HPA-AXIS, GENES, AND ENVIRONMENTAL FACTORS IN RELATION TO EXTERNALIZING BEHAVIORS

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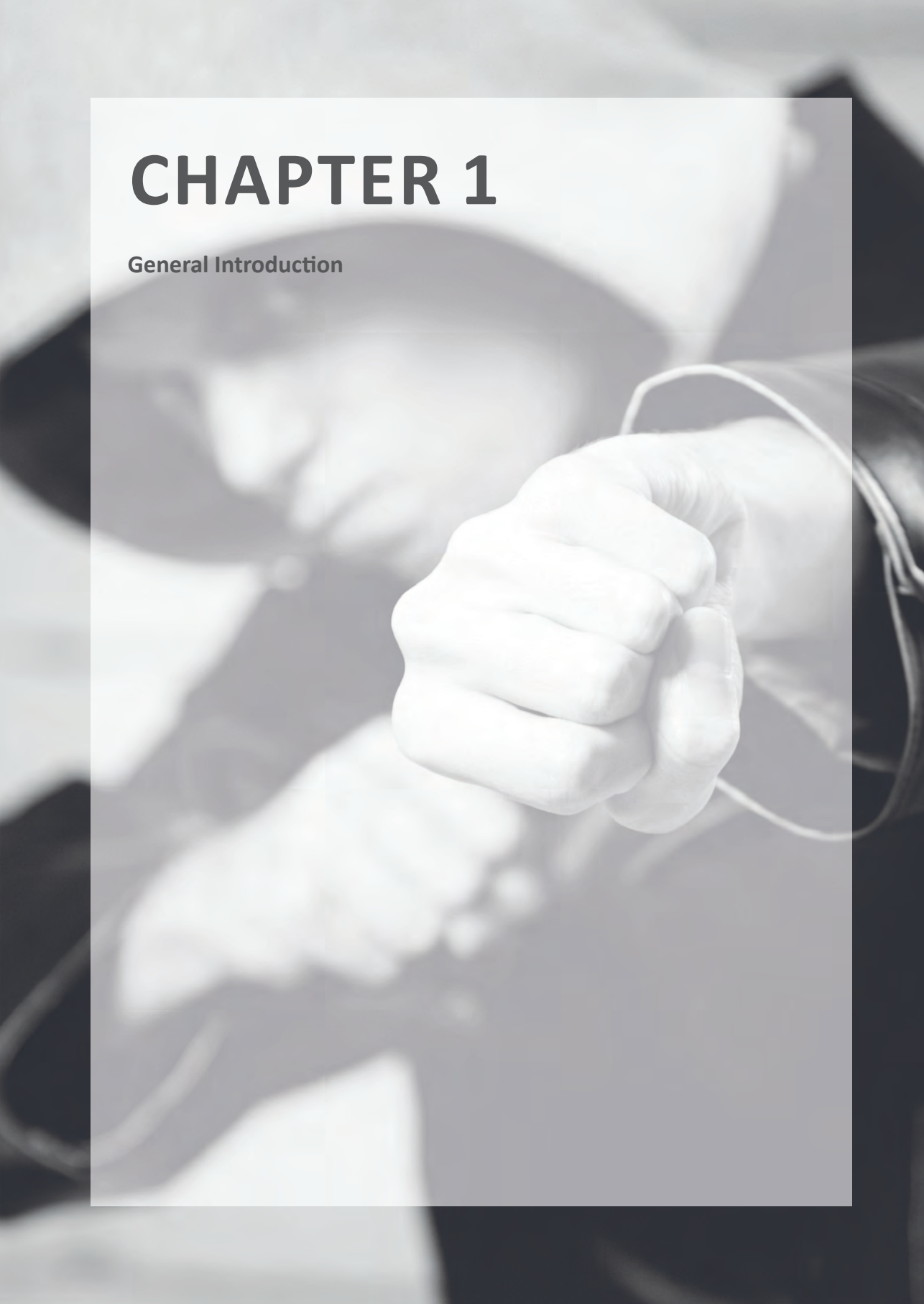
CHAPTER 1

General Introduction



1.1 BACKGROUND

Externalizing behavior problems are very prevalent in children and adolescents and have in common their outward behavior and acting negatively on the *external* environment. These behaviors refer to a class of aggressive and delinquent behaviors, and often also hyperactive impulsive behaviors (Liu, 2004). Besides being directly associated with negative outcomes, externalizing behavior problems are an important predictor of future delinquency, crime, and violence (Liu, 2004; Moffitt & Caspi, 2001). Children or adolescents with externalizing behavior problems are often a burden for their families and society, and to a lesser extent, for themselves (Angold et al., 1998). Prevalence estimates of different types of externalizing behavior problems in the Netherlands vary considerably (between 5% and 20%), depending on how behavior problems are measured (questionnaires versus structured interviews), definition and type of behavior (for example, for delict the highest prevalence is for robbery, truancy, and alcohol abuse), gender (higher prevalence in boys than in girls), and age (peak at age 15-17) (Junger, Mesman, & Meeuw, 2003). All in all, externalizing behavior problems have been increasingly viewed as a public health problem (Liu, 2004).

Externalizing behavior problems may be conceptualized as categories or as dimensions (Walton, Ormel, & Krueger, 2011). The Diagnostic and Statistical Manual of mental disorders fourth edition, text revision (DSM-IV-TR) takes a categorical approach to disorders. The typical externalizing disorders according to the DSM-IV-TR are oppositional defiant disorder (ODD) and conduct disorder (CD); further, attention deficit hyperactivity disorder (ADHD) is often placed among the externalizing disorders (APA, 2000). A major strength of the DSM-IV-TR is that is offers conventional cut-off points on dimensional symptoms, creating dichotomous diagnoses which facilitate treatment decisions (Lahey, Van Hulle, Singh, Waldman, & Rathouz, 2011). According to DSM-IV-TR criteria, ODD, CD, and ADHD affect approximately 3.3 percent, 3.2 percent, and 5 percent respectively of children and/or adolescents worldwide (Canino, Polanczyk, Bauermeister, Rohde, & Frick, 2010; Faraone, Sergeant, Gillberg, & Biederman, 2003).

In contrast, one can take a dimensional approach when studying externalizing behavior problems. A well-known example of this dimensional approach is Achenbach's multivariate and taxonomic model of psychopathology. Achenbach developed a series of questionnaires for parents (Child Behavior Checklist (CBCL)) (Achenbach, 1991a), teachers (Teacher Report Form (TRF)) (Achenbach, 1991b), and adolescents themselves (Youth Self Report (YSR)) (Achenbach, 1991c) that evaluated children's and adolescents' behavioral and emotional functioning. These three questionnaires contain two broadband scales: one for internalizing behavior problems and one for externalizing behavior problems. Since there is broad support for a dimensional latent structure across indicators of externalizing

behavior problems (Boyle et al., 1996; Hudziak, Achenbach, Althoff, & Pine, 2009; Walton et al., 2011), we take a dimensional approach for the measurement of externalizing behavior problems in the present thesis. Moreover, a dimensional approach is particularly useful for studying externalizing behavior problems in a population-based study.

As mentioned before, externalizing behavior problems refer to a class of aggressive, delinquent, and hyperactive behaviors (Liu, 2004). Achenbach's broadband Externalizing scale combines the Aggressive behavior scale and the Delinquent behavior scale (Achenbach, 1991a, 1991b, 1991c). In addition, it is possible to create a DSM-oriented scale of ADHD symptoms based on Achenbach's questionnaires (Achenbach, Dumenci, & Rescorla, 2003), distinguishing symptoms of hyperactivity/impulsivity and symptoms of inattention. Below, we will describe aggressive, delinquent, and hyperactive behaviors in more detail.

Aggressive behaviors consist of physical or verbal behaviors that harm or threaten others, like demanding attention, property destruction, and fighting (Achenbach, 1991a). In addition, the harm must be intended, an aggressive child or adolescent believes that the behavior will harm the victim (Anderson & Bushman, 2002). In general, boys are more often aggressive than girls. In addition, boys often engage in physical aggression and girls often engage in relational aggression (e.g., slander) (Liu, 2004). The CBCL measures aggressive behavior with items like 'fights a lot', 'attacks other people', and 'disobedient at home' (Achenbach, 1991a).

Secondly, delinquent behaviors consist of various antisocial acts like different forms of property crime (e.g., theft, breaking and entering, vandalism) and violence (e.g., aggravated assault, robbery, homicide) (Loeber, Burke, & Pardini, 2009). Delinquency often, but not always, refers to behaviors that violate criminal laws (Liu, 2004). However, many delinquent acts are not detected and therefore it seems better to refer to 'antisocial behavior' when speaking about delinquent behaviors. Like aggression, delinquency is found to be more common in boys than in girls (Liu, 2004). The CBCL measures delinquent behavior with items like 'destroys other things', 'cheats and lies', and 'runs away' (Achenbach, 1991a).

Thirdly, hyperactive behaviors consist of high locomotor activity in behaviors or high levels of restlessness (Ballard et al., 1997). Together with impulsive behaviors, they form one of the two main domains of the combined subtype of ADHD. The other main domain of the combined subtype of ADHD involves inattention behaviors (APA, 2000). Like aggressive and delinquent behaviors, hyperactive, impulsive and inattentive behaviors are found to be more common in boys than in girls (Liu, 2004). The CBCL measures hyperactive and impulsive behaviors with items like 'can't sit still', 'impulsive', and 'talks much', and inattentive behaviors with items like 'can't concentrate' and 'fails to finish things he/she starts' (Achenbach et al., 2003).

1.2 ETIOLOGY

Externalizing behavior problems have a multifactorial etiology. Concerning risk factors for developing externalizing behavior problems, we will distinguish four domains of risk factors: child, parenting and caregiving, sociocultural, and peer-related (Deater-Deckard, Dodge, Bates, & Pettit, 1998).

Concerning risk factors at the *child* level, the most prominent risk factors for externalizing behavior problems are genetic factors. Additive genetic influences (sum of effects of individual genes over loci) explain almost 60 percent of variance in externalizing behavior problems (59.0%), conduct problems (57.6%), and oppositional defiant problems (59.1%) (Burt, 2009). For attention-deficit/hyperactivity problems genetic effects are even stronger: dominant genetic influences (nonadditive or gene-to-gene interactive effects) explain 44.4% of variance in attention-deficit/hyperactivity problems and additive genetic influences explain another 25.9% of variance. In addition, shared environmental effects explain between 10.1% and 15.3% of the variance in externalizing behavior problems, conduct problems, and oppositional defiant problems, whereas shared environmental effects were negligible for attention-deficit/hyperactivity problems (Burt, 2009). The fact that attention-deficit/hyperactivity problems did not show a significant influence of shared environmental effects may be due to methodological issues (Wood, Buitelaar, Rijsdijk, Asherson, & Kuntsi, 2010). Furthermore, nonshared environmental effects explain between 25.8% and 30.8% of the variance in externalizing behavior problems, conduct problems, oppositional defiant problems, and attention-deficit/hyperactivity problems (Burt, 2009). Genetic factors can be operationalized by the presence of specific alleles or SNPs (e.g., 7-repeat allele DRD4; Hohmann et al., 2009) or by lifetime parental externalizing behavior problems (i.e. familial loading of externalizing behavior problems or FLE; Ormel et al., 2005). Since Burt's review (2009) indicate that the familial aggregation of externalizing disorders is mainly due to genetic factors, it can be assumed that familial loading reflects largely genetic risk, although a contribution of shared environmental influences cannot be ruled out. Besides being affected by genetic factors, a second important risk factor at the child level is gender, since externalizing behavior problems are more common in boys than in girls (Liu, 2004). Third, biological risk factors may contribute to the development of externalizing behavior problems (Brennan & Raine, 1997; Raine, 2002). For example, recent evidence suggests a potential role for the hypothalamus-pituitary-adrenal (HPA)-axis in externalizing behavior problems in that low basal cortisol levels may be related to externalizing behavior problems (Alink et al., 2008). Fourth, pre- and perinatal factors may be related to future externalizing behavior problems (Allen, Lewinsohn, & Seeley, 1998; Burke, Loeber, & Birmaher, 2002; Raine, 2002). These factors include factors related to 1) the prenatal environment (e.g., maternal stress during pregnancy, smoking and alcohol use during pregnancy), 2) intrapartum events (e.g., birth difficulties), 3) the immediate

postpartum environment and health problems (e.g., prematurity, anoxia), and 4) the later neonatal environment (e.g., general infant health after birth) (Allen et al., 1998). In addition, temperamental characteristics of the child form risk factors of externalizing behavior problems (Burke et al., 2002). These include high levels of emotionality and neuroticism as well as low levels of effortful control (Muris & Ollendick, 2005). Finally, exposure to negative life events (e.g., being the victim of interpersonal violence or child abuse) may lead to externalizing behavior problems (Burke et al., 2002; Danielson et al., 2006).

Parenting and caregiving risk factors may also involve exposure of the child to negative life events like conflict and violence within the home (Deater-Deckard et al., 1998), so there might be some overlap with the previous domain. In addition, adverse family structural characteristics (e.g., living in a single-mother home), and parental characteristics like parental stress may be related to externalizing behavior problems (Deater-Deckard et al., 1998). Also, the present domain concerns caregiving risk factors. For example, inconsistent parenting, poor parental coping skills, unduly harsh punishing, lack of clear rules, lack of parental supervision, and a lack of emotional support may be risk factors for externalizing behavior problems (Burke et al., 2002; Campbell, 1995; Ellis & Nigg, 2009; Jester et al., 2005; Keown & Woodward, 2002). Finally, as described in the section about risk factors at the child level, parental externalizing behavior problems form a risk factor for developing externalizing behavior problems in their offspring (Ormel et al., 2005). Although there is a genetic component in parental psychopathology, a contribution of shared environmental influences cannot be ruled out.

Sociocultural risk factors for externalizing behavior problems include poverty (Deater-Deckard et al., 1998). However, socio-economic status (SES) may be a more useful risk factor in this regard (Lansford et al., 2006). That is, SES is often used as a 'container variable' representing several aspects of the family context, including poverty, disadvantaged neighborhoods, unemployment, exposure to racial prejudice, and satisfaction with standard of living (Burke et al., 2002; Stafford & Marmot, 2003).

Peer-related risk factors have been shown to be important factors in the development of externalizing behavior problems (Deater-Deckard et al., 1998). For example, antipathetic relationships (relationships based on mutual dislike) (Card, 2010), peer rejection (Burke et al., 2002; Menting, van Lier, & Koot, 2011), and association with deviant peers (Allen, Porter, & McFarland, 2006; Burke et al., 2002) have been implicated in the etiology of externalizing behavior problems.

There is strong evidence that risk factors from all four domains provide significant unique contributions to the development of externalizing behavior problems (Deater-Deckard et al., 1998). However, the story does not end here. There is also support for a cumulative-risk model (Deater-Deckard et al., 1998), that is, the risk for externalizing behavior problems increases with each added risk factor. Also, according to the

developmental model, children's exposure to risk factors may increase with development, and risk factors may intensify or weaken across developmental stages (Loeber et al., 2009). In addition, several studies provide evidence of gene-environment interactions in predicting externalizing behavior problems (Bakermans-Kranenburg & van Ijzendoorn, 2006; Propper, Willoughby, Halpern, Carbone, & Cox, 2007; Robbers et al., 2012; Thapar et al., 2005).

In the present thesis, we will study the separate and combined effects of several risk factors to create a more comprehensive view of the etiology of externalizing behavior problems. First, we will elucidate the relationship between the HPA-axis and externalizing behavior problems by incorporating several other risk factors. Second, the role of gene-environment interactions in externalizing behavior problems will be investigated. We will introduce this approach in more detail below. Thereafter, we will describe the research questions of this thesis, and the sample and methods used. Finally, we present the outline of the present thesis.

1.2.1 The hypothalamus-pituitary-adrenal (HPA)-axis

The hypothalamus-pituitary-adrenal (HPA)-axis is a central component of the body's neuroendocrine response to stress. The hypothalamus secretes corticotropin-releasing hormone (CRH), which, in turn, stimulates the release of adrenocorticotropic hormone (ACTH) in the anterior pituitary. ACTH, in turn, stimulates the adrenal cortex to secrete cortisol. Cortisol is known as the major end product of the HPA-axis in humans (Tsigos & Chrousos, 2002). Yet, activity of the HPA-axis does not end with the production of cortisol. The HPA-axis is controlled by negative feedback regulation that tends to normalize secretion of cortisol (Tsigos & Chrousos, 2002). Elevated cortisol levels reduce ACTH levels as a consequence of negative feedback regulation, followed by a reduction in cortisol levels; in case of reduced cortisol levels, there is less negative feedback regulation at the pituitary, followed by an elevation in ACTH levels (Gold, Drevets, & Charney, 2002).

In normal nonstressful situations, cortisol secretion follows a circadian rhythm characterized by high levels in the morning followed by a decrease throughout the rest of the day. Generally, cortisol levels rise in about half an hour after awakening, which is known as the cortisol awakening response (CAR). Due to the stability of morning cortisol levels, the CAR can serve as a reliable marker of HPA-axis activity (Pruessner et al., 1997).

1.2.1.1 Psychopathology. One of the risk factors at the child level for externalizing behavior problems concerns low levels of arousal. Low HPA-axis activity is associated with low levels of arousal of the central nervous system (Chrousos & Gold, 1998; van Goozen, Matthys, Cohen-Kettenis, Buitelaar, & van Engeland, 2000). According to the stimulation-seeking theory, low arousal represents an unpleasant condition which may lead to stimulus-seeking behavior to attain higher and more pleasant levels of arousal (Raine,

1996; Zuckerman, 1979), which would predispose to externalizing behavior problems. A meta-analysis showed that there was a weak but significant relationship between low basal cortisol levels and externalizing behavior problems, whereas cortisol reactivity was not consistently related with externalizing behavior problems (Alink et al., 2008). This weak relationship and also the fact that several previous studies were not able to reveal a relation between externalizing behavior problems and low HPA-axis activity (Dabbs, Jurkovic, & Frady, 1991; Klimes-Dougan, Hastings, Granger, Usher, & Zahn-Waxler, 2001; Schulz, Halperin, Newcorn, Sharma, & Gabriel, 1997; Sondeijker et al., 2007) could be due to studies not accounting for issues of gender and comorbidity. First, as a consequence of the large amount of studies in clinical or high-risk samples, studies concerning HPA-axis activity and externalizing behavior problems have largely been conducted with boys. In general, girls show higher basal cortisol levels (Klimes-Dougan et al., 2001; Rosmalen et al., 2005) and higher CAR (Pruessner et al., 1997; Rosmalen et al., 2005; Wüst, Wolf, et al., 2000) than boys. Second, high basal cortisol levels may be related to internalizing behavior problems (Goodyer, Park, Netherton, & Herbert, 2001; Ryan, 1998). Thus, both gender and comorbidity with internalizing behavior problems may moderate the relationship between HPA-axis activity and externalizing behavior problems.

1.2.1.2 Environmental risk factors. Assuming that there is a relationship between HPA-axis activity and externalizing behavior problems (Alink et al., 2008), the question is which factors explain that HPA-axis activity varies from individual to individual. Next to genetic factors (Wüst, Federenko, Hellhammer, & Kirschbaum, 2000), (early) environmental risk factors may be related to HPA-axis activity. In the present thesis, we will focus on the role of pre- and perinatal risk factors, parenting, and SES.

First, pre- and perinatal risk factors are relevant for understanding the etiology of externalizing behavior problems. Complications during pregnancy and delivery (i.e. Obstetric Complications (OCs)), such as maternal physical problems during pregnancy and delivery, prematurity, macrosomia (i.e. birth weight higher than 4,500 g), and acute anoxia or hypoxia, are involved in the etiology of externalizing behavior problems (Allen et al., 1998; Batstra, Hadders-Algra, Ormel, & Neeleman, 2004; Buschgens et al., 2009; Nosarti, Allin, Frangou, Rifkin, & Murray, 2005; Raine, 2002). However, little attention has been paid to the mechanism by which OCs may cause externalizing behavior problems. Allen et al. (1998) proposed a biological model in which neurobiological deficits may explain the relationship between OCs and externalizing behavior problems. Among other neurobiological deficits, impaired functioning of the hypothalamus-pituitary-adrenal (HPA)-axis has often been suggested as a potential mediator in the relationship between early stress (from before birth to early childhood) and externalizing behavior problems (Alink et al., 2008; Gunnar, Fisher, & Early Experience, 2006; Huizink, Robles de Medina,

Mulder, Visser, & Buitelaar, 2003; van Goozen, Fairchild, Snoek, & Harold, 2007). However, this potential mediating role of the HPA-axis have not been studied.

Second, parenting and caregiving risk factors may be related to future externalizing behavior problems. Longitudinal studies in school-age children suggest that positive parenting (i.e. warmth, involved parenting, sensitivity) lead to decreases in externalizing behavior problems (Trentacosta et al., 2008), whereas lack of positive parenting lead to increases in externalizing behavior problems (Caspi et al., 2004; Miner & Clarke-Stewart, 2008). Further, negative parenting (i.e. hostility, rejection, harsh discipline) has been reported to lead to increases in externalizing behavior problems (Caspi et al., 2004; Leve, Kim, & Pears, 2005; Miner & Clarke-Stewart, 2008). Yet, how can parenting affect HPA-axis activity? Several reviews indicate that a high-stress environment (such as an environment with negative parenting) leads to increases in children's basal cortisol levels in the short-term (De Bellis, 2001; Grassi-Oliveira, Ashy, & Stein, 2008; Gunnar, 1992), whereas in the long-term decreases in basal cortisol levels are observed (Chrousos & Gold, 1992; De Bellis, 2001; Fries, Hesse, Hellhammer, & Hellhammer, 2005; Grassi-Oliveira et al., 2008; Gunnar & Vazquez, 2001; McEwen & Stellar, 1993; Miller, Chen, & Zhou, 2007). Although relatively few studies investigated the association between positive parenting and basal HPA-axis activity, there is some evidence that positive parenting may be related to low basal cortisol levels (Dockray & Steptoe, 2010; Engert, Efanov, Dedovic, Dagher, & Pruessner, 2011).

Third, SES is a sociocultural or contextual risk factor that is related to externalizing behavior problems (Lansford et al., 2006). As said before, SES is often used as a 'container variable' representing several aspects of the family context. Since low SES may be taken overall as representing a high-stress environment and high SES a low-stress environment, relationships between SES and basal HPA-axis activity are hypothesized to be in the same direction as relationships between parenting and HPA-axis activity. In this regard, the evolutionary-developmental theory of biological sensitivity to context (BSC) (Boyce & Ellis, 2005) offers a conceptual framework for understanding individual differences in biological sensitivity to the environment. According to the developmental programming part of this theory, both children who experience high-stress environments in early life, and children who experience supportive, low-stress environments in early life tend to develop a highly reactive stress response system. In addition, children who experience moderate stress environments tend to develop a low reactive stress response system. Although initial formulation of BSC theory mainly seemed to apply to stress reactivity, the developmental programming part of BSC theory has recently been described in much greater detail, now also involving basal cortisol levels (Del Giudice, Ellis, & Shirtcliff, 2011). Yet, the BSC theory involving basal cortisol levels have rarely been tested.

1.2.2 Gene-environment (GxE) interaction

Gene-environment interactions occur when the effects of genes are dependent on environmental conditions, i.e. are stronger in some environments than in other ones; or vice versa, when environmental factors have a stronger influence in individuals with a specific genetic make-up. So far, research on gene-environment interactions in externalizing behavior problems (and more specifically ADHD) has focused on two types of environmental factors, familial and psychosocial influences (Bakermans-Kranenburg & van Ijzendoorn, 2006), and pre- and perinatal factors (Nigg, Nikolas, & Burt, 2010).

Concerning familial and psychosocial influences, we will focus on parenting in the present thesis. As described above, positive aspects of parenting are related to lower levels of externalizing behavior problems, whereas negative aspects of parenting are related to higher levels of externalizing behavior problems (Caspi et al., 2004; Leve et al., 2005; Miner & Clarke-Stewart, 2008; Trentacosta et al., 2008). When it comes to gene-environment interaction, the question is whether there are any genes that moderate the relationship between parenting and externalizing behavior problems. Particularly relevant is the work of Bakermans-Kranenburg and Van Ijzendoorn (2006), who found a six-fold increase in externalizing behavior problems in children with the DRD4 7-repeat allele exposed to insensitive parenting compared to children without these combined risks.

Concerning pre- and perinatal factors, the focus will be on smoking and alcohol use during pregnancy, and OCs. Smoking and alcohol use during pregnancy (Hill, Lowers, Locke-Wellman, & Shen, 2000) and OCs (Allen et al., 1998; Banerjee, Middleton, & Faraone, 2007) are related to both broadband externalizing behavior problems and ADHD symptoms. Yet, gene-environment interaction studies that involve pre- and perinatal risk factors often concern ADHD symptoms (Nigg et al., 2010) instead of broadband externalizing behavior problems. For that reason, we will also investigate gene-environment interaction on ADHD symptoms instead of on broadband externalizing behavior problems.

Genetic factors can be operationalized by familial loading of externalizing behavior problems (FLE), that is, lifetime parental externalizing behavior disorders (Ormel et al., 2005). Since quantitative genetic studies indicate that the familial aggregation of externalizing disorders is mainly due to genetic factors (Burt, 2009), it can be assumed that familial loading reflects largely genetic risk, although a contribution of shared environmental influences cannot be ruled out. In addition, genetic factors can be operationalized by the presence of specific alleles or SNPs. The DRD4 7-repeat allele and various SNPs of the DAT1 (*SLC6A3*/ dopamine transporter gene) appear to be involved in the etiology of ADHD, as evidenced by a recent review and meta-analysis (Gizer, Ficks, & Waldman, 2009).

1.3 AIMS OF THE PRESENT THESIS

The present thesis has two main aims: 1) To elucidate the relationship between HPA-axis activity and externalizing behavior problems by examining the additive or interactive effects of several environmental and other moderating factors. 2) To explore to which extent and how gene-environment interactions may explain externalizing behavior problems. These aims are divided into five research questions:

1. Is the relationship between HPA-axis activity and externalizing behavior problems dependent on comorbidity with internalizing behavior problems and gender? (aim 1)
2. Is the relationship between HPA-axis activity and externalizing behavior problems mediated by obstetric complications? (aim 1)
3. Do parenting behaviors and family context explain differences in HPA-axis activity? (aim 1)
4. Do genes which are potentially involved in externalizing behavior problems interact with parenting behaviors in predicting externalizing behavior problems? (aim 2)
5. Do genes which are potentially involved in ADHD symptoms interact with obstetric complications in predicting ADHD symptoms? (aim 2)

1.4 THE TRAILS STUDY

The data reported in this thesis have been collected in the context of the TRacking Adolescents' Individual Lives Survey (TRAILS). TRAILS is a prospective study of Dutch adolescents, with the aim to chart and explain the development of mental health from early adolescence into adulthood, both at the level of psychopathology and the levels of underlying vulnerability and environmental risk. Adolescents will be measured bi- or triennially at least until they are 25 years old. The studies described in the present thesis are based upon data from the first (T1), second (T2), and/or third (T3) assessment wave of TRAILS, which ran from March 2001 to July 2002, September 2003 to December 2004, and September 2005 to December 2007, respectively.

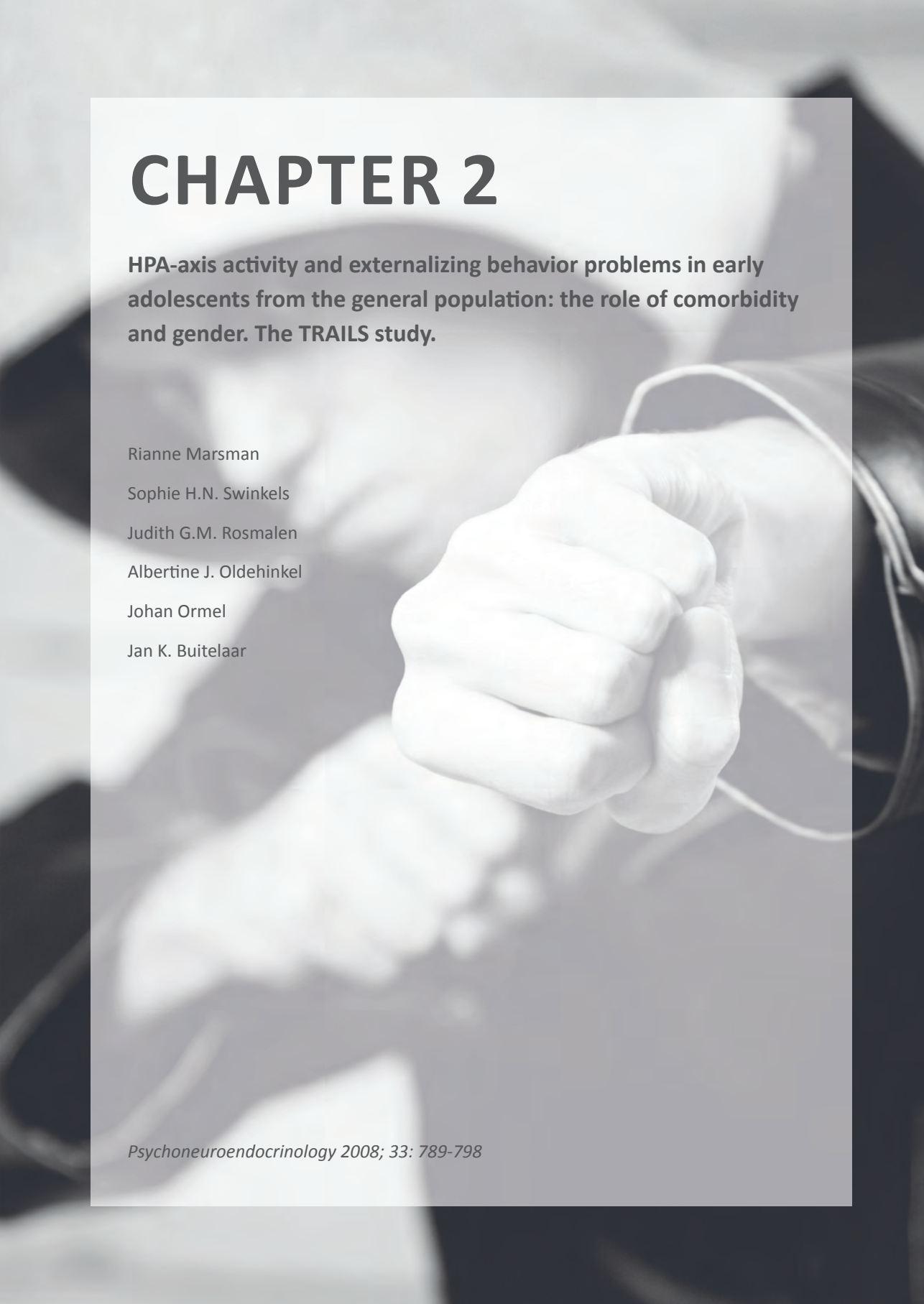
TRAILS participants were selected from five municipalities in the north of The Netherlands, including both urban and rural areas. Children born between October 1, 1989, and September 30, 1990 (first two municipalities), or October 1, 1990, and September 30, 1991 (last three municipalities), were eligible for inclusion, providing that their schools were willing to cooperate and that they were able to participate in the study. Of all individuals approached for participation in the study (n = 3145), 6.7% were excluded. The exclusion criteria were 1) an incapability to participate because of mental retardation or serious physical illness or handicap, and 2) no availability of a Dutch-speaking parent or parent surrogate, and no feasibility to administer a part of the measurement in the

parent's own language. Of all eligible 2935 children, 76.0% (N = 2230, mean age = 11.09, SD = 0.56, 50.8% girls) were enrolled in the study. Parental written informed consent was obtained after the procedures had been fully explained. Responders and non-responders did not differ with respect to the prevalence of teacher-rated behavior problems, nor regarding associations between sociodemographic variables and mental health outcomes (de Winter et al., 2005). Of the 2230 baseline participants, 96.4% (N = 2149, 51.0% girls) participated in the first follow-up assessment (T2), which was held 2 to 3 years after T1 (mean number of months 29.44, SD = 5.37, range 16.69-48.06). Mean age at T2 was 13.56 (SD = 0.53). At T3, the response rate was 81.4%, and mean age was 16.13 (SD = 0.59). Sample sizes differ for the separate studies in the present thesis, depending on the availability of complete data on the measures that were used in the analyses.

