Infant attachment and stress regulation : a neurobiological study

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Printed by Mostert en van Onderen!, Leiden

Cover design by Nanette Lindeman

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 9 december 2010
klokke 10.00 uur

door

Maartje Pieternel Caroline Maria Luijk

geboren te Spijkenisse
in 1983

Promotiecommissie

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The Generation R Study is conducted by the Erasmus Medical Center in close collaboration with the School of Law and Faculty of Social Sciences of the Erasmus University Rotterdam, the Municipal Health Service Rotterdam area, Rotterdam, the Rotterdam Homecare Foundation, Rotterdam and the Stichting Trombosedienst & Artsenlaboratorium Rijnmond (STAR), Rotterdam. We gratefully acknowledge the contribution of general practitioners, hospitals, midwives and pharmacies in Rotterdam. The first phase of the Generation R Study is made possible by financial support from the Erasmus Medical Center, Rotterdam, the Erasmus University Rotterdam and the Netherlands Organization for Health Research and Development (ZonMw). The present study was supported by additional grants from the Netherlands Organization for Scientific Research (grant no. 400-04-182, grant no. 452-04-306 (VIDI), and NWO SPINOZA prize).

Contents

1 General introduction

Attachment and stress regulation: a study on vulnerability and plasticity

Vulnerability and plasticity are key concepts in infant development (Belsky, Hsieh, & Crnic, 1998; Rutter, 2006). Depending on the environment, vulnerabilities can predispose children to adverse outcomes. Plasticity on the other hand, is thought to induce both adverse *and* favorable outcomes, depending on the environment. For full comprehension of the attachment relationship formation, which is a developmental milestone in infancy, the identification of potential vulnerability and plasticity factors is essential, as insecure and disorganized attachments are major risk factors for later-life psychopathology (Fearon et al., 2010; Sroufe, Egeland, Carlson, & Collins, 2005). Parental and, more recently, neurobiological aspects have been associated with attachment quality (De Wolff & Van IJzendoorn, 1997; Fox & Hane, 2008), and also the interaction between these factors is of great interest to attachment researchers (Bakermans-Kranenburg & Van IJzendoorn, 2007). In the current thesis, vulnerability and plasticity in attachment and stress regulation are studied in the largest population based attachment cohort to date.

Importance of quality of care

Early experiences have been shown to influence the behavioral and physiological organization of infants. Studies in humans and other animals document that deprivation of care has a major impact on the infant's developing system of stress regulation (Boyce, Champoux, Suomi, & Gunnar, 1995; Caldji et al., 1998; Carlson & Earls, 1997; Levine & Wiener, 1988; Liu et al., 1997; Meaney, 2001; Plotsky & Meaney, 1993). In the first year of life, regulation and coping are primarily externally organized, which makes the caregiver's responses to the infant's distress an important source of coping (Van Bakel & Riksen-Walraven, 2004). The availability of responsive, sensitive care is thought to promote infant attachment security and to mediate the infant's response to stressors (Gunnar & Donzella, 2002). Through their history of care, infants learn to what extent the caregiver is emotionally available in times of stress. Variation in parental availability is expected to lead to differences in attachment quality in the infant (Sroufe, 1997). Maternal sensitive responsiveness to the baby's signals is considered to be an important determinant of attachment security (Ainsworth, Blehar, Waters, & Wall, 1978; Bakermans-Kranenburg, Van IJzendoorn & Juffer, 2003), whereas extreme insensitivity and, potentially, psychopathology elevate the risk for insecure attachment (Cummings & Davies, 1994; Out, Bakermans-Kranenburg & Van IJzendoorn, 2009).

The case of depression. Maternal depression has been associated with attachment quality. Depression is thought to compromise sensitive parenting behavior, which in turn can undermine the development of a secure attachment relationship. However, the empirical evidence for this association is not unequivocal (see Cummings & Davies, 1994). Research on severe and chronic depression, as well as studies with clinical samples showed significant associations between maternal depression and attachment insecurity (e.g. Teti, Gelfand, Messinger, & Isabella, 1995). In community-based samples, however, the effect of maternal depressive symptoms on attachment quality is less clear; meta-analyses reported small or even insignificant effect sizes (Atkinson et al., 2000; Van IJzendoorn, Schuengel & Bakermans-Kranenburg, 1999). Other studies suggested that pre- and postnatal depression might influence mother-child interaction (Lundy et al., 1999; Righetti-Veltema, Bousquet & Manzano, 2003). Depression may also negatively influence infants' physiological regulation. More specifically, in several studies maternal depression was related to higher levels of stress hormones in infants, which could indicate both environmental and biological mechanisms of transmission (Ashman, Dawson, Panagiotides, Yamada, & Wilkinson, 2002; Essex, Klein, Cho, & Kalin, 2002; Halligan, Herbert, Goodyer, & Murray, 2004; Lupien, King, Meaney, & McEwen, 2000; Young, Vazquez, Jiang, & Pfeffer, 2006).

Attachment and stress regulation: cortisol

Hertsgaard, Gunnar, Erickson, and Nachmias (1995) suggested that assessment of cortisol levels may be particularly useful in attachment research. The neuroendocrine system is stimulated when coping behaviors are inadequate or coping sources are unavailable, which are crucial aspects of unresponsive maternal care and subsequent insecure attachment relationships. Cortisol is released as a result of many aspects of an organism's interaction with the environment, including response to novelty and psychological stressors (Gunnar, 1994; Kirschbaum & Hellhammer, 1989, 1994). In normal situations, production of cortisol follows a diurnal rhythm with high levels at awakening, an increase in secretion shortly after awakening, followed by a decline throughout the day (Kirschbaum & Hellhammer, 1989; Watamura, Donzella, Kertes, & Gunnar, 2004). This diurnal rhythm in basal cortisol levels is relatively stable in adults, but early in life the Hypothalamic-Pituitary-Adrenal (HPA) system shows instability, and it continues to mature throughout infancy and childhood (De Weerth & Van Geert, 2002; De Weerth, Zijl, & Buitelaar, 2003; Watamura et al., 2004).

Various studies have tested the effect of stressful events on HPA-axis functioning in infants, most of them focusing on cortisol levels around the stressful Strange Situation Procedure (SSP; Ainsworth, Blehar, Waters & Wall, 1978) as related to infant attachment classification. Several non-clinical studies on physiological reactions to the SSP documented children's tendency to show elevated cortisol levels after the procedure. The most consistent finding is that

no or only little adrenocortical activation is observed in securely attached infants (Gunnar, Brodersen, Nachmias, Buss, & Rigatuso, 1996; Spangler & Grossmann, 1993). Several studies reported increases in cortisol levels for disorganized infants (Hertsgaard et al., 1995; Spangler & Grossmann, 1993; Spangler & Schieche, 1998), whereas results for both insecure-avoidant and insecure-resistant groups are equivocal. In some studies, both insecure groups were found to have raised cortisol levels after the SSP (Spangler & Grossmann, 1993), others found increased cortisol levels only for insecure-resistant children (Spangler & Schieche, 1998).

Until now, studies have only investigated attachment in relation to stress reactivity, neglecting the relation between attachment and infant diurnal rhythm of cortisol excretion (but see Adam & Gunnar, 2001, for diurnal rhythm and attachment status in adults). However, variation in cortisol reactivity for the different attachment categories may be related to systematic differences in diurnal rhythms.

Genetics of stress regulation

Associations between attachment quality and cortisol levels implicate that cortisol levels are, at least partly, determined by the caregiving environment (Meaney, 2001; Gunnar & Quevedo, 2007). Genetic factors have not received much attention, although there is ample evidence that these play a role in explaining variance in HPA-axis activity (Bartels et al., 2003; Steptoe et al., 2009; Wüst et al., 2004a). Recently, specific candidate genes involved in explaining variability in cortisol levels have been identified. Several studies focused on the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), which mediate many of the effects of mineralocorticoids and glucocorticoids, respectively. Within GR, several molecules, so-called chaperones and co-chaperones, play a critical role. An important co-chaperone is the FKBP5 gene. Genetic variants of the GR, MR and FKBP5 gene (single nucleotide polymorphisms; SNPs), appear to contribute to interindividual variability in HPA-axis and are crucial in the onset and recovery from stress. This in turn is essential for healthy physiological and behavioral regulation (Binder, 2009; Ising et al., 2008; Kumsta et al., 2007; Wüst et al., 2004b). As the infant-parent attachment relationship can be considered the infant's most important emotion regulation system (Bowlby, 1969/1982, Cassidy, 1994), the role of a genetic factor influencing homeostasis might be of great importance.

Genetics of attachment

Next to a genetic factor involved in stress regulation, the 'usual suspects' (Ebstein, 2010), genetic variants in the dopaminergic, serotonergic, oxytonergic, and neuronal plasticity systems, may play a role in the quality of infants' attachment behavior. The dopaminergic system is involved in attentional, motivational, and reward mechanisms (Robbins & Everitt, 1999). Common variations in dopaminergic genes DRD4 48 bp VNTR, DRD4 -521C/T, DRD2/ANKK1 and

COMT Val158Met are associated with regulation of dopamine levels (D'Souza & Craig, 2006). Carrying the minor allele of these polymorphisms (respectively, DRD4 48 bp 7-repeat; DRD4 -521 C; DRD2/ANKK1 T[A1]) has been related to variations in infant temperament (Ebstein, 2006) and ADHD (Faraone & Khan, 2006). A protective effect has been reported for COMT heterozygotes (Val/Met) showing dopamine levels associated with optimal neurobehavioral outcomes, compared with both homozygous groups (Wahlstrom, White & Luciana, 2010). The serotonin system is involved in affect and emotion. The short variant of the serotonin transporter gene 5-HTT (5-HTTLPR) is associated with less efficient transcription and serotonin uptake in the synapse (Greenberg et al., 1999; Heils et al., 1996), and is related to psychiatric disorders (Ebstein, 2006; Rutter, 2006). The oxytonergic system is related to social and parenting behaviors, and both oxytocin levels and variants in the oxytocin receptor gene (OXTR rs53576 and rs2254298; in particular the minor A-allele) are associated with the formation of social bonds in both human and animal studies (Bakermans-Kranenburg & Van IJzendoorn, 2008; Carter et al., 2009; Feldman et al., 2010, Insel, 2010). Finally, brain-derived neurotrophic factor (BDNF) is a protein associated with neuronal growth and survival (Gizer, Ficks & Waldman, 2009). The gene coding for this protein, also called BDNF, contains a polymorphism influencing secretion of BDNF in the brain. This polymorphism (especially the minor Met-allele) is associated with ADHD (Gizer, Ficks & Waldman, 2009) and responses to stress and adversity; children with the Met-allele exposed to early deprivation manifest increased anxiety (Casey et al., 2009). Several studies have been undertaken to identify potential attachment genes (Bakermans-Kranenburg & Van IJzendoorn, 2004; 2007; Lakatos et al., 2000; Spangler, Johann, Ronai & Zimmerman, 2009), providing confusing results which call for replication in a large, ethnically homogeneous sample (Burmeister, McInnis & Zollner, 2008).

Gene-environment interaction

Both environmental factors and genes may affect the attachment relationship and infant stress regulation. The most important effects on child development are probably hidden in interactions between genetic and environmental factors (Barry, Kochanska & Philibert, 2008; Belsky et al., 2009). Gene-environment interactions can take various forms. One is a double risk model (or diathesis stress model; Rutter, 2006), in which some individuals are at heightened risk – because of their genetic make-up – for negative outcomes in the face of adversity, whereas persons without the genetic vulnerability are less affected by adversity (e.g. Caspi et al, 2002). Another specific type of gene-environment interaction is known as differential susceptibility (Belsky, Bakermans-Kranenburg & Van IJzendoorn, 2007; Belsky et al., 2009), where certain genes are thought to render individuals more responsive than others to both positive *and* negative environmental experience. In other words: 'the very same individuals who may be most adversely

affected by many kinds of stressors, may simultaneously reap the most benefit from environmental support and enrichment' (Belsky et al., 2007). In this model, individuals are thought to vary in their plasticity to environmental influences, and studies on GxE interaction in attachment may benefit from a shift from a conventional model of vulnerability genes, or 'risk alleles', to a focus on plasticity or susceptibility.

Attachment in Generation R

The influences of environmental and genetic factors on attachment and stress regulation were studied in the Generation R study. The Generation R study was designed to identify early environmental and genetic determinants of growth, development and health from fetal life into young adulthood in Rotterdam, the Netherlands (Jaddoe et al., 2007; 2008). Detailed measurements of the child's development were obtained in a rather homogeneous subgroup: The Generation R Focus Study. Only children of Dutch national origin were included in this group, meaning that the children, their parents and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research center regularly for various somatic and behavioral assessments (see Figure 1). The Generation R study provides ample information for investigating research questions on environmental and biological factors involved in attachment and stress regulation, and is the largest study with data on attachment, observed parenting and biological markers to date.

