Table 3).

To test whether our results could not be explained by the child's 5-HTTLPR genotype, we first tested whether the child's genotype was associated with maternal sensitivity. Repeated measurements analyses showed that there was no main effect of the child's 5-HTTLPR on maternal sensitivity (B = 0.05 (95% C.I. = -0.03, 0.13), p = .2), see Supplementary Table S2. Next, we included both maternal and child's genotype as predictors of maternal sensitivity in the analyses. Results showed that maternal 5-HTTLPR genotype remained a significant predictor of sensitivity over and beyond the child's genotype: B = 0.12 (95% C.I. = 0.03, 0.21), p = .01. Also, we found no evidence for an interaction-effect between the child's 5-HTTLPR and child fearfulness on maternal sensitivity. Furthermore, no evidence for an interaction-effect between maternal and child's 5-HTTLPR was found (see Supplementary Table S2).

Table 1. Sample descriptives (n=767)

	Mean*	(SD)*
Mothers		
5-HTTLPR (%)		
LL (n=257)	33.5	
LS (n=371)	48.4	
SS (n=139)	18.1	
Sensitivity at 14 months, mean (range)[a]	0.0	(-4.16, 2.58)
Sensitivity at 36 months, mean (range)[b]	0.0	(-2.75, 2.86)
Sensitivity at 48 months, mean (range)[c]	0.0	(-2.56, 2.42)
Psychopathology symptoms	0.36	(0.21)
Family stress	1.18	(0.16)
Educational level (% lower)	34.5	
Parity (% nulli)	63.5	
Age at intake	31.8	(3.74)
Non-parental care, hours per week	16.0	(9.93)
Children		
5-HTTLPR (%)[d]		
LL (n=205)	26.7	
LS (n=295)	38.5	
SS (n=124)	16.2	
Child's social fearfulness, mean (range)[e]	0.0	(-2.72, 3.67)
Child's gender (% boys)	50.1	
Age at 14mo visit, months, median (95% range)	14.5	(13.4, 17.1)
Age at 3 years visit, months, median (95% range)	37.3	(35.5, 41.4)
Age at 4 years visit, months, median (95% range)	51.1	(49.8, 55.1)

* Unless otherwise indicated
[a] n = 537, [b] n = 574, [c] n = 524, [d] n = 624, [e] n = 604

To test the specificity of the findings for 5-HTTLPR, the analyses were repeated using COMT and OXTR. No main effects or interaction effects with social fear on maternal sensitivity were found (see Supplementary Table S3).

We found no evidence of possible confounding by ethnicity: using a subsample (n=607) of mother-child dyads of genetically Caucasian descent, effects of 5-HTTLPR on sensitivity remained essentially the same (B=0.13 (95% C.I. = 0.04, 0.21), p=.003).

Table 2. Associations between 5-HTTLPR and maternal sensitivity

	Maternal sensitivity (per SD)			
	Unadjusted Model		Adjusted Model	
	B (95% C.I.)	p	B (95% C.I.)	p
Repeated measurements analyses				
5-HTTLPR	0.12 (0.04, 0.19)	.003	0.11 (0.03, 0.18)	.005
5-HTTLPR (general model)				
LL	0.00 (ref)	-	0.00 (ref)	-
LS	0.16 (0.04, 0.29)	.008	0.17 (0.06, 0.29)	.004
SS	0.21 (0.06, 0.37)	.007	0.19 (0.04, 0.35)	.01
Per time-point analyses				
Sensitivity at 14 months (n=537)				
5-HTTLPR	0.13 (0.01, 0.25)	.04	0.12 (-0.00, 0.24)	.06
Sensitivity at 3 years (n=574)				
5-HTTLPR	0.08 (-0.04, 0.19)	.2	0.07 (-0.05, 0.18)	.3
Sensitivity at 4 years (n=524)				
5-HTTLPR	0.16 (0.04, 0.28)	.008	0.16 (0.04, 0.28)	.009

Note: The adjusted model was adjusted for psychopathology symptoms, family stress, maternal educational level, parity, age at intake, amount of non-parental care, and child's gender.
Unless otherwise specified, additive models were used.

Table 3. The moderating effects of social fearfulness on the association between 5-HTTLPR and maternal sensitivity

	Maternal sensitivity (per SD)			
	Unadjusted Model		Adjusted Model	
	B (95% C.I.)	p		
Repeated measurements analyses				
social fearfulness x 5-HTTLPR	-0.08 (-0.18, 0.02)	.1	-0.08 (-0.17, 0.01)	.08
Per time-point analyses				
Sensitivity at 36 months (n=532)				
social fearfulness x 5-HTTLPR	-0.08 (-0.20, 0.04)	.2	-0.08 (-0.20, 0.03)	.2
Sensitivity at 48 months (n=453)				
social fearful x 5-HTTLPR	-0.07 (-0.19, 0.06)	.3	-0.08 (-0.20, 0.05)	.2

Note: The adjusted model was adjusted for psychopathology symptoms, family stress, maternal educational level, parity, age at intake, amount of non-parental care, and child's gender.
Furthermore, all models included the main effects of social fearfulness and 5-HTTLPR.
Unless otherwise specified, additive models were used.

DISCUSSION

The present study investigated the effect of 5-HTTLPR on maternal sensitivity in a large population-based sample of mother-child dyads, using repeated measurements of sensitivity at different ages of the child. Mothers carrying *S*-alleles showed more sensitive behavior towards their children than mothers carrying *L*-alleles. No evidence for a moderating effect of child social fearfulness on the association between 5-HTTLPR and maternal sensitivity was found.

The findings of a direct effect of 5-HTTLPR on maternal sensitivity are in line with the observations of Mileva-Seitz and colleagues (2011) who also found that the *S*-allele was associated with more sensitive parenting. The 5-HTTLPR polymorphism may exert its influence on parenting through its associations with maternal characteristics because the 5-HTTLPR polymorphism is associated with various aspects of cognitive functioning. Both rodent and human studies have suggested that *S*-allele carriers show improved cognitive functioning on a variety of tasks including cognitive flexibility, reversal learning, attention, and inhibition (Brigman et al., 2010; Homberg & Lesch, 2011; Jedema et al., 2010). Especially cognitive flexibility and attention are important components of parenting behavior as sensitive parenting depends on the ability to accurately perceive children's signals and to respond to them in an adequate and prompt way (Ainsworth et al., 1978). For example, it has been shown that maternal attention deficit/hyperactivity disorder (ADHD) negatively impacts on maternal parenting practices (Chronis-Tuscano et al., 2008; Murray & Johnston, 2006). Also, poor working memory is predictive of observed reactive parenting (Deater-Deckard et al., 2010). Besides an effect on parenting via maternal characteristics the 5-HTTLPR polymorphism may also exert a direct influence on parenting through underlying neural and hormonal influences. Both oxytocin and vasopressin appear to be of major importance for understanding differences in parenting behavior across species (Galbally et al., 2011; Swain et al., 2007). The two hormones are secreted by the hypothalamic paraventricular nucleus (PVN) which is innervated by serotonergic fibers (Skuse & Gallagher, 2011). Furthermore, serotonin receptors are present in the PVN. Studies have indicated that through its receptors, serotonin influences the release of oxytocin and vasopressin (Jorgensen et al., 2003). Therefore, through its associations with the oxytocin and vasopressin systems, 5-HTTLPR may influence maternal sensitive parenting.

Children inherit genes of the mother associated with sensitive parenting. These inherited genetic variants may evoke certain parenting behaviors (evocative rGE) (Rutter & Silberg, 2002). We showed that no effect of child genotype on sensitive parenting was observed. Moreover, the effect of maternal 5-HTTLPR genotype on

sensitivity was driven by the maternal genotype beyond the child's genotype, thereby confirming the independent effect of the maternal genotype on maternal sensitivity.

However, as parents and children share the same genes associated with both parenting behavior and child outcome (passive rGE), parenting behaviors may also be a marker for genetic heritance rather than a causal factor for child development. Therefore, passive gene-environment correlation needs to be carefully looked at in future studies assessing associations between parenting styles and child outcome.

Next to strengths, our study also has some limitations: Our results may be somewhat biased due to the overrepresentation of higher educated mothers. Second, the Generation R Focus Study is a relatively homogenous population-based cohort that mainly consists of low risk families. While the homogeneity of the sample is advocated for validly testing genetic effects, results may be less generalizable to samples including high-risk families. Furthermore, we did not differentiate between L and Lg although Lg is considered a low expressing genotypic variant of the 5-HTTLPR polymorphism (Hu et al., 2006). However, in Caucasian samples the percentages of Lg have been found to be rather low (Zalsman et al., 2006).

In conclusion, we showed that the maternal 5-HTTLPR polymorphism most likely is associated with maternal sensitive parenting. This finding contributes to growing knowledge that parental behavior is a multifactorial concept. As noted by Swain and colleagues (2007), parenting can be viewed as an interaction among genes, past parenting, current experience, psychological state, neurobiological systems, and environmental constraints. Acknowledging and providing further insights into the multifactorial processes underlying parenting will provide a better understanding of parenting. In particular, investigation of possible mediators of the association between 5-HTTLPR and maternal sensitivity, such as cognitive flexibility and attention, may provide valuable insights into underlying biological pathways and provide further evidence for an association between 5-HTTLPR and parenting. Moreover, as for many complex traits it remains challenging to find and recognize true genetic associations. Therefore, replication of the current association between 5-HTTLPR and sensitive parenting remains warranted.

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SUPPLEMENTARY MATERIAL

Table S1. Correlations among the variables

	Variable	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Maternal 5-HTTLPR	-												
2	Child's 5-HTTLPR	.51***	-											
3	Sensitivity at 14mo	.08*	-.02	-										
4	Sensitivity at 3 year	.05	.07	.16***	-									
5	Sensitivity at 4 year	.11***	.07	.07	.32***	-								
6	Social fearfulness	-.03	-.01	.04	-.00	-.01	-							
7	Psychopathology symptoms	.03	.00	-.05	.00	.02	.01	-						
8	Family stress	-.01	-.01	-.11**	-.05	-.09**	.02	.33***	-					
9	Educational level (ref=higher)	-.03	-.03	-.07	-.17***	-.20***	.02	.07*	.13***	-				
10	Parity (ref=0)	.04	-.01	.02	.07*	.04	.05	-.08**	.03	.02	-			
11	Age at intake	.04	-.07*	.05	.04	.00	.02	-.10**	-.08**	-.24***	.35***	-		
12	Non-parental care	.03	.04	-.00	.07*	.03	.00	-.15***	-.14***	-.30***	-.00	.19***	-	
13	Child's gender (ref=girl)	.02	.04	-.09**	-.05	.00	.08**	.05	.09**	.05	.01	-.08**	-.06	-

*p<0.1, **p<0.05, ***p<0.01

Table S2. The role of maternal and child genotype in the associations between 5-HTTLPR, social fearfulness and maternal sensitivity.

	Maternal sensitivity (per SD)					
	Model 1		Model 2		Model 3	
	B (95% C.I.)	p	B (95% C.I.)	p	B (95% C.I.)	p
Repeated measurements analyses						
Main effects						
maternal 5-HTTLPR	0.12 (0.04, 0.20)	0.005			0.12 (0.03, 0.22)	0.01
child 5-HTTLPR			0.05 (-0.03, 0.12)	0.3	-0.01 (-0.11, 0.08)	0.8
Interaction effects						
social fearfulness x maternal 5-HTTLPR	-0.08 (-0.17, 0.01)	.08			-0.08 (-0.20, 0.04)	0.2
social fearfulness x child 5-HTTLPR			-0.07 (-0.18, 0.03)	0.1	-0.04 (-0.16, 0.09)	0.6
Maternal 5-HTTLPR x child 5-HTTLPR					0.003 (-0.16, 0.17)	0.9

Note: Due to restriction by availability of the child's genotype, a total of n = 624 mother-child dyads were included in the analyses.
Model 1 included only the maternal 5-HTTLPR, Model 2 included only the child's 5-HTTLPR, and Model 3 included both maternal and child 5-HTTLPR.
All models were adjusted for psychopathology symptoms, family stress, maternal educational level, parity, age at intake, amount of non-parental care, and child's gender.
All main effects were also included in the models testing interaction effects.

Table S3. Associations between COMT, OXTR and maternal sensitivity

	Maternal sensitivity (per SD)			
	Unadjusted Model		Adjusted Model	
	B (95% C.I.)	p	B (95% C.I.)	p
COMT	0.02 (-0.06, 0.10)	.6	0.02 (-0.06, 0.10)	.7
COMT (general model)				
ValVal	0.00 (ref)	-	0.00 (ref)	-
ValMet	0.03 (-0.10, 0.16)	.6	0.02 (-0.10, 0.15)	.7
MetMet	0.04 (-0.12, 0.20)	.6	0.03 (-0.13, 0.19)	.7
social fearfulness x COMT	0.01 (-0.10, 0.12)	0.9	0.00 (-0.10, 0.11)	.9
OXTR	-0.00 (-0.09, 0.09)	0.9	-0.01 (-0.10, 0.08)	.8
OXTR (general model)				
GG	0.00 (ref)	-	0.00 (ref)	-
GA	0.06 (-0.06, 0.18)	.3	0.05 (-0.07, 0.17)	.4
AA	-0.08 (-0.29, 0.12)	.4	-0.10 (-0.30, 0.10)	.3
social fearfulness x OXTR*				
social fearfulness x GA	0.07 (-0.09, 0.23)	.4	0.07 (-0.08, 0.22)	.8
social fearfulness x AA	0.01 (-0.14, 0.30)	.4	0.02 (-0.19, 0.23)	.4

Note: The adjusted model was adjusted for psychopathology symptoms, family stress, maternal educational level, parity, age at intake, amount of non-parental care, and child's gender. Furthermore, all models included the main effects of social fearfulness and 5-HTTLPR.
Unless otherwise specified, additive models were used.
*For the interaction between social fearfulness and OXTR a general genetic model was used as the association between OXTR and sensitivity was not linear.

CHAPTER 8

Grandparental divorce, parenting, and the risk of child psychopathology: a three-generational approach

ABSTRACT

Background: Parental divorce and marital discord are associated with an increased risk of offspring psychopathology and an increased likelihood of the offspring to also experience divorce or marital problems. According to the spill-over theory, ineffective parenting may mediate the associations between divorce, marital discord and offspring's development. Parenting practices have also been shown to be stable across generations. In this study we aimed to test a model bringing together the existing research on divorce and parenting to investigate intergenerational continuity of divorce and marital discord and negative parenting behaviors over three generations.

Methods: We examined our hypotheses with Structural Equation Modeling (SEM), using data from a large (n = 3963), longitudinal birth cohort. We examined the effects of grandparental divorce (G1) on grandchild (G3) problem behavior, and whether effects were mediated by parental marital discord (G2), and by maternal parenting behaviors of G1 and G2.

Results: We found that grandparental divorce was associated with grandchild problem behaviors at the child's age of six years, as reported by the mother and the child. These effects were mediated by grandmaternal rejective parenting, maternal harsh parenting, and G2 marital discord. All findings were independent of G2 socio-economic and demographic variables.

Conclusions: Negative effects of grandparental divorce may be associated with grandchild psychopathology through continuities in negative parenting behaviors and marital discord.

INTRODUCTION

Children exposed to parental divorce are at increased risk for a variety of developmental problems, including externalizing and internalizing problems (Averdijk et al., 2012), and lower academic achievement (Evans et al., 2001). The negative effects of parental divorce can even endure into adulthood, as adult offspring of divorced parents also have an increased risk of psychopathology (Amato & Booth, 1997; Amato & Keith, 1991; Rodgers et al., 1997) and lower academic achievement (Amato & Keith, 1991; Larson & Halfon, 2013). Furthermore, there is accumulating evidence that parental divorce increases the risk that offspring will see their own marriages end in divorce or experience marital discord, thereby sustaining a familial cycle of divorce, marital discord and associated problems (Amato, 1996; Amato & Booth, 2001; Amato & Keith, 1991; Conger et al., 2000; Perren et al., 2005; Webster et al., 1995).Yet, few studies have moved beyond the examination of two successive generations and examined the effects of grandparental divorce or marital discord on grandchild development. While these studies underscore that adults with a family history of grandparental divorce or marital discord are at increased risk of experiencing psychopathology and marital discord themselves (Amato & Cheadle, 2005; Caspi & Elder, 1988), more research is warranted before any firm conclusion can be drawn. Also, the investigation of underlying mechanisms by which divorced grandparents place their children and their children's children at risk for developmental problems is warranted (Fincham, 1994; Patterson, 1998).