1.5 OUTLINE OF THE PRESENT THESIS

The next three chapters describe studies that aim to elucidate the relationship between HPA-axis activity and externalizing behavior problems by incorporating several environmental and other moderating factors. In *chapter 2*, we investigate the role of gender and comorbidity with internalizing behavior problems in the relationship between HPA-axis activity and externalizing behavior problems. Given the existence of a relationship between HPA-axis activity and externalizing behavior problems, we turn to potential environmental factors that affect HPA-axis activity. In *chapter 3*, we investigate the role of OCs on HPA-axis activity, determining the potential mediating role of HPA-axis activity in the relationship between OCs and externalizing behavior problems. In *chapter 4*, we examine the role of positive and negative aspects of parenting, as well as the role of SES on HPA-axis activity, using the developmental programming part of the evolutionary-developmental theory of biological sensitivity to context.

Thereafter, two chapters focus on the role of gene-environment interactions in explaining externalizing behavior problems and ADHD symptoms. In *chapter 5*, we investigate the effect of gene-environment interaction on future externalizing behavior problems. Environmental factors concern both positive and negative aspects of parenting, and genetic factors concern both FLE and the presence of the DRD4 7-repeat allele and the absence of the DRD4 4-repeat allele. In *chapter 6*, we explore the relevance of gene-environment interaction on ADHD symptoms. In this study, environmental factors concern smoking and alcohol use during pregnancy, and OCs, and genetic factors concern two SNPs of the DAT1/*SLC6A3*. Finally, we present a summary and a discussion of the results in *chapter 7*.



CHAPTER 2

HPA-axis activity and externalizing behavior problems in early adolescents from the general population: the role of comorbidity and gender. The TRAILS study.

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ABSTRACT

Contradictory findings on the relationship between HPA-axis activity and externalizing behavior problems could be due to studies not accounting for issues of comorbidity and gender. In a population-based cohort of 1768 (10- to 12 year-old) early adolescents, we used a person-oriented approach and a variable-oriented approach to investigate whether comorbidity with internalizing behavior problems and gender moderate the relationship between HPA-axis activity (cortisol awakening response and evening cortisol levels) and externalizing behavior problems. We found that: (1) In early adolescents with pure externalizing behavior problems, there was a particularly strong effect of gender, in that girls showed significantly higher total cortisol levels after awakening (AUC_G levels) and a significantly higher cortisol awakening response (AUC_I levels) than boys. (2) Girls with pure externalizing behavior problems showed a significantly higher cortisol awakening response (AUC_I levels) than girls without behavior problems or girls with comorbid internalizing behavior problems. This effect was absent in boys. (3) Externalizing behavior problems, in contrast to internalizing behavior problems, were associated with higher evening cortisol levels. This effect might, however, result from girls with externalizing behavior problems showing the highest evening cortisol levels. Overall, we were unable to find the expected relationships between comorbidity and HPA-axis activity, and found girls with pure externalizing behavior problems to form a distinct group with regard to their HPA-axis activity. There is need for prospective longitudinal studies of externalizing behavior problems in boys and girls in relation to their HPA-axis activity. It would be useful to consider how other risk factors such as life events and family and parenting factors as well as genetic risks affect the complex relationship between externalizing behavior problems and HPA-axis activity.

2.1 INTRODUCTION

The hypothalamus-pituitary-adrenal (HPA)-axis is a central component of the body's neuroendocrine response to stress, with cortisol as its major end product (Tsigos & Chrousos, 2002). Low HPA-axis activity is associated with low levels of arousal of the central nervous system (Chrousos & Gold, 1998; van Goozen et al., 2000), which would predispose to externalizing behavior problems. According to the stimulation-seeking theory, low arousal represents an unpleasant condition which may lead to stimulus-seeking behavior to attain higher and more pleasant levels of arousal (Raine, 1996; Zuckerman, 1979). Several studies on the relationship between HPA-axis activity and externalizing behavior problems have shown that children or early adolescents (aged 7 to 12) with externalizing behavior problems have low levels of cortisol (both basal and in response to stressors) (Ryan, 1998). However, other studies did not find a relationship between externalizing behavior problems and low basal cortisol levels (Dabbs et al., 1991; Klimes-Dougan et al., 2001; Schulz et al., 1997; Sondeijker et al., 2007).

Relatively few studies on the relationship between HPA-axis activity and externalizing behavior problems have measured cortisol levels in response to awakening. The increase in cortisol levels in about half an hour after awakening, the cortisol awakening response (CAR), can serve as a reliable marker of HPA-axis activity (Pruessner et al., 1997). There is evidence that the CAR is genetically influenced (Wüst, Federenko, et al., 2000) and associated with chronic stress (Pruessner, Hellhammer, Pruessner, & Lupien, 2003; Schlotz, Hellhammer, Schulz, & Stone, 2004; Wüst, Federenko, et al., 2000; Wüst, Wolf, et al., 2000). In contrast, afternoon and evening basal cortisol levels are more environmentally influenced (Bartels, de Geus, Kirschbaum, Sluyter, & Boomsma, 2003; Schreiber et al., 2006; Wüst, Federenko, et al., 2000) and hence susceptible to environmental stressors. Consequently, the CAR and evening basal cortisol levels appear to reflect independent characteristics of HPA-axis activity (Rosmalen et al., 2005).

This paper focuses on the role of gender and comorbidity as potential explanations for the inconsistent results on the relationship between externalizing behavior problems and HPA-axis activity. Gender is thought to be associated with HPA-axis activity, in that girls show higher basal cortisol levels (Klimes-Dougan et al., 2001; Rosmalen et al., 2005) and higher CAR (Pruessner et al., 1997; Rosmalen et al., 2005; Wüst, Wolf, et al., 2000) than boys. An association between gender and HPA-axis activity does not, however, exclude the possibility that gender acts as a moderator in the relationship between HPA-axis activity and externalizing behavior problems (Baron & Kenny, 1986). As a consequence of the large amount of studies in clinical or high-risk samples, studies concerning externalizing behavior problems have largely been conducted with boys. Thus, in samples consisting of predominantly boys, these studies may have mistakenly concluded that externalizing

behavior problems are associated with low HPA-axis activity. So far, few studies have examined the moderating effect of gender on the relationship between HPA-axis activity and externalizing behavior problems. These studies concluded that low cortisol may be a biological marker for *male* externalizing behavior problems in particular (Loney, Butler, Lima, Counts, & Eckel, 2006; Shirtcliff, Granger, Booth, & Johnson, 2005).

Whereas externalizing behavior problems are thought to be inversely related with basal cortisol levels, internalizing behavior problems are thought to be positively related with basal cortisol levels (Goodyer et al., 2001; Ryan, 1998). The hypothesis of additive effects would predict higher than expected basal cortisol levels in the comorbid condition compared to the pure externalizing problem condition. McBurnett et al. (1991) showed that comorbid externalizing and internalizing behavior problems lead to higher cortisol levels than pure externalizing behavior problems in a sample that included boys only. However, later studies that investigated mixed-gender samples could not replicate this finding (Oosterlaan, Geurts, Knol, & Sergeant, 2005; Shirtcliff et al., 2005), which may indicate a gender-specific effect of comorbidity. As an alternative to the hypothesis of additive effects, comorbidity might be characterized by unique characteristics not found in pure conditions. Comorbidity between externalizing and internalizing behavior problems, which occurs more frequently than chance would predict (Boylan, Vaillancourt, Boyle, & Szatmari, 2007), has shown to be more heterogeneous in terms of clinical presentation and in etiology when compared to pure externalizing behavior problems. Since pure externalizing behavior problems are more genetically influenced than comorbid externalizing and internalizing behavior problems (Gjone & Stevenson, 1997), pure externalizing behavior problems may be more strongly related to the genetically influenced CAR than comorbid externalizing and internalizing problems. Comorbidity probably results from a combination of genetic and environmental risk factors (Boylan et al., 2007; Gjone & Stevenson, 1997) and may be related to both the genetically influenced CAR and to the environmentally influenced evening cortisol levels.

The purpose of the present study is to disentangle main effects of externalizing behavior problems and gender on HPA-axis activity from possible moderating effects of gender and comorbidity in a population-based cohort of 10- to 12-year-old early adolescents. To analyze the effects of comorbidity, we will follow both a person-oriented and a variable-oriented approach to data-analysis. In the person-oriented approach, comorbidity is considered as unique to the individual, whereas in the variable-oriented approach, comorbidity is considered as an aggregation of externalizing and internalizing behavior problems. In this way, both approaches may provide different information that can be complementary (Ormel et al., 2005; von Eye, Bogat, & Rhodes, 2006). The first hypothesis is that the potential inverse relationship between externalizing behavior problems and total cortisol levels after awakening is specific for boys. Second, we

hypothesize that comorbid externalizing and internalizing behavior problems lead to higher total cortisol levels after awakening than pure externalizing behavior problems. The third hypothesis is that pure externalizing behavior problems are more strongly related to the CAR than comorbid externalizing and internalizing problems; in addition, we hypothesize that comorbid behavior problems are related to evening cortisol levels as well.

2.2 METHODS

2.2.1 Sample

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective cohort study of Dutch (early) adolescents, with the aim to chart and explain the development of mental health from early adolescence into adulthood, both at the level of psychopathology and the levels of underlying vulnerability and environmental risk. Early adolescents will be measured biennially at least until they are 25 years old. The present study involves data from the first (T1) assessment wave of TRAILS, which ran from March 2001 to July 2002. If both parents and early adolescents agreed to participate, parental written informed consent was obtained after the procedures had been fully explained. Of all early adolescents approached for enrollment in the study (*N* = 3145), 76.0% (N = 2230, mean age = 11.09, *S.D.* = 0.56, 50.8% girls) early adolescents participated in the study. Responders and non-responders did not differ with respect to the prevalence of teacher-rated behavior problems, nor regarding associations between sociodemographic variables and mental health outcomes. Detailed information about sample selection and analyses of non-response bias has been reported elsewhere (de Winter et al., 2005).

2.2.2 Procedure

Well-trained interviewers visited one of the parents or guardians (preferably the mother, 95.6%) at their homes to administer an interview covering a wide range of topics, including developmental history and somatic health, parental psychopathology and care utilization. In addition to the interview, the parent was asked to fill out some questionnaires concerning the child's mental health and behavior. Early adolescents filled out questionnaires at school, in the classroom, under the supervision of one or more TRAILS assistants. Besides, intelligence and a number of biological and neurocognitive parameters were assessed individually (at school, except for saliva samples, which were collected at home). Teachers were asked to fill out a brief questionnaire for all TRAILS-participants in their class. Measures that were used in the present study are described more extensively below.

2.2.3 Measures

2.2.3.1 Behavioral problems. Behavioral problems were assessed with the Child Behavior Checklist (CBCL) (Achenbach, 1991a; Verhulst, van der Ende, & Koot, 1996) and the Youth Self-Report (YSR) (Achenbach, 1991c; Verhulst, van der Ende, & Koot, 1997). The CBCL is a measure of parent-reported emotional and behavioral problems in 4- to 18-year-old children and the YSR is a self-report questionnaire that was modeled on the CBCL. The CBCL and the YSR contain 113 and 112 items, respectively. These items are rated as 0 (not true), 1 (somewhat or sometimes true) or 2 (very true or often true). Both the CBCL and the YSR contain two broadband scales: one for internalizing behavior problems and one for externalizing behavior problems. For each of the two broadband scales, we used the mean of the standardized CBCL and YSR scores. The composite scores on externalizing and internalizing behavior problems were analyzed and compared using a person-oriented approach and a variable-oriented approach.

In the person-oriented approach, our large sample size allowed us to set a strict cut-off (P50) for creating a "supernormal" Control group. The 84th percentile for externalizing and internalizing behavior problems was used as a cut-off to assign adolescents into the groups with behavior problems. For both dimensions, the 84th percentile has been identified as the "borderline range" cut-off discriminating between adaptive and maladaptive behavior (Achenbach & Rescorla, 2001). Early adolescents were assigned to one of the following groups: (1) Control group (externalizing < P50 and internalizing < P50), (2) Pure EXT group (externalizing ≥ P84 and internalizing < P84), (3) Pure INT group (externalizing < P84 and internalizing ≥ P84), and (4) Comorbid group (externalizing ≥ P84 and internalizing ≥ P84).

In the variable-oriented approach, we adopted the framework described by Essex et al. (2006) and used a severity measure (severity = [E+I]/2) as an indice for comorbidity and a directionality measure (directionality = [E-I]/2) for determining whether the possible behavior problems are mainly externalizing or internalizing, where E indicates the standardized externalizing behavior problems and I indicates the standardized internalizing behavior problems.

2.2.3.2 Cortisol. TRAILS participants collected cortisol samples (saliva) at home, using the Salivette sampling device (Sarstedt, Rommelsdorfer Str., D-51588 Nümbrecht, Germany), which was handed to the parent at the parent interview, accompanied by a verbal and a written instruction. The Salivette tube consists of a plastic sampling vessel with a suspended insert containing a sterile neutral cotton wool swab that has to be chewed for about 45 s and then returned to the insert. Participants were instructed to collect three saliva samples: the first sample shortly after waking up (still lying in bed), the second sample 30 min later, and the third sample at 2000 h. Both the sampling and the preceding day should be normal (school) days, without special events or stressful circumstances.

Since the in TRAILS participating schools started at approximately the same time, the sampling-time variation of the morning samples among the early adolescents is expected to be limited and the estimated corresponding times are 0700 h for the first sample ($Cort_{0700}$) and 0730 h for the second sample ($Cort_{0730}$). Participants were instructed not to collect saliva when they were ill, had a cold, had a headache, or were menstruating. Furthermore, they were requested not to take any medication, if possible. Any deviations from this protocol, either in terms of sampling times or in terms of other requirements, were indicated on an accompanying form. Concerning the sampling procedure itself, subjects were instructed to keep a glass of water next to their bed and to thoroughly rinse their mouth with tap water before sampling saliva, and not to consume sour products or brush their teeth shortly before that. Saliva samples were stored by the participants in their freezer directly after sampling and mailed to the institute as soon as possible. Participants who did not return the salivettes within a couple of months were sent a reminder letter. In total, we received saliva samples of 1768 early adolescents (79.3% of all TRAILS participants). Non-responders did not differ from responders in terms of gender (48.4% male vs. 49.4% male for non-responders vs. responders, respectively, χ^2 (df = 1) = 0.716) or pubertal development (average Tanner score = 1.92 vs. 1.86, t = -1.394; p = 0.164); non-responders were slightly older (11.16 years vs. 11.08 years, t = -3.084; p < 0.01) and had a higher mean BMI (18.50 kg/m² vs. 17.92 kg/m², t = -3.224; p < 0.01) (Rosmalen et al., 2005).

The saliva samples were stored at -20 ºC until analysis. Previous studies suggest that salivary cortisol levels are stable for prolonged periods of time at -20 ºC (Aardal & Holm, 1995). After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the laboratory (Department of Clinical and Theoretical Psychobiology, University of Trier, Germany) for analysis. Procedures of determination of cortisol levels are described more extensively elsewhere (Rosmalen et al., 2005).

2.2.4 Statistical analyses

We excluded 22 early adolescents because they used corticosteroid-containing medication. For each time point, single cortisol samples with values that were above 3 S.D. of the mean of the particular time point were excluded from the analysis in order to reduce the impact of outliers ($Cort_{0700}$ 21 excluded, 1666 valid measurements in the final dataset; $Cort_{0730}$ 11 excluded, 1683 valid measurements in the final dataset; $Cort_{2000}$ 18 excluded, 1689 valid measurements in the final dataset). After this exclusion, cortisol levels followed a normal distribution ($Cort_{0700}$ skewness = 0.700, kurtosis = 0.632; $Cort_{0730}$ skewness = 0.426, kurtosis = 0.239; $Cort_{2000}$ skewness = 1.217, kurtosis = 2.014).

With regard to the morning cortisol levels we used Area Under the Curve (AUC) measures. The computation of the AUC is a frequently used method in endocrinological research to assess the overall secretion over a specific time period (Area Under the Curve

with respect to ground, AUC_G), and to estimate circadian changes over a specific time period (Area Under the Curve with respect to increase, AUC_I) (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). Pruessner et al. (2003) recommend employing both formulas when analyzing data sets with repeated measures. We used the following formulas for calculating the (1) total cortisol after awakening: $AUC_G = (Cort_{0730}\text{-}Cort_{0700}) \times 0.5/2 + Cort_{0700} \times 0.5$, and (2) cortisol awakening response (CAR): $AUC_I = (Cort_{0730}\text{-}Cort_{0700}) \times 0.5/2$. The former correlates 0.71 with $Cort_{0700}$ and 0.86 with $Cort_{0730}$ and the latter is in this design mathematically equal to one quarter of the difference between awakening level and level 30 minutes later (Rosmalen et al., 2005). According to their conceptual meaning, findings with respect to AUC_G levels will be interpreted in line with previous studies on basal cortisol samples and findings with respect to AUC_I levels will be interpreted in line with previous studies on the CAR. Furthermore, we used $Cort_{2000}$ levels for our interest in basal cortisol levels in the evening.

In a previous study on the present sample, gender and the quadratic effect of sampling month are identified as potential confounders in the relationship between HPA-axis activity and psychopathology. Age, pubertal development, and BMI are not related to HPA-axis activity (Rosmalen et al., 2005).

In the person-oriented approach, a two-way analysis of covariance (ANCOVA) was performed on AUC_G levels, AUC_I levels, and evening cortisol levels, with group (four levels: Control, Pure EXT, Pure INT, Comorbid) and gender (two levels) as factors, and the quadratic effect of sampling month as covariate. When a main effect of group was found, planned contrasts were examined: Control group versus Pure EXT group, Control group versus Comorbid group, and Pure EXT group versus Comorbid group. When an interaction effect between group and gender was found, we performed a one-way ANCOVA in each of the groups, with gender as factor, and quadratic effect of sampling month as covariate and a one-way ANCOVA in both genders, with group as factor, and quadratic effect of sampling month as covariate. Again, when a significant main effect of group was observed in one of the genders, planned contrasts as described above were examined.

In the variable-oriented approach, three stepwise multiple regression analyses were performed with AUC_G, AUC_I or $Cort_{2000}$ as the dependent variables. In the first step, the potential confounders gender and the quadratic effect of sampling month were entered into the model. In the second step, severity, directionality, and the interaction terms between these factors and gender were entered. When an interaction effect between severity (or directionality) and gender was found, we performed a multiple regression analysis for boys and girls separately, with the quadratic effect of sampling month entered in the first step and severity (or directionality) entered in the second step.

2.3 RESULTS

2.3.1 Person-oriented approach

Table 2.1 shows age, gender, and mean standardized scores for externalizing and internalizing behavior problems for the Control, Pure EXT, Pure INT, and Comorbid group. Age differed significantly among the four groups ($p<0.05$), though differences were small. As we expected, groups had dissimilar gender distributions ($p<0.001$). The Comorbid group showed more severe internalizing behavior problems than the Pure INT group ($p<0.001$) and more severe externalizing behavior problems than the Pure EXT group ($p<0.001$).

2.3.1.1 AUC_G levels. Table 2.2 shows the results of the two-way analyses of covariance. As expected, we found a main effect of gender ($F(1,902) = 17.0$, $p<0.001$) and a main quadratic effect of sampling month ($F(1,902) = 10.2$, $p<0.01$) on AUC_G levels (Table 2.2). In addition, the interaction effect of group x gender approached significance ($F(3,902) = 2.4$, $p=0.062$). This trend and the effects of gender and sampling month accounted for 2.5% of the adjusted variance.

Analyses for boys and girls separately revealed no main effects of group. In contrast, analyses for the separate groups revealed a main effect of gender in the Pure EXT group ($F(1,140) = 11.7$, $p<0.01$) (adjusted $R^2 = 7.3\%$), but not in the Control group, the Pure INT group, and the Comorbid group. In the Pure EXT group, girls showed significantly higher AUC_G levels than boys (Figure 2.1 A).

2.3.1.2 AUC_I levels. We found the expected quadratic main effect of gender ($F(1,902) = 4.5$, $p<0.05$). In addition, we found a significant main effect of group ($F(3,902) = 2.9$, $p<0.05$) and a significant interaction effect of group x gender ($F(3,902) = 3.5$, $p<0.05$). These effects accounted for 0.8% of the adjusted variance.

With respect to the main effect of group, planned contrasts revealed that the Pure EXT group showed significantly higher AUC_I levels compared to both the Control group ($p<0.01$) and the Comorbid group ($p<0.05$) (Figure 2.1 B). In addition, the Control group did not differ from the Comorbid group.

With respect to the interaction effect of group x gender, analyses for boys and girls separately revealed a significant main effect of group in girls only ($F(3,457) = 3.5$, $p<0.05$) (adjusted $R^2 = 2.1\%$). Planned contrasts revealed that girls from the Pure EXT group showed significantly higher AUC_I levels compared to both girls from the Control group ($p<0.01$) and the Comorbid group ($p<0.01$) (Figure 2.1 B). The Control group did not differ from the Comorbid group. Analyses for the separate groups revealed a main effect of gender in the Pure EXT group ($F(1,140) = 9.1$, $p<0.01$) (adjusted $R^2 = 5.1\%$), but not in the Control group, the Pure INT group, and the Comorbid group. In the Pure EXT group, girls showed significantly higher AUC_I levels than boys (Figure 2.1 B).

TABLE 2.1 Age, gender, and mean of the standardized CBCL and YSR scores on internalizing and externalizing behavior problems, for the Control, Pure EXT, Pure INT, and Comorbid group.

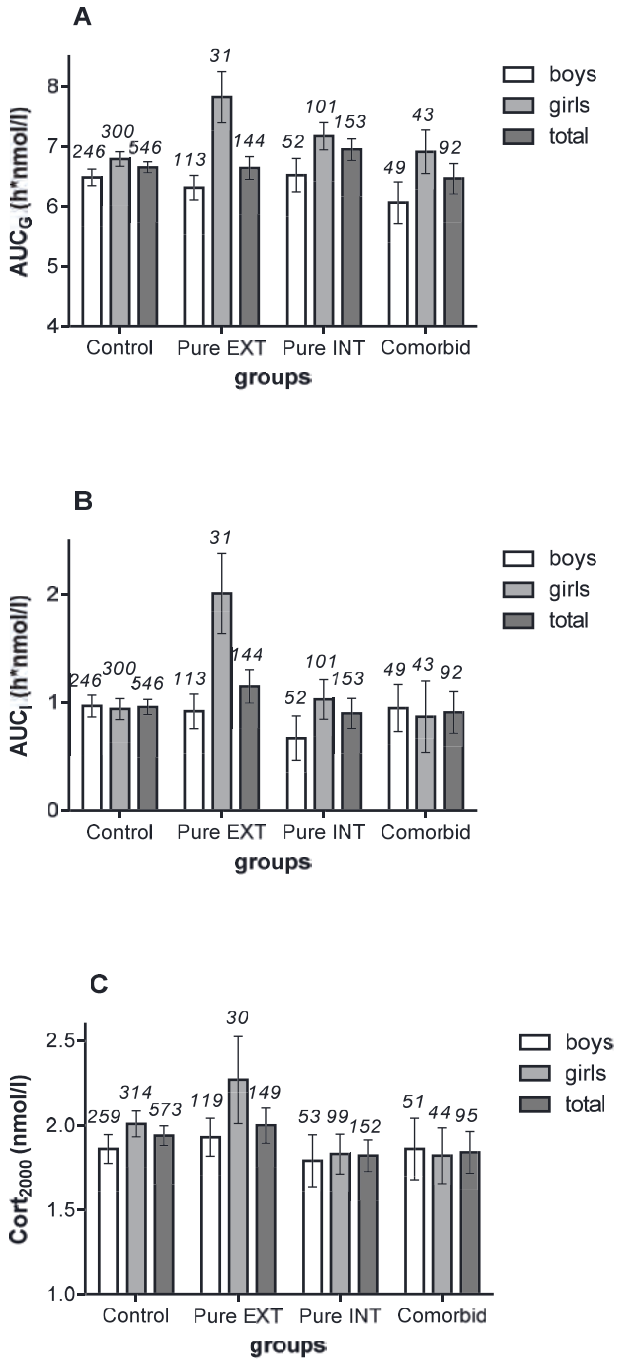
	% / M ± SD				Statistical test value	p-Value
	Control (*n=546*)	Pure EXT (*n=144*)	Pure INT (*n=153*)	Comorbid (*n=92*)		
Demographic characteristics						
Age (years)	11.1 ± .5	11.2 ± .5	11.0 ± .6	11.1 ± .6	F(3,931) = 3.7	0.011
Gender (% boys)	45.1	78.5	34.0	53.3	X=67.9, df=3	<0.001
Behavior problems						
Internalizing						
above p84			1.34 ± .5	1.66 ± .8	t(234) = -4.1	<0.001
Externalizing						
above p84		1.33 ± .4		1.60 ± .6	t(243) = -4.2	<0.001

TABLE 2.2 ANCOVA results for the effect of group, gender, and the interaction effect of group by gender on AUC_G levels, AUC_I levels, and $Cort_{2000}$ levels, with quadratic effect of sampling month as covariate.

	AUC_G				AUC_I				$Cort_{2000}$			
	df	*F*	*p*	*adj.R²*	*df*	*F*	*p*	*adj.R²*	*df*	*F*	*p*	*adj.R²*
Model				2.5 %				0.8 %				4.5 %
Factors												
group	3	1.3	0.270		3	2.9	0.035*		3	0.8	0.487	
gender	1	17.0	<0.001**		1	4.5	0.034*		1	1.0	0.311	
group x gender	3	2.4	0.062		3	3.5	0.015*		3	0.5	0.655	
Covariate												
sampling month	1	10.2	<0.01**		1	1.4	0.236		1	47.0	<0.001**	
Error	902				902				936			

*Note: * significant at the .05 level; ** significant at the .01 level*

FIGURE 2.1 Error bars showing the mean and 95% confidence intervals for AUC_G levels, AUC_I levels, and $Cort_{2000}$ levels for the total group and by gender



2.3.1.3 $Cort_{2000}$ levels. We found the expected main quadratic effect of sampling month ($F(1,936) = 47.0$, $p<0.001$), but no main effect of group or an interaction effect of group x gender. The effect of sampling month accounted for 4.5% of the adjusted variance.

2.3.2 Variable-oriented approach

2.3.2.1 AUC_G levels. Table 2.3 shows the results of the multiple regression analyses. As already shown in the person-oriented approach, gender (ß = -0.102, $p<0.001$) and the quadratic effect of sampling month (ß = 0.113, $p<.001$) significantly predicted AUC_G levels. The interaction effect of severity x gender approached significance (ß = -0.044, $p=0.081$). The addition of the interaction effect increased the adjusted R^2 from 2.3% to 2.4%. Analyses for boys and girls separately revealed that the effect of severity on AUC_G levels approached significance in girls (ß = 0.065, $p=0.071$), but not in boys. In girls, the addition of the effect of severity increased the adjusted R^2 from 0.9% to 1.1%.

2.3.2.2 AUC_I levels. The quadratic effect of sampling month significantly predicted AUC_I levels (ß = 0.071, $p<0.01$). Besides, the interaction effect of severity x gender approached significance (ß = -0.045, $p=0.078$). The addition of the interaction effect increased the adjusted R^2 from 0.5% to 0.6%. As also reported for AUC_G levels, the effect of severity on AUC_I levels approached significance in girls (ß = 0.069, $p=0.052$), but not in boys. In girls, the addition of the effect of severity increased the adjusted R^2 from 1.6% to 1.8%.

2.3.2.3 $Cort_{2000}$ levels. Besides being affected by the quadratic effect of sampling month (ß = 0.202, $p<0.001$), evening cortisol levels were also affected by directionality (ß = 0.056, $p<0.05$). The addition of the effect of directionality did not increase the adjusted R^2 (retained 4.1%). The positive beta coefficient indicates that externalizing behavior problems, in contrast to internalizing behavior problems, are associated with higher evening cortisol levels.

2.4 DISCUSSION

This study was designed to disentangle main effects of externalizing behavior problems and gender on HPA-axis activity from possible moderating effects of gender and comorbidity in a population-based cohort of 10- to 12-year-old early adolescents. We followed a person-oriented approach and a variable-oriented approach, as both approaches may provide different information that can be complementary (Ormel et al., 2005; von Eye et al., 2006).

The first hypothesis was that the potential inverse relationship between externalizing behavior problems and total cortisol levels after awakening is specific for boys. In the person-oriented approach, we did not find the expected group effect on total cortisol after awakening (AUC_G levels) in boys, nor did we find the expected directionality x

TABLE 2.3 Stepwise multiple regression results for the effect of severity, severity x gender, directionality, and directionality x gender on AUC_G levels, AUC_I levels, and $Cort_{2000}$ levels, the covariates gender and quadratic effect of sampling month included in the first step.

Cortisol measures	Step 1	Beta	p-value	adj. R^2	Step 2 (CBCL predictors)	Beta	p-value	adj. R^2
AUC_G	gender	-0.102	0.000**	2.3 %	severity	0.024	0.340	-
	sampling month	0.113	0.000**	2.3 %	severity * gender	-0.044	0.081	2.4 %
					directionality	-0.009	0.734	-
					directionality * gender	-0.036	0.158	-
AUC_I	gender	-0.038	0.134	-	severity	0.029	0.257	-
	sampling month	0.071	0.005**	0.5 %	severity * gender	-0.045	0.078	0.6 %
					directionality	0.017	0.517	-
					directionality * gender	-0.005	0.847	-
$Cort_{2000}$	gender	-0.029	0.229	-	severity	0.002	0.927	-
	sampling month	0.202	0.000**	4.1 %	severity * gender	-0.002	0.931	-
					directionality	0.056	0.031*	4.1 %
					directionality * gender	-0.020	0.412	-

*Note: * significant at the .05 level, ** significant at the .01 level; Adjusted R_2 are reported for significant effects and effects that approached significance.*

gender effect on AUC_G levels in the variable-oriented approach. Hence, we were not able to replicate findings from previous studies that demonstrated an inverse relationship between externalizing behavior problems and cortisol levels in samples consisting of predominantly boys (McBurnett, Lahey, Rathouz, & Loeber, 2000; Oosterlaan et al., 2005; van Goozen et al., 1998) or findings from studies on mixed-gender samples that concluded that low cortisol may be specific for *male* externalizing behavior problems (Loney et al., 2006; Shirtcliff et al., 2005). Our inability to replicate previous findings could be related to the fact that previous studies have largely been conducted on clinical or high-risk samples, whereas the present study was based on a large population-based sample. Early adolescents with more severe externalizing behavior problems are most likely to be found in clinical or high-risk samples rather than in population-based samples. Indeed, low basal cortisol levels were found to be specific for children and adolescents with more severe disruptive behavioral problems (van de Wiel, van Goozen, Matthys, Snoek, & van Engeland, 2004). Moreover, we demonstrated that gender had a particularly strong effect in the Pure EXT group, in that girls from the Pure EXT group showed significantly higher AUC_G levels than boys from the Pure EXT group (Figure 2.1 A). This finding does provides some evidence that the relationship between externalizing behavior problems and hypoactivity of the HPA-axis might be absent in mixed-gender samples.