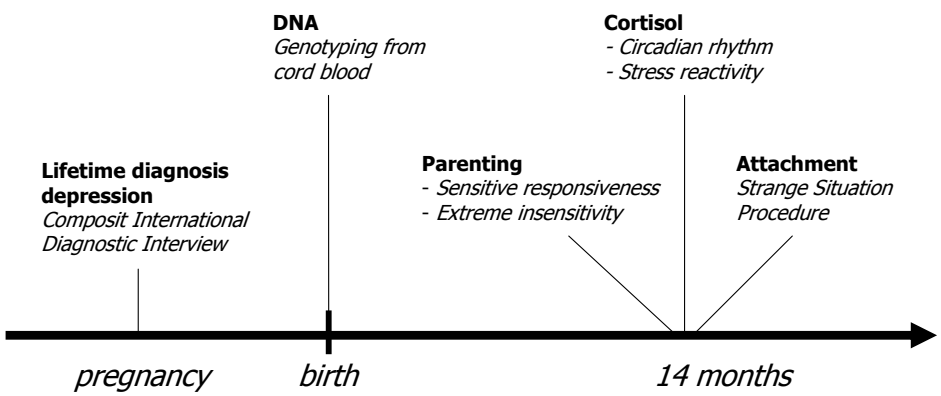

Figure 1. *Assessments in Generation R used in current thesis*

Aims of the study

The general aim of the current thesis is to provide more insight into the role of parental and biological factors in the development of the infant-mother attachment relationship. Both observational and experimental measures were used to assess

these associations, including observed behavior, physiological and genetic markers, and interviews. The main focus of Chapter 2 is the association between quality of attachment and cortisol levels, both cortisol stress reactivity and cortisol circadian rhythm. Moreover, the moderating effect of maternal depression on this association is explored. Chapter 3 extends the current knowledge on cortisol and attachment by adding a genetic component. In Chapter 4 we examine the interaction between genes and early caregiving environment on attachment security. Chapter 5 gives an overview of the molecular genetics of attachment, presenting the findings of an investigation in collaboration with the NICHD Study of Early Child Care and Youth Development (SECCYD). The effects of candidate genes on attachment quality are tested in a large-scale combination of two birth cohorts, providing a unique possibility for immediate replication.

2 Attachment, depression, and cortisol: Deviant patterns in insecure-resistant and disorganized infants

Maartje P. C. M. Luijk, Nathalie Saridjan, Anne Tharner, Marinus H. van IJzendoorn, Marian J. Bakermans-Kranenburg, Vincent W.V. Jaddoe, Albert Hofman, Frank C. Verhulst, and Henning Tiemeier (2010). Developmental Psychobiology, 52, 441-452.

Abstract

Both attachment insecurity and maternal depression are thought to affect infants' emotional and physiological regulation. In the current study, Strange Situation Procedure (SSP) attachment classifications, and cortisol stress reactivity and diurnal rhythm were assessed at 14 months in a prospective cohort study of 369 mother-infant dyads. Maternal lifetime depression was diagnosed prenatally using the Composite International Diagnostic Interview (CIDI). Insecure-resistant infants showed the largest increase in cortisol levels from pre to post SSP; the effect was even stronger when they had depressive mothers. Disorganized children showed a more flattened diurnal cortisol pattern compared to non-disorganized children. Findings are discussed from the perspective of a cumulative risk model.

Introduction

The infant-parent attachment relationship can be considered the infant's most important emotion regulation system (Bowlby, 1969/1982; Cassidy, 1994), since regulation is primarily externally organized in the first year of life. Early experiences are thought to shape the attachment relationship and thereby influence the regulation of behavioral and physiological responses. Most studies of the physiology of attachment relationships focused on measures of heart rate and cortisol during the Strange Situation Procedure (SSP, Ainsworth, Blehar, Waters, & Wall, 1978; e.g. Gunnar, Mangelsdorf, Larson & Hertsgaard, 1989; Oosterman & Schuengel, 2007; Sroufe & Waters, 1977). The current study includes the largest sample to date, which makes it possible to address issues of stress reactivity on the level of the various insecure attachment classifications. Furthermore, we examine the moderating role of maternal depression.

Early experiences have been shown to influence the behavioral and physiological organization of infants. Studies in humans and other animals

document that deprivation of care has a major impact on the infant's developing system of stress regulation (Boyce, Champoux, Suomi, & Gunnar, 1995; Caldji et al., 1998; Carlson & Earls, 1997; Levine & Wiener, 1988; Liu et al, 1997; Meaney, 2001; Plotsky & Meany, 1993). In relatively low-risk populations, differences in quality of care can predict differences in infant stress regulation. In the first year of life, regulation and coping are primarily externally organized. This makes the caregiver's responses to the infant's distress an important source of coping (Van Bakel & Riksen-Walraven, 2004). The availability of responsive, sensitive care is thought to promote infant attachment security and to mediate the infant's response to stressors (Gunnar & Donzella, 2002). Through their history of care, infants learn to what extent the caregiver is emotionally available in times of stress. Variation in parental availability, i.e. consistent sensitivity, inconsistent sensitivity, and consistent insensitivity, may lead to different secure and insecure attachment strategies in the infant (Sroufe, 1997).

Infants of consistently sensitive parents learn to expect their parents to be available in times of stress and have increased chances for developing a secure attachment relationship with their parent, which provides them with a powerful coping mechanism to regulate stressful stimuli. In contrast, infants of inconsistently sensitive or consistently insensitive parents do not come to expect their parents to be available in stressful situations. As a consequence, these children develop insecure attachment relationships with their parents. Insecure-resistant infants maximize their distress signals in order to get their parent's attention, whereas insecure-avoidant infants minimize signs of distress as they have learnt that they might be rejected (Main, 1990). In both cases the insecure children manage to create the best possible proximity to an attachment figure who is not optimally available. When the parent is extremely insensitive or even frightening, parental behaviors may cause a temporary breakdown in the child's strategy to keep close to the attachment figure which leads to dysregulation of negative emotions, as apparent in a disorganized attachment relationship (Main & Solomon, 1990).

Hertsgaard, Gunnar, Erickson, and Nachmias (1995) suggested that assessment of cortisol levels may be particularly useful in attachment research because the neuroendocrine system is believed to be stimulated when coping behaviors are inadequate or coping sources are unavailable. Studies on attachment quality and cortisol have focused mainly on stress reactivity, with assessment of cortisol levels before and after a potentially stressful event. The Strange Situation Procedure has often been used as the stressful event, as it is based on a series of brief infant-caregiver separations and reunions. The SSP is the gold standard procedure to assess the quality of the attachment relationship. Other methods of observing attachment quality, such as the Attachment Q-set (AQS; Waters, 1995) have not been widely used in cortisol research (but see Oosterman & Schuengel, 2007; Van Bakel & Riksen-Walraven, 2004).

Observations of infant behavior in the SSP allow for classification of infant behavior patterns into secure, insecure-avoidant, and insecure-resistant strategies. A secure (B) child seeks contact with the parent upon reunion, either physically or by distance interaction, to be comforted or reassured after the separation and resume exploration of the environment when he/she is settled. Insecure-avoidant (A) children, on the other hand, focus on the environment at the moment of reunion, ignoring the parent or even turning away from the parent. The reunion behavior of an insecure-resistant (C) child is characterized by anxious contact seeking and clinging and at the same time resisting contact with the parent. Resistant children are usually clearly distressed and their interaction with the parent may have an angry quality. On top of these classifications, the level of disorganization can be determined. Disorganized (D) children show a temporary breakdown of their secure, avoidant or resistant strategy of dealing with the return of the parent after separation, for example by simultaneous display of contradictory behaviors such as distress and avoidance (Main & Solomon, 1990).

Cortisol is released as a result of many aspects of an organism's interaction with the environment, including response to novelty and psychological stressors (Gunnar, 1994; Kirschbaum & Hellhammer, 1989; 1994). In normal situations, production of cortisol follows a diurnal rhythm with high levels at awakening, an increase in secretion shortly after awakening, followed by a decline throughout the day (Kirschbaum & Hellhammer, 1989; Watamura, Donzella, Kertes, & Gunnar, 2004). This diurnal rhythm in basal cortisol levels is relatively stable in adults, but early in life the Hypothalamic-Pituitary-Adrenal (HPA) system shows instability, and it continues to mature throughout infancy and childhood (De Weerth & Van Geert, 2002; De Weerth, Zijl, & Buitelaar, 2003; Watamura et al., 2004).

In stressful conditions, cortisol levels may rise. Cortisol response to stress serves an important function in adaptation to novel or stressful circumstances (Gunnar & Donzella, 2002; Van Bakel & Riksen-Walraven, 2004). Various studies have tested the effect of stressful events on HPA-axis functioning in infants, most of them focusing on cortisol levels around the SSP as related to infant attachment classification. Several non-clinical studies on physiological reactions to the SSP documented children's tendency to show elevated cortisol levels after the procedure. The most consistent finding is that no or only little adrenocortical activation is observed in securely attached infants (Gunnar, Brodersen, Nachmias, Buss, & Rigatuso, 1996, Spangler & Grossmann, 1993). Several studies reported increases in cortisol levels for the disorganized infants (Hertsgaard et al., 1995; Spangler & Grossmann, 1993; Spangler & Schieche, 1998).

Results for both insecure-avoidant and insecure-resistant groups are equivocal. In some studies, both insecure groups were found to have raised cortisol levels after the SSP (Spangler & Grossmann, 1993), others found increased cortisol levels only for insecure-resistant children (Spangler & Schieche, 1998). Spangler and Schieche (1998) interpreted their findings for the insecure-resistant group

as supporting an arousal model, assuming associations between behavioral and physiological activation during stress. This model implies that temperamental factors are possibly involved in the physiology of attachment. For example, as found by Gunnar and Donzella (2002), more reactive and irritable children display higher levels of cortisol when faced with a stressor, especially when they have an insecure attachment relationship. However, the aforementioned studies involved relatively small samples, and larger samples with substantial numbers of children in each of the attachment classification groups are needed to draw firmer conclusions on the association between attachment and cortisol reactivity and diurnal rhythm.

Until now, studies have only investigated attachment in relation to stress reactivity, neglecting the relation between attachment and infant diurnal rhythm of cortisol excretion (but see Adam & Gunnar, 2001, for diurnal rhythm and attachment status in adults). However, differences in cortisol reactivity for the different attachment categories may be related to systematic differences in slope of their diurnal rhythms. Although considerable intra- and inter-individual variation is found in cortisol diurnal rhythm in young infants (De Weerth & Van Geert, 2002) some stability after the first birthday has been suggested (Larson, White, Cochran, Donzella, & Gunnar, 1998) and is in fact presumed in studies on cortisol reactivity in the SSP. In the current study diurnal cortisol rhythm is assessed and related to infant attachment classification.

Parental depression may negatively influence infants' physiological regulation. More specifically, in several studies maternal depression was related to higher cortisol levels in infants, which could indicate both environmental and biological mechanisms of transmission (Ashman, Dawson, Panagiotides, Yamada, & Wilkinson, 2002; Essex, Klein, Cho, & Kalin, 2002; Halligan, Herbert, Goodyer, & Murray, 2004; Lupien, King, Meaney, & McEwen, 2000; Young, Vazquez, Jiang, & Pfeffer, 2006). Maternal depression has also been associated with attachment quality. Depression is thought to compromise sensitive parenting behavior, which in turn can undermine the development of a secure attachment relationship. However, the empirical evidence for this association is not unequivocal (see Cummings & Davies, 1994). Research on severe and chronic depression, as well as studies with clinical samples showed a significant association between maternal depression and attachment insecurity (e.g. Teti, Gelfand, Messinger, & Isabella, 1995). In community-based samples, however, the effect of maternal depressive symptoms on attachment quality is less clear; meta-analyses reported small or even insignificant effect sizes (Atkinson et al., 2000; Van IJzendoorn, Schuengel & Bakermans-Kranenburg, 1999). Other studies suggested that pre- and postnatal depression might influence mother-child interaction (Lundy et al., 1999; Righetti-Veltema, Bousquet & Manzano, 2003).

In the current study we examine both cortisol reactivity to a stressor and the diurnal rhythm of cortisol in relation to infants' attachment status. We expect

higher stress reactivity in insecurely attached children than in securely attached children. Furthermore, we expect that infants in the disorganized group differ in their cortisol reactivity from non-disorganized infants. With respect to diurnal rhythm, we expect to find a general pattern with higher morning than evening cortisol values. Since this study is the first to explore cortisol diurnal rhythm in relation to infant attachment status, we have no directed hypothesis on differences among attachment groups. Furthermore, we examine the moderating role of maternal depression on the association between attachment quality and cortisol levels. As maternal depression is related to insecure infant attachment and sub-optimal cortisol outcomes, maternal depression is hypothesized to act as an additional risk factor in the relation between insecure attachment and cortisol.

Method

Setting

The current investigation is embedded within the Generation R Study, a prospective cohort study investigating growth, development and health from fetal life into young adulthood in Rotterdam, the Netherlands, which has been described in detail elsewhere (Jaddoe et al., 2007; 2008). In the Generation R Study, we obtained detailed measurements of the child's development in a rather homogeneous subgroup: The Generation R Focus Study. Only children of Dutch national origin were included in this group, meaning that the children, their parents and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research center regularly for various somatic and behavioral assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study population

In the current investigation, data are presented of the 14-month visit of the Generation R Focus Study. A total of 882 infants and their parents participated between June 2004 and November 2006. In the first part of the visit, that lasted about 30 minutes, anthropometric and physiological measurements were conducted. Then, the Strange Situation Procedure (SSP) was administered, followed by assessments of the infants' motor functioning. In the SSP, twenty-four parents participated with two children (on different days). One child of each sibling pair was randomly excluded to avoid bias due to paired data. Another 29 children were excluded because of technical or procedural problems during the SSP. Of the remaining children, another 108 were not eligible for analysis because they completed the SSP with their fathers. After exclusion of these children, the study population consisted of 721 mother-infant dyads. Of this group, we had complete data on cortisol reactivity for 369 children, and 363 children were included in one or more measures of cortisol diurnal rhythm. Reasons for non-

response were lack of time and failure to obtain saliva samples. A high rate of refusal to chew on cotton swabs is not uncommon in this age group and has been reported before (Goldberg et al., 2003). This is typically found in infants that are not familiar with pacifiers. A non-response analysis was conducted to check for differences between children with and without cortisol data. For cortisol diurnal rhythm, differences between the groups were found for age at assessment, ($p <$ .05) gender ($p < .01$), and breastfeeding at the age of six months ($p < .05$). The group with diurnal cortisol data consisted of younger children, more boys, and the children were breastfed more often at six months of age. For cortisol stress reactivity, the groups differed on age at assessment ($p < .05$) and gender ($p < .01$); again, these children were younger and there were more boys in the group for which the data was available. For both cortisol reactivity and diurnal rhythm, non-response analyses did not show differences on maternal depression. Information about lifetime depression was available for 627 mothers.