According to the 'spill-over' hypothesis, the negative quality of the inter-parental (e.g.) marital relation may 'spill-over' and affect the quality of the parent-child relationship by increasing parental negativity and depleting coping resources (Erel & Burman, 1995). In turn, ineffective parenting is a well-known risk factor for child developmental problems (Bayer et al., 2008; Neppl et al., 2009). While several other mechanisms have been proposed to explain the association between divorce, marital discord and child developmental problems (Davies & Cummings, 1994; Grych & Fincham, 1990), substantial focus has been given to the possible mediating role of ineffective parenting behaviors (Cummings & Davies, 2002; Davies & Cummings, 1994; Grych & Fincham, 1990). Indeed, there is ample evidence that ineffective parenting (partially) mediates the association between parental divorce and marital discord and child psychopathology (Davies et al., 2009; Kaczynski et al., 2006; O'Donnell et al., 2010; Sturge-Apple et al., 2006). However, most studies assessing the mediating role of parenting in the association between parental divorce or marital discord and child development have a cross-sectional or short-term longitudinal (e.g. 1 to 2 years) design. Whether ineffective parenting also underlies the continuity in

divorce or marital discord in the long-term, is less well understood. The few studies describing the mediating role of parenting behavior in the continuity of divorce or marital discord from one generation to the next reported mixed results (Amato & Booth, 2001; Amato & Cheadle, 2005; Conger et al., 2000; Ehrensaft et al., 2011). Furthermore, it is well established that ineffective parenting also shows continuity over generations (Capaldi et al., 2008; Capaldi et al., 2003; Conger et al., 2003), with parents displaying parent behaviors similar to those they have experienced while growing up (Serbin & Karp, 2003). Therefore, it is plausible that continuity in ineffective parenting may also be a mechanism by which grandparental divorce can negatively impact on grandchild's development, but to our knowledge this is not studied before.

The present research brings together the existing research on divorce and ineffective parenting in a single model investigating the intergenerational continuity of divorce and marital discord and negative parenting behaviors over three generations. We aimed at enhancing current insights into the transgenerational stability of divorce and marital discord and their effects on child development over the course of three generations. Also, we aimed at extending current research by assessing whether (stability in) ineffective parenting mediates the effects of parental divorce in the long-term. Ultimately, identifying how these different family processes together transmit risk associated with psychopathology over multiple generations, may help the formulation of effective prevention and intervention strategies.

We hypothesized that divorce in the grandparental generation (G1) predicts marital discord in the parental generation (G2) through G1 rejective parenting by the mother. G2 marital discord, in turn, predicts maternal harsh parenting thereby increasing the risk of grandchild (G3) internalizing and externalizing problems in childhood. We also hypothesized that G1 divorce predicts maternal marital discord, and that G1 rejective parenting predicts G2 harsh parenting. We tested our hypotheses with Structural Equation Modeling (SEM), using data from a large (n = 3963), longitudinal birth cohort. The conceptual model underlying the present study is depicted in Figure 1. All analyses were adjusted for socio-demographic factors, child's gender and child's age. To reduce problems of shared method variance, we used multiple informants: G2 mothers reported on their parents' divorce and parenting variables, while G2 fathers reported on G2 marital discord. Furthermore, G3 outcome was assessed by mother (G2) and child (G3) self-report.

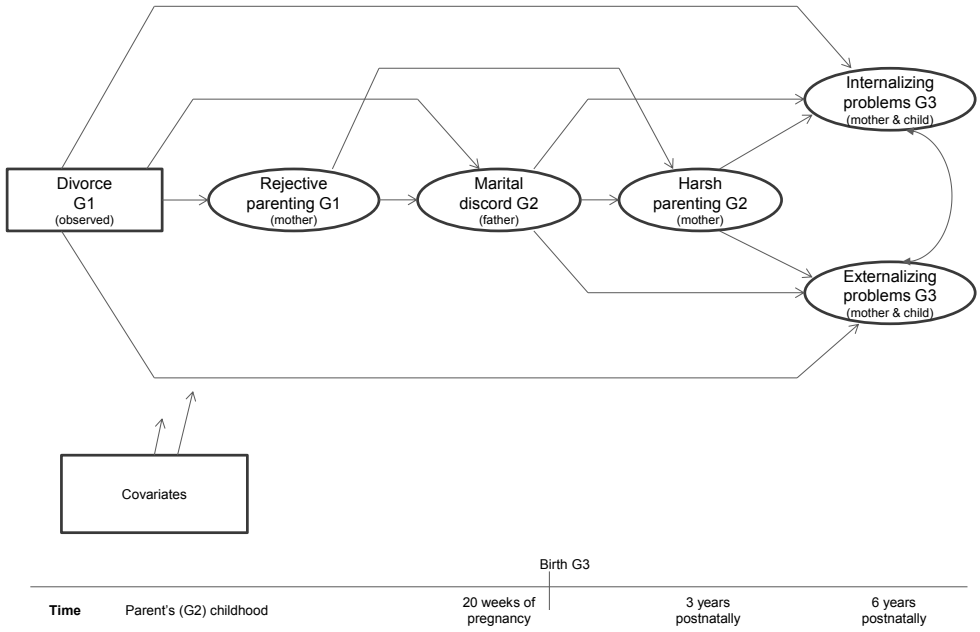

Figure 1. Conceptual Structural Equation Model (SEM)

METHODS

Setting

The study was embedded within the Generation R Study, a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands. The cohort has been described in detail elsewhere (Jaddoe et al., 2012; Tiemeier et al., 2012). Mothers who were resident in Rotterdam at the day of delivery and had a delivery date from April 2002 until January 2006 were contacted. Midwives and obstetricians informed eligible mothers about the study at their first prenatal visit in routine care, and asked them to make an appointment at our research centre.

The study was conducted in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (numbers: prenatal, MEC 198.782/2001/31 and postnatal, MEC 217.595/2002/202). Written informed consent was obtained from all participants.

Study population

Prenatally included mothers with singleton pregnancies leading to a live birth were considered eligible for the current study (n = 8633). A total of n =1922 (22%) mothers were excluded because they were not married or not living together with a partner at the time of inclusion to be able to study associations with parental marital discord. Of the remaining n = 6711 mothers, data on grandparental divorce was available for n = 5407 (86%) mothers. Of these, a total of n = 4003 (74%) mothers reported on child problem behavior when the child was on average six years of age. Data on n = 40 mother-child dyads were subsequently excluded because the child was older than eight years of age at the time of the behavioral assessment. This left 3963 (73%) mothers and their children for analyses. To test consistency, we also tested child self-reports of child behavior (n = 3482 children) as an outcome measure.

Measures

Our Structural Equation Model included grandparental divorce (G1), parental marital discord (G2), grandmaternal (G1) and maternal (G2) parenting behaviors, and child (G3) emotional and behavioral outcome (i.e. as reported by the mother and reported by the child). All these measures were examined via latent constructs, except for grandparental divorce. The items that were used as indicators of the various latent constructs are presented in Table 1. The means or percentages of the items are displayed in the first column of Table 1.

Table 1. Sample descriptives, N=3963

	% yes / Mean (SD)
Grandparental divorce, % yes	22.2
Gender of the child, % boys	49.4
Child's age at outcome assessment (months), by mother	72.14 (4.58)
Child's age at outcome assessment (months), by child	73.37 (4.70)
Child's total behavioral problems at age 18 months	4.52 (1.60)
Maternal psychopathology symptoms	0.41 (0.26)
Maternal age at intake (years)	31.21 (4.38)
Maternal ethnicity, % non - Western	22.5
Maternal educational level, % lower educated	43.6
Family income, % lower	25.3

Marital discord

Grandparental divorce (G1)

At inclusion, grandparental divorce was assessed by self-report questionnaire: mothers (G2) were asked whether their parents (i.e. the child's grandparents (G1)) were divorced or not. If yes, maternal age at parental divorce was assessed.

Parental marital discord (G2)

At 20 weeks of pregnancy, marital discord was assessed with the seventh subscale 'General Functioning' of the Family Assessment Device which is a validated overall self-report measure of health and pathology of a family (Byles et al., 1988). The General Functioning subscale consists of 12 items rated on a 4-points scale. A higher score indicates less well functioning. To reduce shared method variance by maternal report in the current study, we used the partner's report. The internal consistency in the current sample was $\alpha = 0.87$.

When the child was approximately 6 years of age, marital status was assessed again per questionnaire. A total of $n = 342$ (8.6%) of the mothers indicated to have no partner or not to be living with a partner any more. Parental marital discord (G2) and marital status at the child's age of 6 years were significantly correlated ($r_s = 0.12$, $p<0.001$).

Parenting

Grandparental parenting behavior (G1)

Parenting behaviour by the grandparental generation (G1) was assessed with the short form of the 'Own memories on parenting questionnaire' (EMBU), which measures adults perceptions of their parents rearing behaviors (Arrindell et al., 1983; Arrindell et al., 1999). At 30 weeks of pregnancy, mothers (i.e. G2) filled out this self-report inventory for each parent (i.e. for mother's mother (G1) and for mother's father (G1)). Each item is rated on a 4-point scale: 1 (No, never), 2 (Yes, but seldom), 3 (Yes, often), and 4 (Yes, most of the time). In the current study we used the Rejection scale. The Rejection scale includes 7 items assessing punitive, abusive, shaming parenting and favoring siblings over the subject. Higher scores represent more perceived rejective parenting. Good validity and reliability have been reported for the s-EMBU (Arrindell et al., 1999). The internal consistency in the current sample was $\alpha = 0.82$.

Maternal parenting behavior (G2)

Harsh parenting was assessed using an adapted version of the Parent-Child Conflict Tactics Scale (CTS-PC) (Straus et al., 1998) when the child was three years old. Mothers rated their use of disciplining practices during the past two weeks on a 6-point scale ranging from 'never' (0) to 'five times or more' (5). Higher scores reflect a higher incidence of harsh parenting practices. Factor analysis of this adapted CTS-PC has yielded a harsh discipline construct consisting of six items showing good psychometric properties (Jansen et al., 2012). The internal consistency in the current sample was $\alpha = 0.57$.

Child behavioral outcome (G3)

Maternal report

When the child was approximately six years old, mothers filled out the Child Behavior Checklist (CBCL/1,5-5) compromising 99 problem items. By summing the raw scores, seven syndrome scales (Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems and Aggressive Behavior) can be computed. The Internalizing problem score is a summary score for the items on the first four syndrome scales and the Externalizing problem score is a summary score for attention problems and aggressive behavior (see Table 1). A higher score represents a higher severity. Good reliability (mean test-retest Pearson's r = 0.85, interparent agreement r = 0.61) and validity have been reported for the Child Behavior Checklist (Achenbach & Rescorla, 2000). Internal consistencies in the current sample were α = 0.86 and α = 0.90 for the Internalizing and the Externalizing problem scores respectively.

Child report

When children were on average six years old, they visited our research center. During the visit, child self-reports on behavioral problems were obtained using the Berkeley Puppet Interview (BPI), a semi-structured interactive interview technique (Alblow & Measelle, 2003). Two identical hand-puppets made opposing statements about themselves and asked children to indicate which statement described the child best. Fifty statements were scored on a 7-point scale with higher scores indicating more problems (Ringoot et al., 2013). For the purpose of the current study we used the six Symptomatology Scales that target internalizing problems (i.e. the Depression scale, Separation Anxiety scale, and Overanxious scale), and externalizing problems (i.e. Oppositional Defiant, Overt Hostility, Conduct Problems), see Table 1. Throughout the coding process, average interrater reliability for the scales ranged from 0.96 (Overanxious, Overt Hostility, Conduct Problems) to 0.98 (Depression, Separation Anxiety). Internal consistencies in the current sample were α = 0.71 and α = 0.77 for the Internalizing and the Externalizing problem scores respectively.

Covariates

Maternal age, maternal ethnicity, maternal educational level, and family income were determined at enrolment using a postal questionnaire. Maternal ethnicity was dichotomized into 'Western' and 'Non-Western'. Educational level (highest education finished) was dichotomized into 'primary or secondary education' and 'higher education'. Family income was dichotomized into 'less than €2000 per month' and 'more than € 2000 per month'.

Maternal prenatal psychopathology was assessed during the second trimester of pregnancy with the Brief Symptoms Inventory (BSI), a self-report instrument. The BSI is a short version of the Symptom Checklist 90 (SCL-90) that covers a broad spectrum of psychiatric symptoms (Derogatis, 1993; Derogatis & Melisatores, 1983). Good validity and reliability are reported (de Beurs, 2004, 2009). For the purpose of this study the overall summary score, the Global Severity Index score (GSI score) was used. The internal consistency in the current sample was $\alpha = 0.95$.

When the child was 18 months old, mothers filled out the CBCL. We used the Total problems score, derived by summing all syndrome scales, to control for the effect of child problem behavior (G3) on harsh parenting behavior of the mother (G2), i.e. reversed causality. The internal consistency in the current sample was $\alpha = 0.92$.

Statistical analyses

First, we tested whether the proposed latent constructs show good psychometric properties in the current sample using confirmatory factor analyses (CFAs), using MPlus version 7.11 (Muthen & Muthen, 1998-2000). The Maximum Likelihood estimator was used for the internalizing and externalizing CFA measurement models, which is the default in Mplus for analysis with continuous variables. All other measurement models included categorical indicators and the weighted least squares with means and variance adjustment (WLSMV) estimator for categorical data was employed. The categorical indicators were recoded to be dichotomous (0 'never or not true' and 1 'yes or any endorsement of the item') consistent with previous CFAs establishing psychometric properties of the outcome scales (Achenbach & Rescorla, 2000).

Second, structural equation modeling (SEM) using the WLSMV estimator was employed to test the hypotheses that grandparental divorce predicts child internalizing and externalizing problems at age 6 years, and that these relations are mediated by rejective parenting by G1, marital discord by G2, and by harsh parenting by G2 (See Figure 1 for the conceptual model). Inferences about a mediating effect were based upon the indirect effects, i.e. testing whether the path from *X* (independent variable) to *M* (mediator variable) to *Y* (dependent variable) is statistically significant, using the 'Model Constraint' option. Control variables were entered as predictors of all other variables in the model. In addition, our model took into account possible covariance among the two latent child behavior constructs (i.e. internalizing and externalizing problems).

To test consistency of the results and to guard against the possibility of maternal rater bias, the structural model was repeated with child self-reports on internalizing and externalizing problems as the outcome. Additionally, several analyses were conducted to verify the validity of our results: First, to assess whether findings could not be explained by pre-existent child problem behavior leading to more harsh parenting of G2, the paths towards harsh parenting (G2) were additionally adjusted for child problem behavior at the child's age of 18 months. Second, psychological problems were included as a covariate. Because psychological problems and marital discord were assessed at the same time and the temporal directionality could not be inferred, it was not possible to include the GSI as a mediator in the main model.

We used the Comparative Fit Index (CFI), the Tucker-Lewis Index (TLI), and the Root Mean Square Error of Approximation (RMSEA) as our main indices of model fit (Hu & Bentler, 1999). For the CFI and TLI, values greater than 0.95 indicate a good fit. For the RMSEA, values of 0.05 or lower indicate a good fit, values ranging from 0.05 to 0.08 indicate a reasonable fit. We used a p-value of 0.05 - 0.10 to indicate trends and a p-value of <0.05 to indicate significance.