Concerning our second hypothesis, we expected that comorbid externalizing and internalizing behavior problems lead to higher total cortisol levels after awakening (AUC_G levels) than pure externalizing behavior problems, an effect that might be specific for boys (McBurnett et al., 1991). In the person-oriented approach, we did not find a main effect of group nor did we find a main effect of group in boys and girls separately. In the variable-oriented approach, we found an indication that AUC_G levels increase with increasing severity of behavior problems in girls. However, considering the findings of the person-oriented approach, this effect is not likely due to the girls from the Comorbid group, but rather due to the girls from the Pure EXT group (Figure 2.1 A). The fact that we did not find a direction x gender effect underlines the importance of using a person-oriented approach, that is, pure externalizing behavior problems cannot be separated from the person, at least in girls. In short, there appears to be no evidence of an effect of comorbidity on AUC_G levels. Several previous studies were also not able to detect an effect of comorbidity (Oosterlaan et al., 2005; Shirtcliff et al., 2005), suggesting that in externalizing behavior problems, additional internalizing behavior problems do not lead to increased AUC_G levels. Apparently, the relationship between comorbidity and AUC_G levels is more complicated than we assumed, and cannot be explained in a straightforward way by the hypothesis of additive effects of externalizing behavior problems and internalizing behavior problems.

The first part of the third hypothesis was that pure externalizing behavior problems are more strongly related to the genetically influenced CAR (AUC_I levels) than comorbidity,

since pure externalizing behavior problems are thought to be more genetically influenced than comorbid externalizing and internalizing behavior problems (Gjone & Stevenson, 1997). In the person-oriented approach, we indeed found elevated AUC_I levels in the Pure EXT group compared to the Control group and the Comorbid group. Exploration of the gender x group interaction effect revealed that these effects are gender-specific, as they appear in girls but not in boys. In addition, like in the AUC_G analyses, the variable-oriented approach revealed an indication that AUC_I levels increase with increasing severity of behavior problems in girls. Again, considering the findings of the person-oriented approach, this effect may be more due to girls from the Pure EXT group than to girls from the Comorbid group (Figure 2.1 B). We will discuss these findings in girls in more detail later. We also expected that the Comorbid group would show higher AUC_I levels than the Control group. Both in the total group and in analyses for boys and girls separately, this was not the case. It could be that the comorbidity was more environmentally determined, rather than genetically determined, but this was not supported by the analyses on evening cortisol levels. The person-oriented analyses could not confirm the second part of the third hypothesis that comorbid externalizing and internalizing behavior problems are related to evening cortisol levels. In contrast, the variable-oriented approach provided evidence that externalizing behavior problems, in contrast to internalizing behavior problems, may be related to higher evening cortisol levels. Though we did not find a directionality x gender effect, girls with externalizing behavior problems show the highest evening cortisol levels, possibly influencing the strength of this effect (Figure 2.1 C). Moreover, we should not put undue weight on this directionality effect given that this finding did not contribute to an increase of the effect size.

To summarize so far, the key findings of the present study are two-fold. Firstly, we were not able to find the expected relationships between comorbidity and AUC_G levels, AUC_I levels, and evening cortisol levels. Secondly, girls with pure externalizing behavior problems form a distinct group, showing elevated AUC_G levels and AUC_I levels, and possibly influencing the directionality effect on evening cortisol levels. With regard to the former conclusion, as stated before, comorbidity should be considered as more than an addition of externalizing and internalizing behavior problems. Since comorbid externalizing and internalizing behavior problems are probably more clinically and etiologically heterogeneous than pure (externalizing) behavior problems (Boylan et al., 2007; Gjone & Stevenson, 1997), it could be that comorbidity is relatively insensitive to the effects of this single aspect of the neuroendocrine system. Planned contrasts support this explanation, revealing that the Comorbid group did not differ from the Control group with respect to AUC_G levels and AUC_I levels. With regard to the second key finding, there are indications that the CAR (both total levels and with respect to increase) in externalizing behavior problems is strongly dependent on gender. This gender effect was not previously

found since previous researchers almost exclusively studied boys or did not test gender differences. However, our finding is not in line with the study by Pajer et al. (2001) showing low levels of cortisol in girls with conduct disorder. Yet, differences in cortisol sampling (plasma vs. saliva) and operationalizing of behavior problems (conduct disorder vs. broadband externalizing) could account for these apparently contradictory findings. Moreover, the course of externalizing behavior problems could play a role. Moffitt and Caspi (2001) demonstrated that relatively few girls show a life-course-persistent pattern of externalizing behavior problems (ratio = 10 males: 1 female) in comparison to girls with an adolescence-limited pattern (ratio = 1.5 males: 1 female). Moreover, since males with a life-course-persistent pattern of externalizing behavior problems score worse on many risk factors compared to females with an adolescence-limited pattern, it could be hypothesized that elevated AUC_G and AUC_I levels are a particular risk factor for adolescence-limited patterns of externalizing behavior problems in females. However, this possibility needs to be further investigated in longitudinal research.

There are some potential limitations regarding the present study that need to be acknowledged. Firstly, home collection of saliva is much more susceptible to situational influences than collection of saliva in the more controlled conditions at the laboratory. Recent research suggests, however, that home assessment of cortisol in saliva provides the same results as the assessment under highly controlled laboratory conditions (Wilhelm, Born, Kudielka, Schlotz, & Wüst, 2007). Secondly, it has been argued that the dichotomization of quantitative variables, such as the scores of CBCL and YSR, is statistically inferior, rarely defensible and often will yield misleading results (MacCallum, Zhang, Preacher, & Rucker, 2002). We believe that setting a cutoff at the 84th percentile of CBCL and YSR is justified by the important distinction between behavior problems which fall within and outside the clinical range, and which has been confirmed and validated in papers by Achenbach and other working on the CBCL family of rating scales (Achenbach, 1991a; Achenbach & Rescorla, 2001; Verhulst et al., 1996). In the person-oriented approach, the Comorbid group exhibited more severe externalizing behavior problems than the pure externalizing group. Note that our variable-oriented approach took account of this issue by differentiating between overall severity and direction of psychopathology (Essex et al., 2006). As previously discussed, severity of disruptive behavioral problems might be related to HPA-axis activity (van de Wiel et al., 2004). However, our results suggest that this bias does not lead to higher HPA-axis activity in the Comorbid group.

On the other hand, the strength of the study lies in the fact that our findings are based on a very large population-based sample. Moreover, we obtained samples which were not subject to selection bias (de Winter et al., 2005). In addition, few studies have investigated the relationship between HPA-axis activity and externalizing behavior problems in population-based samples, and of these studies, few considered the influence

of gender and comorbidity. Another asset of our study is the use of both person-oriented and variable-oriented analyses which proved to be complementary and took maximal account of the type and distribution of the available data. Note that although our significant findings were mainly based on the person-oriented approach, the results of the variable-oriented approach could be reconciled with the results of the person-oriented approach.

We like to underline that many of our findings were trends and that the effect sizes of our significant findings are relatively weak compared to effect sizes in studies on clinical or high-risk samples. This could be due to subjects included in clinical or high-risk samples being characterized by overall much greater severity of externalizing behavior problems than subjects sampled from the general population, and in turn, this greater severity could explain the size of the effects (van de Wiel et al., 2004). The weak effect sizes could also be due to random measurement errors since we do not know the exact sampling times; hence true relationships may have been underestimated.

In summary, our results indicate that comorbidity and gender need to be considered in studies of HPA-axis activity in relation to externalizing behavior problems. Although we did not find the expected effects with respect to comorbidity, our findings indicate that pure externalizing behavior problems should be differentiated from comorbid behavior problems, especially in girls. Longitudinal research should examine whether the findings in girls with externalizing behavior problems are the result of an adolescent-limited pattern of externalizing behavior problems. Moreover, while comorbidity and gender clarified part of the complex relationship between HPA-axis activity and externalizing behavior problems, there are many other risk factors such as life events and family and parenting factors as well as genetic risks that should be included to study this complex relationship.

CHAPTER 3

Does HPA-axis activity mediate the relationship between obstetric complications and externalizing behavior problems? The TRAILS study.

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ABSTRACT

To examine whether HPA-axis activity mediates the relationship between obstetric complications (OCs) and externalizing behavior problems, and to investigate whether this model is different for boys and girls. In a population-based cohort of 1,768 10- to 12-year-old early adolescents, we assessed the cortisol awakening response and evening cortisol levels. Externalizing behavior problems were assessed using the Child Behavior Checklist and the Youth Self-Report. OCs were retrospectively assessed in a parent interview. OCs significantly predicted externalizing behavior problems, but OCs did not predict HPA-axis activity. Thus, the mediation model was not supported. In addition to the relationship between HPA-axis activity and externalizing behavior problems, which is specific for girls, there is also a relationship between OCs and externalizing behavior problems. However, these two mechanisms are not related to each other indicating that HPA-axis activity is not a mediator in the relationship between OCs and externalizing behavior problems. Future research should focus on understanding the mechanism through which OCs cause externalizing behavior problems.

3.1 INTRODUCTION

Externalizing behavior problems refer to a class of hyperactive and impulsive, oppositional defiant and aggressive behaviors that are very prevalent in children and adolescents and are associated with a huge burden for family and society (Liu, 2004). Externalizing behavior problems have a multifactorial etiology, with a moderate to strong contribution of genetic risk factors (Deater-Deckard & Plomin, 1999), and additional contribution of environmental risk factors, such as family and parenting factors (Jester et al., 2005), and obstetric complications (OCs) (Allen et al., 1998; Batstra et al., 2004; Buschgens et al., 2009; Gutteling et al., 2005; Nosarti et al., 2005; Raine, 2002).

Several studies have revealed that OCs, such as maternal physical problems during pregnancy and delivery, prematurity, macrosomia (i.e. birth weight higher than 4,500 g), and acute anoxia or hypoxia, are involved in the etiology of externalizing behavior problems (Allen et al., 1998; Batstra et al., 2004; Buschgens et al., 2009; Nosarti et al., 2005; Raine, 2002). In addition, emerging evidence suggests that the psychological well-being of the mother during pregnancy, such as maternal emotional difficulties during pregnancy, and prenatal stress or anxiety of the mother, are related to externalizing behavior problems (Allen et al., 1998; Gutteling et al., 2005). Several composite scores on OCs have been introduced (Batstra, Neeleman, Elsinga, & Hadders-Algra, 2006; Milberger, Biederman, Faraone, Guite, & Tsuang, 1997; Prechtl, 1980) in which the classical biological risk factors have been combined with factors concerning the psychological well-being of the mother during pregnancy. This composite score on OCs have also been related to externalizing behavior problems in previous studies, including a TRAILS study (Batstra et al., 2004; Buschgens et al., 2009). However, despite the extensive literature on the relationship between OCs and externalizing behavior problems, little attention has been paid to the mechanism by which OCs may cause externalizing behavior problems. Several researchers suggested a mediating role of neurobiological deficits in the relationship between early childhood adversity and externalizing behavior problems (Alink et al., 2008; Gunnar et al., 2006; van Goozen et al., 2007). In line with these researchers, Allen et al. (1998) proposed a biological model in which neurobiological deficits may explain the relationship between OCs and externalizing behavior problems. Besides causing prefrontal damage, the effects of OCs may impact multiple other brain sites (Raine, 2002). Among other neurobiological deficits, impairment of the hypothalamus-pituitary-adrenal (HPA)-axis has often been suggested as a potential mediator in the relationship between early stress (from before birth to early childhood) and externalizing behavior problems (Alink et al., 2008; Gunnar et al., 2006; Huizink et al., 2003; van Goozen et al., 2007).

Indeed, specific OCs, such as instrumental delivery (i.e. forceps or ventouse), meconium staining of the liquor, prematurity, and maternal stress or anxiety during pregnancy, may adversely affect the function of the child's HPA-axis (Buske-Kirschbaum

et al., 2007; Huizink, Mulder, & Buitelaar, 2004; Mears, McAuliffe, Grimes, & Morrison, 2004; O'Connor et al., 2005; Taylor, Fisk, & Glover, 2000). The HPA-axis is known as a central component of the neuroendocrine stress system, and produces cortisol as its major end product (Tsigos & Chrousos, 2002). On the one hand, several studies suggest that specific OCs are directly associated with elevated cortisol levels in the fetus, child or early adolescent (Buske-Kirschbaum et al., 2007; Huizink et al., 2004; Mears et al., 2004; O'Connor et al., 2005). On the other hand, several studies suggest that specific OCs are associated with elevated cortisol levels in response to a stressor in both infants and young adults (Entringer, Kumsta, Hellhammer, Wadhwa, & Wüst, 2009; Taylor et al., 2000). Moreover, maternal anxiety during pregnancy is associated with an elevated cortisol awakening response (CAR), that is, the rise in cortisol from awakening to 30 min later (O'Connor et al., 2005).

For mediation to occur, HPA-axis activity must also be associated with externalizing behavior problems. In previous TRAILS studies, it was demonstrated that elevated basal cortisol levels are associated with externalizing behavior problems in girls from the general population (Marsman et al., 2008; Sondeijker et al., 2007). Although seemingly contradictory, these findings may be congruent with the general idea that externalizing behavior problems are associated with lower basal cortisol levels (Alink et al., 2008; van Goozen et al., 2007). That is, a large meta-analysis suggests that the effect size for this inverse association is small and specific for boys and for clinical samples (Alink et al., 2008). Thus, this positive association may be specific for girls from the general population. Yet the study by Van Bokhoven et al. (2005) indicated that the positive association may also be inherent to the general population per se, revealing a positive association for boys from the general population. In addition, Pajer et al. (2001) found an inverse association between HPA-axis activity and externalizing behavior problems in a clinical sample of girls, also indicating that the potential positive association is specific for girls with externalizing behavior problems from the general population.

Two main bodies of evidence suggest that the mediation model is gender-specific. First, the relationship between HPA-axis activity and externalizing behavior problems is different for boys and girls. As said before, there is evidence that the inverse association between HPA-axis activity and externalizing behavior problems is specific for boys (Alink et al., 2008), whereas the positive association between HPA-axis activity and externalizing behavior problems is specific for girls (Marsman et al., 2008; Sondeijker et al., 2007). In addition, there may be gender differences in the level of HPA-axis activity. More specifically, gender may be associated with HPA-axis activity, in that girls may show higher cortisol levels than boys (Klimes-Dougan et al., 2001; Rosmalen et al., 2005). Furthermore, there are gender differences in the level of externalizing behavior problems. Boys exceed girls in rates of a life course persistent pattern of externalizing behavior problems, while gender differences are small in an adolescent-limited pattern of externalizing behavior problems

(Moffitt & Caspi, 2001). Second, there may be gender differences in the sensitivity to OCs. Whereas boys experience more OCs than girls (Sandberg, 2002) and are more vulnerable to the effects of OCs than girls (Batstra et al., 2004; Liu, 2004), the relationship between OCs and externalizing behavior problems is possibly specific for girls (Allen et al., 1998). However, there is also evidence that there are no gender differences in the relationship between OCs and externalizing behavior problems (Batstra et al., 2004). In addition, the TRAILS study that found a relationship between OCs and externalizing behavior problems in our sample did not investigate gender differences (Buschgens et al., 2009). In conclusion, gender specificity of the relationship between OCs and externalizing behavior problems is still open to question.

In summary, there are empirical and theoretical reasons to hypothesize that HPA-axis activity serves as a mediator in the relationship between OCs and externalizing behavior problems. Our hypotheses are partly based on previous TRAILS studies that revealed that OCs predict externalizing behavior problems, and that HPA-axis activity is associated with externalizing behavior problems in girls (Marsman et al., 2008; Sondeijker et al., 2007). Therefore, we hypothesize that HPA-axis activity may be the linking mechanism between OCs and externalizing behavior problems. However, to date, no study investigated this model. The mediation model predicts that OCs lead to higher cortisol levels, which in turn lead to more externalizing behavior problems. We hypothesize that this model is specific for girls.

3.2 METHODS

3.2.1 Sample

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective cohort study of Dutch (early) adolescents, with the aim to chart and explain the development of mental health from early adolescence into adulthood, both at the level of psychopathology and the levels of underlying vulnerability and environmental risk. Early adolescents will be measured biennially at least until they are 25 years old. The present study involves data from the first (T1) assessment wave of TRAILS, which ran from March 2001 to July 2002. If both parents and early adolescents agreed to participate, parental written informed consent was obtained after the procedures had been fully explained. The study was approved by the National Dutch Medical Ethics Committee. Of all early adolescents approached for enrolment in the study (N=3,145), 76.0% (N=2,230, mean age = 11.09, SD = 0.56, 50.8% girls) early adolescents participated in the study. Responders and non-responders did not differ with respect to the prevalence of teacher-rated behavior problems, nor regarding associations between sociodemographic variables and mental health outcomes. Detailed information about sample selection and analyses of non-response bias has been reported elsewhere (de Winter et al., 2005; Huisman et al., 2008).

3.2.2 Procedure

Well-trained interviewers visited one of the parents or guardians (preferably the mother, 95.6%) at their homes to administer an interview covering a wide range of topics, including developmental history and somatic health, parental psychopathology and care utilization. In addition to the interview, the parent was asked to fill out some questionnaires concerning the adolescent's mental health and behavior. Early adolescents filled out questionnaires at school, in the classroom, under the supervision of one or more TRAILS assistants. Besides, intelligence and a number of biological and neurocognitive parameters were assessed individually (at school, except for saliva samples, which were collected at home). Teachers were asked to fill out a brief questionnaire for all TRAILS participants in their class. Measures that were used in the present study are described more extensively below.

3.2.3 Measures

3.2.3.1 Behavioral problems. Behavioral problems were assessed with the Child Behavior Checklist (CBCL) (Achenbach, 1991a; Verhulst et al., 1996) and the Youth Self-Report (YSR) (Achenbach, 1991c; Verhulst et al., 1997). The CBCL is a measure of parent-reported emotional and behavioral problems in 4- to 18-year-old children and the YSR is a self-report questionnaire that was modeled on the CBCL. The CBCL and the YSR contain 113 and 112 items, respectively. These items are rated as 0 (not true), 1 (somewhat or sometimes true) or 2 (very true or often true). Both the CBCL and the YSR contain two broadband scales: one for internalizing behavior problems and one for externalizing behavior problems. In the present study, we used the mean of the standardized CBCL and YSR scores on externalizing behavior problems.

3.2.3.2 Obstetric Complications (OCs). Obstetric complications are defined as the broad class of deviations from the expected, normal course of events, including child development during pregnancy, labor/delivery, and the early neonatal period (McNeil, 1988). Data concerning OCs were assessed in the parent interview. OCs included the presence of pregnancy complications (i.e. physical, social or psychological problems during pregnancy), complicated deliveries (i.e. breech presentation, Cesarean section), and hospitalization of the mother (i.e. due to physical problems, postnatal depression) or child (i.e. lack of oxygen, blood transfusion, jaundice) (Gillberg, 1995; Milberger et al., 1997). A composite score on OCs was calculated on the basis of a list of 31 OCs (range 0-14, mean = 1.87, SD = 2.19). If no information was available for six or more items, cases were excluded from further analyses. The distribution of OCs is positively skewed, as most parents report 0 (37.9%, N=609), 1 (19.3%, N=309) or 2 (15.4%, N=247) OCs.

3.2.3.3 Cortisol. TRAILS participants collected cortisol samples (saliva) at home, using the Salivette sampling device (Sarstedt, Rommelsdorfer Str., D-51588 Nümbrecht, Germany), which was handed to the parent at the parent interview, accompanied by a verbal and a written instruction. The Salivette tube consists of a plastic sampling vessel with a suspended insert containing a sterile neutral cotton wool swab that has to be chewed for about 45 s and then returned to the insert. Participants were instructed to collect three saliva samples: the first sample shortly after waking up (still lying in bed), the second sample 30 min later, and the third sample at 20:00 h. Both the sampling and the preceding day should be normal (school) days, without special events or stressful circumstances. Since the TRAILS participating schools started at approximately the same time, the sampling-time variation of the morning samples among the early adolescents is expected to be limited and the estimated corresponding times are 07:00 h for the first sample ($Cort_{0700}$) and 07:30 h for the second sample ($Cort_{0730}$). Participants were instructed not to collect saliva when they were ill, had a cold, had a headache, or were menstruating. Furthermore, they were requested not to take any medication, if possible. Any deviations from this protocol, either in terms of sampling times or in terms of other requirements, were indicated on an accompanying form. Concerning the sampling procedure itself, subjects were instructed to keep a glass of water next to their bed and to thoroughly rinse their mouth with tap water before sampling saliva, and not to consume sour products or brush their teeth shortly before that. Saliva samples were stored by the participants in their freezer directly after sampling and mailed to the institute as soon as possible. Participants who did not return the salivettes within a couple of months were sent a reminder letter. In total, we received saliva samples of 1,768 early adolescents (79.3% of all TRAILS participants). Non-responders did not differ from responders in terms of gender (48.4% male vs. 49.4% male for non-responders vs. responders, respectively, χ^2 (df=1) = 0.132; p = 0.716), OCs (1.67 vs. 1.73, t = -0.569; p = 0.569), and externalizing behavior problems (0.51 vs. 0.51, t = -0.139; p = 0.890).

The saliva samples were stored at -20 ºC until analysis. Previous studies suggest that salivary cortisol levels are stable for prolonged periods of time at -20 ºC (Aardal & Holm, 1995). After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the laboratory (Department of Clinical and Theoretical Psychobiology, University of Trier, Germany) for analysis. Procedures of determination of cortisol levels are described more extensively elsewhere (Rosmalen et al., 2005).

3.2.4 Statistical analyses

A total of 82 early adolescents (4.6%) used medication. Of these, we excluded 22 early adolescents because they used corticosteroid-containing medication. Based on the literature and based on their cortisol values, we found no reason to exclude any of the other participants who used medication. For each time point, single cortisol samples with

values that were above 3 SD of the mean of the particular time point were excluded from the analysis in order to reduce the impact of outliers ($Cort_{0700}$ 21 excluded, 1,666 valid measurements in the final dataset; $Cort_{0730}$ 11 excluded, 1,683 valid measurements in the final dataset; $Cort_{2000}$ 18 excluded, 1,689 valid measurements in the final dataset). After this exclusion, cortisol levels followed a normal distribution ($Cort_{0700}$ skewness = 0.700, kurtosis = 0.632; $Cort_{0730}$ skewness = 0.426, kurtosis = 0.239; $Cort_{2000}$ skewness = 1.217, kurtosis = 2.014).

With regard to the morning cortisol levels we used Area Under the Curve (AUC) measures. The computation of the AUC is a frequently used method in endocrinological research to assess the overall secretion over a specific time period (AUC with respect to ground, AUC_G), and to estimate circadian changes over a specific time period (AUC with respect to increase, AUC_I) (Pruessner et al., 2003). Pruessner et al. (2003) recommend employing both formulas when analyzing datasets with repeated measures. We used the following formulas for calculating the (1) total cortisol levels after awakening (in h x nmol/l): $AUC_G = (Cort_{0730}\text{-}Cort_{0700}) \times 0.5/2 + Cort_{0700} \times 0.5$ (in which 0.5 refers to 0.5 hours) and (2) cortisol awakening response (CAR) (in h x nmol/l): $AUC_I = (Cort_{0730}\text{-}Cort_{0700}) \times 0.5/2$ (in which 0.5 refers to 0.5 hours). The former correlates 0.71 with $Cort_{0700}$ and 0.86 with $Cort_{0730}$ and the latter is in this design mathematically equal to one-quarter of the difference between awakening level and level 30 min later (Rosmalen et al., 2005). According to their conceptual meaning, we used AUC_G levels following previous studies on basal cortisol samples and AUC_I levels following previous studies on the CAR. We calculated the AUC_G and AUC_I for 1,615 participants, from which we received both $Cort_{0700}$ and $Cort_{0730}$. Furthermore, we used $Cort_{2000}$ levels for our interest in basal cortisol levels in the evening (Rosmalen et al., 2005). From 1,689 participants, we received $Cort_{2000}$ levels.

In a previous study on the present sample, gender and the quadratic effect of sampling month were identified as significant predictors of HPA-axis activity. Age, pubertal development, and BMI appeared not to be related to HPA-axis activity (Rosmalen et al., 2005). Prior to analyses, all predictor variables were standardized to minimize multicollinearity. Following the framework described by Baron and Kenny (1986), we tested for potential mediation of HPA-axis activity by a series of four regression analyses. In the first three regression analyses, each of the HPA-axis measures (i.e. AUC_G, AUC_I and $Cort_{2000}$) were the dependent variable. In these regression analyses, the quadratic effect of sampling month was entered in the first block of the model. OCs, gender, and the interaction term between OCs and gender, were entered in the second block of the model. In the fourth regression analysis, externalizing behavior was the dependent variable. Owing to its potential confounding effect, socio-economic status was entered in the first block of the model. Again, OCs, gender, and the interaction term between OCs and gender, were entered in the second block of the model. If OCs were a significant predictor of both HPA-axis measure and externalizing behavior problems, externalizing behavior problems

were regressed on both HPA-axis measure and OCs in a fifth regression. If any of the interaction terms with gender were significant, we performed gender-stratified analyses.

3.3 RESULTS

Table 3.1 shows the mean and standard deviation of the (unstandardized) variables for the total group and by gender. As expected, boys showed more externalizing behavior problems and OCs than girls. In addition, girls showed higher AUC_G levels than boys, while AUC_I levels and $Cort_{2000}$ levels did not differ between boys and girls.

3.3.1 Testing mediation

Table 3.2 shows the results of the regression analyses testing our mediation hypothesis. OCs were not a significant predictor of AUC_G levels, AUC_I levels or $Cort_{2000}$ levels. As in previous TRAILS studies (Marsman et al., 2008; Rosmalen et al., 2005), gender predicted AUC_G levels ($\beta = -0.098$, $p < 0.001$) and the quadratic effect of sampling month predicted AUC_G levels ($\beta = 0.112$, $p < 0.001$), AUC_I levels ($\beta = 0.072$, $p < 0.01$), and $Cort_{2000}$ levels ($\beta = 0.203$, $p < 0.001$).

Socio-economic status (SES) significantly predicted externalizing behavior problems ($\beta = -0.150$, $p < 0.001$) and accounted for 2.4% of the adjusted variance. In addition, OCs ($\beta = 0.052$, $p < 0.05$) and gender ($\beta = 0.210$, $p < 0.001$) predicted externalizing behavior problems. The effects of SES, OCs and gender accounted for 7.0% of the adjusted variance.

Because none of the interaction terms with gender were significant, we did not perform gender-stratified analyses. Given the requirement that the independent variable (OCs) must affect the mediator (HPA-axis activity) to establish mediation (Baron & Kenny, 1986), HPA-axis activity did not emerge as a mediator of the relation between OCs and externalizing behavior problems.

3.4 DISCUSSION

There are empirical and theoretical reasons to expect that HPA-axis activity serves as a mediator in the relationship between OCs and externalizing behavior problems. The present study is the first that investigates this mediation model in a large population-based sample of early adolescents. We found that HPA-axis activity did not mediate the relationship between OCs and externalizing behavior problems.

Although we did not find a mediating effect of HPA-axis activity, our mediator analyses did reveal a direct relationship between OCs and externalizing behavior problems. Consistent with many previous studies (Allen et al., 1998; Batstra et al., 2004; Gutteling et al., 2005; Nosarti et al., 2005; Raine, 2002), including a TRAILS study (Buschgens et al., 2009), a history of OCs was identified as an environmental risk factor for externalizing

TABLE 3.1 Mean and standard deviation of the (unstandardized) variables for the total group and by gender

	Total			Boys			Girls			Boys vs. Girls	
	n	Mean	(SD)	*n*	Mean	(SD)	*n*	Mean	(SD)	*t*	*p*
Externalizing behavior	2000	0.51	(0.32)	980	0.58	(0.35)	1020	0.44	(0.28)	-9.8	<0.001
OCs	2166	1.72	(2.01)	1061	1.80	(2.09)	1105	1.63	(1.93)	-2.0	0.05
HPA-axis activity											
AUC_G	1615	6.72	(2.24)	803	6.48	(2.21)	812	6.95	(2.24)	4.3	<0.001
AUC_I	1615	0.95	(1.73)	803	0.88	(1.64)	812	1.01	(1.82)	1.5	0.13
$Cort_{2000}$	1689	1.95	(1.33)	835	1.90	(1.33)	854	2.00	(1.34)	1.5	0.14

*Note: Externalizing behavior = mean scores on CBCL and YSR (range 0-2); OCs = total obstetric complications. AUC_G and AUC_I in h*nmol/l and $Cort_{2000}$ in nmol/l. The last column lists the results of the independent samples t-tests comparing boys and girls.*

TABLE 3.2 Regression analyses testing for mediation

Predictor		Dependent	Beta	p-value	Adj. R^2 (%)
Model 1		AUC_G			
Block 1	sampling month		0.112	<0.001	1.2
Block 2	OCs		-0.029	0.25	
	gender		-0.098	<0.001	2.1
	OCs*gender		0.019	0.44	
Model 2		AUC_I			
Block 1	sampling month		0.072	<0.01	0.5
Block 2	OCs		-0.007	0.78	
	gender		-0.035	0.17	
	OCs*gender		-0.024	0.33	
Model 3		$Cort_{2000}$			
Block 1	sampling month		0.203	<0.001	4.0
Block 2	OCs		-0.040	0.10	
	gender		-0.026	0.29	
	OCs*gender		-0.030	0.22	
Model 4		Externalizing			
Block 1	SES		-0.150	<0.001	2.4
Block 2	OCs		0.052	0.02	7.0
	gender		0.210	<0.001	7.0
	OCs*gender		0.017	0.81	

Note: Sampling month = quadratic effect of sampling month; OCs = obstetric complications; SES = socio-economic status; Externalizing = externalizing behavior problems. OCs, gender, AUC_G, AUC_I, $Cort_{2000}$, and SES are standardized variables. Adjusted R^2 is reported for significant effects.

behavior problems. This effect was independent of the effect of gender, suggesting that the effect of OCs was not due to the fact that boys experienced more OCs than girls. It must be noted, however, that the effect size of the predictive effect of OCs on externalizing behavior problems is small. Together with gender, which clearly accounted for most of the adjusted variance, OCs predicted only 4.6% of the adjusted variance. In addition, the relationship between OCs and externalizing behavior problems was not gender-specific. This is in line with a study by Batstra et al. (2004) who found that suboptimal obstetric

conditions were related to externalizing behavior problems in both boys and girls. In contrast, Allen et al. (1998) suggested that the association between OCs and externalizing behavior problems may be specific for girls. However, their finding may be the result of a Type I error resulting from multiple testing (Allen et al., 1998).