Procedures and measures

Strange Situation Procedure. Parent-infant dyads were observed in the Strange Situation Procedure (SSP) when the infant was about 14 months of age (M = 14.7 SD = 0.9). The SSP is a widely used and well-validated procedure to measure the quality of the attachment relationship. The procedure consists of 7 episodes of 3 minutes each and is designed to evoke mild stress in the infant to trigger attachment behavior evoked by the unfamiliar lab environment, a female stranger entering the room and engaging with the infant, and the parent leaving the room twice (see Ainsworth et al., 1978, for the protocol). The SSP used in the current study included all these stimuli but to make it fit into a tight time schedule, we shortened the (pre-)separation episodes with one minute keeping the critical reunion episodes intact. Attachment behavior was coded from DVD-recordings according to the Ainsworth et al. (1978) and Main and Solomon (1990) coding systems by two reliable coders, trained at the University of Minnesota. Inter-coder agreement was calculated on 70 SSPs that were coded by both coders. For ABCD classification, inter-coder agreement was 77% ($\kappa = .63$); agreement on disorganization was 87% ($\kappa = .64$). 8% of the cases were discussed with one of two expert coders and classification was assigned after consensus was reached.

Salivary cortisol: diurnal rhythm and stress reactivity. Prior to the 14-month visit of the Generation R Focus Study parents were asked to collect saliva samples from their child at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). Parents received detailed written instructions with pictures concerning the saliva sampling. They were asked to collect five saliva samples during one single weekday at home: immediately after awakening, 30 minutes later, between 11 am and 12 pm, between 3 and 4 pm, and at bedtime; and to note down the sampling times. The child was supposed not to eat or drink 30 minutes before each sampling. The children were otherwise free to follow their normal daily

routines on the sampling day. Parents were asked to keep the samples stored in a freezer until they visited the research centre. If parents forgot to bring the samples, they were asked to send the Salivettes by postal mail. For 397 children (55%) one or more home saliva samples were returned. One child was excluded because it was older than 20 months. To compute a cortisol composite measure, at least the first sample and, depending on the measure, one or two subsequent samples had to be obtained, which left 363 children for the diurnal assessments. None of the children used systemic corticosteroid medication, but 12 children used other corticosteroid-containing medication. Excluding these children did not change the results, so they were included in further analyses. During the visit at the research centre at 14 months of age, three saliva samples were taken; the first prior to the SSP, the second directly after the SSP (which was on average 10 minutes after the first separation of the SSP) and the third about 15 minutes later (M = 16.3, SD = 8.3). For 369 children (51%) three samples were obtained.

Samples were centrifuged and frozen at -80°C. After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the Kirschbaum laboratory (Technical University of Dresden, Biological Psychology, Professor Dr. Kirschbaum) for analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively. For each time point, cortisol values that were above the 99th percentile (>200 nmol/L) were excluded (n = 12) from the analysis to reduce the impact of outliers.

Cortisol analyses. The daytime profile of cortisol secretion was characterized by calculating composite variables of the separate cortisol measurements. In this way we took into account the relation between the separate cortisol values within each child. We determined the area under the curve with respect to ground (AUC_G), which is a measure of total cortisol secretion during the day (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). The AUC_G was established by calculating the total area under the curve from the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis. This takes into account the difference between the single measurements from each other and the distance of these measures from the ground, or zero (Pruessner et al. 2003, p. 918). To correct for differences in length of day, the AUC_G was divided by number of hours between the first cortisol measurement (at awakening) and the last cortisol measurement (before going to bed) (see Watamura et al., 2004). Sleeping hours during the day were not associated with this composite measure. The AUC_G was computed only for children having at least three saliva samples (N = 228). The cortisol awakening response (CAR) was used as an index of HPA axis activity. It was calculated as the difference between cortisol value at awakening and the value 30 minutes after awakening (Kunz-Ebrecht, Kirschbaum, Marmot, & Steptoe, 2004). For CAR, data was available for

N = 258 children. As a measure of the diurnal cortisol decline we calculated the slope by fitting a linear regression line for each child, which predicted the cortisol values from time since awakening. The slope was computed by using the first and last saliva samples and at least one other cortisol measurement. To avoid any effect of the CAR on the slope (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Cohen, Schwartz, Epel, Kirschbaum, Sidney, & Seeman, 2006), the second cortisol sample (30 minutes after awakening) was not included in this measure of the slope. Data were available for N = 248 children. These composite measures of cortisol were moderately intercorrelated (AUC_G-CAR: r =.22, p < .01; AUC_G-slope: r = -.23, p < .01; CAR-slope: r = .51, p < .01).

For stress reactivity a delta was calculated between the last sample ($cortisol_{postSSP}$) and the first sample ($cortisol_{preSSP}$). The second assessment, just after the SSP, was not used, as it was too close to the onset of stress to show an increase. To control for the Law of Initial Values (LIV; Wilder, 1968), which states that the direction of response of a body function depends to a large degree on the initial level of that function, in subsequent analyses this delta was adjusted for the first sample.

Maternal lifetime depression. The Composite International Diagnostic Interview (WHO, 1990) version 2.1 was conducted during a home-visit at 30 weeks of pregnancy to assess lifetime prevalence of psychiatric disorders in the pregnant women. The CIDI is based on the definitions and criteria of the DSM IV; good to excellent psychometric properties have been reported (Andrews & Peters, 1998; Wittchen, 1994). Interviewers had been trained at a WHO training center. The mother's partner was not present during the interview. In the current study we used lifetime diagnoses of unipolar depressive disorder. Unipolar depressive disorder was defined as diagnoses of dysthymia, a single episode of major depression (mild, moderate or severe) and recurrent major depression (mild, moderate or severe).

Results

Attachment

The distribution of the attachment classifications was as follows: 57.8% secure (n = 413), 19.0% avoidant (n = 136), 23.2% resistant (n = 166). Of all children, 22.5% were classified as disorganized (n = 162), 77.5% were non-disorganized (n = 559). No differences were found between the distribution of the complete group (N = 721), and the group for which data on cortisol reactivity or cortisol diurnal rhythm was available (respectively χ^2 (3, N = 721) = 4.11, p = .25; χ^2 (3, N = 721) = 4.15, p = .25).

Table 1 shows the demographic variables for attachment security. No overall differences were found, except for parity. Avoidant children were more often the first child, χ^2 (2, N = 714) = 12.87, p < .01. In Table 2, demographics are shown according to disorganization status. Mothers of non-disorganized children consumed more alcohol during the period they breastfed, χ^2 (1, N = 490) = 5.32, p < .05. Some demographic variables were related to the cortisol measures; age at

Table 1. *Child and parent characteristics of the secure, insecure-avoidant, and insecure-resistant attachment groups*

	Secure (n = 413)	Insecure-avoidant (n = 136)	Insecure-resistant (n = 166)
Child characteristics			
Gender, % female	49.6	42.6	52.1
Parity, % firstborn	59.2	75.7	59.0**
Age at 14 months visit	14.6 (0.9)	14.8 (1.1)	14.7 (0.9)
Time of assessment cortisol$_{preSSP}$	11:28 (1:58)	11:31 (1:57)	11:28 (2:07)
Parental characteristics			
Age at intake mother	31.6 (3.9)	31.7 (3.6)	32.4 (3.7)
Maternal educational level, % low/medium	35.9	35.3	37.0
Marital status, % single	5.7	5.3	1.3
Smoking during pregnancy, %	12.4	14.2	8.0
Alcohol during pregnancy, %	56.4	48.9	61.3
Alcohol during breastfeeding, %	64.2	70.7	58.6

Note. Unless otherwise indicated, values are *M* and (*SD*). ** $p < .01$

Table 2. *Child and parent characteristics of the disorganized and non-disorganized attachment groups*

	Disorganized (n = 162)	Non-Disorganized (n = 559)
Child characteristics		
Gender, % female	51.2	48.1
Parity, % firstborn	58.6	63.3
Age at 14 months visit	14.6 (0.9)	14.7 (0.9)
Time of assessment cortisol$_{preSSP}$	11:06 (1:59)	11:34 (1:59)
Parental characteristics		
Age at intake mother	32.0 (3.8)	31.8 (3.8)
Maternal educational level, % low/medium	33.1	36.8
Marital status, % single	3.8	4.7
Smoking during pregnancy, %	9.3	12.7
Alcohol during pregnancy, %	51.9	57.5
Alcohol during breastfeeding, %	54.5	66.4*

Note. Unless otherwise indicated, values are *M* and (*SD*). * $p < .05$

14-months visit was related to slope ($r = .16, p < .05$), and smoking during pregnancy was related to CAR (F (2, 229) = 3.03, p = .05). Time of cortisol assessment was not related to cortisol measures or attachment classification, in fact, none of the demographic variables were related to both cortisol and attachment measures. Maternal lifetime depression was not related to attachment security, F (2, 618) = 0.96, p = .39, nor to disorganization status, F (1, 625) = 0.14, p = .71.

Attachment and cortisol stress reactivity

To test whether cortisol stress reactivity differed across attachment classifications, an ANCOVA was performed. Because attachment security and attachment disorganization are considered orthogonal dimensions (Van IJzendoorn et al., 1999), they were entered as two separate factors. Maternal lifetime depression was entered as a covariate, as was the first cortisol assessment to control for initial cortisol values. We found a main effect for attachment security, F (2, 308) = 9.03, $p < .01$, $\eta^2 = .06$. Resistant children differed from all other groups, displaying larger deltas, meaning larger differences between pre- and post-stressor assessment (post hoc analysis using Bonferroni criterion; $p < .01$), see Figure 1. In analyses, difference scores for cortisol (deltas) were used. In order to enhance interpretation, in Figure 1 cortisol values are shown. We did not find significant differences in stress reactivity between the disorganized group and the non-disorganized group. No main effect was found for maternal lifetime depression. A significant interaction effect was found for attachment security and maternal depression (F (2, 308) = 4.22, $p < .05$, $\eta^2 = .03$).

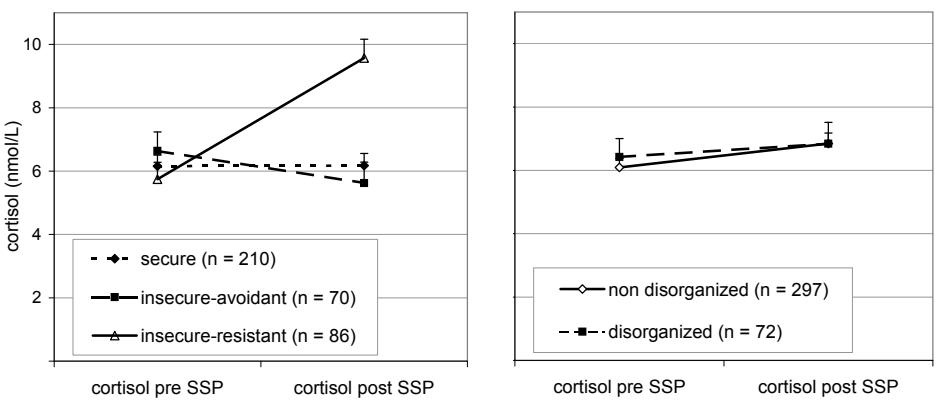

Figure 1. *Insecure-resistant children show high cortisol reactivity compared to the other groups; no differences in cortisol reactivity between disorganized and non-disorganized children*

Locating the interaction effect, we found that resistantly attached infants showed highest cortisol reactivity, in particular when their mothers scored high on depression (r (79) = .21, p (one-tailed) < .05, see Figure 2). In a separate ANOVA, we found no differences in cortisol levels between the groups prior to the SSP (attachment security: p = .53; attachment disorganization: p = .61). When the middle cortisol assessment was aggregated with the first cortisol assessment as a baseline level, similar outcomes were obtained (data not shown).