Data on child behavioral problems as reported by the mother and by the child and on maternal psychopathology were square root transformed to approximate a normal distribution. Missing data were imputed using Multiple Imputation (20 imputation sets) with SPSS version 20.0 for Windows (SPSS, Inc. Chicago, Illinois). Missing data on child self-reports on behavioral problems (12%) were imputed to obtain comparable samples. These 20 datasets were analyzed in MPlus and parameter estimates were averaged over the set of analyses.

Response analyses

Mothers with missing data on grandparental divorce (n = 1304) did not differ on levels of experienced rejective parenting by G1 or on marital discord from mothers without missing data on grandparental divorce (n = 5407). However, mothers with missing data more often had non-western ethnicity (52.5% vs 29.8%, X^2 = 243.44, p<0.001), and were more likely to be lower educated (66.6% vs 48.9%, X^2 = 149.45, p<0.001) than mothers without missing data.

Non-respondents at follow-up (i.e. mothers with missing data on child outcome (n = 1404)) were more likely to experience marital discord (G2) (mean rank 2159.32 vs 1974.38, p<0.001) than respondents (i.e. mothers with data on child outcome (n = 4003)). They did, however, not differ on levels of harsh parenting (G2). Non-respondents more often had non-western ethnicity (50.1% vs 22.7%, X^2 = 393.53, p<0.001), and were more likely to be lower educated (65.5% vs 43.1%, X^2 = 256.97, p<0.001) than respondents.

RESULTS

Descriptive statistics of the mothers and their children are presented in Table 1. Of the total sample, 22.2% (n = 878) of the mothers reported to have experienced parental divorce. Mothers were on average 12 years (SD = 8.01) of age when their parents separated. The majority of the mothers in our study sample had a Western ethnicity (77.5%) and most had a modal or higher family income (i.e. > 2000 euro's per month) (74.7%).

Before testing the structural model, confirmatory factor analyses were performed to establish the validity of our proposed latent factors. The variable loadings on the latent factors and the fit indices are summarized in Supplementary Table S1. For all five measurement models, a reasonably good fit was found with CFI's ranging from 0.97 to 0.99, TLI's ranging from 0.94 to 0.98, and RMSEA's ranging from 0.03 to 0.07. Also, all variable loadings on the hypothesized latent factors were strong and statistically significant (all p<0.001).

Grandparental divorce and child psychopathology as reported by the mother

First present the results of our model using mother reports on child internalizing and externalizing problems (see Figure 2). Structural equation modeling showed a good fit: CFI = 0.97, TLI = 0.97, RMSEA = 0.03, $X^{2\,(609)}$ = 2289.33.

The overall association between grandparental divorce and child externalizing problems was statistically significant (p = 0.007). Grandparental divorce was also directly associated with child externalizing problems (Beta 0.04, p = 0.05). No overall or a direct association between grandparental divorce and child internalizing problems was found.

There were several indirect paths leading to child internalizing and externalizing problems (See Figure 2). Grandparental divorce (G1) was associated with rejective parenting (G1) (Beta 0.30, p < 0.001). In turn, mothers (G2) who had perceived rejective parenting by G1 were more likely to parent their children harshly (Beta 0.26, p < 0.001). Maternal harsh parenting (G2) subsequently placed the child (G3) at increased risk for internalizing and externalizing problems (Beta 0.30, p < 0.001 and Beta 0.38, p < 0.001 respectively). Rejective parenting by G1 was also associated with marital discord (G2) and, in turn, parental marital discord was associated with child externalizing problems (Beta 0.06, p = 0.009).

Next, we tested the specific indirect effects of grandparental divorce on child psychopathology, confirming that G1 divorce is associated with G3 problem behavior through parenting behaviors of G1 and G2 (Beta 0.02, p = 0.001 (internalizing) and Beta 0.03, p < 0.001 (externalizing)). The mediating pathway from G1 divorce to G3

externalizing problems through G1 rejective parenting and G2 marital discord was also confirmed (Beta = 0.002, p = 0.04).

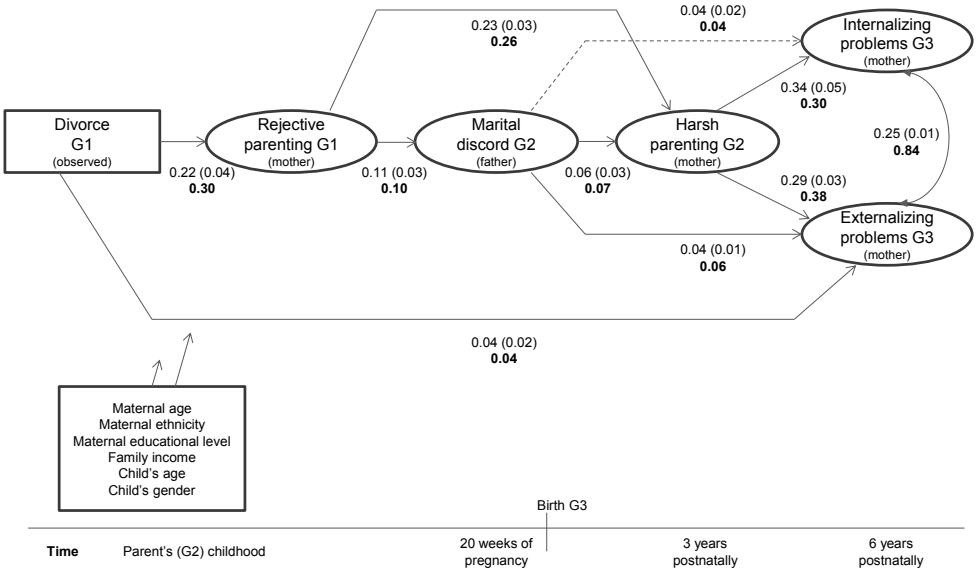

Figure 2. Final Structural Equation Model (SEM) using mother reports of child outcome. Unstandardized and standardized (bold) coefficient estimates (values in parentheses are standard errors). All solid paths are statistically significant at the $p < 0.05$ level. Dashed paths represent trends ($0.10 > p < 0.05$).

Grandparental divorce and child psychopathology as reported by the child

To test consistency of the results across methods and informants, models were repeated with child self-reports on child problem behavior (See Figure 3). Structural equation modeling showed a good fit: CFI = 0.98, TLI = 0.97, RMSEA = 0.03, $X^{2\,(609)} = 2233.90$. No overall or direct associations between grandparental divorce (G1) and child self-reported internalizing and externalizing problems (G3) were found.

However, consistent with the results using maternal reports of child outcome, indirect effects from grandparental divorce to child problem behavior through rejective parenting (G1) and harsh parenting (G2), and through rejective parenting and marital discord (G2) were found. As shown in Figure 3, maternal harsh parenting (G2) was associated with child internalizing and externalizing problems: Beta 0.08, p = 0.03, Beta 0.11, p = 0.002. Also, parental marital discord (G2) was associated with child externalizing problems (Beta 0.05, p = 0.05).

We confirmed the mediating effect of parenting behaviors of G1 and G2 in the association between grandparental divorce and child internalizing and externalizing

problems as reported by the child (i.e. G1 divorce → G1 rejective parenting → G2 harsh parenting → G3 problem behaviors; (Beta 0.004, p = 0.05, and Beta = 0.006, p = 0.01 respectively) by testing the specific indirect effects.

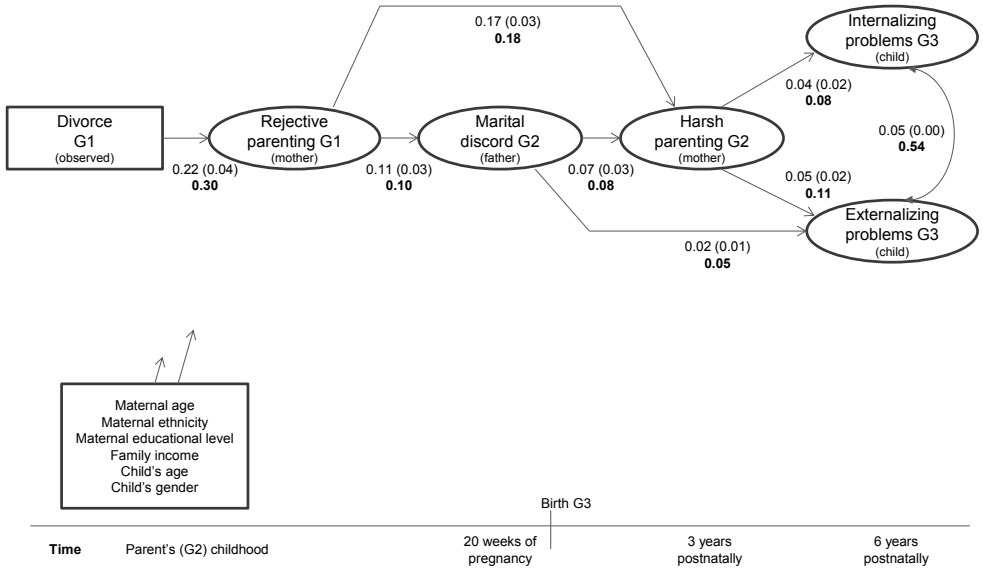

Figure 3. Final Structural Equation Model (SEM) using child reports of child outcome. Unstandardized and standardized (bold) coefficient estimates (values in parentheses are standard errors). All solid paths are statistically significant at the $p < 0.05$ level. Dashed paths represent trends ($0.10 > p < 0.05$).

Additional model testing

Results remained essentially unchanged when we additionally adjusted the associations for pre-existent G3 problem behavior at age 1.5 years (data not shown). When we now included maternal (G2) psychological problems as a covariate, the associations between G1 rejective parenting and G2 marital discord, and between G2 marital discord and G2 harsh parenting attenuated and were no longer statistically significant (See Supplementary material, Figure S1 and Figure S2).

DISCUSSION

The present study investigated the effect of grandparental divorce (G1) on grandchildren's (G3) internalizing and externalizing problems at the age of 6 years, and the mediating effects of marital discord (G2) and parenting behaviors of the

grandparental (G1) and parental (G2) generations. Results showed that grandparental divorce had an effect on G3 problem behavior through perceived rejective parenting by G1 and subsequent harsh parenting by G2 (i.e. the child's mother), and through G1 rejective parenting and subsequent G2 marital discord. These findings suggest that risk associated with divorce in the grandparental generation is transmitted across generations, through continuities in parenting behaviors and marital discord. All findings were independent of G2 socio-economic and demographic variables.

Despite strengths such as a large population-based sample, multiple reporters, and use of longitudinal data, the current study is not without limitations. In order to discuss our findings it is also important to consider these limitations: First, although data was longitudinal, not all data were prospectively assessed: G1 divorce was assessed retrospectively. However, divorce is an objective event and less likely to be substantially biased by recall. Also, G1 rejective parenting was assessed retrospectively and therefore more prone to memory errors. However, G2 marital discord was reported by the spouse, to limit the resulting reporter bias. Second, we had information on G1 divorce, but not on G1 marital discord or G1 psychopathology to further study the grandparental problems. Third, we included data on G1 and G2 mothers only.

The finding that grandparental divorce negatively impacts on grandchild problem behavior through G1 and G2 parenting behaviors and G2 marital discord is in line with the 'spill-over hypothesis', which, in short, implies that negativity from the inter-parent relationship 'spills over' into the parent-child relationship leading to ineffective parenting. In turn, these ineffective parenting behaviors have negative consequences for child development, including an increased risk of internalizing and externalizing problems (Erel & Burman, 1995).

Although our results are in support of the spill-over hypothesis, it is important to note that an alternative explanation may also hold true: Genetic factors may also predispose an individual to inter-personal behavioral problems and negative parenting behaviors. The inheritance of these factors may be the true underlying causal mechanism for the continuities in negative inter-parental and parent-child relations. However, literature assessing genetic influences in the context of divorce is inconsistent: Some studies reported that the concordance of divorce is greater among monozygotic than dizygotic twins, suggesting that genes predispose people to divorce (Jocklin et al., 1996; Mcgue & Lykken, 1992). Other studies, however, showed that the association between parental divorce and child problem behavior was similar for biological and adoptive children thereby making the influence of underlying genetic factors less likely (Brodzinsky et al., 1993; O'Connor et al., 2000). Likewise, Amato and Cheadle (2005) found little support for the assumption of a genetic association between G1 divorce and G3 outcome in their study.

In the current study, the associations including G2 marital discord attenuated once we included G2 psychopathology as a covariate. This is most probably explained by the known bidirectional effect between marital discord and psychopathology, especially depressive symptoms (Beach et al., 2003; Kouros et al., 2008; Whisman & Uebelacker, 2009). Due to the concurrent assessment of psychopathology and marital discord, we were unable to study G2 psychopathology as a mediating factor. This would however be interesting as it is well known that parental depressive symptoms are not only associated with marital discord, but also impact on parenting behaviors with depressed parents showing more rejective and harsh parenting behaviors towards their children than non-depressed parents (Goodman & Gotlib, 1999; Lovejoy et al., 2000). Furthermore, there are indications that the spouse's depressive symptoms may moderate the spill-over from the inter-parental to parent-child relationship: the presence of a depressed spouse may strengthen the spill-over between marital discord and ineffective parenting (Kouros et al., 2014) However, there are also indications that the non-depressed parent or spouse may 'compensate' for the depressed parent's impaired parenting by showing more supportive parenting behavior towards the child (Nelson et al., 2009).

The continuity in parenting behaviors reported in this study is in line with findings from previous studies assessing continuities in harsh or rejective parenting behaviors (Conger et al., 2009; Conger et al., 2003). Several explanations for the observed association between G1 rejective parenting and G2 harsh parenting are possible. These parenting behaviors may be directly transmitted from one generation to the next through observational or experimental learning, as children acquire parenting behaviors through interacting with their parents (Conger et al., 2003; Patterson, 1998) or by genetic inheritance. Also, it has been shown that these parenting behaviors are transmitted through conduct or externalizing problems of G2 parents (Conger et al., 2009; Neppl et al., 2009). These problems in G2 parents may be late effects of negative G2 early home experiences or represent a general genetic liability for externalizing behaviors including harsh parenting. In the current study, associations between G1 rejective parenting and G2 harsh parenting attenuated, but remained significant after adjusting for G2 psychopathology, including externalizing symptoms.

The current study makes an important contribution to current literature: We showed that divorce may have long term consequences, i.e. an increased risk of problem behavior in the third generation, and that continuities in negative parenting behaviors and marital discord may mediate this risk. These findings underscore the need for a family-wide perspective on influences on children's development in families, rather than the focus on the inter-parental or parent-child relationships

separately. Further, together with previous research, these findings may set the stage for a more pro-active prescription of parent training programs, especially if the family history is positive for divorce. There are few interventions that have been shown to be more effective in the reduction of childhood behavioral problems than parent training (Dretzke et al., 2009; Forehand et al., 2013).