We adopted a model inspired by Allen et al. (1998) who suggested a biological model in which neurobiological deficits mediate the relationship between OCs and externalizing behavior problems. They found that this model was particularly appropriate for explaining the relationship between OCs and externalizing behavior problems, as other models were more appropriate for explaining the relationship between OCs and internalizing behavior problems. In addition, this model is very similar to (a part of) the model described by Raine (2002) and Van Goozen et al. (2007). Both studies focused on explaining the relationship between early risk factors and antisocial behavior. Why is it then that we did not find a mediating effect? Although we found a gender-specific relationship between HPA-axis activity and externalizing behavior problems in our previous study (Marsman et al., 2008), there was no evidence of a relationship between OCs and HPA-axis activity. The latter is in contrast to previous studies showing that early adversities may adversely affect the function of the fetal and neonatal HPA-axis (Buske-Kirschbaum et al., 2007; Huizink et al., 2004; Mears et al., 2004; O'Connor et al., 2005; Taylor et al., 2000), leading to elevated cortisol levels (Buske-Kirschbaum et al., 2007; Huizink et al., 2004; Mears et al., 2004; O'Connor et al., 2005). Yet, previous studies have reported on specific OCs, while no study, to our knowledge, has reported on composite scores on OCs in relation to HPA-axis activity. We were unable to test the possibility that specific OCs were related to HPA-axis activity as the number of early adolescents that had a specific OC item was often too small to draw conclusions about that single item. It is also possible that OCs are related to HPA-axis activity, but not to the cortisol measures in our sample. First, concerning the increase in cortisol levels after awakening (CAR or AUC_I levels), only one study found an indication that maternal anxiety during pregnancy may lead to an elevated CAR in pre-adolescent children (O'Connor et al., 2005). However, the significance of findings with regard to the CAR is difficult to determine since studies often use different methodologies (Clow, Thorn, Evans, & Hucklebridge, 2004). Thus, more research is needed to assess the relationship between OCs and the CAR, using similar methodology to determine the CAR. Secondly, we were also unable to find a relationship between OCs and evening cortisol levels. O'Connor et al. (2005) did not find an association between maternal anxiety during pregnancy and evening cortisol levels either. Reason for this non-finding may be that evening cortisol levels display little inter-subject variability (Rosmalen et al., 2005), possibly making it more difficult to detect associations. Finally, we did not find a relationship between OCs and total cortisol levels after awakening (AUC_G levels) despite other studies that found an association between specific OCs and elevated cortisol levels measured in pre-adolescence (Buske-Kirschbaum et al., 2007; O'Connor et al., 2005). The fact that these

studies found elevated cortisol levels in pre-adolescence (Buske-Kirschbaum et al., 2007; O'Connor et al., 2005) seems to exclude the possibility that OCs initially provoke elevations in cortisol, which after down-regulation of the HPA-axis decrease to lower cortisol levels as a long term consequence (De Bellis et al., 1999; Gunnar & Donzella, 2002; Gunnar & Vazquez, 2001). Still, specific OCs may provide the most valid explanation for not finding a relationship between OCs and HPA-axis activity. Future research on high-risk samples may provide useful information on this possibility. If OCs do not account for differences in HPA-axis activity, there may be other risk factors such as life events and family and parenting factors that affect HPA-axis activity.

It is still possible that brain damage is a mediator in the relationship between OCs and externalizing behavior problems (Allen et al., 1998), as HPA-axis is not the only possible mediator in this relationship. One possibility is that OCs lead to neurological impairment in the frontal lobes, which are essential for executive functioning (Beck & Shaw, 2005). In turn, executive function deficits may predispose to externalizing behavior problems (Raine, 2002). A second possibility is that OCs lead to other forms of brain damage, which indirectly leads to externalizing behavior problems. For example, when brain damage causes a physical disability, a child may become less confident in interaction with other children, which then in turn elevates the risk for externalizing behavior problems (Allen et al., 1998). A third possibility is that neurobiological deficits may interact with other factors, like cognitive and emotional functioning (van Goozen et al., 2007) and social risk factors (Raine, 2002). In addition, androgens and the autonomic nervous system may function as potential mediators (van Goozen et al., 2007). All in all, we can conclude that the potential biological mediation of the relationship between OCs and externalizing behavior problems is far more complex than we assumed.

A major strength of the present study is that it was based on a very large population-based sample of early adolescents. This enabled us to test the mediating effect of HPA-axis activity on the relationship between OCs and externalizing behavior problems without the influence of selection biases which are inherent in clinically referred samples (de Winter et al., 2005). A potential limitation concerns the retrospective nature of the information on OCs. However, a previous study concluded that maternal recall may be a surprisingly accurate source of obstetric information and hence an acceptable alternative to more objective indices in a prospective study (Allen et al., 1998). Another limitation concerns the cortisol sampling. First, home collection of saliva is much more susceptible to situational influences than collection of saliva in the more controlled conditions at the laboratory. In addition, home collection relies heavily upon participant adherence (Clow et al., 2004). Recent research suggests, however, that home assessment of cortisol in saliva provides the same results as the assessment under highly controlled laboratory conditions (Wilhelm et al., 2007). Secondly, the present study involves only one day of cortisol assessment. However, we are still confident in the reliability of our cortisol

data. One reason is that the CAR (both overall activity and dynamic of the response) is reasonably stable for individuals across days (Clow et al., 2004; Wüst, Wolf, et al., 2000). In addition, both the sampling and the preceding day were normal school days. In a sample of adults, Hellhammer et al. (2007) revealed that sampling on workdays may reduce the within-subject variation of situational factors because of the uniform schedule on workdays. Since school days are highly scheduled, it is probable that the same applies to the early adolescents in our sample. Moreover, the possible reduction in reliability as a result of one day of cortisol assessment may be counterbalanced by the sample size, which is large enough to off set random fluctuations in individual values.

3.4.1 Conclusions

In addition to our previous study which demonstrated a relationship between HPA-axis activity and externalizing behavior problems in girls (Marsman et al., 2008), we confirmed the relationship between OCs and externalizing behavior problems (Buschgens et al., 2009), although the effect size of this relationship is small. However, these two mechanisms were not related to each other, indicating that HPA-axis activity was not a mediator in the relationship between OCs and externalizing behavior problems. Future research should focus on understanding the mechanism through which OCs cause externalizing behavior problems.

CHAPTER 4

Family environment is associated with HPA-axis activity in adolescents. The TRAILS study.

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ABSTRACT

The purpose of the present study was to investigate the developmental programming part of the theory of biological sensitivity to context using family environmental factors and hypothalamus-pituitary-adrenal (HPA) axis functioning. Specifically, we investigated whether perceived parenting (Rejection and Emotional Warmth) and socio-economic status (SES) predicted basal cortisol levels and the cortisol awakening response (CAR). In a population-based cohort of 1594 adolescents (mean age = 11.08, SD = 0.54) we assessed salivary cortisol, SES and perceived parenting. Perceived parental Emotional Warmth showed an inverse, linear association with basal cortisol levels. In addition, there was a curvilinear relationship between SES and both basal cortisol levels and the CAR. Our findings with regard to basal cortisol levels confirmed our hypothesis: lower basal HPA-axis activity in both high and low SES families compared to intermediate SES families.

4.1 INTRODUCTION

The evolutionary-developmental theory of biological sensitivity to context (BSC) (Boyce & Ellis, 2005) offers a conceptual framework for understanding individual differences in biological sensitivity to the environment. Basically, this theory consists of two parts. According to the first part of the theory, both children who, in early life, experience high-stress environments and children who experience supportive, low-stress environments tend to develop a highly reactive stress response system. In addition, children who experience moderate stress environments tend to develop a low reactive stress response system. We refer to this part of BSC theory as the developmental programming part. As a result, one expects a U-shaped association between environmental factors and reactivity of the stress response system. The developmental programming part of BSC theory was tested in two studies of children (3- to 5-year old and 5- to 7- year old). It was found that family stressors and socio-economic status (SES) predicted a U-shaped association in cardiovascular (i.e. heart rate) and adrenocortical (i.e. cortisol) stress reactivity, consistent with the theory (Ellis, Essex, & Boyce, 2005). According to the second part of the theory, specific beneficial aspects of individual differences are predicted in biological sensitivity to the environment. In a negative environment a highly reactive stress response system is helpful, because one is vigilant to threats and dangers, and in a positive environment one benefits from a highly reactive stress response system, because one is sensitive to social resources and support (Boyce & Ellis, 2005). In addition, a low reactive stress system serves a beneficial function for children in moderate environments, because increased passing of the emotional signals from chronic stressors leads to greater resilience under difficult conditions (Boyce & Ellis, 2005). This part of the theory has been tested and confirmed quite extensively by now (Boyce et al., 2006; Ellis, Shirtcliff, Boyce, Deardorff, & Essex, 2011; Essex, Armstrong, Burk, Goldsmith, & Boyce, 2011; Obradović, Bush, & Boyce, 2011; Obradović, Bush, Stamperdahl, Adler, & Boyce, 2010). The purpose of the present study is to test the developmental programming part of BSC theory on activity of the hypothalamus-pituitary-adrenal (HPA) axis.

The HPA-axis is a central component of the body's neuroendocrine response to stress, with cortisol as its major end product (Tsigos & Chrousos, 2002). Three different aspects of HPA-axis activity are distinguished. Firstly, basal cortisol levels follow a circadian rhythm in healthy humans. We believe it is important to investigate basal cortisol levels since there is evidence that psychopathology in children and adolescents is associated with dysregulations in basal cortisol levels (Lopez-Duran, Kovacs, & George, 2009), thus representing trait characteristics of HPA-axis functioning (Hellhammer et al., 2007). Secondly, the increase in cortisol levels in about half an hour after awakening is another important aspect of HPA-axis activity (Wüst, Wolf, et al., 2000). This cortisol awakening response (CAR) is a specific response to awakening which is distinct from the basal

circadian rhythm of cortisol secretion (Wilhelm et al., 2007). In a recent review several lines of results are presented that support the hypothesis that the magnitude of the CAR is dependent on the anticipation of demands of the upcoming day (Fries, Dettenborn, & Kirschbaum, 2009). Thirdly, challenge-induced cortisol secretion is called stress reactivity. The three measures of HPA-axis functioning are weakly correlated in our dataset (Bouma, Riese, Ormel, Verhulst, & Oldehinkel, 2009), indicating that they reflect different mechanisms (Fries et al., 2009).

Although initial formulation of BSC theory mainly seemed to apply to stress reactivity, the developmental programming part of BSC theory has recently been described in much greater detail, now also involving basal cortisol levels (Del Giudice et al., 2011). In the present study, we will examine how the early environment relates to two aspects of HPA-axis functioning: basal cortisol levels and the CAR.

As said before, the developmental programming part of BSC theory predicts high HPA-axis stress reactivity to develop both in unsupportive, high-stress environments as well as in supportive, low-stress environments (Boyce & Ellis, 2005; Del Giudice et al., 2011). Contrary to developmental programming of HPA-axis reactivity, Del Giudice et al. (2011) predicted that basal cortisol levels are similar between individuals who developed in different environments, while no prediction is done regarding the CAR.

Regarding basal cortisol levels, evidence points in the direction of lower basal cortisol levels in individuals who grew up in stressful family environments, which is inconsistent with Del Giudice et al.'s (2011) predictions. From many reviews written on this topic we conclude that, whereas a high-stress environment leads to increases in children's basal cortisol levels in the short-term (De Bellis, 2001; Grassi-Oliveira et al., 2008; Gunnar, 1992), in the long-term decreases in basal cortisol levels are observed (Chrousos & Gold, 1992; De Bellis, 2001; Fries et al., 2005; Grassi-Oliveira et al., 2008; Gunnar & Vazquez, 2001; McEwen & Stellar, 1993; Miller et al., 2007). That is, persistent adversity over time may lead to lower basal cortisol levels in the long-term, and might have beneficial effects for the organism (Fries et al., 2005).

Relatively few studies investigated the association between positive aspects of the family environment and basal HPA-axis activity. Most studies focused on the potential modifying or buffering effect of positive aspects of family climate on HPA-axis reactivity in infants and young children (Albers, Riksen-Walraven, Sweep, & de Weerth, 2008; Gunnar, 1998; Gunnar & Donzella, 2002; Gunnar, Larson, Hertsgaard, Harris, & Brodersen, 1992). Although the association between positive affect and low basal cortisol levels is well established (Dockray & Steptoe, 2010), to our knowledge, only one study investigated the direct association between a positive (family) environment and basal cortisol levels. That is, a recent study by Engert et al. (2011) carried out in young adults suggests that high perceived parental care was related to low basal cortisol levels and CAR, whereas those with low perceived parental care showed high basal cortisol levels and CAR. Regarding

low perceived parental care, this finding contradicts our hypothesis that individuals who grew up in stressful environments have lower basal cortisol levels. Although evidence for the association between positive aspects of the environment and low basal cortisol levels is sparse, we hypothesize that low basal cortisol levels develop in both low-stress and high-stress environments as compared to moderate stress environments (inverse U-shape). This hypothesis is consistent with the idea that a strong reaction to stimuli from the environment is possible in a system with low basal cortisol levels, due to the lack of a ceiling effect.

Probably, Del Giudice et al. (2011) did not formulate any predictions regarding the developmental programming of the CAR, because of the lack of consistency among studies on the relationship between family environment and the CAR (Fries et al., 2009). One possible explanation for inconsistencies is that the CAR reflects state characteristics of the HPA-axis or, in other words, reflects the anticipated stress of the coming day (Hellhammer et al., 2007). In a study with ballroom dancers a much lower cortisol increase from awakening to 30 min after awakening was found on a competition day compared to a day without competition (Rohleder, Beulen, Chen, Wolf, & Kirschbaum, 2007). In another study, it was found that feelings of loneliness and sadness on a specific day were related to a higher CAR, whereas feelings of tension and anger were not related to the CAR (Adam, Hawkley, Kudielka, & Cacioppo, 2006). In this respect, it is hard to formulate clear hypotheses regarding developmental programming of the CAR.

As far as we know, no study investigated the potential curvilinear relationship between family environment and basal cortisol levels or the CAR. In general, the vast majority of studies focused on linear relationships between negative aspects of the environment in relation to aspects of HPA-axis activity. In this paper, family environment is defined by positive and negative aspects of parenting on the one hand, that is, perceived parental Warmth and perceived parental Rejection, respectively, and socio-economic status (SES) on the other hand. We tested the hypothesis that the association between family environment and basal cortisol levels is inversely U-shaped; investigations with the CAR were largely exploratory.

4.2 METHODS

4.2.1 Sample

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective cohort study of Dutch (early) adolescents, with the aim to chart and explain the development of mental health from early adolescence into adulthood, both at the level of psychopathology and the levels of underlying vulnerability and environmental risk. Adolescents will be measured biennially at least until they are 25 years old. The present study involves data from the first (T1) assessment wave of TRAILS, which ran from March 2001 to July 2002. If both

parents and adolescents agreed to participate, parental written informed consent was obtained after the procedures had been fully explained. Of all adolescents approached for enrollment in the study (N=3145), 2230 (76.0%) adolescents participated in the study. Responders and non-responders did not differ with respect to the prevalence of teacher-rated behavior problems, nor regarding associations between sociodemographic variables and mental health outcomes. Detailed information about sample selection and analyses of non-response bias has been reported elsewhere (de Winter et al., 2005; Huisman et al., 2008). We received at least one saliva sample of 1768 adolescents (79.3% of all TRAILS participants). Non-responders did not differ from adolescents who returned saliva samples in terms of gender (48.4% male vs. 49.4% male for non-responders vs. responders, respectively, χ^2 (df=1) = 0.132; p = 0.716), mean severity scores of behavior problems (-0.0030 vs. -0.0001, t = -0.056; p = 0.955), mean directionality scores of behavior problems (-0.0050 vs. 0.0018, t = -0.226; p = 0.821), perceived parental Rejection (1.40 vs. 1.42, t = -1.369, p = 0.171), and perceived parental Emotional Warmth (3.21 vs. 3.22, t = -0.322, p = 0.748). There was a slight difference in SES between non-responders and responders (-0.34 vs. 0.02, t = -8.350, p < 0.001).

We excluded 22 adolescents because they used corticosteroid-containing medication. For each time point, single cortisol samples with values that were above 3 SD of the mean of the particular time point were excluded from the analysis in order to reduce the impact of outliers ($Cort_{0700}$ 21 excluded; 59 missing values; 1666 valid measurements in the final dataset; $Cort_{0730}$ 11 excluded; 52 missing values; 1683 valid measurements in the final dataset). From 1615 adolescents we received both morning saliva samples, and from 1594 adolescents we received data on SES and parenting (see Section 4.2.3) as well. Therefore, 71,5% (N=1594, mean age = 11.08, SD = 0.54, 50.3% girls) of the adolescents that participated in the TRAILS study were included in the final dataset. The study was approved by the National Dutch Medical Ethics Committee, in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

4.2.2 Procedure

Well-trained interviewers visited one of the parents or guardians (preferably the mother, 95.6%) at their homes to administer an interview covering a wide range of topics, including developmental history and somatic health, parental psychopathology and care utilization. In addition to the interview, the parent was asked to fill out some questionnaires concerning the adolescent's mental health and behavior. The adolescents filled out questionnaires at school, in the classroom, under the supervision of one or more TRAILS assistants. Besides, intelligence and a number of biological and neurocognitive parameters were assessed individually (at school, except for saliva samples, which were

collected at home). Teachers were asked to fill out a brief questionnaire for all TRAILS-participants in their class. Measures that were used in the present study are described more extensively below.

4.2.3 Measures

4.2.3.1 Socio-economic status (SES). SES was based on income level, educational level of both parents, and occupational level of both parents, assessed by a parental questionnaire. These five variables were standardized and combined into one scale with an internal consistency of 0.84 (Veenstra et al., 2005). Several TRAILS studies used this SES-measure (Amone-P'Olak et al., 2009; Herba et al., 2008; Veenstra et al., 2008).

4.2.3.2 Perceived parenting. Adolescent's perception of parental rearing practices was assessed with the EMBU-C (Markus, Lindhout, Boer, Hoogendijk, & Arrindell, 2003), a child version of the EMBU (a Swedish acronym for My Memories of Upbringing). This questionnaire contains a list of 47 items on the factors Rejection, Overprotection and Emotional Warmth. For the present study, we will only use the factors Rejection and Emotional Warmth, because it is difficult to place overprotection on a dimension of positive and negative environment. That is, on the one hand overprotection may be characterized by parents being highly supervising, discouraging independent behavior, and acting in a highly controlling manner, while on the other hand parents may be showing high warmth and emotional involvement (Masia & Morris, 1998). Each item could be rated as 1 = no, never, 2 = yes, sometimes, 3 = yes, often or 4 = yes, almost always; and was asked for both the father and the mother. Rejection is characterized by hostility, punishment, derogation, and blaming of the child. Emotional Warmth refers to giving special attention, praising for approved behavior, unconditional love, and being supportive and affectionately demonstrative. Five items of the Rejection scale were excluded due to low loadings (Oldehinkel, Veenstra, Ormel, de Winter, & Verhulst, 2006). After exclusion of these items, the Rejection scale contains 12 items with Cronbach's $\alpha = 0.84$ for fathers and 0.83 for mothers; and the Emotional Warmth scale contains 18 items with Cronbach's $\alpha = 0.91$ for both fathers and mothers. The answers for both parents were highly correlated ($r = 0.67$ for Rejection and $r = 0.79$ for Emotional Warmth), so we combined them into a single measure as in previous TRAILS papers (Bouma, Ormel, Verhulst, & Oldehinkel, 2008; Oldehinkel et al., 2006; Veenstra, Lindenberg, Oldehinkel, De Winter, & Ormel, 2006). The test-retest stability of a shortened version of the EMBU-C (10-item scales) over a 2-month period has been found to be satisfactory ($r = 0.78$ or higher) (Muris, Meesters, & van Brakel, 2003). There is sufficient support for the factorial and construct validity of this instrument (Dekovic et al., 2006).

4.2.3.3 Cortisol. TRAILS participants collected cortisol samples (saliva) at home, using the Salivette sampling device (Sarstedt, Rommelsdorfer Str., D-51588 Nümbrecht, Germany), which was handed to the parent at the parent interview, accompanied by a verbal and a written instruction. The Salivette tube consists of a plastic sampling vessel with a suspended insert containing a sterile neutral cotton wool swab that has to be chewed for about 45 s and then returned to the insert. Participants were instructed to collect three saliva samples: the first sample shortly after waking up (still lying in bed), the second sample 30 min later, and the third sample at 20:00h. Both the sampling and the preceding day should be normal (school) days, without special events or stressful circumstances. Since all schools participating in TRAILS started at approximately the same time, the sampling-time variation of the morning samples among the adolescents is limited and the estimated corresponding times are 07:00h for the first sample ($Cort_{0700}$) and 07:30h for the second sample ($Cort_{0730}$). In this cohort, 1141 (70,7%) showed a rise in cortisol levels between the awakening sample and the sample 30 min later, in 934 adolescents (57,8%) this awakening response was at least 2.5 nmol/l above individual baseline. Exact procedures and other requirements are described more extensively elsewhere (Rosmalen et al., 2005). Saliva samples were stored by the participants in their freezer directly after sampling and mailed to the institute as soon as possible. The saliva samples were stored at -20 ºC until analysis. Previous studies suggest that salivary cortisol levels are stable for prolonged periods of time at -20 ºC (Aardal & Holm, 1995). After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the laboratory (Department of Clinical and Theoretical Psychobiology, University of Trier, Germany) for analysis. Procedures of determination of cortisol levels are described more extensively elsewhere (Rosmalen et al., 2005).

4.2.3.4 Behavioral problems. Behavioral problems were assessed with the Child Behavior Checklist (CBCL) (Achenbach, 1991a; Verhulst et al., 1996) and the Youth Self-Report (YSR) (Achenbach, 1991c; Verhulst et al., 1997). The CBCL is a measure of parent-reported emotional and behavioral problems in 4- to 18-year-old children and the YSR is a self-report questionnaire that was modeled on the CBCL. The CBCL and the YSR contain 113 and 112 items respectively. These items are rated as 0 (not true), 1 (somewhat or sometimes true) or 2 (very true or often true). Both the CBCL and the YSR contain two broadband scales: one for internalizing behavior problems and one for externalizing behavior problems. For each of the two broadband scales, we used the mean of the standardized CBCL and YSR scores. In our previous study, we adopted the framework described by Essex et al. (2006) and used a Severity measure (Severity = [E+I]/2) as an indice for comorbidity and a Directionality measure (Directionality = [E-I]/2) for determining whether the possible behavior problems are mainly externalizing or internalizing, where E indicates the mean

of the standardized externalizing behavior problems and I indicates the mean of the standardized internalizing behavior problems (Marsman et al., 2008).

4.2.4 Statistical analyses

The cortisol levels followed a normal distribution ($Cort_{0700}$ skewness = 0.70, kurtosis = 0.63; $Cort_{0730}$ skewness = 0.43, kurtosis = 0.24). The computation of the Area Under the Curve (AUC) is a frequently used method in endocrinological research to assess basal cortisol levels (Area Under the Curve with respect to ground, AUC_G), and to estimate the CAR (Area Under the Curve with respect to increase, AUC_I) (Pruessner et al., 2003). Pruessner et al. (2003) recommend employing both formulas when analyzing data sets with repeated measures. We used the following formulas for calculating (1) basal cortisol levels: $AUC_G = (Cort_{0730}\text{-}Cort_{0700}) \times 0.5/2 + Cort_{0700} \times 0.5$ (in which 0.5 refers to 0.5 hours), and (2) the increase in cortisol levels after awakening or the CAR: $AUC_I = (Cort_{0730}\text{-}Cort_{0700}) \times 0.5/2$ (in which 0.5 refers to 0.5 hours). AUC measures are given in h x nmol/l.

Pearson correlation coefficients (r) were used to assess the relationship between the family environmental factors (perceived parental Rejection, perceived parental Emotional Warmth, and SES) and the severity and directionality of behavior problems. Prior to analyses, we standardized our predictor variables to be able to interpret our findings in terms of standard deviation units. Multicollinearity of these predictors was analyzed using tolerance statistics and the variation inflation factor (VIF). Tolerance statistics less than 0.20 and/or VIF of 5 and above indicate a multicollinearity problem (O'Brien, 2007). In the present study, the tolerance statistics ranged from 0.459 to 0.994, and the variance inflation factor (VIF) ranged from 1.001 to 1.911. These values indicate that there are no problems with multicollinearity.

In a previous study on the present sample, gender and the quadratic effect of sampling month were identified as significant predictors of HPA-axis activity (Rosmalen et al., 2005). Only gender may be a potential confounder in our analyses since gender may also be related to differential susceptibility to parenting (Oldehinkel et al., 2006). Age, pubertal development, and BMI appeared not to be related to AUC_I levels and AUC_G levels in the total group (Rosmalen et al., 2005).

Hierarchical regression analyses were conducted on AUC_G levels and AUC_I levels. Gender was entered at Step 1 (0 = girls; 1 = boys). Behavior problems may also be a potential confounder since behavior problems of the adolescents may be associated with the family environmental factors and HPA-axis activity. In a recent review it was found that there is an inverse association between externalizing behavior problems and HPA-axis activity in elementary school-aged children (5 - 12 years old) (Alink et al., 2008). In addition, positive associations between behavior problems, whether or not in interaction with gender, and HPA-axis activity were demonstrated in a previous study on the present

sample (Marsman et al., 2008). However, the direction of the potential associations between family environmental factors and HPA-axis activity may also be reversed, leading to over-correction when adjusting for behavior problems. For this reason, we chose to perform our analyses with and without adjusting for severity of behavior problems, directionality of behavior problems, and their interactions with gender at Step 1. Perceived parental Rejection, perceived parental Emotional Warmth, and SES were entered at Step 2. The variables perceived parental Emotional Warmth and SES were reversed, so that beta indicates the strength of the positive relationship between environmental adversity and cortisol measures. Perceived parental Rejection, perceived parental Emotional Warmth, and SES quadratic terms were entered at Step 3. Quadratic terms were calculated by squaring the standardized scores.

4.3 RESULTS

4.3.1 Correlations

Table 4.1 shows several significant correlations between family environmental factors and behavioral problems. Firstly, we see that perceived parental Emotional Warmth was weakly and negatively correlated with perceived parental Rejection. Secondly, severity of behavior problems was moderately correlated with perceived parental Rejection and weakly and negatively correlated with perceived parental Emotional Warmth. Albeit significant, all other correlations were very weak to negligible (between 0.0 to 0.2).

TABLE 4.1 Correlations between family environmental factors and behavior problems.

	Rejection	Warmth	SES	Severity	Directionality
Rejection	1				
Warmth	-0.356*	1			
SES	-0.071*	0.152*	1		
Severity	0.422*	-0.223*	-0.130*	1	
Directionality	0.077*	-0.106*	-0.086*	0.000	1

*Note: Rejection = perceived parental Rejection, Warmth = perceived parental Emotional Warmth, SES = socio-economic status; Severity = severity behavior problems ((externalizing + internalizing)/2), Directionality = directionality behavior problems ((externalizing - internalizing)/2); * Significant at the 0.01 level.*

4.3.2 Testing for curvilinear effects

Table 4.2 shows the results of the hierarchical regression analyses with adjustment for gender on AUC_G levels and AUC_I levels. Concerning AUC_G levels, Step 1 revealed that girls have higher AUC_G levels than boys. In addition, Step 2 revealed that the more perceived parental Emotional Warmth was observed, the lower AUC_G levels. In step 3, the quadratic effect of SES was a significant predictor of AUC_G levels. This means that both low and high SES was associated with lower AUC_G levels. Together, the effects of gender, perceived parental Emotional Warmth, and the quadratic effect of SES accounted for 1.9% of the adjusted variance in AUC_G levels. The quadratic effect of SES was also a significant predictor of AUC_I levels, indicating that both low and high SES was associated with lower AUC_I levels. The quadratic effect of SES accounted for 0.6% of the adjusted variance in AUC_I levels. The quadratic effects of perceived parental Emotional Warmth and perceived parental Rejection did not predict AUC_G levels or AUC_I levels.

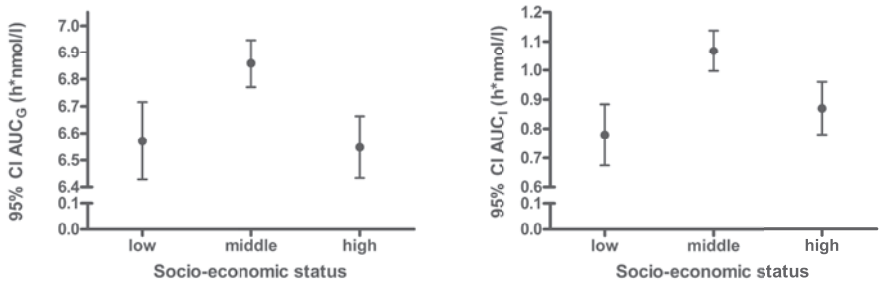
TABLE 4.2 Hierarchical regression analyses.

Cortisol measures		Predictors	Beta	p-value	Adj. R^2
AUC_G					
	Step 1	Gender	-0.11	<0.001	1.1%
	Step 2	Rejection	-0.04	0.118	
		Warmth (reversed)	0.09	0.002	1.6%
		SES (reversed)	0.02	0.562	
	Step 3	$Rejection^2$	0.01	0.833	
		$Warmth^2$	-0.02	0.460	
		SES^2	-0.07	0.008	1.9%
AUC_I					
	Step 1	Gender	-0.04	0.117	
	Step 2	Rejection	-0.03	0.311	
		Warmth (reversed)	0.03	0.299	
		SES (reversed)	-0.02	0.556	
	Step 3	$Rejection^2$	0.03	0.319	
		$Warmth^2$	0.03	0.327	
		SES^2	-0.08	0.002	0.6%

Note: Gender: 0 = girls and 1 = boys. Rejection = perceived parental Rejection, Warmth = perceived parental Emotional Warmth, SES = socio-economic status; Warmth and SES variables were reversed. Beta = standardized beta. Adjusted R^2 is reported for significant effects.

In the hierarchical regression analyses with adjustment for behavior problems, we found again that the more perceived parental Emotional Warmth was observed, the lower AUC_G levels ($\beta = 0.089$, $p < 0.01$). Again, the quadratic effect of SES was a significant predictor of AUC_G levels ($\beta = 0.054$, $p < 0.05$) and AUC_I levels ($\beta = -0.082$, $p < 0.01$), indicating that both low and high SES were associated with lower AUC_G levels and AUC_I levels. To illustrate our findings, we divided the SES measure into three groups, below the 25th percentile, 25-75 percentile, and above the 75th percentile, representing a low, middle, and high SES group respectively. Figure 4.1 shows that both curvilinear effects represent an inverse U-shaped curvilinear association. Table 4.3 shows the corresponding cortisol levels at waking up and 30 minutes later.

FIGURE 4.1 The curvilinear relationship between SES and CAR.



Note: The left figure shows the relationship between SES and basal cortisol levels (AUC_G) and the right figure shows the relationship between SES and the cortisol awakening response (AUC_I).

TABLE 4.3 Cortisol levels among the three SES groups.

	SES-low	SES-middle	SES-high
$Cort_{0700}$	11.65	11.53	11.33
$Cort_{0730}$	14.79	15.85	14.95

Note: SES = socio-economic status; $Cort_{0700}$ = cortisol levels directly after waking up (in nmol/l); $Cort_{0730}$ = cortisol levels half an hour after waking up (in nmol/l)

4.4 DISCUSSION

Inspired by the hardly ever tested developmental programming part of the evolutionary-developmental theory of BSC (Boyce & Ellis, 2005), the present study tested the potential inverse U-shaped association between three family environmental factors (i.e. perceived parental Emotional Warmth, perceived parental Rejection, and SES) and two measures

of HPA-axis functioning (i.e. basal cortisol levels, or AUC_G, and the cortisol awakening response, CAR, or AUC_I). An inverse U-shaped association was observed between SES and both HPA-axis measures. Perceived parental Emotional Warmth in childhood was linearly associated with decreased basal cortisol levels, whereas perceived parental Rejection was not related to HPA-axis activity at all. An explanation for not finding an association between perceived parental Rejection and HPA-axis activity may be that in our population-based sample perceived parental Rejection does not represent a very high-stress environment, while the absence of parental Rejection does not necessarily represent a low-stress environment.