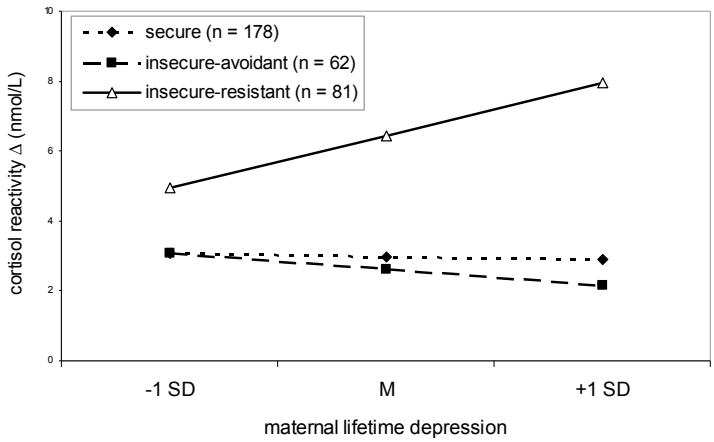

Figure 2. *Stronger effect of maternal lifetime depression on cortisol reactivity of insecure-resistant children compared to insecure-avoidant and secure children*

Attachment and cortisol diurnal rhythm

The excretion of cortisol did show the expected diurnal pattern, with high levels at awaking and a decline throughout the day. In the cortisol diurnal curves of the infants, most children showed no morning rise. We performed an ANCOVA to test the effect of attachment quality on the cortisol measures AUC_G, slope, and CAR. Again, attachment security and attachment disorganization were entered as factors, and maternal depression was included as a covariate. A main effect of disorganization was found for slope (F (1, 213) = 3.99, p < .01, η^2 = .03), indicating a more flattened slope for children with a disorganized attachment classification (slope disorganized group = -0.84, SE = 0.11; slope non-disorganized group = -1.16, SE = 0.06; Figure 3). Also, for AUC_G, an interaction effect was found for attachment security and disorganization, (F (2, 195) = 3.34, p = .04, η^2 = .03). Disorganized-secure infants showed higher cortisol excretion (AUC_G = 10.49, SE = 1.27) than disorganized-insecure infants (AUC_G = 7.48, SE = 1.27 for children with a secondary avoidant classification, and AUC_G = 7.66, SE = 1.05 for children with a secondary resistant classification). Compared to the non-disorganized group, cortisol excretion in the disorganized group was more divergent, dependent on the second classification. No effects were found for CAR or maternal lifetime depression.

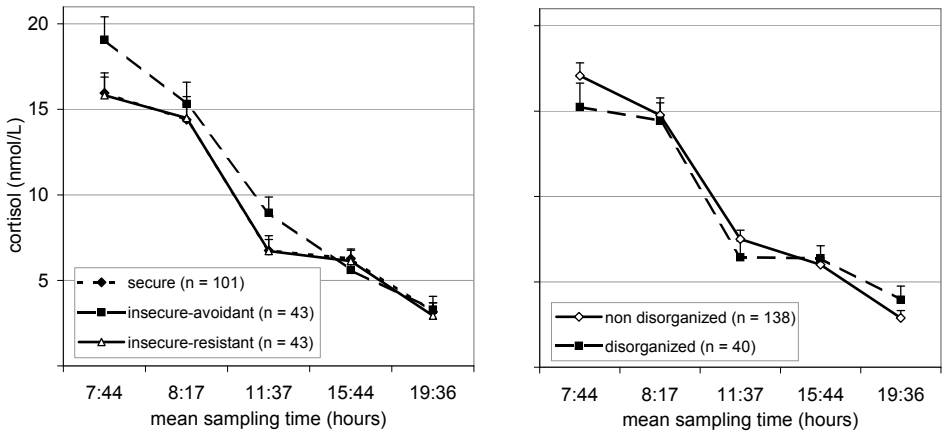

Figure 3. *No differences in cortisol diurnal rhythm for secure, insecure-avoidant, and insecure-resistant children; flattened slope for disorganized children compared to non-disorganized children*

Discussion

In a large cohort study with pertinent data on 369 mother-infant dyads, we found that infant attachment quality was related to cortisol stress reactivity, as assessed before and after the SSP. Resistant infants differed from all other groups, showing the largest increase in cortisol excretion after the SSP. Cortisol diurnal rhythm showed the expected diurnal pattern, with disorganized infants displaying a more flattened slope than non-disorganized infants. Maternal lifetime depression appeared to be a risk factor further elevating cortisol reactivity in infants with a resistant attachment relationship.

Cortisol reactivity and insecure-resistant attachment

Infants with a resistant attachment relationship showed the largest difference between pre and post SSP cortisol assessments compared to all other groups. This result converges partly with the outcomes of previous studies. Resistant infants were found to show higher cortisol levels after a stressful stimulus in some previous studies (Spangler & Schieche, 1998), but not in others (Gunnar et al., 1989; Nachmias, Gunnar, Mangelsdorf, Parritz, & Buss, 1996). In our study, infants classified as disorganized did not show increased reactivity, contrary to some of the previously reported results (Hertsgaard et al., 1995; Spangler & Grossmann, 1993). It may be the case that in previous studies reporting high reactivity in disorganized infants (Hertsgaard et al., 1995; Spangler & Grossmann, 1993) the majority of the infants had a secondary *resistant* classification; meta-analytic evidence confirms the suggestion that resistant infants have a strongly elevated chance of becoming classified as disorganized (Van IJzendoorn et al., 1999).

According to Weinfield, Sroufe, Egeland and Carlson (2008), resistant infants' history of erratic responsiveness renders them less able to direct attachment behaviors at caregivers when appropriate. Their 'maximizing' strategy might result in more physiological arousal than the 'minimizing' strategy of avoidant infants. Spangler and Schieche (1998) also proposed that resistant infants' high activation of the attachment system could not be terminated because they were not able to use the attachment figure effectively. Resistant infants 'maximize' their attachment behavior while they are at the same time unable to find a state of homeostasis in interaction with their caregiver (Cassidy & Berlin, 1994).

In contrast to the resistant infants, infants with secure or avoidant attachment classifications did not show significant increases in cortisol levels. This is partly convergent with previous literature. Both Hertsgaard et al. (1995) and Spangler and Schieche (1998) did not find increases in cortisol in avoidant infants. Minimizing the display of negative emotions might protect avoidant infants against elevated physiological reactivity in mildly stressful settings. Securely attached infants showed hardly any heightened cortisol responses in previous studies. They exhibit appropriate behavioral strategies in coping with the separation (Spangler & Schieche, 1998). According to Bowlby (1973, p. 150), these behavioral strategies can be regarded as an 'outer ring' of life-maintaining systems. When this 'outer ring' is in homeostasis, an adaptation of the 'inner ring', or physiological system, is not necessary.

Another, complementary, explanation can be found in temperamental characteristics of the infant. The concept of regulation plays a central role in both attachment and temperament theory (Vaughn, Bost, & Van IJzendoorn, 2008). Temperamental characteristics of the infants have been found to play a role in stress physiology (e.g., Dettling, Parker, Lane, Sebanc, & Gunnar, 2000). In addition, previous studies documented the association between lowered temperamental reactivity in avoidant children, and heightened temperamental reactivity in resistant children (Vaughn et al., 2008). Interpreting our finding of elevated cortisol reactivity in resistant but not in avoidant children, we speculate that the dual risk of temperamental reactivity and an insecure-resistant attachment relationship may be responsible for the increased cortisol secretion after stress in resistant children. Avoidant infants are supposed to be buffered against elevated cortisol reactivity to mild stress because of their less reactive and somewhat more aloof temperament.

Diurnal rhythm and disorganization

Daytime cortisol showed the expected diurnal pattern, with higher levels at awakening and lower levels at the end of the day (e.g. Mantagos, Moustogiannis, & Vagenakis, 1998; Price, Close, & Fielding, 1983; Spangler, 1991). However, De Weerth and Van Geert (2002) state that while at group level there is evidence for the presence of a diurnal rhythm of cortisol from the early age of 2 months, individuals

can vary greatly in the age at which they acquire the rhythm, which according to Gunnar and Donzella (2002) can be up to 4 years of age. To our knowledge, no previous studies related attachment quality to cortisol diurnal rhythm. In the current study, disorganized infants showed a more flattened slope of the diurnal rhythm than non-disorganized children. A flattened daytime pattern of cortisol –in its extreme form hypocortisolism– has often been found among children growing up in orphanages with structural neglect of basic emotional needs (see Gunnar & Vazquez, 2001, for a review). As a disorganized attachment relationship is thought to originate from extremely insensitive or even frightening parenting, this may cause similar physiological dysregulation in the disorganized group. Furthermore, higher diurnal cortisol excretion was found for disorganized-secure infants, whilst disorganized-insecure groups showed lower cortisol excretion. The interaction effect might indicate the intricate nature of these sub-groups. Cortisol excretion in children with a secondary insecure classification might be decreased in order to prevent enduring activation of the HPA-axis, whereas a secondary secure classification may indicate differential activation of the infants' endocrinological system, causing higher levels of excretion. Replications are essential to confirm these outcomes as our study is the first to be able to differentiate between these sub-groups.

Cortisol reactivity and maternal depression

Although several studies report maternal depression to affect both diurnal and reactivity cortisol levels in offspring (Azar, Paquette, Zoccolillo, Baltzer, & Tremblay, 2007; Brennan et al., 2008; Lupien et al., 2000; Young et al., 2006), in our study involving a non-clinical population such main effects were not found. Nevertheless, a clear interaction effect was found: infants with a resistant attachment relationship and a depressed mother displayed the strongest cortisol reactivity. The interaction between depression and attachment insecurity suggests a double risk model. In the case of resistant infants, the uncertainty about the mothers' availability is suggested to be associated with heightened attachment behavior, increasing the infant's monitoring of the caregiver and decreasing the exploratory competence (Cassidy & Berlin, 1994). In addition, infants of depressed mothers were found to experience reduced sensitivity and increased intrusiveness in interaction with their mothers (Goodman & Gotlib, 1999). Resistant attachment and maternal depression appear to compromise physiological regulation in an additive fashion.

Limitations

Some limitations of the current study need to be discussed. First, the Generation R Focus Study is a relatively homogeneous sample. However, the use of a homogeneous sample may have only led to an underestimation, and not an overestimation of the effects. Second, cortisol was sampled at 14 months of age.

Cortisol levels at this age do show some intra- individual instability (De Weerth & Van Geert, 2002). However, data on the development of cortisol secretion throughout infancy and childhood are scarce, and we did find evidence for an established pattern. Again, instability may have led to an underestimation of the differences among attachment groups. Third, a relatively large part of the participants could not be included in cortisol analyses, due to various reasons. Clearly informing parents about sampling could help to gain more and better saliva samples, however, sampling might remain difficult in 14-month olds. Lastly, a slightly shortened version of the SSP was used, in order to make it fit into the schedule of the visit. This minimal procedural change did not appear to modify the stress of the SSP, since the number of infants for whom the situation appears to be most stressful (resistant and disorganized classifications) was not lower in the current study compared to the standard distribution.

Conclusion

We documented the vulnerability of resistant infants in physiological stress regulation, especially in combination with care from a mother with a lifetime diagnosis of depression. Because of their small numbers in most attachment studies, resistant infants have been understudied as a separate insecure group. Our finding of elevated physiological stress reactivity in resistant children makes clear that this group can and should be differentiated from the other insecure attachment groups. We also showed that disorganized infants differed from non-disorganized infants in their diurnal cortisol rhythm, as they displayed a more flattened daily curve. This finding stresses the disturbed nature of disorganized attachments as one of the most important risks for developmental psychopathology. Our large-sample study suggests the differential physiological concomitants of avoidant, resistant, and disorganized attachments. Because infant attachment patterns have been shown to be relatively stable in stable environments (Fraley, 2002) insecure attachments may have long-term consequences for mental health, in particular in combination with other risk factors such as parental depression. Here we found that insecure-resistant and disorganized attachments can go 'under the skin' and may lead to deviating cortisol reactivity and daily patterns. From a biological perspective (Sapolsky, 2004) adverse early experiences can make humans and other animals more prone to stress and stress-related diseases, and attachment relationships may mediate the intergenerational transmission (Meaney, 2001) of this elevated vulnerability to emotional dysregulation.

3 FKBP5 and resistant attachment predict cortisol reactivity in infants: Gene-environment interaction

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Abstract

Quality of the parent-infant attachment relationship influences physiological stress regulation. Genetic factors also contribute to the stress regulatory HPA-axis. Quality of attachment as an index of the rearing environment (measured with the Strange Situation Procedure, SSP), and HPA-axis related SNPs (*Bcl*I, rs41423247; *TthIII*I, rs10052957; GR-9β, rs6198; N363S, rs6195; ER22/23EK, rs6189 and 6190; and FKBP5, rs1360780) were hypothesized to be related to cortisol reactivity in the stressful SSP. In this large population based sample, FKBP5 rs1360780, but not GR haplotype, was related to cortisol reactivity. Moreover, we found a significant interaction effect for insecure-resistant attachment and FKBP5 rs1360780, indicating a double risk for heightened cortisol reactivity levels in infants with one or two T-alleles of the FKBP5 SNP *and* an insecure-resistant attachment relationship with their mother. Findings are discussed from the perspective of gene-environment interaction.

Introduction

The infant-parent attachment relationship plays a major role in the infant's early life, particularly for socio-emotional development and emotion regulation (Bowlby, 1969/1982; Cassidy, 1994). The quality of the attachment relationship not only influences regulation on the behavioral level, but also affects physiological regulation. The physiological system is activated in stressful contexts, especially when coping behaviors are inadequate or coping resources are unavailable (Hertsgaard et al., 1995). Most studies on the physiology of infant attachment relationships focused on measures of heart rate and cortisol during the Strange Situation Procedure (SSP, Ainsworth et al., 1978; e.g. Gunnar et al., 1989; Oosterman & Schuengel, 2007; Sroufe & Waters, 1977), a mildly stressful procedure with two brief separations from the caregiver in an unfamiliar environment. Differences

in physiology during this procedure have been predominantly attributed to the quality of attachment which is an index of the rearing environment. Genetic factors have not received much attention, although there is ample evidence that genetic factors play a role in explaining variance in HPA-axis activity (Bartels et al., 2003; Steptoe et al., 2009; Wüst et al., 2004a). In the current study, both quality of attachment and genetic variations associated with HPA-axis activity were examined in relation to cortisol reactivity. In addition, the interaction between genetic factors and attachment quality on cortisol reactivity was investigated.