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Summary of confirmatory factor analysis measurement models

Measurement model	% yes / Mean (SD)	Estimates Unstandardized (SE)	Standardized
Model 1: Children's outcomes (G3) maternal report			
Internalizing			
Emotionally reactive	0.99 (0.86)	1.00 (0.00)	1.15
Anxious or depressed	0.84 (0.81)	0.84 (0.01)***	1.03
Somatic complaints	0.90 (0.80)	0.90 (0.01)***	1.12
Withdrawn behaviour	0.75 (0.73)	0.75 (0.01)***	1.02
Externalizing			
Attention	0.87 (0.81)	0.87 (0.01)***	1.07
Aggressive behaviour	2.01 (1.20)	2.06 (0.02)***	1.72
Internalizing with Externalizing		0.24 (0.01)***	0.84
CFI=0.98; TLI=0.96; RMSEA=0.07; x^2 (8)= 175.95			
Model 2: Children's outcomes (G3) child report			
Internalizing			
Depression	4.23 (0.49)	1.00 (0.00)	4.23
Seperation Anxiety	4.33 (0.65)	1.08 (0.06)***	4.33
Overanxious	4.57 (0.63)	1.49 (0.08)***	4.57
Externalizing			
Oppositional Defiant	3.64 (0.50)	1.00 (0.00)	3.64
Overt Hostility	4.02 (0.49)	1.07 (0.05)***	4.02
Conduct Problems	4.68 (0.51)	1.34 (0.05)***	4.68
Internalizing with Externalizing		0.05 (0.00)***	0.54
CFI=0.97; TLI=0.94; RMSEA=0.06; x^2 (15)=3598.93			
Model 3: Harsh parenting (G2)			
I shook my child	7	1.00 (0.00)	0.67
I shouted or screamed angrily at my child	77	1.09 (0.09)***	0.73
I called my child names	5	1.23 (0.10)***	0.82
I threatened to give a slap but I didn't do it	29	0.81 (0.06)***	0.54
I angrily pinched my child's arm	15	0.83 (0.07)***	0.56
I called my child stupid or lazy or something like that	6	0.95 (0.09)***	0.64
CFI= 0.98; TLI=0.96; RMSEA=0.03; x^2 (9)= 50.40			
Model 4: Rejective parenting (G1)			
My mother was angry with me without me knowing why	22	1.00 (0.00)	0.73
My mother beat me more than I deserved	14	1.09 (0.04)***	0.79
My mother beat me in front of others	47	0.66 (0.03)***	0.48
I was treated as the black sheep of the family by my mother	11	1.16 (0.04)***	0.84
I had the feeling that my mother loved my siblings more than me	22	0.92 (0.04)***	0.66
My mother treated me in such a way that I felt ashamed	18	1.03 (0.03)***	0.75
My mother punished me severely even for small things	21	1.05 (0.03)***	0.76
CFI=0.97; TLI=0.95; RMSEA=0.06; x^2 (14)= 50.39			

Model 5: Marital discord (G2)			
If there are any problems we cannot count on each other	28	1.00 (0.00)	0.80
It is difficult making plans to do something together	47	1.00 (0.02)***	0.80
We do not accept each other	62	0.81 (0.02)***	0.65
We cannot talk to each other about any sadness	48	1.03 (0.02)***	0.83
We cannot express our feelings to each other	54	1.05 (0.02)***	0.84
We avoid talking about our worries and problems	59	1.00 (0.02)***	0.80
We do not feel accepted	52	0.99 (0.02)***	0.80
There are a lot of unpleasant and painful feelings	40	0.91 (0.02)***	0.73
We are not able to make decisions	48	1.09 (0.02)***	0.87
Decision-making is a problem	51	0.98 (0.02)***	0.79
We do not trust each other	27	1.14 (0.02)***	0.92
We do not get on well with each other	23	1.15 (0.02)***	0.93
CFI= 0.99; TLI=0.98; RMSEA=0.06; x^2 (54)= 900.62			
Predictor variable			
Divorce, % yes	22.2		

****p<0.001*

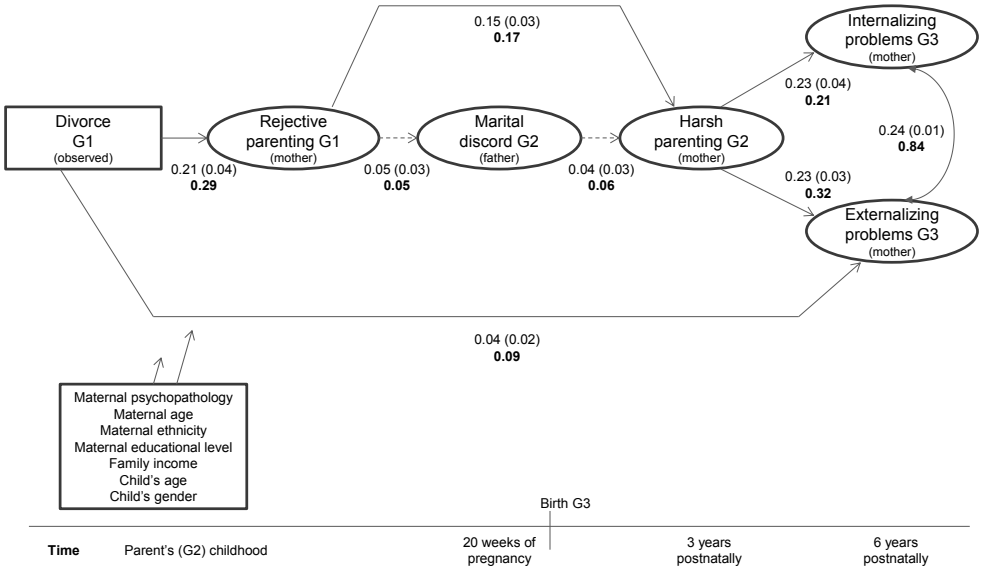

Figure S1. Structural Equation Model (SEM) using mother reports of child outcome. Unstandardized and standardized (bold) coefficient estimates (values in parentheses are standard errors). All solid paths are statistically significant at the $p < 0.05$ level. Dashed paths represent trends ($0.10 > p < 0.05$).

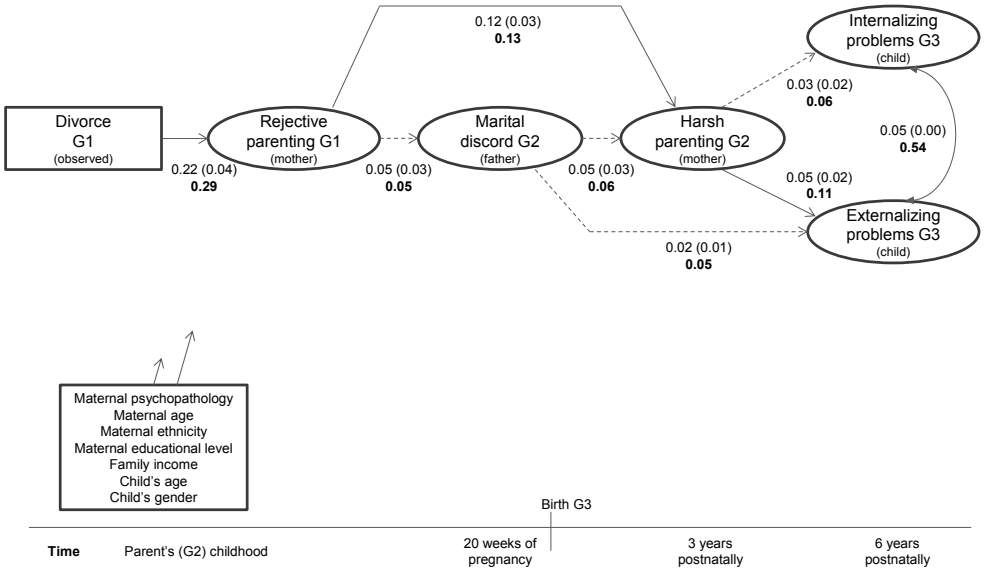

Figure S2. Structural Equation Model (SEM) using child reports of child outcome. Unstandardized and standardized (bold) coefficient estimates (values in parentheses are standard errors). All solid paths are statistically significant at the $p < 0.05$ level. Dashed paths represent trends ($0.10 > p < 0.05$).

EPILOGUE

CHAPTER 9

General discussion

RATIONALE

Parental psychopathology is a major risk factor for child psychopathology and a wide range of other developmental problems, including cognitive problems. In turn, child psychopathology is predictive of psychopathology in adulthood, thereby sustaining a familial cycle of psychopathology over the course of multiple generations. The main challenge for researchers, policy makers, therapists and the society is to interrupt the intergenerational cycle of psychopathology. Therefore, a better understanding of the nature of continuities in psychopathology across multiple generations, and a better understanding of the mechanisms underlying the transmission of psychopathology is needed. Ultimately this knowledge will help us to design prevention and intervention programs for families at risk for psychopathology.

The overall aim of this thesis, to enhance insights in the intergenerational transmission of psychopathology, was addressed by formulating two specific aims: The first specific aim was to increase our understanding of the nature of associations between parental psychopathology, grandparental psychopathology and child psychopathology. The second specific aim was to increase our understanding of the complex roles of genes and parenting behaviors in the intergenerational transmission of psychopathology.

In this chapter we will describe the main findings of this thesis and discuss them in a broader context. Throughout, we will address some important methodological considerations pertaining to the studies included in this thesis and to longitudinal, developmental research in general. For the specific discussion of the results, methodological issues and limitations per study we refer to the respective chapters. Last, we will address the implications for future research and clinical practice.

ASSOCIATIONS BETWEEN GRANDPARENTAL, PARENTAL AND CHILD PSYCHOPATHOLOGY

In **Part I** of this thesis we included two studies that examined the nature of continuities in psychopathology across successive generations. In the first study, we modeled trajectories of maternal depressive symptoms over time and assessed their associations with child psychopathology. In the second study we studied associations between grandparental psychopathology and child psychopathology.

Given the high prevalence and burden of depressive disorder among women in their childbearing years, much of the research assessing the transmission of psychopathology from parents to their children focused on maternal depressive

symptoms. Although our understanding of the associations between maternal depressive symptoms and child psychopathology has increased tremendously over the last years, further understanding is complicated by the fact that much research did not account for the heterogeneity of maternal depressive symptoms as these symptoms can vary in severity and duration. In our study, described in chapter 2, we repeatedly assessed maternal depressive symptoms from mid-pregnancy through the first 3 years postpartum and, using a semi-parametric modeling technique, identified four distinct trajectory groups of maternal depressive symptoms: a trajectory of mothers reporting 'no' depressive symptoms (35%), a 'low' trajectory (54%), a 'moderate' trajectory (11%), and a trajectory including mothers reporting 'high' levels of depressive symptoms. All trajectories remained rather stable over time with the exception of a significant increase in depressive symptoms at 2 and 6 months postnatally, which was noted for the 'low', 'moderate' and 'high' trajectories. Mothers assigned to the higher trajectories had more socioeconomic disadvantages and were more often of non-Western ethnicity. The children of mothers assigned to the higher trajectory groups had significantly more mother and father reported internalizing and externalizing problems at age 3 years, independent of the socioeconomic and ethnic risks. These associations remained after adjusting for concurrent maternal depressive symptoms. Moreover, we showed that the modelling of trajectories is of added value in predicting child psychopathology over using severity scores or 'cut-off' scores to define chronicity of maternal depressive symptoms. Our findings, together with other research, suggest that maternal depressive symptoms follow four to six distinct trajectory groups that remain rather stable over time, but differ in level of severity. Luckily, only a small proportion of mothers is found to experience high chronic symptoms (Campbell et al., 2007; Gross et al., 2009; Kingsbury et al., 2015; Luoma et al., 2015; Mars et al., 2015; Mora et al., 2009; Skipstein et al., 2010; Sutter-Dallay et al., 2012; van der Waerden et al., 2015). In line with other research, children of mothers assigned to the higher trajectory groups are found to experience higher levels of psychopathology, which may be explained by a higher co-occurrence of environmental risks and a higher genetic risk in mothers assigned to higher trajectory groups. Our finding (see chapter 2) and those of others (Mars et al., 2015; van der Waerden et al., 2015), show that the level of children's psychopathology is independent of maternal concurrent depressive symptoms. This might indicate that a history of maternal depressive symptoms might be a marker of genetic and environmental risk processes that persist regardless of the mother's current mood state.

A history of parental psychopathology is one of the most important risk factors for child psychopathology. Therefore, most intergenerational research has focused

on documenting the associations between parental and child psychopathology. Much less is known about the patterns of transmission of psychopathology across three successive generations. The study of grandparental psychopathology is, however, important because a positive history of grandparental psychopathology may be a proxy of genetic and environmental risks which the grandparents may have passed on to their offspring, the child's parents, thereby also placing the grandchild at increased risk for psychopathology. In the study described in chapter 3, we investigated the associations between grandparental psychopathology and child internalizing and externalizing problems while controlling for parental psychopathology. We found that three-year-olds with 1 or more grandparents with a history of depressive disorder or anxiety disorder had an increased risk for parent-reported problem behaviors, independent of their parent's history of psychopathology and their parent's current mood state. This interesting finding is also supported by others (Hancock et al., 2013; Olino et al., 2008). In their study, Hancock and colleagues also took the time the children had spent with their grandparents into account to exclude a direct effect from the grandparents on the grandchildren, but this did not affect their findings. We studied the mediating effects of parenting stress and observed maternal sensitive parenting, but found no indications of underlying mediating mechanisms. Based on earlier research, the roles of partner conflict and impaired parenting practices, such as harsh or rejective parenting, may be subject to further investigation (Cummings et al., 2005; Lovejoy et al., 2000). Furthermore, as discussed in chapter 3, genetic and in utero factors may partly explain the effects reported in our study. In conclusion, our findings suggest that to better identify children and families at risk for psychopathology it is important to include a history of grandparental psychopathology next to a history of parental psychopathology. Moreover, these findings suggest that genetic and environmental risks associated with psychopathology may contribute to the development of child psychopathology, over and above the influence of parental psychopathology (Barker et al., 2012; Goodman & Gotlib, 1999).

The findings of the studies described in **Part I** of this thesis indicate that:

- Perinatal maternal depressive symptoms follow distinct trajectory groups that are rather stable over time, with few mothers experiencing high or clinically relevant symptoms.
- More severe and chronic maternal depressive symptoms occur in a context of environmental risks.
- Children of mothers assigned to higher trajectory groups (i.e. mothers with more severe and chronic depressive symptoms) are at risk for higher levels of psychopathology, independent of concurrent maternal symptoms.
- Children with a history of grandparental anxiety or depressive disorder are at increased risk for psychopathology, independent of a history of parental psychopathology or concurrent parental psychopathology.

Assortative mating

The transmission of genetic and environmental risk factors for psychopathology over generations may be complicated by the notion that persons vulnerable for psychopathology tend to marry persons with a history of psychopathology or with a family history of psychopathology, increasing the child's risk for psychopathology (Dietz et al., 2009). Assortative mating is shown for externalizing psychopathology, including substance abuse and antisocial personality disorders (Rhule-Louie & McMahon, 2007), but there is also evidence for assortative mating among persons with increased risk for depressive and anxious disorders (Mathews & Reus, 2001; Merikangas & Spiker, 1982; van Grootheest et al., 2008). Furthermore, assortative mating has been shown for environmental risks associated with psychopathology such as educational attainment (Abdellaoui et al., 2015).

There are several mechanisms, that are not mutually exclusive, by which assortative mating can be explained (Rhule-Louie & McMahon, 2007). The first mechanism, primary of phenotypic assortment, refers to the selection of a partner based on phenotypic resemblance. In other words, partner selection is based on the direct observation and selection of traits (Watson et al., 2014). The second mechanism is called secondary or social homogamy, meaning that assortment is based on selecting partners with similar socioeconomic or cultural backgrounds (Taylor et al., 2000). In the Netherlands, for example, persons with similar religious background are more likely to mate. Two other mechanisms accounting for partner similarity are contagion, where a partner may develop the trait under investigation

in response to the partner that already has the trait. Also, partners may become more similar the longer they live together based on mutual influences between the partners or by sharing the same factors such as diet and economic status.

Assortative mating has important consequences for several reasons. Individuals with psychopathology who have partners with psychopathology, may develop more severe symptoms (Merikangas, 1982). Moreover, the partner's psychopathology may contribute to relationship difficulties and impaired parenting practices (Keller et al., 2008; McLeod, 1994), while the presence of a 'healthy' parent may buffer the effects of the other parent's psychopathology on the child (Gere et al., 2013; Kahn et al., 2004). From a genetic point of view, the presence of phenotypic assortative mating leads to an increase in the genetic additive variance for the genes associated with the phenotype and for genetically correlated phenotypes (Plomin & Deary, 2015). For heritability studies using twin designs this means that due to the increase in additive genetic variance in each following generation, genetic correlations between dizygotic twins are higher as expected by chance, thereby inflating estimates of shared environmental effects, while the correlations between monozygotic twins do not alter. This phenomenon may lead to biased estimates of the magnitude of genetic and environmental factors if assortative mating is not accounted for (Vinkhuyzen et al., 2012). For genome wide association studies (GWAs), it is important to realize that assortative mating for a trait leads to reduction of heterozygosity only at loci associated with the trait. On the contrary, in the case of inbreeding, the reduction in heterozygosity is expected throughout the genome and will lead to more homozygous deleterious genes in the offspring (Plomin & Deary, 2015).