Concerning perceived parental Emotional Warmth, we did provide evidence that a supportive environment in the form of perceived parental Emotional Warmth was associated with low basal cortisol levels (AUC_G levels). The association found is consistent with the right part of the inverse U-shape and fits with prior research showing an association between positive aspects of family climate and hypoactivity of the HPA-axis (Albers et al., 2008; Engert et al., 2011; Gunnar, 1992, 1998; Gunnar & Donzella, 2002). Moreover, whereas most previous studies considered a positive climate as a moderator of HPA-axis reactivity to a stressor (Albers et al., 2008; Gunnar, 1992, 1998; Gunnar & Donzella, 2002), the present study found a direct association between a positive family climate and HPA-axis functioning. The direct association is in line with studies that suggest an association between positive affect and low basal cortisol levels (Dockray & Steptoe, 2010) and the study by Engert et al. (2011), who demonstrated that high perceived parental care is directly associated with decreased basal cortisol levels in young adults. This finding does not rule out the possibility that perceived parental Emotional Warmth may also act as a modifier in the relationship between stress and HPA-axis reactivity. In contrast to our predictions, low perceived parental Emotional Warmth was not associated with low basal cortisol levels. Again, it could be that the absence of perceived parental Emotional Warmth does not necessarily represent a high-stress environment.

In line with our hypothesis, we found evidence for an inverse U-shaped relationship between SES and basal cortisol levels (AUC_G levels). SES is often used as a 'container variable' representing several aspects of the family context, and thus may be a more useful index representing a supportive and adverse environment on both sides of the continuum. In addition, it could be that SES is a more persistent and chronic factor in the life of a young adolescent. Belsky et al. (2007) suggested that parenting may be a mediating factor between SES and a child's health, indirectly indicating that SES is a more stable and persistent factor than parenting.

In the present study, we chose to consider the three family environmental factors separately, and not all together. The fact that the three family environmental factors are (weakly) correlated with each other may provide support for combining them into a single measure. However, factors should not be combined unless there is reasonable evidence

from prior research to combine them (Larose, 2005). For example, high perceived parental Rejection does not necessarily mean that there is no perceived parental Emotional Warmth and vice versa. For that reason, we decided to look at the family environmental factors separately. Furthermore, testing the effects of those three factors in the same model reveals the effect of each factor controlled for the other two factors. We can therefore safely conclude, that SES has a quadratic association with basal cortisol levels over and above the linear association of perceived Emotional Warmth.

A curvilinear association was also found between SES and the CAR. It must be noted, however, that the amount of explained variance in the model with the CAR was small. The small but significant correlation between basal cortisol levels (AUC_G) and the CAR (AUC_I) (Rosmalen et al., 2005) makes it even more difficult to interpret this finding. The significant association between the CAR and SES squared might very well be ascribed to trait characteristics of HPA-axis functioning present in this measure of state characteristics (Hellhammer et al., 2007). Acknowledging the exploratory nature of studying associations with the CAR, this finding should be replicated in order to confirm the existence of an inverse curvilinear relationship between SES and the CAR.

As this is one of the first studies investigating the developmental programming part of BSC (Ellis et al., 2005), confirmation of our findings with respect to basal cortisol levels is also needed before firm conclusions can be drawn. Another question that needs to be addressed in future studies, is whether individual differences in basal cortisol levels and CAR reflect differences in biological sensitivity to context. Although different measures of stress-reactivity have been shown to reflect differences in sensitivity in young children (Boyce et al., 2006; Ellis, Shirtcliff, et al., 2011; Essex et al., 2011; Obradović et al., 2011; Obradović et al., 2010), for as far as we know, individual differences in basal cortisol levels and the CAR have not been subjected to investigation in light of BSC theory. In the present study we investigated possible programming effects in 11-year-olds. However, increasing evidence suggests that developmental programming might continue into adolescence (Laceulle, Nederhof, Karreman, Ormel, & van Aken, in press; Romeo, 2010; Schmidt et al., 2007). Another step would therefore be to investigate at what stage of development programming of basal cortisol levels and the CAR are finished.

A limitation of the present study is that we collected only two cortisol samples after awakening (which are part of the CAR), and used the aggregate measure (AUC_G) as a measure of basal HPA-axis activity. Although the fact that the AUC_G and AUC_I are only weakly correlated (Rosmalen et al., 2005) underlines that these are distinct measures, and several other studies used the AUC_G as an indication of basal HPA-axis activity (Bonifazi et al., 2006; Boschloo et al., 2011; Marsman et al., 2008; Vedhara et al., 2006), we see that it would have been better to determine this AUC_G measure on more measures than just the two measures after awakening. Del Guidice et al. (2011) did not specify at which time basal cortisol samples should be collected. According to the meta-analysis by Miller et al.

(2007), exposure to chronic stress is associated with significantly lower concentrations of morning cortisol. In addition, many reviews on this topic show that a high-stress environment leads to decreases in basal cortisol levels in the long-term (Chrousos & Gold, 1992; De Bellis, 2001; Fries et al., 2005; Grassi-Oliveira et al., 2008; Gunnar & Vazquez, 2001; McEwen & Stellar, 1993). Since the results with morning cortisol samples (i.e. stress related with decreased levels) fit with these reviews and our hypotheses, we think that morning cortisol samples are especially valuable in the present study. Although we also collected one sample at 20:00h in the evening, there are two main reasons for not including this sample in the present study. The first is a theoretical one. Since the results with evening cortisol samples (i.e. stress related with increased levels) (Miller et al., 2007) doesn't fit with the reviews and hypotheses in our study (i.e. stress related with decreased levels), inclusion of the $Cort_{2000}$ sample separately requires additional hypotheses. This is beyond the scope of this paper. The second reason is a methodological one. We believe that it is highly problematic to include the $Cort_{2000}$ sample in the AUC_G measure, because there is too much time between the second sample in the morning (07.30h) and the evening sample (20.00h), given the documented systematic interindividual differences in the circadian rhythm after awakening. For example, girls have steeper slopes and more curvature to their rhythm than boys (Shirtcliff et al., 2011). In addition, in the same sample of early adolescents between 9 and 15 years old, it was found that age was related to circadian rhythm. That is, the slope becomes flatter as children age (Shirtcliff et al., 2011). On balance, we believe that including the evening sample would lead to a greater systematic error in our data than excluding the evening sample would do.

A future direction we would like to point out is investigating the developmental mechanisms behind the (inverse) U-shaped associations between stress axes functioning and family environmental factors. It is very likely that different mechanisms underlie development of similar basal and reactivity profiles in children from low-stress compared to children from high-stress environments. From animal studies, evidence is available that a lack of stress inoculation results in high reactivity profiles, for example in unhandled animals compared to animals who experienced the mild stress of regular handling, while stress sensitisation seems to underlie the high reactivity profiles following severe stress (Macrì, Zoratto, & Laviola, 2011). Whether similar mechanisms underlie the inverse U-shape with basal HPA-axis activity reported in the present study should be investigated, as well as the generalizability of findings in animal models to humans.

In the present study, findings with and without adjusting for behavior problems were the same. One could argue that individuals with less behavioral problems report a more positive family environment, resulting in a bias in the relationship between family environment and HPA-axis activity. Adjusting for behavior problems reveals the 'pure association' between parenting and HPA-axis activity, since behavior problems may act as a confounder. In our previous study, we already found associations between behavior

problems and HPA-axis activity (Marsman et al., 2008). In addition, the fact that we found significant correlations between perceived parental Rejection on the one hand, and severity and directionality of behavior problems on the other hand, underlines the potential value of adjusting for behavior problems.

The interpretation of the results of this study is limited by its cross-sectional design. We were unable to verify whether parenting and SES represent long-lasting environments. Another limitation concerns the cortisol sampling. Firstly, home collection of saliva is much more susceptible to situational influences than collection of saliva in the more controlled conditions at the laboratory. In addition, home collection relies heavily upon participant adherence (Clow et al., 2004). However, home collection is more ecologically valid than assessment under laboratory conditions and provides the same results (Wilhelm et al., 2007). The major strengths of the present study are the large sample size and the child-report of parenting, since the child's perception of parenting is likely to be more relevant for the child's stress system than parent reports.

In conclusion, this study suggests that there may exist an inverse U-shaped relationship between socio-economic status on the one hand and basal cortisol levels and possibly the CAR on the other hand. In addition, the present study underlines the importance of taking into account positive aspects of the environment. Though the effect sizes of the findings were relatively small, our findings with regard to SES confirmed our hypothesis that was derived from the evolutionary-developmental theory of BSC. Whereas this theory suggests a curvilinear relationship between family environment and stress *reactivity*, we found evidence of an inverse curvilinear relationship between family environment and other measures of HPA-axis functioning.

CHAPTER 5

DRD4 and familial loading interact with perceived parenting in predicting externalizing behavior problems in early adolescence. The TRAILS study.

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ABSTRACT

Although externalizing behavior problems show in general a high stability over time, the course of externalizing behavior problems may vary from individual to individual. Our main goal was to investigate the predictive role of parenting on externalizing behavior problems. In addition, we investigated the potential moderating role of gender and genetic risk (operationalized as familial loading of externalizing behavior problems (FLE), and presence or absence of the DRD4 7-repeat and 4-repeat allele, respectively). Perceived parenting (rejection, emotional warmth, and overprotection) and FLE were assessed in a population-based sample of 1768 10- to 12-year-old adolescents. Externalizing behavior problems were assessed at the same age and two and a half years later by parent report (CBCL) and self-report (YSR). DNA was extracted from blood samples. Parental emotional warmth predicted lower, and parental overprotection and rejection predicted higher levels of externalizing behavior problems. Whereas none of the parenting factors interacted with gender and the DRD4 7-repeat allele, we did find interaction effects with FLE and the DRD4 4-repeat allele. That is, the predictive effect of parental rejection was only observed in adolescents from low FLE families and the predictive effect of parental overprotection was stronger in adolescents not carrying the DRD4 4-repeat allele.

5.1 INTRODUCTION

Among the factors that may influence the severity and course of externalizing behaviors are various aspects of parenting. Longitudinal studies in school-age children suggest that positive parenting (i.e., warmth, involved parenting, and sensitivity) leads to decreases in externalizing behavior problems (Trentacosta et al., 2008), whereas lack of positive parenting leads to increases in externalizing behavior problems (Caspi et al., 2004; Miner & Clarke-Stewart, 2008). Further, negative parenting (i.e., hostility, rejection, and harsh discipline) has been reported to lead to increases in externalizing behavior problems (Caspi et al., 2004; Leve et al., 2005; Miner & Clarke-Stewart, 2008). Longitudinal studies in adolescence are limited but reveal that positive parenting predicts decreases in externalizing behavior problems (Reitz, Deković, & Meijer, 2006) and negative parenting predicts increases in externalizing behavior problems (Leve et al., 2005). Cross-sectional studies in adolescence show similar associations as reported in longitudinal studies (Akse, Hale, Engels, Raaijmakers, & Meeus, 2004; Buschgens et al., 2010; Kim, Hetherington, & Reiss, 1999; Veenstra et al., 2006; Yahav, 2007). The goal of the present study is to extend prior findings on the role of parenting on externalizing behaviors in three ways: (1) by using longitudinal data on early adolescence, (2) by examining the moderating role of gender on the effects of parenting, and (3) and by examining the moderating role of genetic risk on the effects of parenting.

Though there is some evidence that the influence of parenting on externalizing behavior problems may depend on gender (Leve et al., 2005; Miner & Clarke-Stewart, 2008; Rothbaum & Weisz, 1994), many of the studies on the relationship between parenting and externalizing behavior problems have not taken gender differences into account (Buschgens et al., 2010; Caspi et al., 2004; Reitz et al., 2006; Trentacosta et al., 2008; Yahav, 2007). Although a cross-sectional study could not demonstrate a gender-specific association between parenting and externalizing behavior problems (Veenstra et al., 2006), a longitudinal study that followed participants from childhood through adolescence found that harsh discipline directly predicted externalizing behavior problems in boys, whereas it predicted girls' externalizing behavior problems only when it was accompanied by an individual vulnerability (i.e., low fear/shyness or high impulsivity) (Leve et al., 2005). Concerning positive parenting, a longitudinal study that followed children from age 2 to 9 found that low parental sensitivity predicted externalizing behavior problems more strongly in boys than in girls (Miner & Clarke-Stewart, 2008). A meta-analysis by Rothbaum and Weisz (1994) also showed that negative parenting was more strongly linked to externalizing behavior problems for boys than for girls, especially among preadolescents. In the present study, we have the possibility to test the potential moderating effect of gender in a sample that contains data on both positive and negative parenting.

A second potential moderator is the genetic risk for externalizing behavior problems. Firstly, genetic risk will be operationalized by familial loading of externalizing behavior problems (FLE), that is, lifetime parental externalizing behavior disorders (Ormel et al., 2005). Since quantitative genetic studies indicate that the familial aggregation of externalizing disorders is mainly due to genetic factors (Burt, 2009), we assume that familial loading reflects largely genetic risk, although a contribution of shared environmental influences cannot be ruled out. Previous studies based on the present sample found that FLE is related to externalizing behavior problems (Buschgens et al., 2009; Buschgens et al., 2010; Ormel et al., 2005). Moreover, one of these studies found that the interaction between FLE and parenting was cross-sectionally associated with various forms of externalizing behavior problems (Buschgens et al., 2010). More specifically, parental rejection or parental overprotection in combination with FLE were associated with more teacher-rated hyperactivity-impulsivity symptoms. In the same line, we will assess gene-environment interaction by investigating the interaction between FLE and parenting on future externalizing behavior problems. Almost three decades ago, it has been proposed that FLE interacts with an adverse environment in predicting externalizing behavior problems (Cadoret, Cain, & Crowe, 1983). In this classical stress-vulnerability view of gene-environment interaction, outcomes are worse when genetic risk coincides with an adverse environment.

Secondly, genetic risk will be operationalized by the presence of the DRD4 7-repeat allele (i.e., the allele associated with externalizing behavior problems). Particularly relevant to the present study is the work of Bakermans-Kranenburg and Van Ijzendoorn (2006) who found a six-fold increase in externalizing behavior problems in children carrying the DRD4 7-repeat allele exposed to insensitive parenting compared to children without these combined risks. Also, Sheese et al. (2007) demonstrated that children carrying the DRD4 7-repeat allele who also experienced low quality of parenting showed high levels of sensation seeking. In addition, a recent study highlights the potential moderating role of the DRD4 7-repeat allele with positive parenting (Knafo, Israel, & Ebstein, 2011). One study found a decrease in externalizing behavior problems in African American children with the short DRD4 polymorphism (i.e., 2-5 repeats) exposed to warm-responsive parenting (Propper et al., 2007). However, a review by Bakermans-Kranenburg and Van Ijzendoorn (2007) shows that susceptible children (i.e., carrying the DRD4 7-repeat allele) may show lower levels of externalizing behavior problems in favorable environments (i.e., sensitive parenting). Thus, as in the classical stress-vulnerability view of gene-environment interaction, outcomes may be worse when genetic risk coincides with an adverse environment. However, findings concerning a positive environment are less straightforward, since externalizing behavior problems may decrease when genetic risk coincides with a positive environment (Bakermans-Kranenburg & van Ijzendoorn, 2007),

or externalizing behavior problems may decrease when an absence of the genetic risk coincides with a positive environment (Propper et al., 2007). All these studies have in common that they are based on samples of schoolage children. In the present study, we will assess gene-environment interaction by investigating the interaction between the DRD4 7-repeat allele and parenting on future externalizing behavior problems in adolescents.

The effect of the DRD4 7-repeat allele on attention deficit-hyperactivity disorder (ADHD) is well-established in meta-analysis (Gizer et al., 2009). Although prior studies generally demonstrate the potential role of the DRD4 7-repeat allele in moderating the relationship between parenting and externalizing behavior problems, the DRD4 4-repeat allele may also serve as a potential moderator. This 4-repeat allele differs from the 7-repeat allele in secondary messenger (i.e., cAMP) activity and is more sensitive to dopamine stimulation (Asghari et al., 1995). Absence of the 4-repeat allele may be related to lower executive function (Fossella et al., 2002), which is related to behavior problems (Espy, Sheffield, Wiebe, Clark, & Moehr, 2011). Also, a meta-analysis by Li et al. (2006) shows that the presence of one or two 4-repeat alleles have a protective effect for ADHD.

In summary, the goal of the present study was to investigate the main and interactive effects of parenting, gender and genetic risk on future externalizing behavior problems in a population-based sample of adolescents. Our first hypothesis was that negative parenting (i.e., parental rejection and parental overprotection) leads to higher levels of externalizing behavior problems two and a half years later, whereas positive parenting (i.e., parental warmth) leads to lower levels of externalizing behavior problems two and a half years later. Second, we hypothesized that the relationship between parenting and externalizing behavior problems is specific for boys rather than for girls. Our third hypothesis was that the presence of high genetic risk (i.e., FLE or the DRD4 7-repeat allele) would interact with negative parenting in that high genetic risks lead to higher levels of externalizing behavior problems in the presence than in the absence of negative parenting. In addition, we explored whether genetic risk interacts with positive parenting in predicting lower levels of externalizing behavior problems, as well as whether the DRD4 4-repeat allele interacts with parenting in predicting externalizing behavior problems.

5.2 METHODS

5.2.1 Sample

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective study of Dutch adolescents, with the aim to chart and explain the development of mental health from early adolescence into adulthood, both at the level of psychopathology and the levels of underlying vulnerability and environmental risk. Adolescents will be measured bi- or

triennially at least until they are 25 years old. The present study involves data from the first (T1), second (T2), and third (T3) assessment wave of TRAILS, which ran from March 2001 to July 2002, September 2003 to December 2004, and September 2005 to December 2007, respectively.

TRAILS participants were selected from five municipalities in the north of The Netherlands, including both urban and rural areas. Children born between October 1, 1989, and September 30, 1990 (first two municipalities), or October 1, 1990, and September 30, 1991 (last three municipalities), were eligible for inclusion, providing that their schools were willing to cooperate and that they were able to participate in the study. Of all eligible 2935 children, 76.0% (*N* = 2230, mean age = 11.09, S.D. = 0.56, 50.8% girls) were enrolled in the study. Parental written informed consent was obtained after the procedures had been fully explained. Responders and non-responders did not differ with respect to the prevalence of teacher-rated behavior problems, nor regarding associations between sociodemographic variables and mental health outcomes. Detailed information about sample selection and analysis of non-response bias has been reported elsewhere (de Winter et al., 2005; Huisman et al., 2008). Of the 2230 baseline participants, 96.4% (*N* = 2149, 51.0% girls) participated in the first follow-up assessment (T2), which was held 2 - 3 years after T1 (mean number of months 29.44, S.D. = 5.37, range 16.69-48.06). Mean age at T2 was 13.56 (S.D. = 0.53). At T3, the response rate was 81.4%, and mean age was 16.13 (S.D. = 0.59). The TRAILS study was approved by the Central Committee on Research Involving Human Subjects (Dutch CCMO).

5.2.2 Procedure

At T1, well-trained interviewers visited one of the parents or guardians (preferably the mother, 95.6%) at their homes to administer an interview covering a wide range of topics, including development history and somatic health, parental psychopathology and care utilization. In addition to the interview, the parent was asked to fill out some questionnaires concerning the child's mental health and behavior. Adolescents filled out questionnaires at school, in the classroom, under the supervision of one or more TRAILS assistants. Teachers were asked to fill out a brief questionnaire for all TRAILS-participants in their class. T2 involved only questionnaires, to be filled out by the adolescents, their parents and their teachers. As in T1, the adolescents filled out their questionnaires at school, supervised by TRAILS assistants. At T3, blood or buccal cells were collected for DNA analysis. Measures that were used in the present study are described more extensively below.

5.2.3 Measures

5.2.3.1 Parenting. Adolescent's perception of parental rearing practices was assessed with the EMBU-C (Markus et al., 2003), a child version of the EMBU (a Swedish acronym for

My Memories of Upbringing). This questionnaire contains a list of 47 items on the factors Rejection, Overprotection and Emotional Warmth. Each item could be rated as 1 = no, never, 2 = yes, sometimes, 3 = yes, often or 4 = yes, almost always; and was asked for both the father and the mother. Rejection is characterized by hostility, punishment, derogation, and blaming of the child. Overprotection denotes fearfulness and anxiety for the child's safety, guilt engendering, and intrusiveness. Emotional Warmth refers to giving special attention, praising for approved behavior, unconditional love, and being supportive and affectionately demonstrative. Five items of the Rejection scale were excluded due to low loadings (Oldehinkel et al., 2006). After exclusion of these items, the Rejection scale contains 12 items with Cronbach's $\alpha = 0.84$ for fathers and 0.83 for mothers; the Overprotection scale contains 12 items with Cronbach's $\alpha = 0.70$ for fathers and 0.71 for mothers; and the Emotional Warmth scale contains 18 items with Cronbach's $\alpha = 0.91$ for both fathers and mothers. The answers for both parents were highly correlated ($r = 0.67$ for Rejection, $r = 0.81$ for Overprotection, and $r = 0.79$ for Emotional Warmth), so we combined them into a single measure as in previous TRAILS papers (Bouma et al., 2008; Oldehinkel et al., 2006; Veenstra et al., 2006). The test-retest stability of a shortened version of the EMBU-C (10-item scales) over a 2-months period has been found to be satisfactory ($r = 0.78$ or higher) (Muris et al., 2003). There is sufficient support for the factorial and construct validity of this instrument (Dekovic et al., 2006).

5.2.3.2 Behavioral problems. At both T1 and T2, behavioral problems were assessed with the Child Behavior Checklist (CBCL) (Achenbach, 1991a; Verhulst et al., 1996) and the Youth Self-Report (YSR) (Achenbach, 1991c; Verhulst et al., 1997). The CBCL is a measure of parent-reported emotional and behavioral problems in 4- to 18-year-old children and the YSR is a self-report questionnaire that was modeled on the CBCL. The CBCL and the YSR contain 113 and 112 items respectively. These items are rated as 0 (not true), 1 (somewhat or sometimes true) or 2 (very true or often true). Both the CBCL and the YSR contain two broadband scales: one for internalizing behavior problems and one for externalizing behavior problems. As in our previous studies, we used the mean of the CBCL and YSR scores on externalizing behavior problems (Marsman, Rosmalen, Oldehinkel, Ormel, & Buitelaar, 2009; Marsman et al., 2008). This broadband scale of externalizing behavior problems is composed of two narrow-band syndromes: delinquent behavior and aggressive behavior.

5.2.3.3 Familial loading. At T1, lifetime parental psychopathology was assessed by means of the TRAILS Family History Interview (FHI), administered at the parent interview. Five spectra (or dimensions) of psychopathology were assessed: depression, anxiety, substance dependence, persistent antisocial behavior, and psychosis. Each spectrum was introduced by a vignette (available on request) describing the main DSM-IV characteristics of the

spectrum, followed by a series of questions assessing lifetime occurrence, professional treatment, and medication use. Biological parents were interviewed separately using a single informant, typically the mother. For each spectrum, we assigned each parent to one of the following categories: 0 = (probably) never had an episode, 1 = (probably) yes, or 2 = yes and treatment and/or medication. For antisocial behavior, the last category was: 2 = (probably) yes and police involvement. Prevalence rates in mother and fathers respectively were, for depression: 27% and 15%; for anxiety: 16% and 6%; for substance dependence: 3% and 7%; and for antisocial behavior: 3% and 7%. In the present study, we used the familial loading of externalizing behavior problems (FLE). As externalizing behavior problems we combined substance dependence and antisocial behavior. The empirical justification for the construction of the familial loadings has been reported elsewhere (Ormel et al., 2005). Two groups were created as in a previous TRAILS study: adolescents from low FLE families (82.2%) and adolescents from high FLE families (17.8%) (Buschgens et al., 2009).

5.2.3.4 DRD4. DNA was extracted from buffy coats or buccal swabs (Cytobrush®) with the use of a manual salting out procedure similar to the protocol described by Miller and colleagues (1988). The 48 bp direct repeat polymorphism in exon 3 of DRD4 was genotyped on the Illumina BeadStation 500 platform (Illumina Inc., San Diego, CA, USA). The genotyping assay was carried out in a CCKL quality-certified laboratory and has been validated earlier. Three percent blanks as well as duplicates between plates were taken along as quality controls during genotyping. Determination of the length of the alleles was performed by direct analysis on an automated capillary sequencer ABI3730, Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) using standard conditions (Nederhof, Creemers, Huizink, Ormel, & Oldehinkel, 2011). Information on length of polymorphisms was available for 1451 subjects. Allele frequencies and genotype distribution of the DRD4 are presented in Table 5.1. DRD4 genotypes were grouped according to the presence of at least one 4-repeat allele or at least one 7-repeat allele, respectively. No deviation from Hardy-Weinberg equilibrium was observed ($X2 = 0.38$, d.f.=1, $p=0.54$).

5.2.3.5 Socio-economic status (SES). SES was based on income level, educational level of both parents, and occupational level of both parents, assessed by a parental questionnaire. These five variables were standardized and combined into one scale with an internal consistency of 0.84 (Veenstra et al., 2005). Several TRAILS studies used this SES-measure (Amone-P'Olak et al., 2009; Herba et al., 2008; Veenstra et al., 2008). In the present study, SES may act as a confounder, since SES is related to both parenting factors and externalizing behavior problems (Marsman et al., 2011).

TABLE 5.1 Allele frequencies and genotype distribution of the DRD4 gene.

Allele / genotype	*n*	%
Allele		
2	254	8.75
3	162	5.58
4	1836	63.27
5	24	0.83
6	15	0.52
7	584	20.12
8	27	0.93
Total	2902	100.00
Genotype		
2/2	11	0.76
2/3	14	0.96
2/4	159	10.96
2/5	2	0.14
2/6	4	0.28
2/7	52	3.58
2/8	1	0.07
3/3	3	0.21
3/4	108	7.44
3/5	1	0.07
3/7	30	2.07
3/8	3	0.21
4/4	589	40.59
4/5	8	0.55
4/6	8	0.55
4/7	359	24.74
4/8	16	1.10
5/5	1	0.07
5/7	10	0.69
5/8	1	0.07
6/7	3	0.21
7/7	62	4.27
7/8	6	0.41
Total	1451	100.00

5.2.4 Data analysis

Pearson correlation coefficients (r) were calculated between gender, SES, the parenting factors, FLE, DRD4 7-repeat allele, and externalizing behavior problems at T1 and T2. Subsequently, we conducted hierarchical multiple linear regression analyses on externalizing behavior problems at T2. Prior to regression analysis, all predictor variables were standardized to avoid multicollinearity. In the first analysis, gender and SES were added in the first step, since both may act as covariates. In the second step, parental Overprotection, parental Emotional Warmth, parental Rejection, FLE, and DRD4 7-repeat allele were added. In the third step, interaction terms between parenting factors and gender, interaction terms between parenting factors and FLE, and interaction terms between parenting factors and the DRD4 7-repeat allele were added.

Externalizing behavior problems at T1 are probably not only associated with externalizing behavior problems at T2, but also with parenting factors at the same time (Akse et al., 2004; Buschgens et al., 2010; Kim et al., 1999; Veenstra et al., 2006; Yahav, 2007). However, the direction of the association between externalizing behavior problems at T1 and parenting factors is unclear, leading to potential over-correction when adjusting for externalizing behavior problems at T1. For this reason, we chose to perform our analyses with and without adjusting for externalizing behavior problems at T1. The second analysis was the same as the first analysis, with the only difference being that externalizing behavior problems at T1 was added to step 1. In the next two analyses, we explored the effect of the DRD4 4-repeat allele by substituting the DRD4 7-repeat variable by the DRD4 4-repeat variable. When an interaction effect was found in both the analysis with adjustment for externalizing behavior problems at T1 and the analysis without adjustment for externalizing behavior problems at T1, we performed gender stratified analyses or genetic risk-stratified analyses.

5.3 RESULTS

Table 5.2 shows the correlations between the predictors and externalizing behavior problems. All predictors significantly correlated with externalizing behavior problems at T1. Except for the DRD4 7-repeat allele, predictors also significantly correlated with externalizing behavior problems at T2. Boys have more externalizing behavior problems than girls at T1. Higher SES is associated with lower externalizing behavior problems at T1 and T2. In addition, parental overprotection and parental rejection was associated with more externalizing behavior problems at T1 and T2, whereas parental emotional warmth was associated with less externalizing behavior problems at T1 and T2. Also, familial loading of externalizing psychopathology was associated with externalizing behavior problems at T1 and T2. The presence of the DRD4 7-repeat allele was associated with

gender and externalizing behavior problems at T1 but not with externalizing behavior problems at T2. Furthermore, externalizing behavior problems at T1 were associated with externalizing behavior problems at T2.

TABLE 5.2 Correlations, means and standard deviations of predictors and outcome variables

	1	2	3	4	5	6	7	8	9
1. Gender	-								
2. SES	0.02	-							
3. Overprotection	-0.06**	-0.09**	-						
4. Warmth	0.10**	0.14**	0.18**	-					
5. Rejection	-0.11**	-0.07**	0.36**	-0.36**	-				
6. FLE	-0.01	-0.17**	0.01	-0.04*	0.00	-			
7. DRD4 7-repeat	-0.05*	-0.02	-0.04	0.02	-0.04	-0.04	-		
8. EXT T1	-0.21**	-0.14**	0.23**	-0.25**	0.41**	0.10**	-0.06*	-	
9. EXT T2	-0.04*	-0.13**	0.18**	-0.17**	0.26**	0.13**	-0.01	0.59**	-
Mean	-	-	1.86	3.21	1.42	-	-	0.26	0.26
S.D.	-	-	0.38	0.50	0.33	-	-	0.16	0.17

*Note: Gender: 1 = boys, 2 = girls; SES: 1 = low, 2 = intermediate, 3 = high; Overprotection = parental overprotection; Warmth = parental emotional warmth; Rejection = parental rejection; FLE = familial loading of externalizing psychopathology: 1 = low FLE, 2 = high FLE; DRD4 7-repeat: 1 = absence of the 7-repeat allele in DRD4, 2 = presence of the 7-repeat allele in DRD4; EXT T1 = externalizing behavior problems at T1; EXT T2 = externalizing behavior problems at T2; * Significant at the 0.05 level; ** Significant at the 0.01 level. Means and standard deviations are shown of unstandardized variables.*

The model without correction for externalizing behavior problems at T1 is presented in the left column of Table 5.3. Regarding hypothesis 1, parental overprotection and parental rejection were significantly related to more externalizing behavior problems at T2, and parental emotional warmth was significantly related with less externalizing behavior problems at T2. In addition, FLE was significantly related to more externalizing behavior problems at T2, whereas the DRD4 7-repeat allele was not related to externalizing behavior problems at T2. Regarding hypothesis 2, the interaction term between gender and rejection was significant and negative. In addition, with respect to hypothesis 3, the interaction terms between the DRD4 7-repeat allele and parenting factors did not significantly predict externalizing behavior problems at T2. However, the interaction term between FLE and parental rejection was also significant and negative. Together SES, the parenting factors, FLE, and the interaction effects accounted for 12.6% of the adjusted variance in externalizing behavior problems at T2.

Results of the model with correction for externalizing behavior problems at T1, presented in the right column of Table 5.3, were largely the same as the results of the model without correction for externalizing behavior problems. The only differences were that we found also a main effect of gender, whereas the main effect of rejection and the interaction effect between gender and rejection were no longer present. As expected, externalizing behavior problems at T1 accounted for a lot of variance in externalizing behavior problems at T2 as this model explained 37.1% of the adjusted variance.