Studies on the association between attachment quality and cortisol levels have focused mainly on stress reactivity, with assessment of cortisol levels before and after the stressful SSP. The SSP is the gold-standard procedure to assess the quality of the infant-caregiver attachment relationship. The SSP allows for classification of the relationship as secure, insecure-avoidant, or insecure-resistant. Securely attached (B) children seek contact with the parent upon reunion, either physically or by distance interaction, to be comforted or reassured after the separation and resume exploration of the environment when they are settled. Based on their interactions with the caregiver, they have learned that she/he is available in times of stress. In contrast, infants of inconsistently sensitive or consistently insensitive parents do not come to expect their parents to be available in stressful situations, with insecure (avoidant or resistant) attachment relationships as a result. Children with insecure-avoidant (A) attachments focus on the environment at the moment of reunion, ignoring the parent or even turning away from them. The reunion behavior of an insecure-resistant (C) child is characterized by anxious contact seeking and clinging and at the same time resisting contact with the parent. On top of these classifications, attachment disorganization can be observed and rated. Disorganized (D) children show a temporary breakdown of their secure, avoidant or resistant strategy of dealing with the return of the parent after separation (Main & Solomon, 1990).

In several non-clinical studies, children tended to show elevated cortisol levels in reaction to the SSP. The most consistent finding is that only little or no adrenocortical activation is observed in securely attached infants, and increased cortisol levels for the disorganized infants (Gunnar et al., 1996; Hertsgaard et al., 1995; Spangler & Grossmann, 1993). Results for the insecure-avoidant and insecure-resistant groups are inconsistent. In some studies, both insecure groups were found to have elevated cortisol levels after the SSP (Spangler & Grossmann, 1993), others found increased cortisol levels only for insecure-resistant children (Spangler & Schieche, 1998). In a previous study on the current sample (Luijk et al., 2010) we found increased cortisol levels for insecure-resistant children.

Associations between attachment quality and cortisol reactivity implicate that cortisol reactivity levels are, at least partly, determined by the caregiving environment (Gunnar & Quevedo, 2007; Meaney, 2001). Evidence for the contribution of genetic factors has been mixed (Ouellet-Morin et al., 2008;

Steptoe et al., 2009; Wüst et al., 2004a), and it has been noted that 'the genetic and environmental contributions to cortisol reactivity in early childhood have yet to be documented' (Ouellet-Morin et al., 2008, p. 212). This interplay between genetic and environmental factors was recently studied by Frigerio and colleagues (2009), who found independent effects of candidate genes (5HTT, GABRA6, DRD4, and COMT) and attachment quality on alpha amylase, another potential biomarker for physiological arousal. They did not, however, find effects of attachment quality, genetics, or their interaction on cortisol reactivity. In view of these diverging findings, they note that replication in larger samples is required.

Recently, specific candidate genes that play a role in explaining variability in cortisol reactivity have been identified. Several studies focused on the glucocorticoid receptor (GR) that mediates many of the effects of glucocorticoids. Genetic variants of the GR gene, e.g. single nucleotide polymorphisms (SNPs), appear to contribute to interindividual variability in HPA-axis activity by affecting a cell's sensitivity for glucocorticoids (DeRijk & De Kloet, 2008; Wüst et al., 2004b). Five different SNPs within the GR have been investigated in previous research; *Bcl*I (rs41423247), *TthIII*I (rs10052957), GR-9β (rs6198), N363S (rs6195) and ER22/23EK (rs6189 and 6190). No effects of GR on basal cortisol excretion have been found (Rautanen et al., 2006; Rosmond et al., 2000). However, HPA-axis reactivity as assessed using a social stressor, the Trier Social Stress Test, showed that carriers of the N363S G allele had increased cortisol responses. On the other hand, carriers of the *Bcl*I G allele and GR-9β G allele showed an attenuated response (Ising et al. 2008; Kumsta et al., 2007; Wüst et al., 2004b).

Importantly, blocks of specific SNP combinations are usually found within genes, resulting in several haplotypes, that is, groups of specific SNPs in a gene that tend to be inherited together. These haplotypes can have different effects compared to 'isolated' SNPs (DeRijk et al., 2008). In the current study, effects of GR haplotypes on cortisol reactivity will be tested (Van den Akker et al., 2006; 2008). GR activation is regulated by a large molecular complex. In this complex, several molecules, so-called chaperones and co-chaperones, play a critical role. Altering the composition of the (co-)chaperones influences sensitivity of GR to cortisol and thus affects HPA-axis responsivity (Binder et al., 2004; Binder, 2009). The FKBP5 co-chaperone of GR has been associated with changes in HPA-axis activity by altering the negative feedback system (Ising et al., 2008). The feedback loop is crucial in recovery from stress, which in turn is essential for healthy physiological and behavioral regulation. As the infant-parent attachment relationship can be considered the infant's most important emotion regulation system (Bowlby, 1969/1982, Cassidy, 1994), the role of a genetic factor influencing homeostasis might be of great importance. FKBP5 has several SNPs, and for these SNPs the most consistent findings were reported for rs1360780. For individuals carrying one or two copies of the minor (T) allele, i.e. the allele that is less frequent in the population, positive associations have been found with major depression, bipolar

disorder, post-traumatic stress disorder and a faster response to antidepressant treatment (for a review, see Binder, 2009). With respect to HPA-axis activity, this SNP did not show an effect on basal cortisol levels (Binder et al., 2004), but it did show an effect on cortisol responses to the Trier Social Stress Test (Ising et al., 2008). Participants who were homozygous for the minor allele (TT genotype) showed an impaired recovery from stress compared to carriers of the CC or CT genotype.

In the current study we expand the findings on attachment security and cortisol reactivity from previous studies (Gunnar et al., 1996; Hertsgaard et al., 1995; Spangler & Schieche, 1998) by adding a genetic component. Carriers of the minor alleles of the haplotypes of GR and the FKBP5 SNP were expected to show altered cortisol reactivity levels. Furthermore, it is hypothesized that the association between attachment security and stress reactivity is moderated by GR and FKBP5. A combination of insecure-resistant attachment and carrying one or more 'risk alleles' of GR and the FKBP5 SNP was expected to lead to higher cortisol reactivity.

Method

Setting

The current investigation is embedded within the Generation R Study, a prospective cohort study investigating growth, development and health from fetal life into young adulthood in Rotterdam, The Netherlands, which has been described in detail elsewhere (Jaddoe et al., 2007; 2008). In the Generation R Study, we obtained detailed measurements of the child's development in a rather homogeneous subgroup: The Generation R Focus Study. Only children of Dutch national origin were included in this group, meaning that the children, their parents and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research center regularly for various somatic and behavioral assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study population

DNA was collected from cord blood samples at birth. At the age of 14 months, infants and their mothers participated in the Strange Situation Procedure (SSP). In 589 infants, information on GR and FKBP5 genotypes and quality of attachment was available. Of this group, cortisol was sampled in 310 children. Unsuccessful sampling was mainly due to refusal to chew on cotton swabs, which is not uncommon in this age group and has been reported before (Goldberg et al., 2003). This is typically found in infants that are not familiar with pacifiers. A non-response analysis was conducted to check for differences between children with and without cortisol data. Differences between the groups were found for gender

(p = .01); the group with cortisol data consisted of more boys. Also, educational level of the mother differed (p = .03); mothers in the group for which data was available were less highly educated. No differences were found in the distribution of attachment classifications or genotype (.09 < p < .87).

Procedures and measures

Strange Situation Procedure. Parent-infant dyads were observed in the Strange Situation Procedure (SSP, Ainsworth et al., 1978) when the infant was about 14 months of age (M = 14.7, SD = 0.9). The SSP is a widely used and well-validated procedure to measure the quality of the attachment relationship. The procedure consists of seven episodes of 3 minutes each and is designed to evoke mild stress in the infant to trigger attachment behavior evoked by the unfamiliar lab environment, a female stranger entering the room and engaging with the infant, and the parent leaving the room twice (see Ainsworth et al., 1978, for the protocol). The SSP used in the current study included all these stimuli but to make it fit into a tight time schedule, we shortened the (pre-) separation episodes with one minute keeping the critical reunion episodes intact. Attachment behavior was coded from DVD-recordings according to the Ainsworth et al. (1978) and Main and Solomon (1990) coding systems by two reliable coders, trained at the University of Minnesota. Inter-coder agreement was calculated on 70 SSPs that were coded by both coders. For ABCD classification, inter-coder agreement was 77% (κ = .63); agreement on disorganization was 87% (κ = .64). Eight percent of the cases were discussed with one of two expert coders and classification was assigned after consensus was reached. We also coded the percentage of time the infant was crying during the SSP (intercoder reliability ICC = .98) to include as a covariate in the analyses.

Salivary cortisol: stress reactivity. During the visit at the research centre at 14 months of age, three saliva samples were taken using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany); the first prior to the SSP, the second directly after the SSP (which was on average 10 minutes after the first separation of the SSP) and the third about 15 minutes later (M = 16.3, SD = 8.3). None of the children used systemic corticosteroid medication, but 12 children used other corticosteroid-containing medication. Excluding these children did not change the results, thus we included them in further analyses.

Samples were centrifuged and frozen at -80°C. After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the Kirschbaum laboratory (Technical University of Dresden, Biological Psychology, Professor Dr. Kirschbaum) for analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively. For each time point, cortisol values that were above the 99th percentile (>200 nmol/L) were excluded (n = 12) from the analysis to reduce the impact of outliers.

Cortisol analyses. For stress reactivity a delta was calculated between the last sample ($cortisol_{postSSP}$) and the first sample ($cortisol_{preSSP}$). The second assessment, just after the SSP, was not used, as it was too close to the onset of stress. To control for the Law of Initial Values (LIV; Wilder, 1968), which states that the direction of response of a body function depends to a large degree on the initial level of that function, in subsequent analyses this delta was adjusted for the first sample.

Genotyping. DNA was collected from cord blood samples at birth. To check for potential contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases, which were excluded. All participants were genotyped for polymorphisms in the glucocorticoid receptor gene, *Bcl*I (rs41423247), *TthIII*I (rs10052957), GR-9β (rs6198), N363S (rs6195) and ER22/23EK (rs6189 and 6190); and the FKBP5 gene (rs1360780). Table 1 shows the allele frequencies for the GR SNPs. Genotyping was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 min), then 40 cycles of 94° C (15 s) and 60° C (1 min)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97-99% of the samples. To confirm the accuracy of the genotyping results 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes. For the glucocorticoid receptor gene we used the genotype data for each of the 5 polymorphisms to infer the haplotypes present in the population using the program PHASE, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (Stephens et al., 2001). For each haplotype, three genotype combinations were distinguished as carrying 0, 1, or 2 copies of the haplotype allele. Haplotype 1 carries the major alleles of the polymorphisms; therefore, the reference allele is defined as carrying 2 copies of haplotype 1. The FKBP5 SNP was presented in a similar way; frequency of the minor allele was indicated (0, 1 or 2 copies). Distribution for FKBP5 was as follows: 147 CC (47.4%), 139 CT (44.8%), 24 TT (7.7%). Table 2 shows the specific nucleotide variations and distribution of the GR haplotypes and FKBP5. Genotype frequencies were in Hardy Weinberg equilibrium (χ^2s [1, N = 310] < 1.28, ps > .26). GR haplotypes and the FKBP5 SNP were not correlated.

Table 1. *Allele frequencies and minor allele frequencies for GR SNPs*

GR SNP	Allele frequency (%)[a]			MAF (%)
	0	1	2	
*Bcl*I (rs4142347)	41	46	13	36
*TthIII*I (rs10052957)	46	44	10	32
GR-9β (rs6198)	68	29	3	18
N363S (rs6195)	91	9	0	4
ER22/23EK (rs6189/6190)	92	8	0	4

[a] % of copies of the minor allele. MAF = minor allele frequency. All GR SNPs were in HWE (χ^2s [1, N = 310] < 0.66, ps > .42).

Statistical analyses. First, we checked whether demographic variables were related to cortisol, genotype, and attachment classification using ANOVAs and Chi-square tests. An ANCOVA was performed to test the association between attachment quality and cortisol reactivity levels, controlling for initial cortisol values. Because attachment security and attachment disorganization are considered orthogonal constructs (Van IJzendoorn et al., 1999), they were entered as two separate factors. The relation between attachment quality and genotypes was tested using a Chi-square test. A regression analysis was used to test the main effects of genotypes on cortisol reactivity, correcting for initial cortisol values. Associations were also tested for individual GR SNPs, which yielded similar results (data available upon request). Using a regression analysis, we tested for a potential interaction effect of insecure-resistant attachment, FKBP5, and GR on cortisol stress reactivity. For this analysis, infants with an insecure-resistant attachment classification were contrasted to infants with a non-resistant classification. In the first step, the first cortisol assessment ($cortisol_{preSSP}$) was entered to control for initial values. In the second step, resistant versus non-resistant attachment classification was entered. In the third step, GR haplotypes and FKBP5 were entered. In the fourth step, interactions between GR haplotypes, and FKBP5 with resistant attachment classification were entered. Except for the first cortisol assessment, all variables were centered based on the N for which cortisol reactivity data was available.