THE ROLES OF GENES AND PARENTING IN THE TRANSMISSION OF PSYCHOPATHOLOGY

While genetic factors are proposed to account for an important part of the association between parental psychopathology and child development, relatively few genes responsible for the heritability of psychopathology and related traits are identified yet. This is most likely explained by the fact that traits such as depression, anxiety, and aggression are so-called complex traits where many genes with small to modest effects and environmental factors combine in complex ways to cause the trait to develop to any particular state. Ineffective parenting is an important example of an environmental factor proposed to play a significant role in the transmission of psychopathology (Goodman & Gotlib, 1999; Lovejoy et al., 2000). The studies included in **Part II** of this thesis aimed at enhancing insights in the main and mutual

roles of genes and parenting underlying the transmission of psychopathology and their effects on child cognitive development.

In the study described in chapter 4 of this thesis, we aimed at identifying new genes influencing language acquisition. The study of language acquisition in the context of psychopathology is important as research has shown that impaired expressive language in toddlers is associated with an increased risk of internalizing and externalizing problems (Hawa & Spanoudis, 2014). While genetic influences on language development are presumed (Hayiou-Thomas et al., 2012; Rice, 2012, 2013), little is known about the specific genetic determinants of language acquisition. We performed a genome wide association study (GWAs) and follow-up study of expressive vocabulary scores in up to 10.819 independent toddlers of European descent investigating an one-word stage (15 – 18 months) and a two-word stage (24- 30 months). Based on the combined analysis of all samples, we identified a new locus (rs7642482) on chromosome 3p12.3, near ROBO2, associated with the early phase of language acquisition (p=1.3x10^{-8}). ROBO2, or roundabout 2, is an axon-guidance receptor and involved in the outgrowth of axons, thereby playing an important role in brain development (Lin et al., 2009). Earlier studies have reported associations between ROBO2 genes and proteins and autism spectrum disorders. For example, rare ROBO2 deletions have been reported in individuals with autism, as well as decreased ROBO2 expression in lymphocytes (Anitha et al., 2008) and in the anterior cingulate cortex (Suda et al., 2011). Autism spectrum disorder is, among others, characterized by language problems, varying from non-speaking to a normal or high level of ability. However, the majority of individuals with autism will have difficulty using language effectively. The contribution of genetic variants to early language acquisition in healthy children was supported by the Genome Complex Trait Analysis (GCTA) performed in our study (meta-GCTA $h^2_{15\text{-}18\text{-}months}$=0.13). Findings of earlier studies using twin designs to estimate the heritability of language also support the contribution of genetic factors in language acquisition (Bishop et al., 2006; Dale et al., 1998; Kovas et al., 2005). However, before any specific conclusions can be drawn, the findings of our study need further investigation and replication. Also, it is important to bear in mind that next to genetic influences, environmental factors including parenting behaviors are of importance for spoken language development. Although not investigated in the current thesis, research has shown that maternal sensitivity is associated with improved language development (Leigh et al., 2011; Nozadi et al., 2013). The mother's impaired ability to adequately respond to the child's talk could also underlie associations between maternal psychopathology, especially depressive disorder, and impaired language development (Sohr-Preston & Scaramella, 2006).

In chapter 5 and chapter 6 we described two studies that investigated the interaction between genes and environment in the etiology of young children's internalizing problems and working memory problems, respectively. To further increase our understanding of the role of candidate gene by environment interactions (cGxE) in the etiology of childhood psychopathology, we extended the selection of our environmental factors beyond the traditional 'stressful life events' (Moffitt et al., 2005). We selected two environmental factors, other than stressful life events, that were biologically plausible in the context of the selected candidate genes and outcomes. In the first GxE study (chapter 5), we investigated the association between a polymorphism in the promotor region of the serotonin transporter gene (5-HTTLPR) and child internalizing problems in the presence of maternal smoking during pregnancy. The 5-HTTLPR was chosen as a candidate gene because serotonin is an important neurotransmitter that modulates anxiety and mood, among other brain functions (Murphy & Lesch, 2008). Furthermore, serotonin is also involved in the development of brain circuits modulating emotional behavior (Gaspar et al., 2003). Maternal smoking during pregnancy was hypothesized to moderate the association between 5-HTTLPR and internalizing problems, as smoking is known to negatively affect the developing brain (Falk et al., 2005) and, more specifically, to impair serotonergic synaptic functioning (Slotkin et al., 2006; Xu et al., 2001). We found that 3-year old children carrying the short allele (s-allele) and whose mothers continued to smoke during pregnancy were at increased risk for internalizing problems as reported by each parent. Also, the maternal 5-HTTLPR genotype had an additional, independent effect on child internalizing problems. No main effect of 5-HTTLPR genotype on internalizing problems or on maternal smoking was observed. These findings show that the child's risk for internalizing problems is increased based on the interaction between genetic vulnerability, i.e. carrying the 5-HTTLPR s-allele, and exposure to maternal smoking during pregnancy. As argued in chapter 5, no moderating effect of paternal smoking was observed, supporting a true intra-uterine effect of maternal smoking and not a non-causal effect of smoking through socio-economic, behavioral, and genetic factors. Several other studies, predominantly focusing on externalizing psychopathology including attention deficit hyperactivity disorder (ADHD), have also investigated the moderating role of prenatal cigarette smoking on the association between genetic factors and child psychopathology. However, so far, reported results are inconsistent (Altink et al., 2008; O'Brien et al., 2013; Thakur et al., 2012) and include a negative replication of our findings (Geels et al., 2012).

In the study described in chapter 6, we assessed the moderating role of harsh parenting on the association between a polymorphism (Val^{158}Met) of the Catechol-O-Methyltransferase (COMT) gene on child's working memory problems. Working memory is important for psychosocial and academic functioning (Gathercole et al., 2006; McQuade et al., 2013), and deficits in working memory have been implicated in psychiatric disorders including schizophrenia and ADHD (Johnson et al., 2013; Westerberg et al., 2004). The Val^{158}Met polymorphism is shown to be associated with cognitive functioning, including working memory performance, with adults and in children carrying the Met allele having superior performance (Diamond, 2011; Dickinson & Elvevag, 2009; Mier et al., 2010). However, there are indications that under circumstances of a rise in dopamine levels Met/Met carriers may deteriorate on cognitive performance whereas Val/Val carriers may improve. Psychosocial stress is an example of such a circumstance. We used both linear gene-association regression analyses and family based association testing (FBAT) to test our hypothesis that harsh parenting, by inducing chronic stress and subsequently increasing dopamine levels, moderates the association between COMT and working memory performance in healthy children. We consistently found that maternal harsh parenting may differentially impact on the association between COMT and child working memory performance: compared to ValVal carriers, MetMet carries performed worse on working memory in the presence of high levels of harsh parenting. In the presence of low levels of maternal harsh parenting, MetMet carriers outperformed the Val homozygotes on working memory. We did not find main effects of the COMT polymorphism on working memory problems. Neither did we find an effect of COMT on harsh parenting, making a gene-environment correlation accounting for the findings less likely. While an earlier study reported similar results when assessing the moderating effect of childhood maltreatment on the association between COMT and working memory performance in a sample of adults (Green et al., 2014), we have not located similar studies in (healthy) childhood populations. Therefore, replication in strongly warranted before any firm conclusions can be drawn.

That genetic factors may not only interact with environmental factors, but also predict the environment, was shown by our findings described in chapter 7. We included a study that assessed whether maternal genetic variation at 5-HTTLPR was associated with observed parenting, which is often proposed as a mediating factor in the intergenerational transmission of psychopathology. Sensitive parenting, the ability to accurately perceive children's signals and respond to them in an adequate, prompt way (Ainsworth et al., 1978), is critical for a child's mental, social, and cognitive development. While research has shown that genetic influences may be important determinants of sensitive parenting (Neiderhiser et al., 2004; Plomin

et al., 1994), research on molecular genetic determinants of parenting is relatively sparse compared to the abundant research on non-genetic determinants of parenting behavior (Swain et al., 2007). Genetic factors may influence parenting behavior through their impact on parental, but also child characteristics. We found that mothers carrying the s-allele were more sensitive towards their children. Importantly, this effect was not explained by the child's 5-HTTLPR genotype. Our findings are in line with an earlier study also reporting that the s-allele of 5-HTTLPR is associated with higher levels of positive parenting (Mileva-Seitz et al., 2011). However, the results are in contrast to a study also using a Dutch sample that reported that the s-allele of 5-HTTLPR was related to less sensitive parenting (Bakermans-Kranenburg & Van IJzendoorn, 2008). An explanation for the seemingly divergent finding may be that those authors assessed the association between 5-HTTLPR and sensitivity in a sample of toddlers at high risk for externalizing problems. If risk exposure differs among samples, findings for candidate genes may be inconsistent due to an underlying GxE (i.e. the 5-HTTLPR genotype in interaction with the stress of parenting a child with externalizing problems) (Caspi et al., 2003). Possible GxE in which mothers carrying the s-allele showed greater sensitivity under circumstances of low stress, but showed decreased sensitivity under conditions of high stress is demonstrated by two earlier studies (Mileva-Seitz et al., 2011; Sturge-Apple et al., 2012). In our study, we assessed whether child social fearfulness moderated the association between 5-HTTLPR and maternal sensitivity, but we found no evidence of a GxE effect. We also showed that only maternal 5-HTTLPR was related to maternal sensitivity, and that the child's genotype was not. The latter would have suggested that the association between 5-HTTLPR and sensitive parenting could have arisen because the child evoked the parenting behavior from the mother based on his or hers genes.

The last study included in this thesis assessed the roles of grandparental divorce, parental marital discord and maternal ineffective parenting behaviors over the course of three generations in relation to child psychopathology (see chapter 8). While parenting behaviors are often the focus of studies assessing mediating factors in the transmission of psychopathology, substantial research has shown that parental divorce or marital discord are also negatively associated with child development (Cummings & Davies, 2002). Furthermore, according to the spill-over hypothesis, negativity of the inter-parental relationship may 'spill-over' and negatively affect the quality of the parent-child relationship (Erel & Burman, 1995). Child outcome is subsequently affected by an impaired parent-child relationship. Both marital discord and ineffective parenting behaviors are shown to be transmitted over generations (Amato & Booth, 2001; Perren et al., 2005; Serbin & Karp, 2003), with romantic partners having a similar quality of their relationship as their parents

had and showing similar parenting styles as their parents had. Yet, few studies have assessed whether continuities in romantic relationships and in parenting behaviors mediate risk of psychopathology over three generations. This is important, as continuities in marital discord and parenting practices may both be targets for interventions. We found that divorce in the grandparental generation was indeed predictive of (grand)child internalizing and externalizing psychopathology. In line with the spill-over hypothesis, effects were mediated by continuities in impaired, negative parenting and through continuity in marital discord. All effects were independent of parental psychopathology and socio-economic variables. However, a possible alternative explanation for our findings may also hold true; genetic factors associated with negative personality traits and child psychopathology may also predispose individuals to inter-personal problem behaviors and to impaired parenting behaviors. Earlier research is inconsistent regarding a genetic association between grandparental or parental divorce and child psychopathology (Amato & Cheadle, 2005; Jocklin et al., 1996; O'Connor et al., 2000). Also, the complex associations between psychopathology and marital discord warrants further study as inter-correlations have been shown, but it is not clear whether psychopathology predicts marital discord or vice versa (Kouros et al., 2008; Whisman & Uebelacker, 2009). In our study we did not have information on grandparental psychopathology and data on parental psychopathology and marital discord was cross-sectional. As a result, we could not make inferences regarding the specificity of our findings for grandparental divorce. Neither were we able to include parental psychopathology as a mediator rather than a confounder. Nevertheless, the findings underline a family wide perspective including both inter-parental and parent-child relations when it comes to risk assessment in families of children at risk for psychopathology.

The findings of the studies described in **Part II** of this thesis indicate that:

- Genetic variants are involved in early language acquisition.
- Children's vulnerability for internalizing problems might be a result of an interaction between variation in 5-HTTLPR and in utero exposure to maternal prenatal smoking.
- The COMT polymorphism may differentially impact on child working memory performance as a function of maternal harsh parenting: children carrying the Met-allele show superior performance under low levels of harsh parenting, while Val-allele carriers show superior performance in the presence of high levels of harsh parenting.
- Variation at maternal 5-HTTLPR may be implicated in maternal sensitive parenting, with mothers carrying the s-allele showing increased sensitive parenting.
- Grandparental divorce is associated with grandchild internalizing and externalizing problems through continuities in impaired parenting of the grandparental and parental generations, and through marital discord of the parental generation.

Challenges in interpreting results of Gene x Environment research

One of the first questions pertaining to GxE research is whether the reported results are valid. The skepticism about the validity of GxE results is mostly due to the frequent non-replication of initial results (Dick et al., 2015; Duncan et al., 2014). An example of a debate that is still ongoing, is whether the initial finding of Caspi et al. (2003), that the association between the 5-HTTLPR polymorphism and depression is moderated by stressful life events (SLE), holds true. Since the original publication, three meta analyses have been published that studied the validity of the original report (Karg et al., 2011; Munafo et al., 2009; Risch et al., 2009). The first two meta-analyses were compatible with false-positive findings (Munafo et al., 2009; Risch et al., 2009). However, it has been argued that the authors of these two meta-analyses used very stringent in- and exclusion criteria (i.e. they only included studies that investigated an interaction between 5-HTTLPR and number of SLE on depression), favoring studies that used self-report assessment of SLE, whereas studies using objective measures of stress were excluded (Uher & McGuffin, 2008). A later meta-analysis aimed at including all available studies that assessed relationships between 5-HTTLPR, stress, and depression did find evidence for a 5-HTTLPR x stress interaction in the vulnerability for depression (Karg et al.,

2011). The authors argued that evidence was specifically strong for studies using chronic stressors and objective measures to assess the stressors (Karg et al., 2011). More recently, a protocol for a fourth meta-analysis on the interaction between 5-HTTLPR and stress in the development of depression has been published, keeping the debate ongoing (Culverhouse et al., 2013). Next to differences in definitions of environmental factors and phenotypes and in assessment methods (including taking into account the timing of the environmental variable, subjective versus objective reports, population stratification), publication bias, multiple testing and insufficient power also contribute to false-positive findings and lack of replication of GxE findings (Dick et al., 2015; Duncan & Keller, 2011; Duncan et al., 2014). Scholars questioning the validity of GxE findings, including the authors of the first two meta-analyses on 5-HTTLPR, stress and depression(Munafo et al., 2009; Risch et al., 2009) usually take a statistical approach to study the validity of reported GxE findings; they rely solely on consistently, replicated results (i.e. meta-analyses) where replication elements should match the original report's elements. However, there is also an construct-validity approach towards questioning the validity of any given GxE finding. This approach advocates to focus not only on statistical evidence, but also on convergent evidence; results have to emerge repeatedly despite variation in sample characteristics, measures of phenotype and environmental factors, and that are validated across human epidemiological studies, experimental neuroscience, and animal research (Caspi et al., 2010; Wankerl et al., 2010). Scholars taking this approach argue that research conducted among humans (including observational and experimental neurobiological research), mice, and rodents have consistently shown that variation at the serotonin transporter gene indeed moderates the organism's reactivity to stress (Caspi et al., 2010).