TABLE 5.3 Hierarchical multiple linear regression analysis predicting externalizing behavior problems at T2

Regression model without externalizing at T1 as predictor					Regression model with externalizing at T1 as predictor				
	Predictors	Beta	p	Adj. R^2		Predictors	Beta	p	Adj. R^2
Step 1				2.6%	Step 1				36.1%
	Gender	-0.045	0.084			Gender	0.092	**<0.001**	
	SES	-0.159	**<0.001**			SES	-0.069	**0.001**	
						EXT T1	0.602	**<0.001**	
Step 2				12.3%	Step 2				36.8%
	Gender	0.005	0.856			Gender	0.099	**<0.001**	
	SES	-0.099	**<0.001**			SES	-0.051	**0.021**	
						EXT T1	0.571	**<0.001**	
	Overprotection	0.129	**<0.001**			Overprotection	0.070	**0.005**	
	Warmth	-0.133	**<0.001**			Warmth	-0.052	**0.040**	
	Rejection	0.182	**<0.001**			Rejection	0.010	0.723	
	FLE	0.094	**<0.0001**			FLE	0.053	**0.013**	
	DRD4	0.009	0.730			DRD4	0.033	0.120	

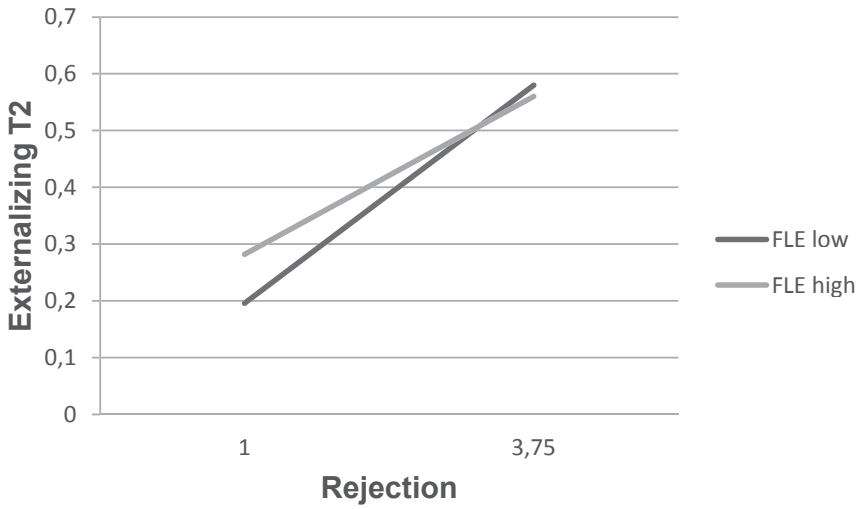
TABLE 5.3 Continued

Regression model without externalizing at T1 as predictor					Regression model with externalizing at T1 as predictor				
	Predictors	Beta	p	Adj. R^2		Predictors	Beta	p	Adj. R^2
Step 3				12.6%	Step 3				37.1%
	Gender	0.006	0.820			Gender	0.100	**<0.001**	
	SES	-0.100	**<0.001**			SES	-0.050	**0.023**	
						EXT T1	0.571	**<0.001**	
	Overprotection	0.125	**<0.001**			Overprotection	0.065	**0.009**	
	Warmth	-0.128	**<0.001**			Warmth	-0.047	0.063	
	Rejection	0.192	**<0.001**			Rejection	0.016	0.558	
	FLE	0.089	**<0.001**			FLE	0.052	**0.016**	
	DRD4	0.007	0.773			DRD4	0.031	0.144	
	Gender * Overprotection	-0.012	0.671			Gender * Overprotection	0.008	0.740	
	Gender * Warmth	0.006	0.839			Gender * Warmth	-0.025	0.324	
	Gender * Rejection	0.066	**0.032**			Gender * Rejection	0.038	0.147	
	FLE * Overprotection	0.028	0.323			FLE * Overprotection	0.025	0.299	
	FLE * Warmth	-0.032	0.267			FLE * Warmth	-0.017	0.495	
	FLE * Rejection	-0.071	**0.017**			FLE * Rejection	-0.067	**0.008**	
	DRD4 * Overprotection	0.013	0.657			DRD4 * Overprotection	0.004	0.876	
	DRD4 * Warmth	0.003	0.909			DRD4 * Warmth	-0.013	0.602	
	DRD4 * Rejection	-0.031	0.305			DRD4 * Rejection	-0.028	0.281	

Note: Gender: 1 = boys, 2 = girls; SES: 1 = low, 2 = intermediate, 3 = high; Overprotection = parental overprotection; Warmth = parental emotional warmth; Rejection = parental rejection; FLE = familial loading of externalizing psychopathology: 1 = low FLE, 2 = high FLE; DRD4 7-repeat: 1 = absence of the 7-repeat allele in DRD4, 2 = presence of the 7-repeat allele in DRD4; EXT T1 = externalizing behavior problems at T1. Bold p-values are significant at the 0.05 level.

Given the significant negative interaction effect between FLE and rejection in both analyses, we performed separate regression analyses for adolescents from low FLE families and for adolescents from high FLE families. These analyses showed that the effect of parental rejection on higher externalizing behavior problems at T2 was present in low FLE families ($\beta = 0.172$, $p < 0.001$) but not in high FLE families ($\beta = 0.085$, $p = 0.16$) . Overall, parenting factors explained twice as much variance in externalizing behavior problems in low (Adj. $R^2 = 9.9\%$) versus high FLE families (Adj. $R^2 = 4.8\%$). Figure 5.1 shows the regression lines for adolescents from the low and high FLE families.

FIGURE 5.1 Regression lines predicting externalizing behavior problems from parental rejection for adolescents from the low and high FLE families.

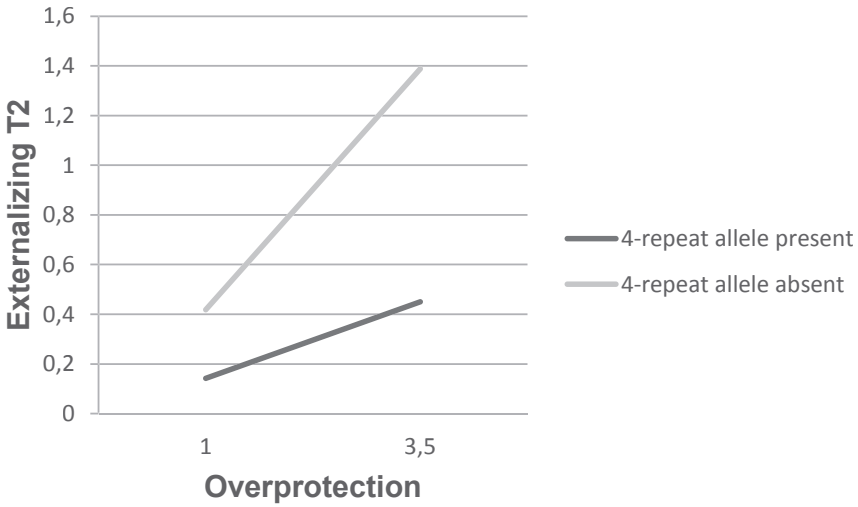


Note: Rejection = parental rejection within the minimum and maximum range values.

Next, we investigated the potential moderating role of the DRD4 4-repeat allele (results not shown). There was no main effect of the DRD4 4-repeat allele in both analyses ($\beta = 0.003$, $p = 0.91$ in analysis without externalizing at T1 as predictor and $\beta = -0.019$, $p = 0.37$ in analysis with externalizing at T1 as predictor). However, these analyses revealed a significant interaction effect between the DRD4 4-repeat allele and overprotection, both without correction for externalizing behavior problems at T1 ($\beta = -0.084$, $p < 0.01$) and with correction for externalizing behavior problems at T1 ($\beta = -0.051$, $p < 0.05$). This interaction effect between the DRD4 4-repeat allele and overprotection in the third step was found over and above the main effect of overprotection (without correction for externalizing

behavior problems at T1; β = 0.131, p <0.001; with correction for externalizing behaviour problems at T1; β = 0.070, p =0.005). Stratified analyses showed that the effect of parental overprotection on higher externalizing behavior problems at T2 was stronger in adolescents not carrying the DRD4 4-repeat allele (β = 0.388, p < 0.001) than in adolescents carrying the DRD4 4-repeat allele (β = 0.123, p <0.001) . Parenting factors explained more variance in externalizing behavior problems in the group not carrying the 4-repeat allele (Adj. R^2 = 14.4%) than in the group carrying the 4-repeat allele (Adj. R^2 = 11.1%). Figure 5.2 shows the regression lines for adolescents carrying and not carrying the DRD4 4-repeat allele. Adolescents with an absent 4-repeat allele and high levels of perceived parental overprotection showed the highest levels of externalizing behavior problems at T2.

FIGURE 5.2 Regression lines predicting externalizing behavior problems from parental overprotection for adolescents carrying and not carrying the 4-repeat allele of the DRD4.



Note: Overprotection = parental overprotection within the minimum and maximum range values.

5.4 DISCUSSION

In the present longitudinal study we demonstrated that parenting is a significant predictor of externalizing behavior problems in 10- to 12-year-old adolescents from the general population. In addition, we found a significant interaction between parental rejection and FLE in predicting externalizing behavior problems, such that the effect of parental rejection is only present in the absence of FLE. We were unable to replicate main or interactive effects of the DRD4 7-repeat allele in predicting future externalizing behavior

problems. However, the DRD4 4-repeat allele interacted with parental overprotection in predicting future externalizing behavior problems.

Consistent with our first hypothesis, parental overprotection and parental rejection lead to higher levels of externalizing behavior problems two and a half years later, whereas parental emotional warmth leads to lower levels of externalizing behavior problems two and a half years later. The present study extended an earlier cross-sectional study by showing that overprotection also predicts externalizing behavior problems two and a half years later (Yahav, 2007). The finding of parental rejection was only present in the model without correction for externalizing behavior problems at T1. In that way, this finding does not fit with a previous longitudinal study in adolescence that looked at the predictive role of harsh discipline (Leve et al., 2005). Possibly, operationalization and measurement of negative aspects of parenting are essential in determining their effects. Our finding of emotional warmth was consistent with the outcome of the longitudinal study by Reitz and colleagues (2006) that was also conducted in early adolescence and looked at the effect of parental involvement and decisional autonomy.

Regarding our second hypothesis, the present study revealed no evidence that the effect of parenting is specific for boys. This is in contrast to previous studies reporting that the relationship between parenting and externalizing behavior problems is stronger for boys than for girls (Leve et al., 2005; Miner & Clarke-Stewart, 2008; Rothbaum & Weisz, 1994). However, all of these studies examined specific parenting factors (e.g. harsh discipline, approval, and restrictiveness) that differed from the factors used in the present study (i.e., rejection, overprotection, and emotional warmth). A cross-sectional study that looked also at rejection, overprotection, and emotional warmth, could not demonstrate a gender-specific association between parenting and externalizing behavior problems (Veenstra et al., 2006). Moreover, the meta-analysis by Rothbaum and Weisz (1994) did not include longitudinal designs and may have become outdated since only studies published between 1940 and 1992 were considered. They suggested that the reason for the potential gender-specific relationship may lie in the fact that boys have higher levels of externalizing behavior problems than girls, leading to different effects of parenting. Although we found higher initial levels of externalizing behavior problems in boys, we found as well that girls showed higher levels of externalizing behavior problems at T2 when we corrected for the effect of externalizing behavior problems at T1. Since both boys and girls show considerable variance in externalizing behavior problems, it is likely that our study would detect a moderator effect if it was present. Previous studies that found moderator effects may have been biased by a lack of variance in one of the genders. To conclude, we found evidence that parental rejection, parental overprotection, and parental emotional warmth predicts externalizing behavior problems in a similar way in boys and girls.

As for the third hypothesis, the present study revealed an interaction effect between parental rejection and FLE in predicting externalizing behavior problems. Contrary to the classical stress-vulnerability view of gene-environment interaction, however, the relationship between negative parenting (i.e., parental rejection) and externalizing behavior problems was present in low but not in high FLE families. The results suggest a competing risk model. That is, the effect of FLE was present when parental rejection was low and the effects of parental rejection was present when FLE was low. Sonuga-Barke and colleagues (2009) also provided evidence for alternative pathways in showing effects of maternal expressed emotion on emotional problems in children and adolescents with the low-risk genotype. They suggest that a high-risk genotype may produce a general insensitivity to environmental factors. The same model may apply to adolescents from high FLE families being not sensitive to parental rejection. However, an alternative explanation for these findings is that low statistical power impeded our ability to detect an effect of parental rejection in adolescents from high FLE families. Contrary to the interaction effect between parental rejection and FLE, there were no interaction effects between parental warmth and parental overprotection on the one hand, and FLE on the other hand, in predicting externalizing behavior problems. A reason for this might be that there is not enough variance in these parenting factors, since the present study was based on a population-based sample of adolescents. That is, possibly a ceiling effect prevented us from detecting effects in these more positive parenting factors. Despite the interaction effect between parenting and FLE in predicting future externalizing behavior problems, we were unable to find an interaction effect between parenting and the DRD4 7-repeat allele. We initially tested the DRD4 7-repeat allele since it had been shown to interact with parenting factors in previous studies and since it is hypothesized that dopaminergic genes influence the sensitivity to salient environmental cues (Bakermans-Kranenburg & van Ijzendoorn, 2006, 2007; Propper et al., 2007; Sheese et al., 2007). An explanation for not finding this interaction effect may be that the DRD4 7-repeat allele makes someone more sensitive to parenting factors at childhood age than in adolescence. It could also be that other genes or alleles are involved in adolescence. On an exploratory basis, we investigated the potential moderating role of the DRD4 4-repeat allele and parenting on future externalizing behavior problems. We demonstrated that adolescents with an absent 4-repeat allele and high levels of perceived parental overprotection showed the highest levels of externalizing behavior problems at T2. While a previous study found that the absence of the 4-repeat allele was related to lower executive function (Fossella et al., 2002), there are, to our knowledge, no previous studies that investigated interaction effects between the 4-repeat allele and parenting. The present finding confirms and extends previous conclusions that the presence of the 4-repeat allele has a protective effect (Li et al., 2006). That is, we found that this protective effect was present in an environment with low and high perceived parental overprotection. The absence of

the 4-repeat allele seems to make individuals more vulnerable in an environment with high perceived parental overprotection. This is in accordance with the classical stress-vulnerability view of gene-environment interaction. However, confirmation of this finding in independent datasets is needed before firm conclusions can be drawn.

Some limitations of the present study should be noted. First, we did not measure parenting at the second measurement. Consequently, we cannot assess potential bidirectional associations between parenting and externalizing behavior problems. Indeed, Miner and Clarke-Stewart (2008) found that children's externalizing behavior problems affect later parenting. Second, our measure of parenting was based on a single self-report questionnaire by the adolescent and thus reflects perceived parenting. However, there is sufficient support for the reliability, and the factorial and construct validity of this instrument (Dekovic et al., 2006; Muris et al., 2003). In future research, however, it would be useful to incorporate measures of parents' and siblings' perception of parenting. In addition, a genetic systems approach should be adopted by testing the overall effect of relevant dopaminergic genes (Gardner, Bertranpetit, & Comas, 2008).

There are also several strengths of the present study. Regarding psychosocial moderators, Nigg and colleagues (2010) state that different methods provide evidence for gene-environment interaction in predicting ADHD. This paper presents a unique approach by combining a quantitative method with a molecular-genetic method. Second, it is a very large longitudinal population-based study, whereas many previous studies were cross-sectional and were based on socially and economically disadvantages samples, which were thus more likely to display externalizing behavior problems. In addition, the nature of this sample provided us the opportunity to investigate the potential moderating role of gender, FLE and presence of the DRD4 7-repeat allele and the DRD4 4-repeat allele. Third, we used a sample of adolescents, whereas most of the longitudinal studies that focused on the effect of parenting on future externalizing behavior problems are limited to childhood.

Despite the common notion that adolescents are less dependent on their caregivers and spend increasing time outside the family with their peer group, we demonstrated that parenting is a significant predictor of externalizing behavior problems during early adolescence. Parental rejection interacted with FLE in predicting externalizing behavior problems, in that the effect of parental rejection was only present in the absence of FLE. These results suggest a competing risks model. That is, the effect of genetic risk was present when environmental adversity was low and the effect of environment was present when genetic risk was low. Yet, replication of this finding is necessary before drawing firm conclusions. Although the DRD4 7-repeat allele did not interact with parenting in predicting externalizing behavior problems, the findings related to the DRD4 4-repeat allele provide a basis for additional molecular-genetic studies examining the interactive influence of risk genes and parenting on the course of externalizing behaviors.

CHAPTER 6

Interaction between the *SLC6A3* gene and early risk factors on ADHD symptoms in adolescence. The TRAILS study.

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Submitted for publication

ABSTRACT

The purpose of the present study was to investigate the main and interaction effects of two risk alleles of the *SLC6A3* gene and three early risk factors (maternal smoking and alcohol use during pregnancy, and obstetric complications (OCs)) on continuous parent-reported and self-reported ADHD symptom measures. In this longitudinal population-based study (n=1141 and n=1336 for parent-reported and self-reported data, respectively), adolescents were measured at three measurement waves (mean age 11.09, 13.56, and 16.13). Maternal smoking during pregnancy and OCs were related to more parent-reported ADHD symptoms. In addition, adolescents carrying the risk allele of SNP rs40184 that had a history of severe OCs had the highest level of parent-reported inattention symptoms, whereas adolescents carrying the non-risk allele of SNP rs40184 and prenatal alcohol exposure had the highest level of self-reported hyperactivity/impulsivity symptoms. There is more need for purposeful built gene-environment interaction studies to unravel the genetic architecture of ADHD.

6.1 INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is among the most common disorders in childhood and adolescence, affecting at least 5% of children worldwide according to the DSM-IV criteria (Faraone et al., 2003). Although ADHD is defined as a disorder in the categorical classification system DSM-IV-TR (APA, 2000), there is broad support for a dimensional latent structure across indicators for inattention, hyperactivity/impulsivity, and ADHD (Boyle et al., 1996; Lubke, Hudziak, Derks, van Bijsterveldt, & Boomsma, 2009). Therefore, we take a dimensional approach for the measurement of ADHD symptoms in the present population-based study.

Family, twin, and adoption studies reveal a strong contribution of genetic risk factors to the etiology of ADHD (Burt, 2009; Faraone et al., 2005). Several candidate genes (*SLC6A3*, DRD4, DRD5, 5-HTT, HTR1B, and SNAP25) appear to be involved in the etiology of ADHD, as evidenced by a review and meta-analysis (Gizer et al., 2009). This study will focus on the role of the dopamine transporter gene *SLC6A3*, since it is involved in dopaminergic neurotransmitter signaling, which seems to be altered in ADHD patients (Volkow et al., 2007). Further, methylphenidate, the primary drug treatment in ADHD, acts on dopamine transporters in the striatum and reduces ADHD symptoms (Faraone, Spencer, Aleardi, Pagano, & Biederman, 2004; Medori et al., 2008). The response to methylphenidate is associated with polymorphisms of the *SLC6A3* gene (Froehlich et al., 2011; Kooij et al., 2008).

Next to genetic factors, ADHD symptoms are also affected by environmental risk factors, including those that are active very early in development. Maternal smoking during pregnancy and, to a lesser extent, maternal alcohol use during pregnancy, have frequently been related to ADHD symptoms (Banerjee et al., 2007; Linnet et al., 2003). In addition, complications during pregnancy and delivery (obstetric complications, OCs) have been related to ADHD (Banerjee et al., 2007; Ben Amor et al., 2005), and in particular to hyperactivity/impulsivity symptoms (Freitag et al., 2011). Furthermore, children with low birth weight and / or born prematurely are at greater risk for developing ADHD (Thapar, Cooper, Jefferies, & Stergiakouli, 2011).

Last but not least, the interactive effects of genes and environment have been implicated in the etiology of ADHD (Buitelaar, 2005; Ficks & Waldman, 2009; Freitag, Rohde, Lempp, & Romanos, 2010; Nigg et al., 2010). So far, research on gene-environment interactions in ADHD has focused on two types of environments, pre- and perinatal factors, and familial and psychosocial influences (Nigg et al., 2010). In the present study, the focus will be on pre- and perinatal moderators since previous results concerning interaction effects with pre- and perinatal factors have been inconsistent (Nigg et al., 2010).

The present study was designed to advance our knowledge of the main and interaction effects of the *SLC6A3* gene and pre- and perinatal risk factors on ADHD

symptoms in several ways. First, we will summarize prior research on the interaction between the *SLC6A3* gene and early risk factors on ADHD symptoms. Second, following several studies that demonstrate the importance of using the core symptom dimensions of ADHD (i.e. inattention and hyperactivity/impulsivity) as outcome measures (Becker, El-Faddagh, Schmidt, Esser, & Laucht, 2008; Bidwell et al., 2011; Freitag et al., 2011; Kahn, Khoury, Nichols, & Lanphear, 2003; Nikolas & Burt, 2010; Waldman et al., 1998), we will also use these dimension scores as outcomes in a large population-based cohort of adolescents which have been measured at three time points. The longitudinal design of this study provides data on the course of ADHD symptoms during adolescence. In the present study we will analyze the (a) main effects of the *SLC6A3* gene, (b) main effects of early environmental risk factors, and (c) the interaction effect between both on ADHD symptoms. Firstly, we hypothesize to find a main effect of the *SLC6A3* gene on ADHD symptoms. Secondly, we hypothesize to find main effects of maternal smoking and OCs on ADHD symptoms; the latter may be specifically related to hyperactivity/impulsivity symptoms. Thirdly, hypotheses involving the interaction between the *SLC6A3* gene and early environmental risk factors on ADHD outcomes will be formulated after presenting a short overview of previous studies on the interaction between the *SLC6A3* gene and early risk factors on ADHD outcomes.

6.2 METHODS

6.2.1 Sample

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective study of Dutch adolescents, with the aim to chart and explain the development of mental health from early adolescence into adulthood, both at the level of psychopathology and the levels of underlying vulnerability and environmental risk. Adolescents will be measured bi- or triennially at least until they are 25 years old. The present study involves data from the first (T1), second (T2), and third (T3) assessment wave of TRAILS, which ran from March 2001 to July 2002, September 2003 to December 2004, and September 2005 to December 2007, respectively.

TRAILS participants were selected from five municipalities in the north of The Netherlands, including both urban and rural areas. Children born between October 1, 1989, and September 30, 1990 (first two municipalities), or October 1, 1990, and September 30, 1991 (last three municipalities), were eligible for inclusion, providing that their schools were willing to cooperate and that they were able to participate in the study. Of all eligible 2935 children, 76.0% (N = 2230, mean age = 11.09, SD = 0.56, 50.8% girls) were enrolled in the study. Parental written informed consent was obtained after the procedures had been fully explained. Responders and non-responders did not differ with

respect to the prevalence of teacher-rated behavior problems, nor regarding associations between sociodemographic variables and mental health outcomes. Detailed information about sample selection and analysis of non-response bias has been reported elsewhere (de Winter et al., 2005; Huisman et al., 2008). Of the 2230 baseline participants, 96.4% (N = 2149, 51.0% girls) participated in the first follow-up assessment (T2), which was held 2 to 3 years after T1 (mean number of months 29.44, SD = 5.37, range 16.69-48.06). Mean age at T2 was 13.56 (SD = 0.53). At T3, the response rate was 81.4%, and mean age was 16.13 (SD = 0.59). We received complete parent-reported and self-reported data (all three measurement waves) on ADHD symptoms (based on CBCL and YSR) from 1402 participants and 1613 participants, respectively. We received complete parent-reported and self-reported data as well as data on early risk factors and the *SLC6A3* gene from 1141 participants and 1336 participants, respectively. The TRAILS study was approved by the Central Committee on Research Involving Human Subjects (Dutch CCMO).

6.2.2 Procedure

At T1, well-trained interviewers visited one of the parents or guardians (preferably the mother, 95.6%) at their homes to administer an interview covering a wide range of topics, including development history and somatic health, parental psychopathology, and care utilization. In addition to the interview, the parent was asked to fill out some questionnaires concerning the child's mental health and behavior. Adolescents filled out questionnaires at school, in the classroom, under the supervision of one or more TRAILS assistants. Teachers were asked to fill out a brief questionnaire for all TRAILS-participants in their class. T2 and T3 also involved questionnaires, to be filled out by the adolescents, their parents and their teachers. As in T1, the adolescents filled out their questionnaires at school, supervised by TRAILS assistants. At T3, blood or buccal cells were collected for DNA analysis. Measures that were used in the present study are described more extensively below.

6.2.3 Measures

6.2.3.1 ADHD symptoms. At T1, T2, and T3, behavioral problems were assessed with the Child Behavior Checklist (CBCL) (Achenbach, 1991a; Verhulst et al., 1996) and the Youth Self-Report (YSR) (Achenbach, 1991c; Verhulst et al., 1997). The CBCL is a measure of parent-reported emotional and behavioral problems in 4- to 18-year-old children and the YSR is a self-report questionnaire that was modeled on the CBCL. The CBCL and the YSR contain 113 and 112 items respectively. These items are rated as 0 (not true), 1 (somewhat or sometimes true) or 2 (very true or often true). In our opinion, it is necessary to analyze the results of parent and adolescents separately, since agreement between both is poor (Barkley, Fischer, Edelbrock, & Smallish, 1991; Faraone et al., 2003). Following Achenbach,

Dumenci, and Rescorla (2003) DSM-oriented scales of ADHD inattention and ADHD hyperactivity/impulsivity symptoms were created on both the CBCL and the YSR items. The validity of these scales have been found to be high in terms of significant associations with DSM clinical diagnosis (Achenbach & Rescorla, 2001). The inattention scale included item 4. Fails to finish, 8. Can't concentrate, and 78. Inattentive, with Cronbach's $\alpha = 0.78$ for CBCL items at T1, 0.80 at T2, and 0.81 at T3; and with Cronbach's $\alpha = 0.54$ for YSR items at T1, 0.63 at T2, and 0.70 at T3, and the hyperactivity/impulsivity scale included item 10. Can't sit still, 41. Impulsive, 93. Talks much, and 104. Loud, with Cronbach's $\alpha = 0.75$ for CBCL items at T1, 0.72 at T2, and 0.71 at T3; and with Cronbach's $\alpha = 0.56$ for YSR items at T1, 0.61 at T2, and 0.60 at T3. Since the symptoms of the inattention scale and hyperactivity/impulsivity scale were not normally distributed, arcsin transformation was applied to the symptom scales. That is, the raw scores (range 0-6 inattention scale, range 0-8 hyperactivity/impulsivity scale) were converted to a proportional score and normalized with angular transformations to arcsin values (Freeman & Tukey, 1950). After transformation, skewness and kurtosis were, respectively, between -0.42 and 0.89, and between -0.49 and 0.59.

6.2.3.2 Pre- and perinatal factors. Pre- and perinatal factors were assessed retrospectively in the parent interview. Maternal smoking during pregnancy was categorized into three groups: nonsmokers (69.5%), between 1 and 10 cigarettes a day (23.6%), and more than 10 cigarettes a day (6.9%). Retrospective recall of maternal smoking during pregnancy was found to be consistent with antenatal records (Rice et al., 2007). Secondly, maternal alcohol use during pregnancy was categorized into three groups: nonusers (81.3%), less than 1 glass a week (13.9%), and more than 1 glass a week (4.8%). Although the consistency between retrospective recall of maternal alcohol use during pregnancy and antenatal records is hard to determine because alcohol use during pregnancy is not routinely recorded in medical records (Rice et al., 2007), it is also believed that mothers probably report higher levels of prenatal alcohol use when interviewed retrospectively (Jacobson, Chiodo, Sokol, & Jacobson, 2002). Thirdly, Obstetric Complications (OCs) are defined as the broad class of deviations from the expected, normal course of events, including child development during pregnancy, labor/delivery, and the early neonatal period (McNeil, 1988). OCs included the presence of pregnancy complications (i.e. physical, social or psychological problems during pregnancy), complicated deliveries (i.e. breech presentation, Caesarean section), and hospitalization of the mother (i.e. due to physical problems, postnatal depression) or child (i.e. lack of oxygen, blood transfusion, jaundice) (Gillberg, 1995; Milberger et al., 1997). Following a previous TRAILS study (Buschgens et al., 2009), a composite score on OCs was calculated on the basis of a list of 31 OCs (range 0 to 14, mean = 1.87, *SD* = 2.19). If no information was available for 6 or more items, cases

were excluded from further analyses. Since the distribution of OCs was highly skewed, three groups were created: no complications (0 complications; 37.9%), mild complications (1 to 4 complications; 52.1%), and severe complications (5 or more complications;10.0%).

6.2.3.3 Genotyping. DNA was extracted from buffy coats or buccal swabs (Cytobrush®) with the use of a manual salting out procedure similar to the protocol described by Miller and colleagues (1988). Unfortunately, the 40 base pair VNTR polymorphism located in the 3' untranslated region (UTR) of the *SLC6A3* gene was not genotyped. Instead, 2 SNP's from the 3' end region were used: SNP rs40184 (risk allele G; Gizer et al., 2009) and SNP rs1042098 (risk allele A; Brookes et al., 2006). Pairwise LD estimates between the 3' UTR VNTR and SNP rs40184 and SNP rs1042098 are 0.92 and 0.91, respectively (Brookes et al., 2006).

6.2.4 Data analysis

First, an overview of previous studies that investigated the interaction between the *SLC6A3* gene and early risk factors on ADHD outcomes will be presented. Second, to determine main effects of the *SLC6A3* gene SNPs and pre- and perinatal factors, hierarchical regression analyses were conducted on the mean level (based on three time points) of transformed inattention symptoms, and transformed hyperactivity/impulsivity symptoms (transformation procedures described in section on ADHD symptoms). These analyses were performed twice, with symptoms based on a parent report (CBCL) and symptoms based on a self report (YSR). Potential covariates gender, birth weight, and SES (Linnet et al., 2003) were entered at Step 1. The SNPs rs40184 and rs1042098, the pre- or perinatal risk factors (i.e. smoking and alcohol use during pregnancy, and OCs) were entered at Step 2.

Third, to determine potential interaction effects, repeated measures ANOVA were conducted on transformed inattention symptoms and transformed hyperactivity/impulsivity symptoms, at three time points. Again, these analyses were performed twice, with symptoms based on a parent report (CBCL) and symptoms based on a self report (YSR). The SNPs rs40184 and rs1042098, the pre- or perinatal risk factors (i.e. smoking and alcohol use during pregnancy, and OCs), and the interaction terms between SNP and pre- or perinatal risk factor were used as independent factors. As in the hierarchical regression analyses, gender, birth weight, and SES were used as covariates in these analyses. Since we performed analyses on 2 outcomes (inattention symptoms, and hyperactivity/impulsivity symptoms), 2 informants (parent-report and self-report), and with 2 SNPs (rs40184 and rs1042098), we performed a total of 8 analyses. In both regression analyses and repeated measures ANOVA, Bonferroni correction was used to adjust for multiple testing (0.05/8 = 0.00625).

6.3 RESULTS

6.3.1 Overview previous studies

In Table 6.1 we present the results from previous studies on the interaction between the *SLC6A3* gene and early risk factors on ADHD outcomes. Firstly, there is evidence from population-based studies that maternal smoking during pregnancy amplifies the effect of the 10-repeat allele of *SCL6A3* on hyperactivity/impulsivity symptoms for the total sample (Kahn et al., 2003) and for boys only (Becker et al., 2008). In addition, maternal smoking during pregnancy interacts with the 9-repeat allele of *SCL6A3* in predicting total ADHD symptoms (Neuman et al., 2007). However, case-control studies or clinical studies without control group were unable to demonstrate an interaction effect between maternal smoking during pregnancy and the *SLC6A3* gene in predicting ADHD (Altink et al., 2009; Brookes et al., 2006; Langley et al., 2008). Secondly, the results from two clinical studies that investigated the interaction between the *SLC6A3* gene and alcohol use during pregnancy were not consistent (Brookes et al., 2006; Langley et al., 2008). There are no population-based studies on the interaction effects of the *SLC6A3* gene and prenatal exposure to alcohol and obstetric complications (OCs) on ADHD symptoms. Based on these results, we hypothesize to find an interaction effect between maternal smoking during pregnancy and the *SLC6A3* gene on ADHD hyperactivity/impulsivity symptoms. Because there are, to our knowledge, no other population-based studies on the interaction with other early risk factors (i.e. maternal alcohol use during pregnancy and OCs), these analyses will be exploratory.