Table 2. *Distribution of GR haplotypes and FKBP5 and main effects on cortisol reactivity*

GR haplotype	Nucleotide variations					Haplotype copies (%)			HF (%)	*B* (95% *CI*)	*p* value
						0	1	2			
Wildtype	G	GG	A	C	A	33	49	18	42	0.12 (-4.29; 4.54)	.96
*Bcl*I	G	GG	A	**G**	A	62	33	5	22	0.49 (-3.98; 4.97)	.83
*TthIII*I + *Bcl*I	**A**	GG	A	**G**	A	72	27	1	14	0.68 (-3.89; 5.24)	.77
GR-9β + *TthIII*I	**A**	GG	A	C	**G**	75	24	1	13	1.07 (-3.49; 5.64)	.64
N363S	G	GG	**G**	C	A	91	9	0	4	1.46 (-3.51; 6.43)	.56
ER22/23EK + GR-9β + *TthIII*I	**A**	**AA**	A	C	**G**	92	8	0	4	-0.36 (-5.37; 4.66)	.89
FKBP5						Genotype frequency (%)			MAF (%)		
						CC	CT	TT			
rs1360780					C/T	47	45	8	30	1.28 (0.31; 2.25)	.01

Note. The nucleic acid changes are indicated in bold; C = Cytidine, G = Guanine, A = Adenine, T = Thymine. FKBP5 SNP was in HWE (χ^2 [1, N = 310] = 1.28, p = .26). MAF = minor allele frequency, HF = haplotype frequency.

Results

Distribution of attachment

In the group for which both cortisol reactivity data was available (N = 310), distribution of attachment classifications was as follows: 56.7% secure (n = 174), 18.6% insecure-avoidant (n = 57), 24.8% insecure-resistant (n = 76). Of all children, 18.4% were classified as disorganized (n = 57), 81.6% were non-disorganized (n = 253), a common distribution in non-clinical populations (Van IJzendoorn et al., 1999). Participant characteristics are displayed in Table 3. Time of cortisol assessment was not related to cortisol measures or attachment classification, in fact, none of the demographic variables were related to cortisol, genotype, and attachment classification at the same time.

Table 3. *Sample characteristics (N=310)*

Child characteristics	
Child gender, % girls	43.5
Parity, % firstborn	61.9
Birth weight in grams	3543 (483)
Age at 14 months visit	14.6 (0.8)
Time of assessment cortisol prior to SSP	12:07 (2:00)
Parental characteristics	
Age at intake mother	31.7 (4.1)
Maternal educational level, % low/medium	39.9
Hours working, mother	28.2 (12.9)
Marital status, % single	5.6
Smoking during pregnancy, %	11.0
Alcohol during pregnancy, %	55.2
Breastfeeding at 6 months, %	31.0

Note. Unless otherwise indicated, values are mean (*SD*).

Attachment quality, HPA-axis genes and cortisol reactivity

Infants with an insecure-resistant attachment relationship showed the highest cortisol reactivity levels from pre SSP to post SSP (F (2, 300) = 17.60, $p < .01$, η^2 = .11, see Table 4). We did not find significant differences in stress reactivity between the disorganized group and the non-disorganized group, nor an interaction effect of attachment security and attachment disorganization. Attachment quality was not related to glucocorticoid haplotypes ($.25 < p < .97$) or the FKBP5 SNP (rs1360780) (p = .14). Haplotypes of the glucocorticoid receptor were not related to cortisol reactivity ($.56 < p < .96$). The FKBP5 SNP was however related to cortisol reactivity (B = .13, CI = 0.31; 2.25, p = .01, see Table 1), indicating that infants with FKBP5-CT and TT genotypes showed increased cortisol reactivity.

The more T alleles infants carried, the stronger was their cortisol reactivity. Using a regression analysis, we tested for a potential interaction effect of insecure-resistant attachment, FKBP5, and GR haplotypes on cortisol stress reactivity. Main effects of GR haplotypes ($.14 < p < .91$) and interactions between GR haplotypes and resistant attachment did not reach significance ($.32 < p < .85$).

Table 4 . *Cortisol values pre SSP, post SSP and cortisol reactivity Δ (nmol/L)*

	Cortisol pre SSP	Cortisol post SSP	Cortisol reactivity Δ[a]	
Secure	6.26 (5.06)	6.15 (4.25)	-1.21 (0.87)	
Insecure-avoidant	6.27 (5.04)	5.42 (3.60)	-0.61 (0.62)	
Insecure-resistant	5.89 (4.97)	9.92 (8.91)	4.04 (0.65)	**
Disorganized	6.27 (7.02)	7.08 (8.09)	1.07 (0.37)	
Non-disorganized	6.15 (4.44)	6.89 (5.29)	0.41 (0.74)	

Note. Unless otherwise indicated, values are M (SD). Cortisol reactivity Δ corrected for initial cortisol values. [a] Values are M (SE). ** F (2, 300) = 17.60, $p < .01$

In Table 5, the most parsimonious model is presented. Main effects for resistant attachment and FKBP5 were significant (β = .30, $p < .001$; β = .19, $p < .001$, respectively), as was the interaction between FKBP5 and resistant attachment (β = .12, $p < .05$). The model explained 32% of the variance. Infants with a resistant attachment relationship and the FKBP5-CT genotype showed more increased cortisol reactivity than resistant infants with the CC genotype, and resistant infants with the FKBP5-TT genotype showed the largest increases in cortisol reactivity. The results remained essentially the same when infant crying during the SSP was included as a covariate.

Table 5. *Regression analysis predicting cortisol reactivity from FKBP5 and insecure-resistant attachment, controlling for initial cortisol values*

	B (95% *CI*)	β	*p*	F_{change}	R^2	R^2_{change}
Step 1				75.48	0.20	0.20
Cortisol$_{preSSP}$	-0.51 (-0.63;-0.40)	-0.42	<.001			
Step 2				36.52	0.28	0.09
Resistant attachment	2.10 (1.44; 2.76)	0.30	<.001			
Step 3				8.10	0.30	0.02
FKBP5	1.88 (0.85; 2.90)	0.19	<.001			
Step 4				5.29	0.32	0.01
FKBP5 * Resistant attachment	1.20 (0.17; 2.22)	0.12	.022			

Note. $R^2 = .32$. Final model F (4, 302) = 34.72, $p < .01$. β is a standardized coefficient and denotes SD change in cortisol reactivity per SD change in the predictor. The statistics are derived from the final block of the regression model.

Discussion

An insecure-resistant attachment relationship predisposes infants to heightened cortisol reactivity levels. Also, the minor allele of the FKBP5 SNP was associated with cortisol reactivity in an additive fashion; the more T alleles, the higher levels of cortisol reactivity. Furthermore, an interaction between insecure-resistant attachment and FKBP5 was found. This represents a double risk for heightened cortisol reactivity levels in infants who carry one or two T-alleles of the FKBP5 SNP and at the same time have an insecure-resistant attachment relationship with their mother.

Insecure-resistantly attached infants have been found to display high cortisol levels after a stressful stimulus in some studies (Spangler & Schieche, 1998), but not in others (Gunnar et al., 1989; Nachmias et al., 1996). Resistant infants' high activation of the attachment system may not be terminated soon after the reunion with the caregiver because they are unable to use the attachment figure effectively, which makes it difficult for these children to find a state of homeostasis (Cassidy & Berlin, 1994). In the current study, no effects of disorganization on stress reactivity were found. In a previous study on the same sample (Luijk et al., 2010), we found evidence for an association between disorganized attachment and flattened cortisol diurnal rhythm, which may indicate a different stress mechanism in the disorganized group. As determinants of attachment disorganization differ from those of attachment security, genetic susceptibility as well as physiological and behavioral developmental outcomes might be only partly overlapping.

FKBP5 rs1360780 has been associated with altered stress reactivity in an adult sample (Ising et al., 2008); individuals homozygous for the minor allele (TT genotype) showed an impaired recovery from stress. Furthermore, Binder et al. (2008) found that the FKBP5 SNP moderated the relation between child abuse and adult post-traumatic stress disorder (PTSD), and alterations in attachment quality or HPA-axis sensitivity were suggested as possible mechanisms for this association. Combined with findings from the current study, evidence grows for the contribution of this minor allele to differences in GR sensitivity, and to differential activation of the feedback loop of the HPA-axis when confronted by a psychological stressor (Binder, 2009). The negative feedback system is essential in recovery from stressful situations, for example the SSP. A balanced stress recovery system that promotes homeostasis is of great importance. Presumably, both the minor allele of the FKBP5 SNP and an insecure-resistant attachment relationship prevent adequate termination of the stress reaction. This lack of homeostasis could put the child at developmental risk; long-term negative outcomes have been shown for both insecure attachment (Fearon et al., 2010; Warren et al., 1997) and, indirectly, for carriers of the FKBP5 SNP (Binder et al., 2008). It should be noted however, that associations in the current study are correlational, and that the underlying mechanisms need further elaboration.

In the current study GR haplotypes were not related to cortisol reactivity. However, these results should be interpreted with some caution. Whereas we had sufficient power to analyze the two most frequent haplotypes, the haplotypes including the SNPs N363S and ER22/23EK displayed very low frequencies in the current sample. Few studies have investigated the association between GR haplotypes and cortisol reactivity, and report consistent but small effects of GR SNPs in adults (for a review, see Wüst et al., 2004a). In infants, these associations have remained largely uncharted. GR haplotypes and FKBP5 were not related to attachment security or attachment disorganization. This is consistent with findings from previous studies, as main effects of candidate genes on attachment quality have not been reliably established (Bakermans-Kranenburg & Van IJzendoorn, 2007). Recently, Frigerio and colleagues (2009) examined effects of attachment quality and candidate genes on alpha amylase and cortisol. They reported gene-environment interaction effects for alpha amylase, but no effects for cortisol. The current study investigated specific HPA-axis related genes in a large, population based sample, and provides evidence for effects of FKBP5, attachment quality and their interaction on cortisol reactivity.

The findings from the present study support the idea of interplay between genetic and environmental factors in explaining developmental outcomes (Rutter et al., 2006). Resistant attachment and FKBP5 predispose infants to increased cortisol reactivity both independently as well as in interaction. The current outcomes provide support for a double-risk model (Belsky et al., 2007) as the combination of environmental (indexed by resistant attachment) and genetic (FKBP5) risks increase stress reactivity in an additive way, even in a rather homogeneous, low-risk sample. In a more diverse sample the gene-environment interaction effect might even be larger. Furthermore, it should be noted that careful assessment of the environment is essential for establishing G x E interactions. In the current study, the quality of the attachment relationship offers an observation-based but indirect index of the environment. Detailed direct observation of parenting quality in the natural setting may offer a more complete assessment, but was beyond the scope of the current study.

In sum, the present study shows HPA-axis related genes and attachment quality to be associated with stress reactivity both independently and in interaction. The combination of an insecure-resistant attachment relationship and carrying the minor allele of the FKBP5 gene is related to increased stress reactivity in infants.

4 The association between parenting and attachment is moderated by a polymorphism in the mineralocorticoid receptor gene: Evidence for differential susceptibility

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Manuscript submitted for publication

Abstract
Maternal sensitive responsiveness and extreme insensitivity only partly explain the variance in attachment security. Differences in attachment security may well be rooted in the interplay of genetic variations and environmental factors. The association between parenting (observed sensitive responsiveness and extreme insensitivity) and attachment security (assessed with the Strange Situation Procedure) was hypothesized to be moderated by genes involved in the regulation of the stress response: the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) genes. A significant GxE interaction was found: Infants carrying the minor MR allele (G) were significantly more securely attached if their mothers showed more sensitive responsiveness, *and* significantly less securely attached if their mothers showed more extremely insensitive behaviors. These associations were not significant for carriers of the AA genotype of MR. Findings are discussed from a differential susceptibility perspective.

Introduction
Attachment security is a developmental milestone, defined as the child's need to seek proximity to and comfort from a potentially protective caregiver in times of stress (e.g., illness, danger, Bowlby, 1969/1982). Maternal sensitive responsiveness to the baby's stress and distress signals is considered to be an important determinant of attachment security (Ainsworth, Blehar, Waters, & Wall, 1978; Bakermans-Kranenburg, Van IJzendoorn & Juffer, 2003), whereas extreme insensitivity bordering on neglectful parenting, elevates the risk for insecure attachment (Out, Bakermans-Kranenburg & Van IJzendoorn, 2009).

Maternal sensitive responsiveness and extreme insensitivity only partly explain the variance in attachment security, and attachment differences may also be rooted in genetic differences. Main effects of genetic factors on attachment

security have been found to be elusive, both in behavioral and molecular genetic studies (Bakermans-Kranenburg & Van IJzendoorn, 2007; Bokhorst et al., 2003; O'Connor & Croft, 2001; Roisman & Fraley, 2008). However, genetic effects on child development are probably hidden in interactions with environmental factors (Barry, Kochanska & Philibert, 2008; Belsky et al., 2009).