A second question pertaining to the validity of GxE findings, is whether the reported finding is truly the result of a GxE, and not of an underlying GEr. Gene environment correlations have been categorized as passive, active, or evocative GEr (Jaffee et al., 2013; Plomin & Rutter, 1998), with passive and evocative GEr being the two primary types of GEr. In the case of passive GEr, parents transmit the genetic risk variant and expose the child to the environment that is created as a result of them carrying the same genetic variant. We will explain this further by taking GxE research with maternal prenatal smoking as an environmental factor as an example. Research has shown that maternal smoking in itself is highly heritable, and thus genetic factors play a substantial role in the etiology of smoking behavior. At the same time, child psychopathology is also heritable and similar genetic variants might be involved. Thus, maternal smoking may reflect a genetic predisposition to psychopathology rather than a causal risk factor. For ADHD, this notion is supported

by research showing that the association between maternal prenatal smoking and ADHD is fully accounted for by unmeasured familial confounding, including genetic factors (Skoglund et al., 2014). Also, higher rates of psychopathology have been reported in both parents of children with ADHD whose mothers continued to smoke during pregnancy (Sengupta et al., 2015). For cognition and externalizing psychopathology it has also been suggested that shared genetic factors account for the smoking behavior of the mothers as well as the cognitive or behavioral outcome of the children (Gaysina et al., 2013; Kuja-Halkola et al., 2014). While in our study (see chapter 5) we adjusted all analyses for a broad measure of parental psychopathology and repeated analyses using paternal smoking during pregnancy as an environmental variable, we cannot fully exclude that maternal smoking is a proxy for genetic vulnerabilities that together with the vulnerability of carrying the 5-HTTLPR s-allele accounts for the increased risk for internalizing psychopathology. The same principle (i.e. the possibility of underlying GEr) holds true for the findings of our study presented in chapter 6.

Studies assessing (postnatal) parenting variables in relation to child outcome are further complicated by the fact that evocative GEr also might explain the findings. Evocative GEr occurs when a child, based on his or her genetic make-up, evokes certain behaviors from the environment including parenting behavior. That the child's genotype is indeed an important determinant of parenting behavior, through evocative GEr, is confirmed by several reviews (Avinun & Knafo, 2014; Klahr & Burt, 2014). Furthermore, various studies using adoption designs and also including information on the birth mother, have shown that genetically at risk children evoke certain parenting behaviors from their adoptive parents due to their own (inherited) inhibited or disruptive behaviors (Elam et al., 2014; O'Connor et al., 1998). For example, O'Connor et al. (1998) showed that the negative control adoptive children received from their adoptive mothers was associated with biological mothers' antisocial behavior, suggesting that the parenting the children received in their adoptive families was evoked by a genetic risk. Using a relatively small sample of twins, Pener-Tessler et al. (2013) showed that boys (n = 116) carrying the s-allele of 5-HTTLPR experienced more observed positive parenting, independent of the maternal genotype. This association was mediated by self-control, with boys carrying the s-allele having higher levels of maternal rated self-control which, in turn, evoked higher levels of positive maternal parenting. These findings urge the need for more studies on (molecular) evocative GEr in relation to parenting and child outcome to provide further insights into the etiology and the causality of reported findings. It is, however, important to bear in mind that children may not only evoke behaviors from the family system, but also from others in their social environment such as their peers.

Shared genes underlying phenotypic associations in observational, intergenerational research

Gene-environment correlations may not only underlie GxE findings, but may underlie almost all associations between a parent's phenotype and their biologically child's phenotype. This is because behavioral traits, such as various forms of psychopathology, but also 'environmental' phenotypes, such as parenting behaviors, are genetically influenced: As for psychopathology, there is recent evidence suggesting that one general underlying dimension summarizes an individuals' liability to develop any form of psychopathology: the General Psychopathology factor, or p-factor (Caspi et al., 2014; Lahey et al., 2011). An etiological hypothesis is that a genetic liability underlies p, with multiple genes enhancing risk for any form of psychopathology rather than for a specific form (Caspi et al., 2014). This hypothesis is supported by results from molecular genetic studies and genetically informed observational studies, reporting genetic overlap between different forms of psychopathology and related phenotypes (Gatt et al., 2015; Lichtenstein et al., 2009; Savage et al., 2015). There is also substantial evidence of genetic influences on various forms of parenting behaviors (Collins et al., 2000; Kendler, 1996; Neiderhiser et al., 2004). Importantly, there is evidence that the same genetic factors involved in psychopathology are also involved in the rearing or family environment (McAdams et al., 2013; Narusyte et al., 2011). Because parents share on average 50% of their genes with their children and because of genetic pleiotropy, shared genes (i.e. underlying GEr) may be an important cause of associations between various parental phenotypes and child phenotypes. For example, if the same genetic factors are involved in parental harsh parenting and in child externalizing problems we cannot infer that there is a true environmental association between harsh parenting and externalizing problems. The association could also be explained by shared genetic factors (passive or evocative GEr). Other examples of associations between phenotypes that may be explained by shared genes, also pertaining to the studies included in this thesis, are; parental psychopathology and child psychopathology, maternal smoking behavior and child psychopathology, maternal parenting behaviors and child cognitive ability, and parental marital discord and child psychopathology. Accordingly, we cannot rule out the possibility that the associations described in this thesis may be, in part, explained by shared genetic factors, rather than representing true causal findings. While we tried to account for possible confounds in our studies as best as possible, the gold standard to control for unobservable, confounding factors (including shared genetic factors) is the randomized controlled trial (RCT).

In a RCT, the study subjects are randomly assigned to a condition of interest (e.g. the phenotypic or environmental risk factor). However, in many cases an RCT

is infeasible. For example, you cannot randomly assign people to smoke cigarettes in pregnancy or not. Neither can you randomly assign children to a new set of parents; parents who exhibit harsh parenting behaviors and parents who do not, or to parents with and without marital problems. Alternatively, family-based quasi-experimental designs including multiple family relationships that differ in their genetic risk and environmental exposures, can be implemented in observational research. In short, these designs are genetically informed and can therefore estimate the separate contribution of genetic and environmental factors to the associations under investigation. Examples of such designs are adoption studies, in vitro fertilization studies, sibling comparisons, twin studies, and children of twins (CoT) studies (see for a review about these designsD'Onofrio et al., 2013). From studies using these designs it follows that parental depression and antisocial behavior have an environmental effect on child psychopathology, after adjusting for familial confounds including genetic liability (McAdams et al., 2014). However, in the case of parental smoking and child behavioral and cognitive outcome, there is strong evidence that associations are explained by genetic factors passed down from mother to child (D'Onofrio et al., 2013). It also follows from these studies that associations between parenting behaviors and other family characteristics such as marital conflict and child psychopathology can be partly explained by the sharing of common genes. Interestingly, as discussed earlier, the genetic effects do not only run from parent to child (passive GEr), but children can affect their parent's behavior just as much (evocative GEr) (Avinun & Knafo, 2014; McAdams et al., 2014).

In conclusion, inferring causality in observational, developmental research is very challenging, especially because GEr may underlie many associations between parental (environmental) phenotypes and child phenotypes. The use of family-based quasi-experimental designs can help us better understand which associations are environmental and which are (partly) influenced by genes. Ultimately, the environmental risks associated with child psychopathology which are least genetically influenced may form targets for intervention and prevention programs, as those environmental risks may be best modifiable.

Recommendations for observational, intergenerational research designs

Besides including quasi-experimental designs in the study of intergenerational transmission of psychopathology as discussed above, I would like to make some other recommendations for the design of observational, intergenerational studies (Cairns et al., 1998; Serbin & Karp, 2004). First, the data should be prospective rather than retrospective. While there is no debate about the inclusion of prospective assessments over retrospective assessment, it is logistically not always possible to include

prospective assessments, for example in our study described in chapter 8 where we did not have prospective data available for the grandparental generation. However, not every type of retrospective assessment is problematic per se. For instance, the retrospective recall of an objective and significant event (e.g. parental divorce), is less likely to be subject to bias. The retrospective recall of past behavior is, however, subject to recall bias, a form of information bias. Recall bias occurs when individuals report about a past behavior or event, which is distorted based on their current mood state or personal characteristics (Newson et al., 2011). For example, an individual that is currently depressed may remember his or hers upbringing more negatively than an individual that is non-depressed. In the study described in chapter 8, where we included a retrospective report of mothers' perceived rejective parenting by their mothers (i.e. mother's mothers). In order to reduce recall bias we included a control variable assessing the mother's current mood state in the analyses. Interestingly, there is evidence suggesting relatively high stability of reports of perceived parental rearing as measured with the EMBU questionnaire (Richter & Eisemann, 2001), and showing that reports of experienced rearing were not indicative of fluctuations in depression severity of the respondents (Gerlsma et al., 1993). This points to the fact that retrospective reports of parenting should not be immediately minimized as biased recalls due to mood or other personal characteristics, but can also be representative of actual experienced parenting.

The second recommendation for observational, intergenerational studies is that the data should be measured at multiple time intervals (i.e. multilevel), and be obtained from multiple measurement sources or domains. It is a challenge for large cohort studies to not solely rely on self-reports, but to include multilevel data and data obtained from multiple measurement sources or domains. The pitfall of relying on self-reports is the possibility of introducing shared-method-variance. This occurs when similar informants are used to assess both the predictor variable and the outcome. For example, if a mother experiences depressive symptoms or has personality characteristics that include a pessimistic mood, this might influence her report of her child's behavior; the report of her child's behavior might be colored by her own mood state. This can then cause spurious associations between psychosocial predictors and psychopathology. In the studies included in this thesis we used self-reports of parental and child psychopathology, parenting behavior, and cognitive outcomes. However, we tried to avoid shared-method-variance by including reports of other and multiple informants (see Chapters 3, 4 and 8) and we also included observed or multi-level data where possible (see chapters 2, 6 and 7).

Last, when studying similarities across two or more successive generations, it has been stated that associations are most clear when the individuals that are studied are observed at approximately the same age or at the same developmental stage. When individuals are of different ages, for example when correlating a parent's trait score to a child's trait score, two possible confounds can be introduced. One confound is that the trait score assessed in adulthood may measure something quite different than the same trait score assessed in childhood (Patterson, 1998). The second confound may be introduced when the trait scores are measured in different ways for parents and children (Patterson, 1998). However, it is also been argued that individuals do not have to be studied at the same developmental ages depending on the hypothesized underlying process (Dubow et al., 2003). For example, when the underlying process leading to associations between parental and child psychopathology is thought to be social learning, than the child has to be exposed to the parent's behavior somewhere during childhood in order to assess the association. Furthermore, it is also a logistic challenge to maintain contacts with a cohort over so many years to be able to assess two (or more) generations at the same developmental stage. The subjects included in the Generation R Study are not yet followed long enough for us to be able to assess the parents and their children at similar developmental stages (i.e. in young adulthood), but efforts are made to be able to do so in the future. Ultimately, it would be very interesting to be able to follow this cohort until the children become parents themselves, which would allow us to study three successive generations. This would give us the opportunity to prospectively study continuities in mediating variables, for example parenting behaviors.

Like father, like child?

Many of the researchers studying the intergenerational transmission of psychopathology have focused on the transmission from mother to child, especially when the outcome was assessed in young children. This also holds true for most studies included in this thesis. Several reasons for the focus on mothers exist; traditionally, mothers are considered the primary care givers and therefore thought to have to most influential role on early child development. Also, assessing mothers provides researchers with the opportunity of studying intra-uterine effects on child development. Furthermore, logistic or practical reasons play an important role, as mothers are often more willing to participate in studies.

However, nowadays in most Western countries fathers also have an active role in daily family life and actively participate in child upbringing. Consequently, exposure to paternal behaviors including psychopathology and family interactions is likely to also transfer risks associated with psychopathology to their children. Indeed,

research assessing the role of paternal psychopathology on child development underscores that exposure to paternal psychopathology is also associated with an increased risk for child psychopathology and cognitive outcomes, independent of maternal psychopathology (Ramchandani & Psychogiou, 2009). Research including fathers with antisocial personality disorders and twin offspring has shown that children exposed to a father with high levels of antisocial behavior have an increased risk for behavioral problems, especially when the father was present in the family over a longer period of time, rather than being absent (Blazei et al., 2008; Jaffee et al., 2003). This indicates that, next to a genetic vulnerability, fathers also expose their children to an environmental risk associated with their behavioral characteristics, possibly via impaired parenting. That the role of (early) father-child interactions are indeed of importance for child development is shown by recent studies (Malmberg et al., 2016; Moller et al., 2016; Ramchandani & Psychogiou, 2009), but in contrast to research assessing mother-child interactions, this field is still relatively neglected and more research is needed. Future research could also assess differences in parent-child interactions between fathers and mothers, and assess how these differences relate to child development as there are indications that fathers differ from mothers in their parenting styles (Bogels & Perotti, 2011; Meunier et al., 2012).

RECOMMENDATIONS FOR RESEARCH

Based on the findings in this thesis several recommendations for future research can be made. The first recommendation is to adapt a family wide perspective when assessing a child's risk for psychopathology, including all aspects of the family system surrounding the child. The gross of research has focused on a smaller sub-system of the family system, mostly mother-child interactions such as parenting behaviors. However, assessment of all family interactions simultaneously, including father-child interactions and inter-parental interactions, will help understand of how different family processes together mediate risk towards a child. It is well-recognized that interactions in one sub-system influence not only the individuals directly involved, but also the larger family system (Cummings et al., 2005; Lucas-Thompson & Goldberg, 2011). For example, we showed that marital discord can directly impact on child psychopathology, but can also affect ineffective parenting behaviors (chapter 8). Incorporating this family wide perspective in longitudinal research including three successive generations will provide the unique opportunity of studying continuities or discontinuities in family processes. Identification of underlying processes that help sustain or interrupt familial cycles of psychopathology will ultimately help the formulation of effective prevention and intervention programs.

Second, our current understanding of the transmission of psychopathology would benefit from further research on how genetic and environmental factors together mediate the risk for psychopathology from one generation to the next. Although there is an ongoing debate on the validity of GxE studies, in my opinion good quality GxE research (i.e. overcoming heterogeneity in phenotypes and environmental variables, including objective data where possible, large sample sizes, see above) will add to our understanding of how genetic and environmental factors exert influences on child development. The testing of a GxE effect should not have to depend on the existence of genetic main effect on the selected phenotype. Rather, it might well be that the inconsistent findings regarding a genetic main effect might trigger us to consider GxE instead (Caspi et al., 2010). Future GxE research may broaden the G-component by the inclusion of genetic pathways. In path-way based tests, researchers assess predefined gene-sets based on prior biological knowledge (Wang et al., 2010). Also, broadening the E-component by the inclusion of positive environments may help us further understand why some children differentially respond to environmental exposures: Recent studies show that individuals carrying certain genetic variants (susceptibility markers) are more prone to function poorly in stressful environments, but that they are also the ones to benefit the most from supportive or positive environments (see the differential susceptibility hypothesis) (Bakermans-Kranenburg & van, 2015; Belsky & Pluess, 2013). Furthermore, collaboration between researchers from different scientific fields, including neuroscience and animal research will prove fruitful in adding to the 'construct' validity of GxE research and thereby to a general understanding of the biological and pathophysiological underpinnings of various forms of psychopathology.

An intriguing notion of recent research and example of the complex interplay between genes and environment, is that the environment may also alter gene expression. Epigenetics, defined as changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence, offers an emerging and challenging direction for research on the interplay between nature and nurture. Methylation is such an epigenetic mechanism where under the influence of an (early) environmental factor modification of methylation patterns of the genome occurs it subsequent alterations to gene expression. For instance, in humans, a history of child abuse or neglect has also been associated with altered methylation patterns in adult hippocampal tissue (McGowan et al., 2009). Also, childhood abuse has been previously associated with altered methylation patterns of promotor region of the serotonin transporter gene in later life (Beach et al., 2010) and with lower hippocampal volume (Booij et al., 2015). Together, these findings set the stage for further epigenetic research to increase our understanding of the mechanisms by which genetic and environmental factors may influence child development.

As mentioned before, a promising avenue for future research lies in unraveling which 'environmental' behaviors or responses of the environment (e.g. parenting behavior), are in fact evoked by genetically influenced characteristics of the children (GEr). While the bi-directionality of the parent-child relationship is acknowledged (Belsky, 1984), research into this field is still relatively sparse. Importantly, this bi-directionality can also be extended to the social environment of the child where, based on their genes, children evoke behaviors from other family members than their parents, and from their peers. For example, it has been shown that children that are more outgoing based on their genetic make-up, evoke more prosocial behaviors from an unfamiliar peer (DiLalla et al., 2015). Ultimately, the suggestions made for future research on gene-environment interplay will help us better understand psychopathology and improving models for intervention and or treatment.