6.3.2 Descriptives of ADHD symptoms

Table 6.2 shows mean levels of the transformed parent-reported and self-reported ADHD inattention symptoms and ADHD hyperactivity/impulsivity symptoms by measurement wave. In addition, internal consistency was determined for each of the symptoms over the three measurement waves. Internal consistency of parent-reported ADHD symptoms varied between 0.82 and 0.83, and internal consistency of self-reported ADHD symptoms varied between 0.62 and 0.68. Whereas parent reported symptoms appear to decrease over time, self reported symptoms appear to increase over time.

TABLE 6.1 Summary of previous studies on the interaction between the *SLC6A3* gene and early risk factors on ADHD outcomes.

Study	Design	*SLC6A3* markers	*SLC6A3* x smoking	*SLC6A3* x alcohol	*SLC6A3* x OCs
Case-control studies or clinical studies without control group					
Altink et al., 2009	Continuous outcome: attentional control (neuropsychological task) Case-control study (clinical diagnosis of ADHD present) Age 5-17 n = 56 ADHD probands / n = 23 affected siblings / n = 105 controls	*SLC6A3* 3'UTR VNTR (10/10) *SLC6A3* VNTR in intron 8 (6/6) *SLC6A3* haplotype 10/6_10/6	No interaction effect observed	Not investigated (no main effect of alcohol observed)	Not reported
Brookes et al., 2006	Analysis by stratifying the probands into groups based on the binary environmental risk measure. Clinical study without control group (clinical diagnosis of ADHD present) Age 5-15 n = 396 ADHD probands from English and Taiwanese sample (no controls)	*SLC6A3* 3'UTR VTNR *SLC6A3* VTNR in intron 8 rs40184 rs1042098 rs27072 AC repeat D5S678 D5S2005 D5S1981 AAAC-repeat Haplotypes	No interaction effect observed	Significant interaction. Exposed to environmental risk: OR: 3.46 / 2.7. Not exposed to environmental risk: OR: 1.11 / 1.24. (first OR for interaction with *SLC6A3* 10/3 haplotype; second OR for interaction with haplotype frequencies from haplotype-based haplotype relative risk analysis).	Not reported
Langley et al., 2008	Dichotomous outcome: diagnosis. Analysis with pseudocontrols. Clinical study without control group (clinical diagnosis of ADHD present) Age 9 n = 266	*SLC6A3* 3'UTR VNTR (10/10)	No interaction effect observed	No interaction effect observed	Not reported

TABLE 6.1 Continued

Study	Design	*SLC6A3* markers	*SLC6A3* x smoking	*SLC6A3* x alcohol	*SLC6A3* x OCs
Population-based studies					
Becker et al., 2007	Continuous outcomes (inattention, hyperactivity/ impulsivity, and ODD/CD symptoms) Population-based study Age 15 n = 305	*SLC6A3* 3'UTR VNTR (10/10)	Significant interaction on hyperactivity/ impulsivity symptoms ($p = 0.012$) in males (R^2 of complete model = 0.088) No interaction effects observed in females.	Not reported	Not reported
Kahn et al., 2003	Continuous outcomes (inattention, hyperactivity/ impulsivity, and oppositional symptoms) Population-based study Age 5 n = 161	*SLC6A3* 3'UTR VNTR (10/10)	Significant interaction on hyperactivity/ impulsivity symptoms ($p = 0.01$) and on oppositional symptoms ($p = 0.001$). (no effect sizes reported)	Not reported	Not reported

TABLE 6.1 Continued

Study	Design	*SLC6A3* markers	*SLC6A3* x smoking	*SLC6A3* x alcohol	*SLC6A3* x OCs
Neuman et al., 2007	Dichotomous outcomes: any DSM-IV ADHD diagnosis, DSM-IV predominantly inattentive subtype, DSM-IV combined type, and population-defined few symptoms, severe inattentive, and combined latent class subtypes Population-based twin study Age 7 - 18 n = 1540	*SLC6A3* 3'UTR VNTR	Significant interaction on DSM-IV ADHD (OR = 1.84), DSM-IV combined (OR = 2.93), and population-defined severe combined class (OR = 2.56). For the three other outcome variables OR not significant.	Not investigated (no main effect of alcohol observed)	Not reported
Marsman et al., 2012	Continuous outcomes (inattention symptoms, hyperactivity/ impulsivity symptoms; parent and self report) Population-based longitudinal study Age 10 – 18 n = 1336 (T1) / n = 1312 (T2) / n = 1223 (T3)	*SLC6A3* rs40184 *SLC6A3* rs1042098	No interaction effect observed	Interaction with *SLC6A3* rs40184 on YSR hyperactivity/ impulsivity symptoms (p = 0.006). (Effect statistical significant after Bonferroni correction.)	Interaction with *SLC6A3* rs40184 on CBCL inattention symptoms (p = 0.005). (Effect statistical significant after Bonferroni correction.)

TABLE 6.2 Mean levels of ADHD symptoms by measurement wave and internal consistency.

	T1	T2	T3	Internal consistency (α)
Parent-reported				
ADHD inattention symptoms	31.33 (n=2022)	25.93 (n=1902)	25.27 (n=1510)	0.82
ADHD hyperactivity symptoms	25.24 (n=2031)	18.60 (n=1906)	15.59 (n=1502)	0.83
Self-reported				
ADHD inattention symptoms	30.12 (n=2156)	33.89 (n=2066)	34.24 (n=1648)	0.62
ADHD hyperactivity symptoms	29.79 (n=2161)	32.45 (n=2080)	33.17 (n=1643)	0.68

Note: Mean levels of transformed variables are shown, since the original variables were not normally distributed.

6.3.3 Main effects

Hierarchical regression analyses (results not shown) revealed that OCs were associated with significantly higher parent-reported ADHD inattention symptoms ($p<0.001$) and parent-reported ADHD hyperactivity/impulsivity symptoms ($p=0.004$). In addition, smoking during pregnancy was associated with significantly higher parent-reported ADHD inattention symptoms ($p<0.001$) and parent-reported ADHD hyperactivity/impulsivity symptoms ($p<0.001$). Boys showed significantly higher parent-reported ADHD inattention symptoms ($p<0.001$) and higher parent-reported ADHD hyperactivity/impulsivity symptoms ($p<0.001$) than girls. In addition, lower SES was related to significantly more parent-reported ADHD inattention symptoms ($p<0.001$) and more parent-reported ADHD hyperactivity/impulsivity symptoms ($p<0.001$). In the hierarchical regression analyses on self-reported symptoms, only smoking during pregnancy was a significant predictor of self-reported ADHD hyperactivity/impulsivity symptoms ($p = 0.001$). The main effects of SNPs rs40184 and rs1042098 were not significant.

6.3.4 Interaction with SNP rs40184

Table 6.3 and 6.4 show the results of the repeated measures ANOVA with SNP rs40184 as risk factor on parent and self-reported ADHD symptoms, respectively. The interaction term between SNP rs40184 and OCs was associated with parent-reported inattention symptoms ($F(2,1083) = 5.2$, $p = 0.005$) which was significant after Bonferroni correction ($p = 0.00625$). Adolescents with the combination of risk genotypes (AG or GG) and a history of severe complications (OCs = 2) had higher parent-reported inattention symptoms at all three time points, compared to adolescents without this combination (see Figure 6.1).

In the model on self-reported symptoms (Table 6.4), the interaction term between SNP rs40184 and alcohol exposure was significant after Bonferroni correction on ADHD hyperactivity/impulsivity symptoms ($F(2,1268) = 5.1$, $p = 0.006$). Adolescents with the non-risk allele (AA) and with prenatal alcohol exposure showed the highest level of self-reported hyperactivity/impulsivity symptoms.

6.3.5 Interaction with the *SLC6A3* gene rs1042098

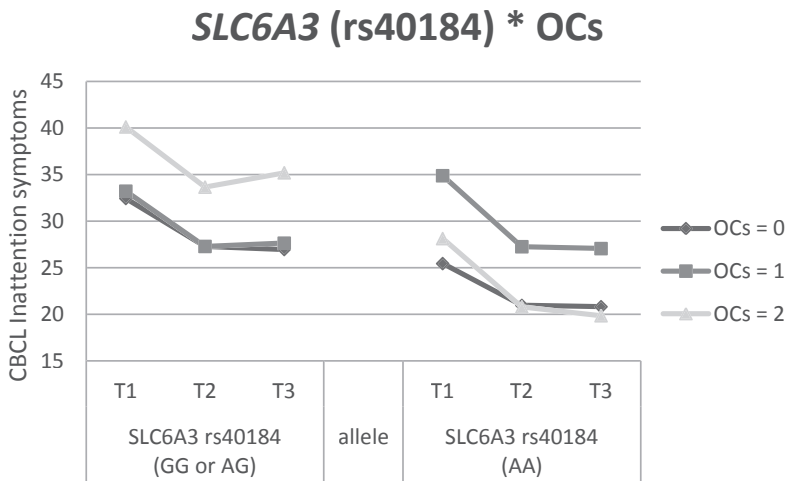
In the repeated measures ANOVA with the *SLC6A3* gene rs1042098 as risk factor on parent-reported ADHD symptoms (results not shown), only the covariates gender (p-values < 0.001) and SES (p-values < 0.001) were associated with ADHD inattention symptoms and ADHD hyperactivity/impulsivity symptoms (boys showed higher symptoms than girls and lower SES was related to higher symptoms). There were no main effects of any pre- or perinatal factor, nor was the interaction between rs1042098 and pre- or perinatal factors significant. In the model on self-reported symptoms, there were no effects of covariates or main or interaction effects between pre- or perinatal factors.

TABLE 6.3 Repeated measures analyses with SNP rs40184, early risk factors, and their interaction terms as factors (CBCL data).

	ADHD inattention symptoms (3)			ADHD hyperactivity symptoms (3)		
	df	F	p	df	F	*p*
Model						
Factors						
SNP rs40184	1	4.0	0.05	1	1.4	0.20
OCs	2	4.1	0.02	2	1.6	0.20
Smoking	2	2.7	0.07	2	2.0	0.14
Alcohol	2	0.1	0.93	2	0.2	0.84
OCs*SNP	2	5.2	**0.005**	2	0.7	0.49
Smoking*SNP	2	0.6	0.57	2	0.4	0.65
Alcohol*SNP	2	1.3	0.26	2	1.2	0.29
Covariates						
Gender	1	51.9	**<0.001**	1	6.3	**0.002**
Birth weight	1	0.0	0.97	1	0.5	0.64
SES	1	24.2	**<0.001**	1	3.2	0.04

Note: SNP=SNP rs40184 (SLC6A3). P-values in bold indicate significance after the Bonferroni correction.

FIGURE 6.1 *SLC6A3* (SNP rs40184) * OCs on CBCL inattention symptoms (p = 0.005)



Note: GG/AG = risk allele.

TABLE 6.4 Repeated measures analyses with SNP rs40184, early risk factors, and their interaction terms as factors (YSR data).

	ADHD inattention symptoms (3)			ADHD hyperactivity symptoms (3)		
	df	*F*	*p*	*df*	*F*	*p*
Model						
Factors						
SNP rs40184	1	0.7	0.40	1	1.9	0.17
OCs	2	3.2	0.04	2	0.4	0.67
Smoking	2	3.5	0.03	2	3.9	0.02
Alcohol	2	2.3	0.10	2	0.4	0.65
OCs*SNP	2	2.6	0.07	2	0.1	0.89
Smoking*SNP	2	0.3	0.78	2	0.4	0.67
Alcohol*SNP	2	4.2	0.02	2	5.1	**0.006**
Covariates						
Gender	1	0.0	0.86	1	4.7	0.03
Birth weight	1	0.2	0.63	1	0.37	0.55
SES	1	1.9	0.17	1	0.29	0.59

Note: SNP=SNP rs40184 (SLC6A3). P-values in bold indicate significance after the Bonferroni correction.

6.4 DISCUSSION

The present study summarizes the current state of research on the interaction between the dopamine transporter gene and early risk factors on ADHD outcome measures. Further, it investigates the main and interaction effects of two risk alleles of the *SLC6A3* gene and three early risk factors (maternal smoking during pregnancy, alcohol use during pregnancy, and total OCs) on continuous ADHD outcome measures.

In accordance with prior studies (Banerjee et al., 2007; Linnet et al., 2003), maternal smoking during pregnancy was found to be related to ADHD symptoms (both inattention symptoms and hyperactivity/impulsivity symptoms). In the present study, we found the same relationship for both parent-reported and self-reported ADHD symptoms, although the relationship between smoking during pregnancy and self-reported ADHD inattention symptoms did not reach Bonferroni-corrected significance. In contrast, alcohol use during pregnancy was not related to ADHD symptoms in this study. This is also in line with prior studies that provide mixed evidence for the association between alcohol exposure during pregnancy and ADHD symptoms (Banerjee et al., 2007; Linnet et al., 2003). Further, OCs were found to be related only to parent-reported symptoms. These findings are also in

accordance with findings in previous studies (Banerjee et al., 2007; Ben Amor et al., 2005; Freitag et al., 2011). Thus far, the main effects of early risk factors were in accordance with our hypotheses. However, there were no main effects of SNPs rs40184 and rs1042098 of the *SLC6A3* gene on ADHD inattention or hyperactivity/impulsivity symptoms.

The more searching question of this study was about the presence of gene-environment interaction. What stands out from previous studies summarized in Table 6.1 is that most studies investigated the interaction between the *SLC6A3* gene and prenatal exposure to smoking as early risk factor. Studies with prenatal alcohol exposure as early risk factor are scarce. Only two clinical studies investigated the interaction between the *SLC6A3* gene and alcohol use during pregnancy and the results were not consistent (Brookes et al., 2006; Langley et al., 2008).

In contrast to our hypothesis and previous population-based studies that investigated the interaction between maternal smoking during pregnancy and the *SLC6A3* gene (Becker et al., 2008; Kahn et al., 2003; Neuman et al., 2007), we did not find any significant interaction effects between maternal smoking during pregnancy and the *SLC6A3* gene. There may be several reasons for this inconsistency. Firstly, it might be that there is no interaction effect between maternal smoking during pregnancy and the *SLC6A3* gene in predicting ADHD symptoms and that the results published are a consequence of false positive findings (Ioannidis, 2005). Secondly, the power to detect gene-environment interactions is low when incidence rates of the environmental risk factor is much lower or higher than 50% (Caspi, Hariri, Holmes, Uher, & Moffitt, 2010). In the present study, incidence of maternal smoking during pregnancy is 30.5%. However, incidence rates in other studies that report an interaction effect were even lower and varied between 21.1% and 26.9% (Becker et al., 2008; Kahn et al., 2003; Neuman et al., 2007). Thirdly, all previous studies that report an interaction effect used the 40 base pair VNTR polymorphism located in the 3' untranslated region (UTR) of the *SLC6A3* gene. Unfortunately, this VNTR was not genotyped in the present sample. In our opinion, confirmation of previous findings on the interaction between prenatal smoke exposure and the *SLC6A3* gene is needed before firm conclusions can be drawn.

We found that the interaction between SNP rs40184 and alcohol exposure in predicting ADHD hyperactivity/impulsivity symptoms was significant after Bonferroni correction. However, the non-risk allele (AA), and not the risk allele (AG or GG), was related to the highest level of self-reported hyperactivity/impulsivity symptoms in adolescents with prenatal alcohol exposure. It is hard to relate this finding to the only study that reported an interaction effect between prenatal alcohol exposure and the *SLC6A3* gene, since that specific study was a clinical study without a control group and the interactions found were between haplotypes of the *SLC6A3* gene (Brookes et al., 2006). Moreover, the incidence of maternal alcohol use in pregnancy was 57.8% in the study by Brookes et al. (2006), while the incidence was 18.7% in the present study. As said before,

the power of detecting gene-environment interaction increases when incidence rates of the environmental risk factor are closer to 50% (Caspi et al., 2010). Thus, our study shows little evidence for an interaction effect between the *SLC6A3* gene and prenatal alcohol exposure on ADHD symptoms.

Concerning OCs, we explored whether a composite score on OCs as early risk factor interacts with the *SLC6A3* gene in predicting ADHD symptoms. To our knowledge, there are no gene-environment interaction studies between OCs and the *SLC6A3* gene on ADHD outcomes. In the present study, we found that SNP rs40184 interacts with OCs in predicting ADHD parent-reported inattention symptoms. More specifically, adolescents carrying one or two copies of the risk allele that had a history of severe complications had the highest level of parent-reported inattention symptoms at all three time points. This is in accordance with the basic idea behind gene-environment interaction, in which there are particular adverse conditions under which particular risk genes have major effects (Nigg et al., 2010). However, the effect found was related to inattention, and not hyperactivity/impulsivity symptoms. This finding is in contrast to ideas that gene-environment interaction involving pre- and perinatal effects may relate primarily to hyperactivity (Nigg et al., 2010). Of course, the finding of the present study that interactions may predict inattention symptoms needs to be replicated by other studies.

A limitation of the present study is the fact that the 40 base pair VNTR polymorphism located in the 3' untranslated region (UTR) of the *SLC6A3* gene was not genotyped. Although pairwise LD estimates between the 3' UTR VNTR and SNP rs40184 (0.92) and SNP rs1042098 (0.91) are high (Brookes et al., 2006), we cannot compare our results with the studies that investigated the homozygosity of the 10-repeat allele as risk factor. In addition, the results with parent-reported ADHD symptoms do not match with the results obtained with self-reported ADHD symptoms. However, it is, in our opinion, useful to determine ADHD symptoms on two informants, since different informants may observe different but valid ADHD behaviors (Hartman, Rhee, Willcutt, & Pennington, 2007). A model that incorporates genetic contributions unique to a rater may provide better fit to the data than a model that did not (Hartman et al., 2007). We believe that the results of parent-reported symptoms are more reliable, since children at age 9 to 11 (comparable with T1 in the present study) are poor informants of ADHD symptoms (Faraone et al., 2003). Moreover, in the present study it was observed that parent-reported symptoms had higher internal consistency than self-reported symptoms. In addition, the studies summarized in Table 6.1 have relied almost solely on parent reports (Brookes et al., 2006; Kahn et al., 2003; Lahey et al., 2011; Neuman et al., 2007) or a combination of parent and teacher reports (Altink et al., 2009). Only one study used a measure that was based on both self and parent reports (Becker et al., 2008). Whereas the value of parent reports may be biased as well (Hartman et al., 2007), the value of self reports needs to be examined in future research.

These limitations must be considered in light of the strengths of the present study. Firstly, this study is based on a very large population-based sample. With the exception of the study by Neuman et al. (2007), the sample sizes of other studies were much smaller. Secondly, the design of this study is unique compared to other studies in that longitudinal data were gathered from different informants (parent and self-reports). Thirdly, we used a continuous outcome measure of ADHD symptoms. Nigg et al. (2010) state that more differentiation of effects may ensue when symptom dimensions are considered. Moreover, studying symptom dimensions provided us the possibility to distinguish between inattention and hyperactivity/impulsivity symptoms.

In conclusion, the present study shows that smoking during pregnancy and OCs significantly predicts ADHD symptoms in a large population-based sample of adolescents. Second, we conclude that previous studies on the interaction between pre- and perinatal factors and the *SLC6A3* gene were inconclusive. We observed only two interaction effects which were statistical significant after Bonferroni correction. First, SNP rs40184 interacts with OCs in predicting ADHD parent-reported inattention symptoms (risk allele and a history of severe complications was related to the highest level of parent-reported inattention symptoms). Second, SNP rs40184 interacts with alcohol use during pregnancy in predicting ADHD self-reported hyperactivity/impulsivity symptoms (non-risk allele and alcohol use during pregnancy was related to the highest level of self-reported hyperactivity/impulsivity symptoms). Although the latter effect was related to hyperactivity symptoms (in concordance with the hypotheses by Nigg et al., 2010), the direction of this finding runs counter to expectations. All in all, replications of the findings is needed. There is more need for purposeful built gene-environment interaction studies instead of pragmatic studies. TRAILS is a pragmatic study, not designed to assess gene-environment interaction because of the low incidence of environmental risks.

CHAPTER 7

Summary and General Discussion

7.1 INTRODUCTION

The aims of the present thesis were to elucidate the relationship between hypothalamus-pituitary-adrenal (HPA)-axis activity and externalizing behavior problems by examining the additive or interactive effects of several environmental and other moderating factors, and to explore to which extent and how gene-environment interactions may explain externalizing behavior problems. Below, findings on those two themes, i.e. HPA-axis and gene-environment interaction, are summarized and discussed. Furthermore, limitations and strengths of the studies described are presented, as well as suggestions for future research. Finally, a conclusion will be presented.

7.2 THE HYPOTHALAMUS-PITUITARY-ADRENAL (HPA)-AXIS

7.2.1 Summary

Individuals with externalizing behavior problems are supposed to have low levels of arousal and HPA-axis activity (Chrousos & Gold, 1998; van Goozen et al., 2000). According to the stimulation-seeking theory, low arousal represents an unpleasant condition which may lead to stimulus-seeking behavior in order to increase arousal levels back to an optimal or normal level (Raine, 1996; Stoff & Susman, 2005; Zuckerman, 1979). Although a meta-analysis showed that there was a weak but significant relationship between low basal cortisol levels and externalizing behavior problems (Alink et al., 2008), we hypothesized that: 1) this relationship was specific for boys (since studies concerning HPA-axis activity and externalizing behavior problems have largely been conducted with boys), 2) comorbid externalizing and internalizing behavior problems lead to higher cortisol levels (since high basal cortisol levels may be related to internalizing behavior problems (Goodyer et al., 2001; Ryan, 1998)), and 3) pure externalizing behavior problems are more strongly related to the genetically influenced CAR than comorbid externalizing and internalizing problems (since pure externalizing behavior problems are more genetically influenced than comorbid externalizing and internalizing behavior problems (Gjone & Stevenson, 1997)) and comorbid externalizing and internalizing behavior problems are more strongly related to the environmentally influenced evening cortisol levels (since comorbidity probably results from a combination of genetic and environmental risk factors (Boylan et al., 2007; Gjone & Stevenson, 1997)). In *chapter 2*, none of these hypotheses could be decisively confirmed; there was only small evidence for the first part of the third hypothesis. Instead, it was demonstrated that elevated cortisol levels (both total basal cortisol levels after awakening and the CAR) are specific for girls with pure externalizing behavior problems, i.e., without comorbid internalizing behavior problems.

The next question is then, which factors are responsible for the fact that HPA-axis activity varies from individual to individual? Next to genetic factors (Wüst, Federenko, et

al., 2000), (early) environmental risk factors may be related to HPA-axis activity. Potential early environmental risk factors that may be related to HPA-axis activity are complications during pregnancy and delivery (i.e. Obstetric Complications (OCs)). OCs were found to be related to externalizing behavior problems (Allen et al., 1998; Batstra et al., 2004; Buschgens et al., 2009; Nosarti et al., 2005; Raine, 2002). Based on a biological model in which neurobiological deficits may explain the relationship between OCs and externalizing behavior problems (Allen et al., 1998), we hypothesized that HPA-axis activity serves as a mediator in the relationship between OCs and externalizing behavior problems. In addition, we hypothesized that this relationship is specific for girls, based on the results from chapter 2. In *chapter 3*, this hypothesis was not confirmed. It was shown that OCs were not related to cortisol levels. However, OCs were related to externalizing behavior problems.

Other environmental risk factors that may be related to HPA-axis activity are parenting and socio-economic status (SES). Inspired by the hardly ever tested developmental programming part of the evolutionary-developmental theory of BSC (Boyce & Ellis, 2005), and the ideas of Del Giudice et al. (2011) who described this theory in much greater detail, now also involving basal cortisol levels, we hypothesized that the association between family environment (i.e., parenting and SES) and basal cortisol levels is inversely U-shaped. We formulated no specific hypothesis with respect to the CAR. These investigations were largely exploratory. In *chapter 4*, our main hypothesis was partly supported. More specifically, we found evidence for a curvilinear relationship between SES and HPA-axis activity (both total basal cortisol levels after awakening and the CAR). In addition, perceived parental emotional warmth in childhood was linearly associated with decreased total basal cortisol levels after awakening.

In sum, the studies described in chapters 2, 3, and 4 of this thesis showed that girls with pure externalizing behavior problems form a distinct group since they show high levels of HPA-axis activity (both total basal cortisol levels after awakening and the CAR), and that parental warmth and SES, but not OCs, were related to HPA-axis activity. Yet it should be noted that many of the hypothesized relationships were absent and that the effect sizes of the findings presented are rather small. Below, we will present some factors that may be related to the absent or small effects in chapters 2 to 4.

7.2.2 Population-based samples versus clinical samples

One of the potential reasons for absent or small effects is the fact that the studies described were based on a population-based sample, and not on a clinical sample. For example, the absence of an inverse relationship between HPA-axis activity and externalizing behavior problems in boys in chapter 2 may be related to the fact that boys with more severe externalizing behavior problems are most likely to be found in clinical samples rather than in population-based samples. Indeed, low basal cortisol levels were

found to be specific for children and adolescents with more severe disruptive behavioral problems (van de Wiel et al., 2004). Also, the absent relationship between OCs and HPA-axis activity in chapter 3 may be related to sample properties. Whereas the composite score on OCs was based on a list of 31 OCs, the range of this scale was only 0 - 14. Since 72.6% of the parents reported 0, 1, or 2 OCs, the distribution of OCs is highly skewed and shows minimal variance. Because of the well-established relationship between OCs and externalizing behavior problems (Batstra et al., 2006), it is likely that clinical samples show more variation in, as well as higher levels of, OCs. In that case, a clinical sample has more power to detect a relationship between OCs and HPA-axis activity. Finally, the absent curvilinear relationships between parenting and HPA-axis activity in chapter 4 may be related to the minimal variation in perceived parenting. It is possible that high levels of perceived parental rejection or low levels of perceived parental emotional warmth does not represent a very high-stress environment. As was the case with OCs, there is a well-established relationship between parenting and externalizing behavior problems. That is, both a lack of positive parenting and high negative parenting lead to increases in externalizing behavior problems (Caspi et al., 2004; Miner & Clarke-Stewart, 2008). Therefore, it is likely to observe higher levels of parental rejection and lower levels of parental emotional warmth in clinical samples than in population-based samples. In that case, again, a clinical sample has more power to detect a relationship between parenting and HPA-axis activity.

7.2.3 Genetically versus environmentally determined HPA-axis

Another issue related to the studies described on HPA-axis activity is the question to which extent the HPA-axis is genetically or environmentally determined. In a recent study by Van Hulle et al. (2012), it was shown that both genetic and environmental factors are related to one's cortisol circadian rhythm. More specifically, both basal morning cortisol levels and morning-to-afternoon slope (also CAR; Wüst, Federenko, et al., 2000) show the highest amount of variation attributable to genetic factors (Van Hulle et al., 2012). In chapters 3 and 4, however, we investigated the relationship between several environmental factors (i.e., OCs, parenting, and SES) and the CAR (i.e., both total cortisol levels after awakening and the increase in cortisol levels after awakening). Hence, true relationships between these environmental factors and HPA-axis activity may have been underestimated or absent due to the fact that the HPA-axis measures we used were based on cortisol levels in the morning. On the other hand, it must be noted that the CAR (both total cortisol levels after awakening and the increase in cortisol levels after awakening) was based on a single day measurement. There is also evidence that the CAR of a single day is determined largely by state factors and only for a small part by trait factors (Hellhammer et al., 2007). If this is the case, effects are less underestimated than expected.

Whereas morning cortisol levels show the highest amount of variation attributable to

genetic factors, evening cortisol levels show the highest amount of variation attributable to shared environmental factors (Bartels et al., 2003; Van Hulle et al., 2012). In chapter 3, we also investigated the relationship between OCs and evening cortisol levels but we were unable to find this relationship. Reason for this non-finding may be that evening cortisol levels display little inter-subject variability (Rosmalen et al., 2005), possibly making it more difficult to detect associations.

As said before, the focus in chapters 3 and 4 was on environmental factors related to HPA-axis activity. In chapter 2, the primary focus was on the relationship between HPA-axis activity and externalizing behavior problems. In chapter 1, we already showed that the most prominent risk factors for externalizing behavior problems are genetic factors, next to environmental factors. In chapter 2, a further differentiation was made between pure externalizing behavior problems and comorbid externalizing and internalizing behavior problems. Pure externalizing behavior problems are more genetically influenced than comorbid externalizing and internalizing behavior problems (Gjone & Stevenson, 1997); Comorbidity probably results from a combination of genetic and environmental risk factors (Boylan et al., 2007; Gjone & Stevenson, 1997). Indeed, we found a relationship between pure externalizing behavior problems and the CAR (both total cortisol levels after awakening and the increase in cortisol levels after awakening), and not between comorbidity and the CAR. As expected, there were no differences between those with pure externalizing behavior problems and those with comorbid externalizing and internalizing behavior problems with respect to the more environmentally based evening cortisol levels.

7.2.4 Measurement of HPA-axis activity

In Section 7.2.3 we already discussed how sampling time of HPA-axis measures may be related to differences in outcomes. A related issue is the fact that we do not know the exact sampling times of the cortisol measures. Participants were instructed to collect the first sample shortly after waking up (still lying in bed), and the second sample 30 minutes later. Thus, absent or small effects in chapters 2 to 4 may also be related to random measurement errors. Since we do not know the exact sampling times, true relationships may have been underestimated.

Also, we used only two (relatively static) aspects of HPA-axis activity in chapter 2 to 4, that is, basal cortisol levels (i.e., total cortisol levels after awakening and evening cortisol levels) and the CAR (i.e., the increase in cortisol levels after awakening). A third aspect of HPA-axis activity is called stress reactivity, or challenge-induced cortisol secretion. The three measures of HPA-axis functioning are weakly correlated in our dataset (Bouma et al., 2009), indicating that they reflect different mechanisms (Fries et al., 2009).

The added value of measuring challenge-induced cortisol secretion, next to basal cortisol levels, lies in the fact that stress reactivity directly measures a susceptibility to

stressful challenge (Boyce & Ellis, 2005). Hence, this measure is very relevant for the studies in chapter 3 and 4 that investigated the relationship between environmental risk factors and HPA-axis activity. On the other side, the measurement of basal cortisol levels and the CAR we used may also be an interesting addition to the existing knowledge that is related to stress reactivity. In chapter 4, for example, we were able to confirm a hypothesis that was derived from the evolutionary-developmental theory of BSC that suggested a curvilinear relationship between family environment and stress reactivity. That is, we found evidence of an inverse curvilinear relationship between family environment and the CAR (i.e., both total cortisol levels after awakening and the increase in cortisol levels after awakening). For the studies described in chapter 2 and 3, there are also arguments for using basal cortisol levels and CAR instead of a stress reactivity measure. The study described in chapter 2 primarily focused on factors that moderated the relationship between HPA-axis activity and externalizing behavior problems. Moreover, a meta-analysis showed that there was a weak but significant relationship between basal cortisol levels and externalizing behavior problems, whereas cortisol reactivity was not consistently related with externalizing behavior problems (Alink et al., 2008). The outcome of this meta-analysis also affects the study described in chapter 3 which investigated the potential mediating role of HPA-axis in the relationship between OCs and externalizing behavior problems. For mediation to occur, HPA-axis activity must also be associated with externalizing behavior problems.