Because attachment is functional for the regulation of stress (Bowlby, 1969/1982; Hertsgaard, Gunnar, Erickson & Nachmias, 1995), we focus on genes involved in the regulation of the stress response: the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) that have been implicated in the variability of HPA axis responses to social stressors (DeRijk & De Kloet, 2008). We hypothesize that polymorphisms in GR and in MR are important candidates for GxE in the case of attachment security. Following the concept of differential susceptibility as a specific type of GxE interaction (Belsky, Bakermans-Kranenburg & Van IJzendoorn, 2007), we hypothesize that a combination of receiving more responsive parenting and carrying minor alleles of GR or MR leads to a more *secure* attachment relationship, whilst a combination of experiencing more extremely insensitive parenting and carrying minor alleles of GR and MR is related to a more *insecure* attachment relationship.

Method

Setting

The current investigation is embedded within the Generation R Study, a prospective cohort study investigating growth, development and health from fetal life into young adulthood in Rotterdam, the Netherlands (Jaddoe et al., 2007; 2008). In the Generation R Study, detailed measurements of the child's development in an ethnically homogeneous subgroup were obtained. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study population

DNA was collected from cord blood samples at birth. At the age of 14 months, infants and their mothers participated in the Strange Situation Procedure (SSP). For 601 infants, information on MR and GR genotypes and quality of attachment was available. Within this group, maternal sensitive responsiveness was observed for 530 children, maternal extreme insensitivity was observed for 543 children. Participant characteristics are displayed in Table 1. A non-response analysis was conducted to check for differences between children with and without sensitivity data. Differences between the groups were found for gestational age ($p < .05$), family income ($p < .05$), and maternal alcohol use during pregnancy ($p < .05$). No differences were found for attachment security or genotype ($.08 < p < .92$). Of the demographic variables, only parity was related to maternal sensitivity, genotype, and attachment security at the same time. Taking parity into account in the analyses did not change the results.

Table 1. *Sample characteristics*

Child characteristics	
Child gender, % female	48.8
Parity, % firstborn	62.1
Birth weight in grams	3507 (540)
Gestational age in weeks	40.0 (1.7)
Apgar score	8.6 (1.1)
Richters Security Score	0.2 (2.6)
Parental characteristics	
Age at intake mother	31.8 (3.8)
Maternal educational level, % low/medium	35.6
Hours working, mother	28.3 (12.4)
Marital status, % single	4.4
Smoking during pregnancy, %	11.0
Alcohol during pregnancy, %	54.2
Breastfeeding at 6 months, %	29.5
Maternal Sensitive Responsiveness	6.6 (1.2)
Maternal Extreme Insensitivity	1.3 (1.0)

Note. Unless otherwise indicated, values are mean (SD).

Procedures and measures

Strange Situation Procedure. Parent-infant dyads were observed in the Strange Situation Procedure (SSP, Ainsworth et al., 1978) when the infant was 14.7 months of age (*SD* = 0.9). The SSP is a widely used and well-validated procedure to measure the quality of the attachment relationship. The procedure consists of seven episodes of 3 minutes each and is designed to evoke mild stress in the infant to trigger attachment behavior evoked by the unfamiliar lab environment, a female stranger entering the room and engaging with the infant, and the parent leaving the room twice (see Ainsworth et al., 1978, for the protocol). The SSP used in the current study included all these stimuli but to make it fit into a tight time schedule, we shortened the (pre-) separation episodes with one minute keeping the critical reunion episodes intact.

Attachment behavior was coded from DVD-recordings according to the Ainsworth et al. (1978) and Main and Solomon (1990) coding systems by two reliable coders, trained at the University of Minnesota. Attachment behaviors may be categorized as secure or insecure. When stressed, secure infants seek comfort from their mothers, which proves effective, enabling the infant to return to play. Insecure-avoidant infants show little overt distress, while turning away from or ignoring mother on reunion. Insecure-resistant infants are distressed and angry,

but ambivalent about contact, which does not effectively comfort and allow the child to return to play. Distribution of attachment classifications was as follows: 57.3% secure (n = 413), 18.9% insecure-avoidant (n = 136), 23.0% insecure-resistant (n = 166). No classification could be assigned for n = 6 (0.8%) children. Inter-coder agreement was calculated on 70 SSPs that were coded by both coders, inter-coder agreement was 77% (κ = .63). Eight percent of the cases were discussed with one of two expert coders and classification was assigned after consensus was reached. Continuous scores for attachment security were computed using Van IJzendoorn and Kroonenberg's (1990) adaptation of Richter's algorithm (Richters, Waters & Vaughn, 1988).

Maternal sensitive responsiveness. Maternal sensitive responsiveness was observed during a psychophysiological assessment in the 14 months lab visit with Ainsworth's rating scales for sensitivity (Ainsworth, Bell, & Stayton, 1974). Scores for sensitive responsiveness were based on the subscale scores for sensitivity and cooperation (r = .87), both scored on 9-point rating scales with higher scores indicating more sensitive responsiveness. The intraclass correlation for sensitive responsiveness (single measure, absolute agreement) was .65 (n = 82).

Maternal extreme insensitivity. Maternal extreme insensitivity was observed during the 14 months lab visit, by coders unaware of the ratings of maternal sensitivity and attachment security. The scale includes 1) parental withdrawal and neglect; and 2) intrusive, negative, aggressive or otherwise harsh parental behaviors (Out, et al., 2009). Discrete extremely insensitive behaviors were coded on a 9-point scale, with higher scores indicating more extreme insensitivity. The intraclass correlation (single measure, absolute agreement) was .63 (n = 36).

Genotyping. DNA was collected from cord blood samples at birth. Participants were genotyped for polymorphisms in the glucocorticoid receptor gene, *Bcl*I (rs41423247), *TthIII*I (rs10052957), GR-9β (rs6198), N363S (rs6195) and ER22/23EK (rs6189 and 6190); and the mineralocorticoid receptor gene (rs5522). To check for potential contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases, which were excluded. Genotyping was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 min), then 40 cycles of 94° C (15 s) and 60° C (1 min)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97-99% of the samples. To confirm the accuracy of the genotyping results 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes. For the glucocorticoid receptor gene we used the genotype data for each of the 5 polymorphisms to infer the haplotypes present in the population using the program PHASE, which

implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (Stephens et al., 2001). For each haplotype, 3 genotype combinations were distinguished as carrying 0, 1, or 2 copies of the haplotype allele. The GR wildtype carries the major alleles of the polymorphisms. Genotype frequencies were in Hardy Weinberg equilibrium (χ^2s [1, N = 568 - 592] < 1.23, ps > .27). GR haplotypes and the MR SNP were not correlated. Due to low minor allele frequencies (3-5%), two haplotypes (N363S and ER22/23EK + GR-9β + *TthIII*I) were not used in further analyses. Table 2 shows the allele frequencies.

Table 2. *Distribution of GR haplotypes and MR and main effects on attachment security*

Haplotype / SNP	Allele frequency (%)[a]			MAF (%)	*r*	*p*
	0	1	2			
GR Wildtype	35	48	17	41	.04	.37
*Bcl*I	60	36	4	22	.05	.27
*TthIII*I + *Bcl*I	73	26	1	14	-.02	.61
GR-9β + *TthIII*I	76	23	1	13	-.06	.13
MR rs5522	79	19	2	11	.05	.20

[a] % of copies of the minor allele. MAF = minor allele frequency. All SNPs were in HWE (χ^2s [1, N = 568 - 592] < 1.23, ps > .27).

Results

GR haplotypes and the MR SNP were not related to attachment security (.13 < p < .61), maternal sensitive responsiveness (.59 < p < .99), or extreme insensitivity (.34 < p < .99). Maternal sensitive responsiveness and extreme insensitivity were only modestly correlated (r = -.14, p <.01). There were no main effects of sensitive responsiveness and extreme insensitivity on attachment security (r = .05, p = .18 and r = -.04, p = .38, respectively). Using a regression analysis, we tested for an interaction effect of maternal sensitive responsiveness, GR haplotypes, and MR on attachment security, controlling for extreme insensitivity. The same model was run for extreme insensitivity, controlling for sensitive responsiveness. In both models, main effects of GR haplotypes and MR did not reach significance (.06 < p < .86). The interaction between the MR SNP and sensitive responsiveness was significant (β = .10, p = .02), and a similar effect was found for the interaction between the MR SNP and extreme insensitivity (β = -.13, p = .005; Table 3). Interactions between GR haplotypes and maternal sensitive responsiveness were not significant (.34 < p < .99); the same was true for extreme insensitivity (.13 < p < .66). Locating the interaction effect, we found that infants carrying the minor MR allele (G) were significantly more securely attached if their mothers showed more sensitive responsive behaviors, and were significantly less securely attached if their mothers showed more extremely insensitive behaviors. These associations were not significant for carriers of the AA genotype of MR (Figure 1).

Table 3. *Regression analyses predicting attachment security from maternal sensitive responsiveness and extreme insensitivity and MR*

Sensitive Responsiveness	*B* (95% *CI*)	β	R^2	R^2_{change}	**Extreme Insensitivity**	*B* (95% *CI*)	β	R^2	R^2_{change}
Step 1			<0.01	<0.01	Step 1			<0.01	<0.01
Extreme insensitivity	-0.06 (-0.30; 0.18)	-0.02			Sensitive responsiveness	0.08 (-0.11; 0.27)	0.38		
Step 2			0.01	0.01	Step 2			0.01	0.01
Sensitive responsiveness	0.09 (-0.09; 0.28)	0.04			Extreme insensitivity	-0.07 (-0.31; 0.16)	-0.03		
MR	0.49 (-0.00; 0.98)	0.09			MR	0.48 (-0.01; 0.97)	0.09		
Step 3			0.02	0.01	Step 3			0.03	0.02
Sensitive responsiveness * MR	0.47 (0.07; 0.88)	0.10*			Extreme insensitivity * MR	-0.68 (-1.16;-0.21)	-0.13**		

Note. Final model for sensitive responsiveness: F (4, 497) = 2.47, $p < .05$, $R^2 = 2\%$; for extreme insensitivity: F (4, 497) = 3.13, $p < .05$, $R^2 = 3\%$. β is a standardized coefficient and denotes SD change in attachment security per SD change in the predictor. The statistics are derived from the final block of the regression models.

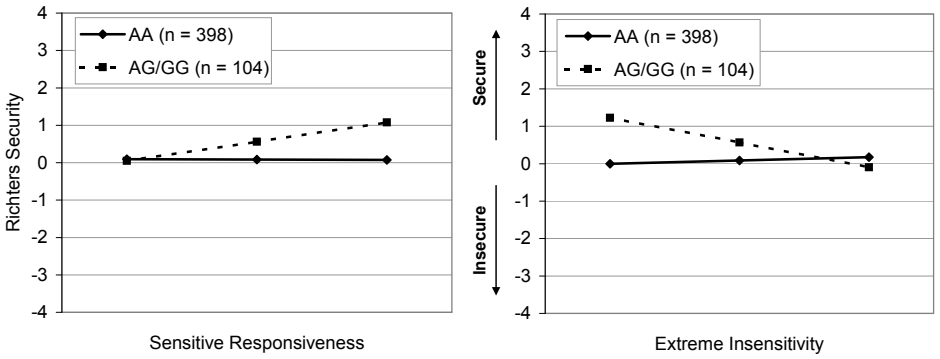

Figure 1. *Interaction between MR genotype and maternal sensitive responsiveness (left) and extreme insensitivity (right) on attachment security*

Discussion

Infants carrying the minor MR allele (G) were more securely attached if their mothers showed more sensitive responsiveness, *and* less securely attached if their mothers showed more extremely insensitive behaviors, whereas these associations were not significant for carriers of the AA genotype of MR. Genetic variation in MR thus seems to modulate infants' sensitivity to care, both in a positive (maternal sensitive responsiveness), as well as in a negative (maternal extreme insensitivity) environment. This supports the differential susceptibility hypothesis (Belsky et al., 2007).

MR is involved in the fast onset of responses and associated with processing of stressful information (DeRijk & De Kloet, 2008). We speculate that infants who are faster and better in processing information on maternal behaviors in stressful circumstances might be more susceptible to the effects of both positive care (sensitive responsiveness) and negative parenting (extreme insensitivity), for better *and* for worse. This potential mechanism should be examined in future biochemical as well as behavioral studies.

The two types of observed maternal behavior might be thought to reflect two extremes on a caregiving continuum. However, conceptually as well as statistically they indicate different, only weakly related dimensions of parenting. It should be noted that in the current, homogeneous middle class sample, quality of maternal care was not associated with attachment security. Generally, maternal care is only weakly to moderately associated with attachment, and null findings have also been reported (Barry et al., 2008).

McGowan and colleagues (2009) showed that exposure to early adversity was associated with epigenetic regulation of the GR receptor. In the area of attachment, epigenetic regulation of the serotonin transporter gene was found to influence the way in which adults cope with loss of attachment figures or other

trauma (Van IJzendoorn et al., 2010). The combination of GxE and epigenetics (Zhang & Meaney, 2010) seems to be the most promising avenue for investigating the complex interplay between genetic and environmental factors in explaining developmental outcomes, and in particular attachment security.