The last, general recommendation is that longitudinal research should include repeated assessments of psychosocial risks and psychopathology. The inclusion of repeated assessments makes it possible to study the development of psychosocial risks and of psychopathology over time. Also, with special statistical modelling, a researcher can simultaneously model the level of severity and of duration of symptoms of psychopathology (see our study included in chapter 2 of this thesis). In the future this will hopefully lead to the (early) identification of subgroups of individuals with psychopathology transmitting the greatest risk to their children. These individuals can then be specifically targeted for prevention and intervention strategies. Most likely, not only depressive symptoms are heterogeneous in nature, but most other forms of psychopathology or adverse behaviors are as well.

CLINICAL IMPLICATIONS

The findings of this thesis do not have a direct implication for clinical practice. However, some suggestions can be made. Clinicians and other health care workers, including general practitioners (GPs) and midwifes, play an important role in the identification of families with an increased risk of child psychopathology. Therefore, a good understanding of risk factors for child psychopathology is important. While parental psychopathology and parental psychosocial risks, such as ineffective parenting, are considered key risk factors for child psychopathology, it follows from this thesis that a history of grandparental psychopathology also increases the risk for child psychopathology even in the absence of parental psychopathology. Also, we have shown that a history of grandparental divorce may increase the child's risk for psychopathology. Therefore, clinicians and other health care workers assessing children at risk for psychopathology should also adopt a family-wide risk assessment

including information on psychopathology and psychosocial risks including those of the grandparental generation next to the key risk factors mentioned before. In my opinion, especially GPs should play a pro-active role in signaling early risks for psychopathology in families with (a desire for) children, because the GP, as a family doctor, possesses a wealth of information regarding the wider family system. In the presence of risk factors for child psychopathology, the GP can counsel (future) parents, but also suggest prevention or intervention therapies for psychological problems, marital problems, or parenting interventions among others.

Several commercial companies offer the ability of genetic profiling, where based on the assessment of multiple genetic risk variants, a risk for psychopathology can be determined. However, genetic profiling in families with known risk has not yet been proven successful and not of added value to the risk prediction based on known risk factors such as parental psychopathology, and psychosocial risks (Janssens & van Duijn, 2008). This is most likely explained by the fact that multiple genes, with small effects are involved in the etiology of psychopathology. Furthermore, there is also research indicating that genetic profiling, or offering biogenetic explanations for psychopathology, increases the stigma of individuals with psychopathology by pointing out a stable and intrinsic state, and causes them to feel 'fundamentally' flawed and guilty for their condition (Rusch et al., 2010). This implies that clinicians should not refer individuals to genetic profiling tests, but should also be careful with offering explanations for the symptoms that solely focus on biogenetic factors.

Concluding remarks

In conclusion, the findings of this thesis underscore the complexity of mechanisms underlying the intergenerational transmission of psychopathology. Individuals may transmit genetic and environmental risks associated with psychopathology to their children, even when they do not express (concurrent) symptoms of psychopathology themselves. The challenge for researchers, but also for clinicians and policy makers, is to adopt a family wide perspective when assessing risk of psychopathology in children and their families. This perspective should include, next to the assessment of parent-child interactions (e.g. parenting behavior), assessment of inter-parental interactions and of characteristics of the grandparental generation, including psychopathology and family interactions. Furthermore, enhancing insights in the genetic and environmental interplay underlying psychopathology will increase understanding of the transmission of psychopathology. Only if combined with insights from environmental research will genetics ultimately help the formulation of effective early prevention and intervention strategies.

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CHAPTER 10

Summary/Samenvatting

SUMMARY

Parental psychopathology is a major risk factor for child psychopathology and a wide range of other developmental problems, including cognitive problems. In turn, child psychopathology is predictive of psychopathology in adulthood, thereby sustaining a familial cycle of psychopathology. Because psychopathology often recurs in the same families over the course of multiple generations, the intergenerational transmission of psychopathology remains a subject of great interest to researchers, clinicians, and policy makers. Ultimately, a clear understanding of the processes underlying the transmission of psychopathology is needed to formulate effective prevention and intervention programs for families at increased risk for psychopathology. Therefore, as described in Chapter 1, the main aim of this thesis was to enhance current insights into the intergenerational transmission of psychopathology. We formulated two specific aims: The first specific aim was to increase our understanding of the nature of associations between grandparental, parental and child psychopathology. The second specific aim was to increase our understanding of the complex roles of genes and parenting behaviors in the transmission of psychopathology. To address these aims, the studies included in this thesis were embedded in the Generation R Study, a prospective population-based cohort from fetal life onwards in the city of Rotterdam, the Netherlands.

In **Part 1** of this thesis two studies addressing the first specific aim were embedded. In Chapter 2 we modeled the heterogeneity of maternal depressive symptoms from pregnancy to the child's age of three years using a latent class modeling technique. We showed that perinatal maternal depressive symptoms follow 4 distinct trajectory groups that are rather stable over time, with a small trajectory group of mothers (1.5%) experiencing symptoms that are clinically relevant. The mothers assigned to the higher trajectory groups were more often of non-Western ethnicity and had more socioeconomic risks such as low educational level as compared to mothers assigned to the lower trajectory groups. The children of the mothers assigned to the higher trajectory groups were at increased risk for psychopathology, independent of the mothers' concurrent depressive symptoms, as compared to children of mothers assigned to lower trajectories. Moreover, in Chapter 3, we showed that children with a positive family history of grandparental anxiety or depressive disorder are at increased risk for psychopathology, independent of a history of parental psychopathology or concurrent parental symptoms.

In **Part 2** of this thesis we included studies that addressed the second specific aim. In Chapter 4, we studied the influence of genetic factors on early language acquisition using a Genome Wide Association Study (GWAS), which is a hypothesis

free approach. We identified a common genetic variant, rs7642482, associated with the early phase of language acquisition. The variant is located near ROBO2, an axon-guidance receptor involved in the outgrowth of axons that has been associated with autism spectrum disorders .

In Chapter 5 and Chapter 6 we studied the mutual roles of candidate genes and environmental risk factors in the etiology of children's internalizing problems and working memory problems, respectively. In Chapter 5 we studied the effect of an interaction between a polymorphism in the promotor region of the serotonin transporter gene (5-HTTLPR) and maternal prenatal smoking on child internalizing problems. We showed that children carrying the short allele (s-allele) were at increased risk for internalizing problems when the mothers continued to smoke during pregnancy. We found no interaction-effect of 5-HTTLPR and paternal prenatal smoking, supporting that the findings are truly in support of an intra-uterine effect of maternal smoking. In the second study (Chapter 6) we showed that the association between a polymorphisms of the COMT-gene (Val158Met) and child working memory problems was moderated by maternal harsh parenting. Using two statistical modelling techniques, a linear gene-association regression analyses and a family based association test (FBAT), we consistently showed that children homozygous for the Met allele perform better on working memory in the presence of low maternal harsh parenting, but perform worse on working memory in the presence of high maternal harsh parenting compared to children homozygous for the Val allele.

That parenting behaviors are also influenced by genetic factors, is shown by our results in Chapter 7. We studied the role of 5-HTTLPR on observed maternal sensitive parenting that was repeatedly assessed at three different time points. We showed that mothers carrying the s-allele were more sensitive towards their children than mothers carrying the l-allele. Findings were not explained by the child's 5-HTTLPR genotype. We also studied whether child fearfulness moderated the association between maternal 5-HTTLPR and sensitive parenting, but found no evidence for this.

In the last study included in this thesis, Chapter 8, we assessed the roles of parental divorce, marital discord and ineffective parenting behaviors over the course of three generations in relation to child psychopathology. Using a structural equation model, we found that grandparental divorce was predictive of child internalizing and externalizing problems through continuities in ineffective parenting of the grandmother and the mother. Also, grandparental divorce predicted maternal marital discord which, in turn, was predictive of child psychopathology. All effects were independent of maternal psychopathology and socio-economic variables.

The results of the studies included in this thesis are discussed in a broader context in Chapter 9. In addition, some methodological challenges pertaining to these studies are discussed. The chapter concludes with implications for future research and clinical applications.

In conclusion, the findings of this thesis underscore the complexity of the mechanisms underlying the intergenerational transmission of psychopathology: Individuals may transmit genetic and environmental risks associated with psychopathology to their children, even when they do not express (concurrent) symptoms of psychopathology themselves. The challenge for researchers, but also for clinicians and policy makers, is to adopt a family wide perspective when assessing risk of psychopathology in children and their families. This perspective should not only include assessment of parent-child interactions, but also of interparental interactions and of characteristics of the grandparental generation, including psychopathology and family interactions. Enhancing insights in the genetic and environmental interplay underlying psychopathology will increase understanding of the transmission of psychopathology. Only if combined with insights from environmental research will genetics ultimately help the formulation of effective early prevention and intervention strategies for children and their families at risk for psychopathology.

SAMENVATTING

Psychopathologie bij ouders is een belangrijke voorspeller voor psychopathologie en andere ontwikkelingsproblemen bij het kind, waaronder cognitieve problemen. Psychopathologie op de kinderleeftijd is wederom voorspellend voor psychopathologie op oudere leeftijd, waardoor er een familiaire cirkel van psychopathologie in stand kan worden gehouden. Omdat psychopathologie vaak voorkomt bij opeenvolgende generaties binnen dezelfde familie, blijft de intergenerationele transmissie van psychopathologie een belangrijk onderwerp voor onderzoekers, clinici en politici. Uiteindelijk is een duidelijk inzicht in de verschillende processen die de transmissie van psychopathologie onderliggen nodig om effectieve preventie- en interventieprogramma's te ontwerpen voor families met een verhoogd risico op psychopathologie. Daarom, zoals beschreven in hoofdstuk 1, was het hoofddoel van dit proefschrift het bevorderen van de huidige inzichten in de intergenerationele transmissie van psychopathologie. We formuleerden twee subdoelen: Het eerste subdoel was om meer inzicht te verkrijgen in de aard van de verbanden tussen psychopathologie bij grootouders, psychopathologie bij ouders en psychopathologie bij het kind. Het tweede subdoel was om meer inzicht te verkrijgen in de complexe rollen van genetische factoren en opvoedingsstijlen die de transmissie van psychopathologie onderliggen. De studies in dit proefschrift werden uitgevoerd binnen het Generation R Onderzoek, een grootschalig prospectief cohortonderzoek onder Rotterdamse kinderen en hun ouders. In dit geboortecohort worden groei, ontwikkeling en gezondheid bestudeerd vanaf de zwangerschap tot in de vroege volwassenheid.

In **Deel 1** van dit proefschrift hebben we twee studies geïncludeerd die het eerste subdoel adresseren. In Hoofdstuk 2 hebben we de diversiteit van depressieve symptomen bij de moeder onderzocht vanaf de zwangerschap tot het kind drie jaar is. Met behulp van latente klassen analyses laten we zien dat perinatale depressieve symptomen bij de moeder ingedeeld kunnen worden in 4 verschillende symptoom-groepen (latente klassen) die vrij stabiel zijn door de tijd heen, maar verschillen in ernst van de depressieve symptomen. Slechts een kleine groep moeders (1.5%) ervaart symptomen die voldoen aan het criterium van een 'depressieve stoornis'. Moeders die toegewezen zijn aan de ernstigere symptoom-groepen zijn vaker van niet-Westerse afkomst en hebben meer socio-economische risicofactoren, zoals een lager opleidingsniveau, dan moeders toegewezen aan de lagere symptoom-groepen. De kinderen van moeders die aan de hogere symptoom-groepen zijn toegewezen, hebben een hoger risico op psychopathologie, onafhankelijk van de huidige symptomen die moeder ervaart. In Hoofdstuk 3 laten we zien dat kinderen

die een positieve familie anamnese hebben voor een angststoornis of depressieve stoornis bij een van de grootouders, een hoger risico hebben op het ontwikkelen van psychopathologie. Dit effect werd niet verklaard door psychopathologie bij de ouders.

In **Deel 2** van dit proefschrift hebben we studies geïncludeerd gericht op het tweede subdoel. In Hoofdstuk 4 hebben we de invloed van genetische factoren op vroege taalontwikkeling onderzocht door middel van genoom brede associatie studie (GWAS). Dit is een hypothese-vrije benadering. We hebben een genetische variant geïdentificeerd, rs7642482, die geassocieerd is met de vroege fase van taalontwikkeling. Deze genetische variant is vlak naast ROBO2 gelokaliseerd, een receptor die betrokken is bij de uitgroei van axonen en eerder is geassocieerd met autisme spectrum stoornissen.

In Hoofdstuk 5 en Hoofdstuk 6 hebben we de gezamenlijke rol van kandidaat genen en omgevingsfactoren in de etiologie van internaliserende problemen en werkgeheugen bij kinderen onderzocht. In Hoofdstuk 5 hebben we het effect van een interactie tussen een polymorfisme in de promotor regio van het serotonine transporter gen (5-HHTLPR) en roken tijdens de zwangerschap door moeder onderzocht op internaliserende problemen bij kinderen. We hebben laten zien dat kinderen die het korte allel van het polymorfisme dragen (s-allele) een verhoogd risico hebben op internaliserend problematiek als hun moeders gerookt hebben tijdens de zwangerschap. We hebben dit interactie-effect niet gevonden tussen 5-HTTLPR en roken van de vader tijdens de zwangerschap. Dit duidt erop dat het om een intra-uterien effect van blootstelling aan sigarettenrook gaat. In de tweede studie (Hoofdstuk 6) hebben we laten zien dat een verband tussen een polymorfisme van het COMT gen (Val158Met) en problemen met het werkgeheugen bij het kind, gemodereerd wordt door blootstelling van het kind aan een hardhandige opvoedingsstijl van de moeder. Door gebruik te maken van twee verschillende statistische technieken, een lineaire gen-associatie regressie en een familie gebaseerde associatie test, hebben we consistent laten zien dat kinderen die homozygoot zijn voor het Met-allel een beter werkgeheugen hebben wanneer ze blootgesteld worden aan lage levels van een hardhandige opvoedingsstijl, maar een slechter werkgeheugen hebben wanneer ze blootgesteld worden aan hoge levels van een hardhandige opvoedingsstijl in vergelijking met kinderen die homozygoot zijn voor het Val-allel.

Dat opvoedingsstijlen ook beïnvloed worden door genetische factoren, laten onze resultaten in Hoofdstuk 7 zien. We hebben het effect van 5-HTTLPR op een sensitieve opvoedingsstijl van moeder onderzocht. De sensitiviteit van moeder was op drie verschillende tijdstippen onderzocht. We hebben laten zien dat moeders die het s-allel dragen meer sensitief zijn naar hun kinderen dan moeders die het l-allel

dragen. De bevindingen werden niet verklaard door het 5-HTTLPR genotype van het kind. We hebben ook onderzocht of het effect nog gemodereerd wordt door angstig gedrag van het kind, maar hier vonden we geen bewijs voor.

In de laatste studie van dit proefschrift, beschreven in Hoofdstuk 8, hebben we onderzocht of scheiding en huwelijksproblematiek en ineffectieve opvoedingsstijlen van de grootouders en de ouders van invloed zijn op het risico op psychopathologie bij het (klein)kind. We hebben gebruik gemaakt van SEM (structural equation modeling) en vonden dat scheiding van de grootouders zowel internaliserende als externaliserende problematiek bij het (klein)kind voorspelt via huwelijksproblematiek bij de ouders van het kind, en door continuïteit in ineffectieve opvoedingsstijlen van de grootmoeders en moeders van de kinderen. De gevonden effecten waren onafhankelijk van psychopathologie bij de moeders en onafhankelijk van socio-economische variabelen.

In Hoofdstuk 9 worden de resultaten van de studies in dit proefschrift bediscussieerd in een bredere context. Ook worden er een aantal methodologische uitdagingen van de studies beschreven. Het hoofdstuk besluit met enkele implicaties voor wetenschappelijk onderzoek en voor de praktijk.