7.2.5 Measurement of environmental factors

Of course, results differ depending on the environmental factors used. In the present thesis, we used both separate variables (i.e., parenting factors in chapter 4) and sums of variables (i.e., OCs and SES in chapters 3 and 4). According to Larose (2005), factors should not be combined unless there is reasonable evidence from prior research to combine them. For example, high perceived parental Rejection does not necessarily mean that there is no perceived parental Emotional Warmth and vice versa. For that reason, we decided to look at the parenting factors separately. Furthermore, testing the effects of those three factors in the same model reveals the effect of each factor controlled for the other two factors. On the other hand, there are good reasons for using composite measures of OCs and SES. Firstly, since OCs rarely occur in isolation (Batstra et al., 2006), several composite measures on OCs have been introduced (Batstra et al., 2006; Milberger et al., 1997; Prechtl, 1980) in which the classical biological risk factors have been combined with factors concerning the psychological well-being of the mother during pregnancy. This composite score on OCs has also been related to externalizing behavior problems in previous studies (Batstra et al., 2004; Buschgens et al., 2009). Secondly, SES is often used as a 'container variable' representing several aspects of the family context. For that reason it is useful to use a composite score that contains information on income level, educational level of both parents, and occupational level of both parents. The high

internal consistency of this variable (.84; Veenstra et al., 2005) provides further evidence for using this measure.

Another issue that is related to the environmental factors is that of the informants used to obtain these measures. Obviously, parents are the ones who should report on OCs and SES. For parenting factors (i.e., are parents warm or rejective?), it is questionable whether parents or children should be informants. Reports from both parents and children may be subject to biases. The use of parent reports may be problematic because of the risk of giving socially desirable answers. On the other hand, the use of child reports may be problematic because these may be more influenced by the child's current mood or circumstances or dependent on the child's linguistic and cognitive abilities (Matson, Andrasik, & Matson, 2010). In chapter 4, we chose to measure parenting based on a child-report instead of a parent report, since the child's perception of parenting is likely to be more relevant for their own stress system than parent reports.

7.3 GENE-ENVIRONMENT (GXE) INTERACTION

7.3.1 Summary

Almost three decades ago, it has been proposed that genetic risk may interact with an adverse environment in predicting externalizing behavior problems (Cadoret et al., 1983). In this classical stress-vulnerability view of gene-environment interaction, outcomes are worse when genetic risk coincides with an adverse environment. Nowadays, there is still evidence of this diathesis-stress model when predicting externalizing behavior problems (Nederhof, Belsky, Ormel, & Oldehinkel, 2012). However, there is also increasing evidence for the differential susceptibility model that suggests not only that outcomes are worse when genetic risk coincides with an adverse environment, but also that outcomes are better when 'genetic risk' coincides with a supportive environment (Belsky, Bakermans-Kranenburg, & van IJzendoorn, 2007; Ellis, Boyce, Belsky, Bakermans-Kranenburg, & van Ijzendoorn, 2011).

There is evidence that both parenting (Leve et al., 2005; Reitz et al., 2006) and genetic risk (familial loading of externalizing behavior problems (FLE); Buschgens et al., 2009; DRD4 7-repeat allele; Schmidt, Fox, Rubin, Hu, & Hamer, 2002) are related to future externalizing behavior problems. So the question arises whether parenting and genetic risk interact in predicting externalizing behavior problems. Several studies showed such an interaction effect in school-age children (Bakermans-Kranenburg & van Ijzendoorn, 2006; Knafo et al., 2011; Propper et al., 2007; Sheese et al., 2007). Next to the potential moderating effect of genetic risk, gender may be a potential moderator in the relationship between parenting and externalizing behavior problems. That is, boys may be sensitive to other environmental risk factors than girls, and the whole pattern of gene-environment interaction effects may differ between boys and girls (Leve et al., 2005;

Miner & Clarke-Stewart, 2008; Rothbaum & Weisz, 1994). Based on the above mentioned studies and models, we hypothesized that: 1) negative parenting leads to higher levels of externalizing behavior problems, whereas positive parenting leads to lower levels of externalizing behavior problems, 2) the relationship between parenting and externalizing behavior problems is specific for boys rather than for girls, and 3) the presence of high genetic risk (i.e., FLE or the DRD4 7-repeat allele) interacts with negative parenting in that high genetic risks lead to higher levels of externalizing behavior problems in the presence than in the absence of negative parenting (in accordance with the diathesis-stress model). In addition, we explored whether genetic risk interacts with positive parenting in predicting lower levels of externalizing behavior problems (in accordance with the differential susceptibility model). In *chapter 5*, the first hypothesis was confirmed. More specifically, parental overprotection and parental rejection lead to higher levels of externalizing behavior problems two and a half year later, whereas parental emotional warmth leads to lower levels of externalizing behavior problems two and a half year later. These relationships appeared not to be gender-specific. Thus, the second hypothesis was not confirmed. As for the third hypothesis, firstly, the present study revealed an interaction effect between parental rejection and FLE in predicting externalizing behavior problems. Contrary to the diathesis-stress model, however, the relationship between parental rejection and externalizing behavior problems was present in low but not in high FLE families. This result suggest a competing risk model. That is, the effect of FLE was present when parental rejection was low and the effects of parental rejection was present when FLE was low. Secondly, we demonstrated that adolescents with an absent 4-repeat allele and high levels of perceived parental overprotection showed the highest levels of future externalizing behavior problems. The absence of the 4-repeat allele seems to make individuals more vulnerable in an environment with high perceived parental overprotection. This is in accordance with the diathesis-stress model.

In *chapter 6*, we also investigated gene-environment interaction. In this chapter, we looked at the interaction effect between the *SLC6A3* gene and three early risk factors (maternal smoking and alcohol use during pregnancy, and obstetric complications (OCs)) on ADHD symptom measures. Both the *SLC6A3* gene (Gizer et al., 2009) and the early risk factors mentioned (Banerjee et al., 2007; Ben Amor et al., 2005; Freitag et al., 2011; Linnet et al., 2003) are related to ADHD symptoms. Also, the interactive effects of genes and environment have been implicated in the etiology of ADHD (Buitelaar, 2005; Ficks & Waldman, 2009; Freitag et al., 2010; Nigg et al., 2010). Based on previous studies and models, we hypothesized that: 1) there is a main effect of the *SLC6A3* gene on ADHD symptoms, 2) there are main effects of maternal smoking and OCs on ADHD symptoms, and 3) there are interaction effects between the *SLC6A3* gene and early environmental risk factors on ADHD (hypotheses were formulated more specifically after presenting a short overview of previous studies on the interaction between the *SLC6A3* gene and early risk

factors on ADHD outcomes). The first hypothesis was not confirmed. However, the second hypothesis was confirmed: maternal smoking during pregnancy and OCs were related to more parent-reported ADHD symptoms. Also, the third hypothesis was confirmed. Firstly, adolescents carrying the risk allele of SNP rs40184 and a history of severe OCs had the highest level of parent-reported inattention symptoms. This finding is in accordance with the diathesis-stress model. Secondly, adolescents carrying the non-risk allele and prenatal alcohol exposure had the highest level of self-reported hyperactivity/impulsivity symptoms. This finding is in accordance with the competing risk model.

In sum, the studies described in chapters 5 and 6 of this thesis provide evidence for gene-environment interaction effects. These effects are in accordance with the diathesis-stress model and the competing risk model. However, we found no evidence of differential susceptibility. Whereas we revealed several main effects of (early) environmental factors, there were no main effects of genes. Below, we will describe some factors that may be related to the findings in chapters 5 and 6.

7.3.2 Ambiguous findings

Although there have been numerous studies on gene-environment interaction since it first came to light (Cadoret et al., 1983), findings are ambiguous. Firstly, where we were unable to replicate previous results, it is possible that results previously published are a consequence of false positive findings (Ioannidis, 2005). Secondly, the power to detect gene-environment interactions is low when incidence rates of the environmental risk factor is much lower or higher than 50% (Caspi et al., 2010). Obviously, in a population-based study like ours that was not purposefully built as a gene-environment interaction study through enrichment of the environmental exposure, incidence of environmental risk is lower than in a clinical sample. Thirdly, results may depend upon the environmental and genetic risk factors chosen. We will now turn to measurement of genetic risk factors.

7.3.3 Measurement of genetic risk

In chapters 5 and 6 we chose to include one single gene, the DRD4 and the *SLC6A3* gene respectively. There are good theoretical grounds for studying DRD4 in relation to externalizing behavior problems (i.e., the dopaminergic system is associated with lower reception effectiveness, which is related to maladjustment such as aggression (Bakermans-Kranenburg & van Ijzendoorn, 2006)) and DRD4 has often been studied in gene-environment interaction studies related to externalizing behavior problems (Bakermans-Kranenburg & van Ijzendoorn, 2006, 2007; Knafo et al., 2011; Propper et al., 2007; Sheese et al., 2007). However, it must be acknowledged that there are several other

genes that may serve as a moderator in gene-environment interaction studies related to externalizing behavior problems, such as DRD2 (Brennan et al., 2011; Nederhof et al., 2012), COMT (Brennan et al., 2011; Nederhof et al., 2012), CHRM2 (Dick et al., 2011), and GABRA2 (Dick et al., 2009). Also, in the study described in chapter 6, the *SLC6A3* gene is not the only possible candidate in a gene-environment interaction study related to ADHD symptoms. There are several other candidate genes (DRD4, DRD5, 5-HTT, HTR1B, and SNAP25) which appear to be involved in the etiology of ADHD (Gizer et al., 2009).

In chapter 5, genetic risk was also operationalized by familial loading of externalizing behavior problems (FLE). Since quantitative genetic studies indicate that the familial aggregation of externalizing disorders is mainly due to genetic factors (Burt, 2009), we assumed that familial loading reflects largely genetic risk, although a contribution of shared environmental influences cannot be ruled out. In sum, we acknowledge that FLE is not a pure measure of genetic risk and that several other genes could have been studied in chapters 5 and 6.

7.4 LIMITATIONS AND STRENGTHS

Specific limitations of the studies described in this thesis have been discussed in chapters 2 to 6. Here we will address two limitations for each of the main themes of this thesis, the HPA-axis (chapters 2 to 4) and gene-environment interaction (chapters 5 and 6) respectively. Firstly, a limitation of the HPA-axis studies is that they lack measures of challenge-induced cortisol secretion. We already discussed this topic in Section 7.2.4. Secondly, a limitation of the HPA-axis studies is that they were all cross-sectional (data from T1). Hence, the results of these studies gave no insight into the causality of the relationships found. Concerning the chapters on gene-environment interaction, one limitation is that the incidence rates of risk environments and risk genes are probably lower in a population-based study. Studies in which genotype and environmental risk are close to 50% (e.g. gene-environment interaction studies with balanced cell sizes) are better powered to test gene-environment interaction effects (Caspi et al., 2010). A second limitation of the studies on gene-environment interaction is that there are several potentially relevant genes (see Section 7.3.3) which were not analyzed in the present study.

The studies described in this thesis have in common that they are based on the TRAILS study, a very large population-based sample of early adolescents. A major strength of this sample is that it enabled us to detect small effects due to the power associated with large samples. Further, the size of the sample allowed us for the investigation of a variety of environments in chapters 3 to 6, that is early risk factors (obstetric complications (OCs), smoking and alcohol use during pregnancy), parenting factors, and SES.

7.5 SUGGESTIONS FOR FUTURE RESEARCH

Several suggestions for future research follow directly from the four limitations described in Section 7.4. Firstly, it is suggested to incorporate a measure of challenge-induced cortisol secretion. The added value of measuring challenge-induced cortisol secretion lies in the fact that stress reactivity directly measures a susceptibility to stressful challenge (Boyce & Ellis, 2005). While there are arguments against using such a measure which are mentioned in chapters 2 and 3 (see Section 7.2.4), this measure is particularly relevant for the study described in chapter 4, in which the evolutionary-developmental theory of BSC forms an important topic. Secondly, it is suggested to use prospective data. Longitudinal data on HPA-axis activity provide insight into the stability or instability of cortisol levels. The more stable the cortisol levels, the more likely that HPA-axis activity is determined by state factors (such as socio-economic status; described in chapter 4). There is already evidence that the CAR of a single day is determined largely by state factors and only for a small part by trait factors (Hellhammer et al., 2007). Thirdly, it is suggested to study clinical samples as well, since it is likely that these are more exposed to environmental risk. As said before, studies in which environmental risk is close to 50% are better powered to test gene-environment interaction effects (Caspi et al., 2010). Fourthly, it is suggested to investigate more genes. In addition to the genes described in Section 7.3.3, it would be interesting to include corticoid receptor genes.

The inclusion of corticoid receptor genes is particularly relevant since it represents a bridge between the two main themes of this thesis: the HPA-axis and gene-environment interaction. Both mineralocorticoid (MR) and glucocorticoid receptors (GR) may influence the HPA-axis during stress and during the circadian cycle (Klok et al., 2011). Also, both common functional MR gene variants (Derijk, 2009; Klok et al., 2011) and GR gene variants (Derijk, 2009) affect the HPA-axis. The inclusion of these gene variants may lead to a more complete understanding of the etiology of externalizing behavior problems.

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NEDERLANDSE SAMENVATTING

INLEIDING

Achtergrond

Kinderen en jongeren met externaliserende gedragsproblemen hebben te weinig controle over hun emoties en uiten dat op een negatieve manier naar buiten toe (*extern = uitwendig*). Hierbij valt te denken aan agressieve gedragingen (zoals vechten), delinquente gedragingen (zoals opzettelijke vernielingen), maar ook hyperactieve en impulsieve gedragingen (zoals niet stil kunnen zitten). Kinderen en jongeren met externaliserende gedragsproblemen zijn vaak een probleem voor hun gezin, de samenleving en, in een mindere mate, voor henzelf. Naar schatting komen externaliserende gedragsproblemen voor bij 5 tot 20 procent van de jongeren in Nederland, afhankelijk van het meetinstrument (vragenlijsten versus interviews), type gedragingen (diefstal komt relatief vaak voor), geslacht (jongens vertonen vaker gedragsproblemen dan meisjes) en leeftijd (er is een piek bij de leeftijd van 15 tot 17 jaar).

Tijdens de adolescentie (veelal gedefinieerd als de periode tussen de 10 en 20 jaar) komen externaliserende gedragsproblemen in toenemende mate voor. Maar waarom vertoont de ene adolescent zoveel meer externaliserende gedragsproblemen als de andere? Er zijn veel verschillende risicofactoren voor externaliserende gedragsproblemen, welke in te delen zijn in vier verschillende domeinen: het domein van het kind, het domein van de ouders en de opvoeding, het sociaal-culturele domein en het domein van de leeftijdsgenoten.

Bij het *domein van het kind* onderscheiden we geslacht, genetische factoren (erfelijkheid), biologische factoren (waaronder de HPA-as; later nader uitgelegd) en pre- en perinatale factoren (risicofactoren tijdens zwangerschap en bevalling). Bij het *domein van de ouders en de opvoeding* kan gedacht worden aan levensgebeurtenissen die binnen het gezin plaatsvinden (zoals ruzies tussen ouders), structurele kenmerken (zoals een eenoudergezin), kenmerken van de opvoeding (zoals een autoritaire opvoeding) en aan externaliserende gedragsproblemen bij de ouders zelf. Met het *sociaal-culturele domein* bedoelen we allerlei begrippen die vallen onder de noemer 'sociaal-economische status' (SES). Denk hierbij aan armoede, werkloosheid van (een van) de ouders en kenmerken van de buurt of wijk waarin de adolescent opgroeit. Ten slotte is het *domein van de leeftijdsgenoten* een belangrijk domein gebleken waaruit risicofactoren voor externaliserende gedragsproblemen naar voren komen. Meer specifiek gaat het hierbij bijvoorbeeld om leeftijdsgenoten die de adolescent in kwestie afwijzen of het hebben van 'foute vrienden'.

In dit proefschrift worden de afzonderlijke en gecombineerde effecten van verschillende risicofactoren onderzocht om op die manier een completer beeld te krijgen van de oorzaken van externaliserende gedragsproblemen bij adolescenten. Allereerst

wordt in hoofdstuk 2 tot en met 4 de rol van de HPA-as nader onderzocht door hierbij verschillende andere risicofactoren te betrekken. Ten tweede wordt in hoofdstuk 5 en 6 de rol van gen-omgevingsinteractie (later nader uitgelegd) bij externaliserende gedragsproblemen tijdens de adolescentie nader onderzocht.

De HPA-as

De 'hypothalamus-pituitary-adrenal-axis' (HPA-axis) is de Engelse aanduiding voor de hypothalamus-hypofyse-bijnier-as (ook wel afgekort als HPA-as). De HPA-as is een centraal systeem in het lichaam van de mens dat een belangrijke rol speelt bij stress. Het eindproduct van dit systeem is cortisol, dat ook wel het stress-hormoon wordt genoemd. Ook in normale, niet-stressvolle situaties is er sprake van cortisoluitscheiding door de bijnier volgens een min of meer vast dag- en nachtritme. 's Ochtends, direct na het ontwaken, stijgt de cortisoluitscheiding gedurende ongeveer een half uur (ook wel de 'cortisol ontwaak reactie' genoemd) waarna de cortisoluitscheiding in normale omstandigheden afneemt gedurende de rest van de dag.

In eerder onderzoek werd vaak beweerd dat adolescenten met externaliserende gedragsproblemen een lage HPA-as activiteit hebben en dus lage cortisol levels. De theorie hierachter is dat zij hierdoor meer geneigd zijn om stimulatie op te zoeken in de vorm van externaliserende gedragsproblemen. In eerdere onderzoeken werden echter voornamelijk jongens meegenomen en werd onvoldoende nagegaan of er sprake was van eventuele comorbide *(= het tegelijkertijd voorkomen van)* internaliserende gedragsproblemen (zoals angst en depressie). In hoofdstuk 2 van dit proefschrift wordt de rol van geslacht en comorbiditeit onderzocht binnen de relatie tussen de HPA-as en externaliserende gedragsproblemen. Vervolgens wordt in hoofdstuk 3 en 4 nagegaan in hoeverre omgevingsfactoren van invloed zijn op de HPA-as. Zo wordt in hoofdstuk 3 onderzocht in hoeverre pre- en perinatale factoren de HPA-as beïnvloeden. Het is al bekend dat er een relatie is tussen pre- en perinatale factoren en externaliserende gedragsproblemen, maar nog niet duidelijk of de HPA-as hierin een mediërende rol speelt. Wanneer dat het geval is, zouden pre- en perinatale factoren leiden tot neurobiologische tekorten en een verstoorde HPA-as, welke vervolgens gerelateerd is aan externaliserende gedragsproblemen. In hoofdstuk 4 wordt onderzocht in hoeverre SES en opvoeding een kromlijnig verband hebben met de HPA-as. De theorie hierachter is die van de 'biologische gevoeligheid voor context', waaruit het idee voortkomt dat extreme omgevingen verschillende voordelen kunnen bieden. Een zeer negatieve omgeving kan zorgen voor een reactieve HPA-as waardoor het beter reageert op mogelijke gevaren en een zeer positieve omgeving kan zorgen voor een reactieve HPA-as waardoor het meer profiteert van bronnen van steun.

Gen-omgevingsinteractie

Gen-omgevingsinteractie gebeurt wanneer de effecten van bepaalde genen afhankelijk zijn van bepaalde omgevingscondities. Volgens het klassieke kwetsbaarheid-stressmodel ontstaat de meest negatieve uitkomst wanneer een genetisch risico samengaat met een negatieve omgeving. Dit model wordt ook nog vaak als uitgangspunt gehanteerd wanneer men externaliserende gedragsproblemen wil voorspellen. Daarnaast is het model van differentiële kwetsbaarheid steeds meer in opkomst. Volgens dit model ontstaat een positieve uitkomst wanneer een genetisch risico samengaat met een positieve omgeving.

In hoofdstuk 5 van dit proefschrift wordt gen-omgevingsinteractie onderzocht door na te gaan in hoeverre opvoeding interacteert met een genetisch risico (het DRD4 gen en aanwezigheid van externaliserende gedragsproblemen bij de ouders zelf) in het voorspellen van toekomstige externaliserende gedragsproblemen. Ook wordt nagegaan of er hoofdeffecten zijn van opvoeding en het genetische risico. Tenslotte wordt in dit hoofdstuk nagegaan of de relatie tussen opvoeding en externaliserende gedragsproblemen afhankelijk is van geslacht, aangezien jongens mogelijk gevoelig zijn voor andere omgevingsinvloeden dan meisjes en gen-omgevingsinteractie mogelijk in zijn geheel verschilt tussen jongens en meisjes. In hoofdstuk 6 wordt eveneens gen-omgevingsinteractie onderzocht. In dit hoofdstuk wordt nagegaan in hoeverre drie vroege omgevingsfactoren (1. roken tijdens de zwangerschap, 2. alcoholgebruik tijdens de zwangerschap en 3. pre- en perinatale risicofactoren) interacteren met een genetisch risico (het *SLC6A3* gen) in het voorspellen van ADHD symptomen. Ook wordt nagegaan of er hoofdeffecten zijn van de vroege omgevingsfactoren en het genetische risico. Tenslotte wordt in dit hoofdstuk een overzicht gegeven van eerdere studies die de interactie tussen vroege omgevingsfactoren en het *SLC6A3* gen onderzochten in relatie tot ADHD (symptomen of diagnose), waarna gen-omgevingsinteractie binnen ons eigen onderzoek wordt bepaald.

HET TRAILS-ONDERZOEK

In alle onderzoeken die in dit proefschrift beschreven staan is gebruik gemaakt van data uit het TRAILS-onderzoek. TRAILS staat voor 'TRacking Adolescents' Individual Lives Survey' en is een grootschalig onderzoek waarin ruim 2000 adolescenten uit Noord-Nederland worden gevolgd van hun 10^{e} tot 25^{e} levensjaar. Het doel van het TRAILS-onderzoek is het in kaart brengen van de geestelijke gezondheid vanaf het begin van de adolescentie tot aan de volwassenheid, zowel op het niveau van psychopathologie als op het niveau van onderliggende kwetsbaarheden en omgevingsrisico´s. Adolescenten worden twee- tot driejaarlijks onderzocht. De onderzoeken die in dit proefschrift staan beschreven hebben betrekking op de eerste (T1), tweede (T2), en/of de derde (T3) meting van TRAILS, welke

respectievelijk liepen van maart 2001 tot juli 2002, september 2003 tot december 2004 en van september 2005 tot december 2007.

In de eerste drie metingen hebben de adolescenten, leerkrachten, ouders en broers en zussen vragenlijsten ingevuld en hebben de adolescenten meegedaan aan verschillende lichamelijke metingen. Op basis van de vragenlijsten zijn onder andere de volgende variabelen gemeten die voor dit proefschrift relevant zijn: de mate van (externaliserend en internaliserend) probleemgedrag (adolescent vragenlijst, oudervragenlijst en leerkrachtvragenlijst), de mate van probleemgedrag (psychopathologie) bij ouders (oudervragenlijst), pre- en perinatale factoren (oudervragenlijst), SES (oudervragenlijst) en ervaren opvoeding (adolescent vragenlijst). Daarnaast is de hoeveelheid cortisol gemeten in het speeksel van de adolescenten en DNA geïsoleerd uit het bloed of het wangslijmvlies van de adolescenten.

RESULTATEN

De HPA-as

In hoofdstuk 2 werd onderzocht of er een relatie was tussen cortisol levels en externaliserende gedragsproblemen en of geslacht en comorbide internaliserende gedragsproblemen hierbij een rol speelden. In dit onderzoek werd gevonden dat meisjes met zuiver externaliserende gedragsproblemen (dus zonder comorbide internaliserende gedragsproblemen) hogere HPA-as activiteit hadden dan jongens met zuiver externaliserende gedragsproblemen. Dit uitte zich zowel in de totale cortisol levels na het ontwaken als in de stijging van cortisol tijdens het ontwaken.

Er is dus een relatie gevonden tussen de HPA-as en externaliserende gedragsproblemen in meisjes. De volgende vraag is dan: welke factoren zijn er verantwoordelijk voor dat cortisol levels van persoon tot persoon verschillen? In hoofdstuk 3 werd onderzocht of pre- en perinatale factoren de HPA-as beïnvloeden. Hoewel wij een verband vonden tussen pre- en perinatale factoren en externaliserende gedragsproblemen, vonden wij geen verband tussen pre- en perinatale factoren en de HPA-as van adolescenten.

In hoofdstuk 4 werd onderzocht of andere omgevingsfactoren de HPA-as beïnvloedden, namelijk SES en opvoeding. We vonden een kromlijnig verband tussen SES en de HPA-as (zowel in de totale cortisol levels na het ontwaken als in de stijging van cortisol tijdens het ontwaken). Dat wil zeggen dat adolescenten die zijn opgegroeid in lage SES-gezinnen én in hoge SES-gezinnen lage HPA-as activiteit vertonen, terwijl adolescenten die in middelmatige SES-gezinnen zijn opgegroeid hoge HPA-as activiteit vertonen. Daarnaast vonden we dat ouderlijke warmte samen ging met lagere HPA-as activiteit bij de adolescenten.

Gen-omgevingsinteractie

In hoofdstuk 5 werd gen-omgevingsinteractie onderzocht door na te gaan in hoeverre opvoeding interacteert met een genetisch risico (het DRD4 gen en aanwezigheid van externaliserende gedragsproblemen bij de ouders zelf) in het voorspellen van toekomstige externaliserende gedragsproblemen. We vonden allereerst hoofdeffecten van opvoeding: ouderlijke overbescherming en ouderlijke afwijzing leidden tot meer externaliserende gedragsproblemen twee en een half jaar later en ouderlijke warmte leidde tot minder externaliserende gedragsproblemen twee en een half jaar later. Deze relaties waren niet specifiek voor jongens of meisjes. Ook vonden we in dit onderzoek twee bewijzen voor gen-omgevingsinteractie. Ten eerste vonden we dat afwezigheid van ouderlijke afwijzing (positieve omgeving) vaker leidde tot externaliserende gedragsproblemen wanneer de ouders zelf externaliserende gedragsproblemen vertoonden (aanwezigheid genetisch risico) dan wanneer de ouders geen externaliserende gedragsproblemen vertoonden (afwezigheid genetisch risico). Deze bevinding is strijdig met het klassieke kwetsbaarheid-stressmodel. Ten tweede bleken adolescenten die geen DRD4 -4R allel hadden (aanwezigheid genetisch risico) die tegelijkertijd ouderlijke overbescherming ervaarden (negatieve omgeving), vaker externaliserende gedragsproblemen te vertonen in de toekomst. Deze bevinding is wel in overeenstemming met het klassieke kwetsbaarheid-stressmodel.

Ook in hoofdstuk 6 werd gen-omgevingsinteractie onderzocht. In dit hoofdstuk werd nagegaan in hoeverre drie vroege omgevingsfactoren interacteren met een genetisch risico (het *SLC6A3* gen) in het voorspellen van ADHD symptomen. We vonden geen hoofdeffect van het *SCL6A3* gen, maar wel van roken tijdens de zwangerschap en pre- en perinatale risicofactoren. Beide vroege omgevingsfactoren gingen samen met meer (door ouders gerapporteerde) ADHD symptomen bij de adolescenten. Ook in dit onderzoek vonden we twee bewijzen voor gen-omgevingsinteractie. Ten eerste bleken adolescenten die het risico allel van de SNP rs40184 van het *SLC6A3* gen hadden (aanwezigheid genetisch risico) wiens moeder ernstige pre- en perinatale complicaties had ervaren (negatieve omgeving) meer (door ouders gerapporteerde) aandachtsproblemen te vertonen. Deze bevinding is in overeenstemming met het klassieke kwetsbaarheid-stressmodel. Ten tweede bleken adolescenten die het niet-risico allel van de SNP rs40184 van het *SLC6A3* gen hadden (afwezigheid genetisch risico) wiens moeder alcohol had gebruikt tijdens de zwangerschap (negatieve omgeving) meer (door adolescenten zelf gerapporteerde) hyperactiviteit- en impulsiviteitsymptomen te vertonen. Deze bevinding is in overeenstemming met het 'strijdige risico's model'.

CONCLUSIE

Samenvattend heeft dit proefschrift in hoofdstuk 2 tot en met 4 laten zien dat meisjes met zuiver externaliserende gedragsproblemen een bijzondere groep vormen, aangezien zij verhoogde HPA-as activiteit vertonen. Zij hebben zowel hogere totale cortisol levels na het ontwaken als een hogere stijging van cortisol tijdens het ontwaken, in vergelijking tot jongens met zuiver externaliserende gedragsproblemen. Zowel ouderlijke warmte als SES bleken gerelateerd te zijn aan HPA-as activiteit. Pre- en perinatale factoren bleken niet gerelateerd aan HPA-as activiteit, maar wel aan externaliserende gedragsproblemen. Echter, er moet erkend worden dat de aan de HPA-as gerelateerde effecten over het algemeen zwak waren en soms strijdig met de vooraf gestelde hypotheses.

Daarnaast hebben de onderzoeken die staan beschreven in hoofdstuk 5 en 6 enig bewijs laten zien voor gen-omgevingsinteractie. Deze effecten waren in overeenstemming met het klassieke kwetsbaarheid-stressmodel en het strijdige risico's model. We hebben geen bewijs gevonden voor het model van differentiële kwetsbaarheid. Naast de gen-omgevingsinteractie effecten, vonden we enige hoofdeffecten van omgevingsfactoren, maar geen hoofdeffecten van genetische factoren.

In toekomstig onderzoek kan er mogelijk een brug worden geslagen tussen de twee hoofdthema's van dit proefschrift: de HPA-as en gen-omgevingsinteractie. Zowel receptoren van mineralocorticoïden (MR) als die van glucocorticoïden (GR) beïnvloeden mogelijk de HPA-as gedurende stress en gedurende het dag- en nachtritme. Genetische varianten van deze receptoren kunnen dus ook de HPA-as beïnvloeden. Het toevoegen van deze genetische varianten maakt de kennis over de oorzaken van externaliserende gedragsproblemen mogelijk nog completer.

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CURRICULUM VITAE

Rianne Marsman werd op 10 april 1982 geboren te Raalte. In 2000 behaalde zij haar vwo-diploma aan het Carmel College Salland te Raalte, waarna zij startte met de studie Psychologie aan de Rijksuniversiteit Groningen. Zij koos voor de afstudeerrichting Ontwikkelingspsychologie en de nevenrichting Klinische Psychologie. In haar derde studiejaar verzorgde zij lessen Statistiek voor eerstejaars Psychologie studenten in de functie van student-assistent. In haar laatste studiejaar behaalde zij de aantekening psychodiagnostiek door twee praktijkstages te lopen. Tevens deed zij in dat jaar onderzoek naar de magnocellulaire dysfunctie van dyslectici en schreef hierover haar scriptie. In 2004 behaalde zij haar doctoraalexamen.

Vanaf april 2005 startte zij als junior onderzoeker, verbonden aan de afdeling Psychiatrie van het Universitair Medisch Centrum St. Radboud. De eerste twee jaren van haar promotieonderzoek was zij werkzaam aan het Universitair Medisch Centrum Groningen om aldaar een bijdrage te leveren aan de dataverzameling van het TRAILS (TRacking Adolescents' Individual Lives Survey) onderzoek. Vervolgens werkte zij twee jaren op de afdeling Psychiatrie van het Universitair Medisch Centrum St Radboud om aldaar de onderzoeksresultaten te beschrijven onder begeleiding van prof. dr. J.K. Buitelaar. Deze onderzoeksresultaten staan beschreven in dit proefschrift. Vanaf april 2009 is Rianne werkzaam als docent bij de opleiding Toegepaste Psychologie bij de Academie Mens en Arbeid van Saxion hogeschool te Deventer waar zij onder meer afstudeeronderzoeken begeleidt, lessen verzorgt en onderwijs ontwikkelt binnen de leerlijn Toegepast Onderzoek en de hoofdstroom Ontwikkelingspsychologie en Orthopedagogiek.