Concluderend onderschrijven de bevindingen van dit proefschrift de complexiteit van de processen die de intergenerationele transmissie van psychopathologie onderliggen. Personen kunnen genetische- en omgevingsfactoren die verband houden met een risico op psychopathologie doorgeven naar de volgende generatie zonder dat ze zelf klinische symptomen van psychopathologie hebben. De uitdaging voor onderzoekers, maar ook voor clinici en politici, is dat men een familie-brede benadering toepast wanneer men het risico op psychopathologie voor een kind en zijn of haar familie wil inschatten. Dit houdt in dat men niet alleen de kwaliteit van ouder-kind relaties moet beoordelen, maar ook de kwaliteit van de relatie tussen ouders. Tevens is het advies om karakteristieken van de generatie van de grootouders mee te nemen, inclusief psychopathologie en de kwaliteit van de verschillende familierelaties. Ook het verder onderzoeken van de gezamenlijke effecten van genetische- en omgevingsfactoren die psychopathologie onderliggen, zal bijdragen aan een beter begrip van de transmissie van psychopathologie. Alleen wanneer inzichten uit genetisch onderzoek gecombineerd worden met omgevingsonderzoek zal dit het formuleren van vroege preventie- en interventieprogramma's ten goede komen.

ADDENDUM

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LIST OF PUBLICATIONS AND MANUSCRIPTS

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Chapter 2

Cents, R.A.M., Diamantopoulou, S., Hudziak, J.J., Jaddoe, V.W.V., Hofman, A., Verhulst, F.C., Lambregtse-van den Berg, M.P., Tiemeier, H. (2013).
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* Both authors contributed equally

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Name PhD student:	Rolieke A.M. Cents
Erasmus MC Departments:	The Generation R Study
	Child & Adolescent Psychiatry/Psychology
Research School:	NIHES
PhD period:	2008 - 2012
Promotors:	Prof.dr. F.C. Verhulst
	Prof.dr. H.W. Tiemeier
Co-promotor:	dr. M.P. Lambregtse – van den Berg

1. PhD training Year		
	Year	**Workload (Hours/ECTS)**
General courses		
- MSc Genetic Epidemiology, Netherlands Institute for Health Sciences (NIHES), Erasmus Medical Center, Rotterdam	2008-2011	70.0
- Biomedical English Writing and Communication, Erasmus Medical Center, Rotterdam	2010-2011	4.0
Specific courses		
- SNPs and Complex diseases, MolMed, Erasmus Medical Center, Rotterdam	2008	1.0
- Biomedical Research Techniques, MolMed, Erasmus Medical Center, Rotterdam	2009	1.2
- Analyzing developmental trajectories, D. Nagin, SRCD, Amsterdam	2010	1.2
Seminars and workshops		
- Attending research meetings Generation R Study Group, Erasmus Medical Center	2008-2011	1.0
- The power of early experiences: On brain plasticity, sensitive periods and biobehavioral recovery from early trauma. Faculty of Social Sciences, Leiden University	2009	0.1
- Joint Eagle and Birth Cohorts Consortium Workshop, Oslo, Norway	2010	0.5
Presentations		
- Generation R research meeting	2009	0.5
- International Congress of the IFPE, Vienna, Austria (oral presentation)	2009	1.0
- Generation R Symposium, Rotterdam (oral presentation)	2010	0.5
- SRCD, Montreal, Canada (poster presentation 2x)	2011	1.0
- Congress of the Netherlands association of Psychiatry, Maastricht (oral presentation)	2012	0.5

(Inter)national conferences		
- International Congress of the IFPE, Vienna, Austria	2009	1.0
- Generation R Symposium, Rotterdam	2010	0.2
- SRCD, Montreal, Canada	2011	1.0
Other		
- Deputy PhD Students in the Generation R Management Team	2009-2011	2.0
- Participant of the EAGLE working group Cognition and Behavior	2009-2011	1.0
- Reviewing papers	2009-2012	0.6

2. Teaching

	Year	Workload (Hours/ECTS)
Supervising practicals and excursions, Tutoring		
- Vaardigheidsonderwijs 'Normale psychische ontwikkeling 0-5 jaar'	2010	1.0
- Minor Child & Adolescent Psychiatry 'De gekte voorbij'	2010 & 2011	3.0
- Lecturing students, Department of Psychology, Erasmus University Rotterdam	2011	0.3
Supervising Master's theses		
- Perceived parenting, parenting stress and child behaviour; how do they relate? The Generation R Study – Casper van Duijnhoven, Medical Student, Erasmus Medical Center	2011	3.0
- Maternal depressive symptoms and child executive functioning – Aimee Oei, Medical Student, Erasmus Medical Center	2011	3.0

Note: 1 ECTS (European Credit Transfer System) equals a workload of 28 hrs.

CURRICULUM VITAE

Rolieke Cents was born on March 21st 1981, in Wijhe, the Netherlands. In 1999 she graduated from secondary school (atheneum) at the Carmel College Salland in Raalte. In the same year she started her study of medicine at the Erasmus Medical Center - University of Rotterdam. During a period of one college year (2002 to 2003) she suspended her study and engaged in an extra-curricular activity as a fulltime board member (Questor and Assessor Interne) of the Rotterdam Medical Student's Association (MFVR). In 2004, she wrote her graduation thesis on 'Health Related Quality of Life in children with Traumatic Brain Injury' at the Hospital for Sick Children in Toronto, Canada, under the supervision of Prof.dr. J. Hutchison and Dr. C. Parshuram. In 2005 she started her rotations and continued her extra-curricular activities as a member of the national medical students representative, the former 'KNMG Studentenplatform' currently known as 'De Geneeskundestudent'.

After obtaining her medical degree in 2007, she started working as a medical doctor (MD) at the Department of Child and Adolescent Psychiatry/Psychology at the Erasmus Medical Center. During this year she applied for a grant at the Sophia Foundation for Scientific Research (SSWO). The subject of the grant was the study of the genetic and non-genetic influences underlying the intergenerational transmission of psychopathology. The grant proposal was awarded (grant 547) and in 2008 she started working on the research project under the supervision of Prof.dr. H. Tiemeier, Dr. M.P. Lambregtse-van den Berg, and Prof.dr. F.C. Verhulst, which resulted in the work described in this thesis. In parallel, she obtained a Master of Science degree in Genetic Epidemiology (2011).

In September 2012, she started her training as a General Practitioner (GP) at the Erasmus Medical Center, which she finished in July 2016. During her GP-training she participated in a masterclass under the supervision of Prof.dr. P.J.E. Bindels with a resulting publication 'Suïcide onder adolescenten' (Huisarts & Wetenschap, March 2016). She continued to work on her thesis in her spare time.

Currently, she is working as a GP in 'Huisartsenpraktijk Arkel', in Arkel. In the future, she hopes to continue her research in the field of child development and psychopathology and combine it with her professional career as a GP.

Rolieke is married to Leonard Seghers who is a resident in Pulmonology. Together they have two sons, Tristan (2009) and Sweder (2014), and a daughter, Dagmar (2012). They live in Nieuw-Terbregge, Rotterdam.

DANKWOORD

Dit dankwoord is het laatste hoofdstuk in dit proefschrift, en daarmee is ook een einde gekomen aan mijn promotie-traject. Een traject dat niet succesvol afgesloten had kunnen worden zonder de bijdrage en steun van diverse mensen:

Een speciaal woord van dank gaat uit naar alle kinderen en hun ouders die al jarenlang deel uitmaken van het Generation R Cohort. Veel dank voor het belangeloos invullen van de vragenlijsten en jullie bezoeken aan het onderzoekscentrum. Door jullie bijdrage zijn mede de onderzoeken in dit proefschrift tot stand gekomen en ligt er nog een enorme schat aan gegevens klaar om onderzocht te worden!

Mijn (co)promotoren: Geachte Prof.dr. F.C. Verhulst, beste Frank, dankjewel voor je snelle, vaak enthousiaste, maar altijd kritische noten bij mijn manuscripten. Ik heb bewondering voor de manier waarop je aan het hoofd van een grote organisatie staat en toch ook zo dicht bij je medewerkers, studenten, en patiënten blijft staan. Geachte Prof.dr. H. Tiemeier, beste Henning, dit proefschrift is eindelijk af! Om jou te citeren: 'Ende gut, alles gut!'. Mede door jouw enorme gedrevenheid, je altijd kritische houding, en je enorme enthousiasme voor de wetenschap, ben je mij altijd blijven stimuleren om op wetenschappelijk gebied het uiterste van mezelf te vergen. Door het steeds weer verleggen van mijn grenzen is dit (naar mijn mening) prachtige proefschrift tot stand gekomen. Geachte dr. M.P. Lambregtse – van den Berg, beste Mijke, goed voorbeeld doet volgen. Ik heb veel respect voor de manier waarop jij alle ballen in de lucht houdt. Bedankt voor je sterke klinische blik, je gedrevenheid, afgewisseld met de gezellige praatjes over het wel en wee van onze kinderen. Beste Frank, Henning, en Mijke, ik hoop van harte dat met het voltooien van dit proefschrift geen einde aan onze samenwerking is gekomen.

Leden van de kleine commissie: Geachte Prof.dr. S.A. Kushner, geachte Prof. dr. J.K. Buitelaar en geachte Prof.dr. S. Claes, hartelijk dank voor uw bereidheid om zitting te nemen in de kleine commissie en voor het kritisch doornemen van dit proefschrift.

Leden van de grote commissie: Geachte Prof.dr. B. Franke en geachte Prof.dr. P. Prinzie, hartelijk dank voor het doornemen van dit proefschrift en de bereidheid op te treden als opponenten tijdens mijn verdediging. Een speciaal woord van dank aan Prof.dr. P.J.E. Bindels, beste Patrick, onze samenwerking begon reeds voor het voltooien van dit proefschrift. Bedankt voor de prettige begeleiding en samenwerking tijdens de masterclass, ik hoop dat we dit in de toekomst zullen voortzetten.

Mijn paranimfen: Lieve Claudia, we leerden elkaar kennen als collega's, maar werden als snel hartsvriendinnen. We kunnen samen lachen en huilen. Ontzettend bedankt voor alle afleiding, grapjes, peptalks en (inhoudelijke) discussies. Ik ben

ontzettend blij dat jij naast me staat tijdens de verdediging. Lieve Theliene, mijn lieve zusje. Ontzettend bedankt voor je interesse, steun, en luisterend oor (ook al praat je zelf ook erg graag). Natuurlijk sta jij vandaag naast mij! En wie weet worden de rollen nog eens omgedraaid.

Mijn co-auteurs: bedankt voor jullie bijdrage aan de verschillende manuscripten. In het bijzonder dank aan Jolien. Ik kan ons SEM model wel dromen! Bedankt voor je eindeloze geduld, je uitleg, en het telkens weer opnieuw 'draaien' van het model. Het is een mooi manuscript geworden. Also, special thanks to Beate. Thanks for your patience. I hope that someday, I will know half of what you know about statistics and genetics.

Dit proefschrift had ook niet tot stand kunnen komen zonder de ondersteuning van alle bureau- en focusmedewerkers. Bedankt aan allen die op welke manier dan ook een bijdrage hebben geleverd en leveren aan het verzamelen, opslaan, bewerken, en bewaren van alle data. Met name dank aan Patricia, Alwin, Claudia en Marjolein. Vooral de GWAS analyses vergezeld van een kinderwagen met baby zal ik niet snel vergeten! En natuurlijk ook dank aan Laureen, de ten alle tijden behulpzame en zeer efficiënte kracht van de kinder- en jeugdpsychiatrie.

Ook een woord van dank aan alle collega's van Generation R. Dank voor jullie gezelligheid en inhoudelijke commentaar. Een speciaal woord van dank aan mijn kamergenootjes en tegenover-kamergenootjes: Rianne, Marina, Nicole, Maartje, Fleur, Ank, Hanneke, Marieke, Nienke, en Nina. Jullie waren en zijn een belangrijke factor in alle ter zake doende, en minder ter zake doende gezelligheid en steun. Fleur, wat ontzettend leuk om jou weer bij Generation R tegen te komen. De klik was er opnieuw! Bedankt voor je support, meedenken en gezelligheid, wie weet kruisen onze paden in de toekomst nog eens. Marieke, wij leerden elkaar pas echt kennen tijdens de huisartsopleiding. Bedankt voor je goede vriendschap en je ongezouten mening die ik altijd erg kan waarderen! Lieve Claudia, Marieke, Hanneke, Nienke, Ank en Nina, ik hoop dat we onze gezellige borrels en etentjes ook in de toekomst blijven voortzetten, ondanks de nog steeds toenemende hectiek van ons (gezins)leven.

Beste Gerard en Jantine, ontzettend bedankt voor de ruimte die jullie mij in mijn eerste opleidingsjaar tot huisarts geboden hebben om me weer in de kliniek te verdiepen, en daarnaast een gezin en een promotietraject draaiende te houden. Alle lieve dames van de huisartsenpraktijk, ook jullie bedankt voor alles. Door jullie heb ik een ontzettend leuk en leerzaam eerste opleidingsjaar gehad, en alle ballen in de lucht kunnen houden!

Beste Peter en Paulien, de ballen bleven ook in mijn derde en laatste opleidingsjaar in de lucht. Ontzettend bedankt voor de fantastische begeleiding in mijn verdere traject tot 'echte' huisarts. Jullie hebben een prachtige praktijk! En ook dank aan

alle lieve dames van Huisartsenpraktijk Arkel; wat is het toch gezellig met jullie! Die gezelligheid, maar ook al die luisterende oren en de humor zijn aspecten niet kunnen en mogen ontbreken op de werkvloer om plezier in je werk te blijven houden.

Lieve vriendinnen en vrienden, dank jullie allemaal voor alle steun, oppeppers, afleiding, maar bovenal voor alle gezelligheid. Inmiddels wonen we allemaal verspreid over het land, maar we weten elkaar altijd weer te vinden! Ontzettend leuk om mee te maken hoe iedereen zijn of haar weg vindt. Mijn 'oudste' vriendinnen; dankjulliewel voor de blijvende interesse en bovenal voor de nuchtere kijk op het leven. Dit is een heerlijke afwisseling in mijn hectische bestaan.

Lieve (schoon)zussen en –broers, en leden van de 'A&C happy family', wat ontzettend mooi en leuk om te zien hoe onze levens zich steeds verder ontvouwen. Ik hoop dat we nog erg lang zo bij elkaar betrokken blijven. Bedankt voor alle vertrouwdheid, warmte en gezelligheid. Jullie zijn een basis waar ik op ieder moment op kan terugvallen.

Lieve Camiel en Ank, bedankt voor alle liefde, alle onuitputtelijke interesse voor alles wat ik doe en meemaak, en alle belangeloze support. Lieve Dineke, ik kan je niet genoeg bedanken voor al het moois dat we nog iedere dag terugzien in onze prachtige kinderen. We denken veel aan je..

Lieve pap en mam, bedankt voor de genen en de opvoeding: Mede door jullie ben ik geworden wie ik nu ben, en heb ik kunnen bereiken wat ik nu bereikt heb. Ontzettend bedankt voor jullie onvoorwaardelijke liefde, alle vertrouwen en de emotionele steun (als wel fysieke steun als het weer eens op oppassen en huis schoonmaken aankwam)! Ik hou van jullie.

Lieve Leonard. Dit is ook een beetje jouw proefschrift: Jij maakt dat ik ben wie ik wil zijn, en doe wat ik wil doen. Jij vult me niet alleen aan, maar haalt het beste in me naar boven. Bedankt voor het zijn wie je bent. Je rust en stabiliteit waren onmisbaar in deze zeer hectische periode van ons leven. Ik hou oneindig veel van je en ben ontzettend trots met jou als man en vader van onze prachtige kinderen.

Lieve Tristan, Dagmar en Sweder, jullie maken ons leven compleet. Mama geniet ieder moment van de ontwikkeling die jullie doormaken (en wat leer ik daarbij nog steeds veel over mezelf!). Jullie zijn echte rakkers! Ik hou ontzettend veel van jullie en ben razend benieuwd waar de genen en de opvoeding jullie gaan brengen!