5 Attachment genes? Associations of dopaminergic, serotonergic, oxytonergic and neuroplasticity candidate genes with attachment security and disorganization

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Abstract

In two birth cohort studies with genetic and attachment data of more than 1100 infants in total, we tested main effects of candidate genes involved in the dopamine, serotonin, oxytocin, and neuroplasticity systems on attachment security and disorganization. We found no additive genetic associations for attachment security and attachment disorganization, assessed with the Strange Situation Procedure. However, specific tests for dopamine and serotonin system genes revealed a co-dominant risk model for COMT Val158Met, very consistent across both samples. Carriers of the Val/Met genotype showed higher disorganization scores (combined effect size $d = 0.20$, $CI = 0.09; 0.32$, $p = .001$). This unexpected finding might be explained by a broader range of plasticity in heterozygotes, which may increase susceptibility to environmental influences. The current study provides uniquely robust results in combining the two largest attachment cohorts with molecular genetic data to date. Future directions in research on the genetics of attachment are discussed.

Introduction

Attachment is defined as the child's need to seek proximity to a favorite, protective caregiver in times of stress (e.g., illness, danger) and to derive comfort from the attachment figure in stressful settings (Cassidy, 2008). Insecure and especially disorganized attachments elevate risk for psychopathology in adolescence and adulthood (Sroufe, Egeland, Carlson, & Collins, 2005). Formation of an attachment relationship, considered essential for offspring survival (Bowlby, 1969/1982; Suomi, 2008), is influenced mainly by the interactive history of an infant and its caregiver

and, to a lesser extent, socio-demographic factors and psychosocial characteristics of the parents (Belsky & Fearon, 2008). An emphasis on environmental origins of attachment-related individual differences is consistent with behavior-genetic studies of twins, which estimate the contribution of genetic factors to attachment security and disorganization to be negligible (Bokhorst et al., 2003; O'Connor & Croft, 2001; Roisman & Fraley, 2008).

Nevertheless, much-cited work by Lakatos and colleagues (2000) a decade ago presented evidence of a direct genetic effect on disorganized attachment involving a 48 base pair variable number tandem repeat (VNTR) in the promoter region of the Dopamine D4 receptor gene (DRD4). In a homogeneous sample of 90 low-risk Caucasian children, the 7-repeat allele was associated with higher risk for disorganized attachment. These results stimulated several replication efforts (Bakermans-Kranenburg & Van IJzendoorn, 2004; Spangler, Johann, Ronai, & Zimmermann, 2009), but none reproduced evidence of a direct association between DRD4 and disorganized attachment (see Bakermans-Kranenburg & Van IJzendoorn, 2007 for a review). Later, Spangler and colleagues (2009) reported a direct genetic association between the short allele of the serotonin transporter gene 5-HTT and increased risk for attachment disorganization. Their findings in 96 low-risk Caucasian infants call for replication in larger samples.

In two large cohorts of infants, we assessed polymorphisms in the dopaminergic, serotonergic, oxytonergic, and neuronal plasticity systems, to examine whether these are associated with the quality of infants' attachment behavior. The dopaminergic system is involved in attentional, motivational, and reward mechanisms (Robbins & Everitt, 1999). Common variations in dopaminergic genes DRD4 48 bp VNTR, DRD4 -521C/T, DRD2/ANKK1 and COMT Val158Met are associated with regulation of dopamine levels (D'Souza & Craig, 2006). Behaviorally, carrying the minor allele of these polymorphisms (respectively, DRD4 48 bp 7-repeat; DRD4 -521 C; DRD2/ANKK1 T[A1]) has been related to variations in infant temperament (Ebstein, 2006) and ADHD (Faraone & Khan, 2006). A protective effect has been reported for COMT heterozygotes (Val/Met) showing dopamine levels associated with optimal neurobehavioral outcomes, compared with both homozygous groups (Wahlstrom, White, & Luciana, 2010).

The serotonin system is involved in affect and emotion. A 44 bp insertion/deletion segment of the serotonin transporter gene 5-HTT (5-HTTLPR) is associated with less efficient transcription and serotonin uptake in the synapse (Greenberg et al., 1999; Heils et al., 1996), and the short allele is related to psychiatric disorders (Ebstein, 2006; Rutter, 2006). The oxytonergic system is related to social and parenting behaviors, and both oxytocin levels and polymorphisms in the oxytocin receptor gene (OXTR rs53576 and rs2254298; in particular for the minor A-allele) are associated with the formation of social bonds in both human and animal studies (Bakermans-Kranenburg & Van IJzendoorn, 2008; Carter, Boone, Pournajafi-Nazarloo, & Bales, 2009; Feldman, Gordon, Schneiderman, Weisman,

& Zagoory-Sharon, 2010; Insel, 2010). Finally, brain-derived neurotrophic factor (BDNF) is a protein associated with neuronal growth and survival (Gizer, Ficks, & Waldman, 2009). The gene coding for this protein, also called BDNF, contains a polymorphism influencing secretion of BDNF in the brain. This polymorphism (especially the minor Met-allele) is associated with ADHD (Gizer et al., 2009) and responses to stress and adversity; children with the Met-allele exposed to early deprivation manifest increased anxiety (Casey et al., 2009).

Combining the two largest attachment cohorts to date provides a unique opportunity to explore effects of candidate genes involved in the dopamine, serotonin, oxytocin, and neuroplasticity systems on attachment security and disorganization. The use of a standardized assessment in two independent, well-powered cohorts of Caucasian infants may lead to robust findings.

Materials and Methods

Setting

This report is based on two investigations, the Generation R Study, a prospective cohort study investigating development from fetal life into young adulthood in Rotterdam, the Netherlands (see Jaddoe et al., 2007; 2008), and the NICHD Study of Early Child Care and Youth Development (SECCYD), a prospective study carried out in 10 sites in the USA following children from birth to age 15 years (NICHD, 2005).

Detailed studies were performed in an ethnically homogeneous sub-sample of children of Dutch national origin from the Generation R Study. These children, their parents and their grandparents were born in the Netherlands, which was a selection criterion in order to reduce the risk of confounding (population stratification) by ethnicity. Children participating in this cohort were born between February 2003 and August 2005. Children visited the research center regularly for various assessments. Detailed measurements of child development also were obtained in the SECCYD, which followed an ethnically diverse sample, though the focus of the present inquiry was on the sub-set of Caucasian participants. Participating children were born in 1991 and regularly visited the local universities that recruited them. Written informed consent was obtained from parents of all participants in both studies, which were approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam and the Internal Review Boards of the SECCYD participating universities, respectively.

Study population

In the Generation R study, DNA was collected from cord blood samples at birth. To check for contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases, which were excluded. SECCYD DNA was obtained from buccal cheek cells when children were 15 years old. In both studies infants and their parent participated in the Strange Situation

Procedure (SSP) at age 15 months. Quality of attachment was available for 829 (Generation R) and 1191 (SECCYD) parent-child dyads; availability of genotype information ranged from n = 640 to n = 690 for specific SNPs in Generation R. In SECCYD, DNA was collected from n = 711 participants, 478 to 522 of whom were Caucasian, provided pertinent genotype information *and* completed the SSP in infancy. Non-response analysis indicated significant differences between the groups with and without genotypic data in Generation R mainly on perinatal variables. Children without genotypic data had lower gestational age, birth weight and Apgar scores (ps < .01) and mothers were more often nulliparous (p < .05). These births may have been more problematic, raising logistical difficulties to sample cord blood for DNA. SECCYD non-response analysis indicated that Caucasians with genotypic and infant attachment data differed from Caucasians lost to follow-up before age 15 years or who did not provide genetic data; those in the current analysis were more likely to be female (p < .05) and have mothers who were somewhat older (p < .01) and more educated (p < .01) at study onset. Table 1 presents characteristics of both samples.

Procedures and measures

Strange Situation Procedure. In both studies, parent-infant dyads were observed in the Strange Situation Procedure (SSP, Ainsworth, Blehar, Waters, & Wall, 1978) when the infant was about 15 months old. In the Generation R study, SSPs were conducted with the primary caregiver; 87% mothers (n = 721) and 13% fathers (n = 108). In SECCYD, SSPs were conducted with mothers. The SSP is a well-validated, widely used procedure to measure the attachment quality. It consists of seven 3-minute episodes designed to evoke mild stress to trigger attachment behavior (Ainsworth et al., 1978). To make it fit a tight time schedule in Generation R (only), two (pre-) separation episodes were shortened by one minute, keeping the critical reunion episodes intact (Luijk et al., 2010).

Attachment behaviors may be categorized as secure (B) or insecure (A, C, D; Main & Solomon, 1990). When stressed, secure (B) infants seek comfort from their mothers, which proves effective, enabling the infant to return to play. Avoidant (A) infants show little overt distress, while turning away from or ignoring mother on reunion. Resistant (C) infants are distressed and angry, but ambivalent about contact, which does not effectively comfort and allow the child to return to play. Examples of disorganized/disoriented (D) behaviors are prolonged stilling, rapid approach-avoidance vacillation, sudden unexplained affect changes, severe distress followed by avoidance, and expressions of fear or disorientation upon return of mother.

Attachment behavior was coded from DVD (Generation R) and videotape (SECCYD) recordings according to established coding systems (Ainsworth, et al., 1978) by two or three highly-trained, reliable coders. Inter-coder agreement was calculated on 70 SSPs in Generation R and 1191 double-coded SSPs in the

SECCYD. For ABCD classification, inter-coder agreement was 77% and 83% (κ = .63 and .69); agreement on disorganized versus non-disorganized attachment classification was 87% and 90% (κ = .64 and .64), respectively.

Richters and associates (1988) developed a method to score attachment in a continuous way. Van IJzendoorn and Kroonenberg (1990) adapted their algorithm, producing a valid Attachment Security Scale which has been widely used (e.g. Kochanska, Aksan, Knaack, & Rhines, 2004). Higher security scores indicate a more secure attachment relationship. Continuous scores for disorganization were derived directly from coding, with higher scores indicating more disorganized behavior. Intercoder reliability (intraclass correlation coefficients [ICC]) for the continuous attachment security and disorganization scales were .88 and .88, respectively, in Generation R (n = 70) and were .92 and .84, respectively, in SECCYD (n = 1191).

Genotyping. Genotyping was performed for genes in the dopaminergic system; DRD4 48 bp VNTR, DRD4 -521C/T (rs1800955), DRD2 (rs1800497), COMT Val158Met (rs4680), the serotonergic system; 5-HTTLPR, and the oxytonergic system; OXTR (rs53576 and rs2254298), and a gene involved in neuroplasticity; BDNF (rs6265). Table 2 displays minor allele frequencies (MAF). Frequency distributions conformed to the Hardy-Weinberg equilibrium (HWE), except for OXTR rs53576 (χ^2 = 4.90; p = .03) in Generation R and DRD4 48 bp VNTR (χ^2 = 14.17; $p < .001$) in SECCYD. The appendix provides detailed information about extraction and genotyping procedures.

Statistical analyses. Preliminary ANOVA and correlational analyses evaluated whether demographic variables were related to genotype and attachment security. Associations between the pertinent gene polymorphisms and attachment security and disorganization were tested using regression analyses applying additive genetic models. For DRD4 48 bp VNTR, DRD2, COMT, and 5-HTT VNTR previous studies have suggested increased risk for carriers of the DRD4 48 bp 7-repeat (Ebstein, 2006), the A1 allele of DRD2 (Berman, Ozkaragoz, Young, & Noble, 2002), and the short allele of 5-HTT (Lesch et al., 1996; Philibert et al., 2007), and a beneficial effect for COMT heterozygotes (Wahlstrom et al., 2010). These models were tested in additional ANOVAs. Attachment security and disorganization, as orthogonal constructs (Van IJzendoorn, Schuengel, & Bakermans-Kranenburg, 1999), were analyzed separately. Assuming a power of 0.80 and significance level of .05 (2-sided) (using Quanto 1.2.4 software, http://hydra.usc.edu/GxE), we were able to detect genetic effects of 1% of explained variance in both outcomes in Generation R and approximately 1.5% in SECCYD.

Results

Distribution of attachment

Distribution of attachment classifications was as follows in Generation R and SECCYD: 58.6% and 69.8% secure (n = 486 and n = 370), 18.2% and 15.7% insecure-avoidant (n = 151 and n = 83), 22.4% and 14.5% insecure-resistant (n = 186 and n = 77). In Generation R, no classification could be assigned for n = 6 (0.7%) children (All SECCYD participants were assigned to their best fitting category). Of all children, 21.0% and 13.4% were classified as disorganized (n = 174 and n = 71), 79.0% and 83.2% were non-disorganized (n = 655 and n = 441). SECCYD excluded 18 (3.4%) difficult to classify cases from the ABCD groupings. Mean Attachment Security Scale scores in Generation R and SECCYD were 0.24 (SD = 2.58) and 1.21 (SD = 3.17); mean disorganization scores were 3.37 (SD = 1.91) and 2.39 (SD = 2.01). Of all background characteristics (see Table 1), only breastfeeding at six months was associated both with attachment quality (security: $p < .05$ and disorganization: $p < .05$) and genotype ($p < .01$) in the Generation R sample. Children breastfed at six months were more secure and less disorganized, and less often carried the minor Val allele of COMT. Taking breastfeeding into account as a covariate did not change the Generation R results. None of the demographic variables in Table 1 was associated with both attachment quality and genotype in SECCYD.

Table 1. *Sample characteristics for Generation R and NICHD SECCYD*

Child characteristics	Generation R	NICHD SECCYD
Child gender, % female	49.3	51.5
Parity, % nulliparous	63.5	47.7
Birth weight in grams	3514 (540)	3537 (496)
Gestational age in weeks	40.0 (1.8)	39.3 (1.4)
Apgar score, % < 7	4.8	--
Parental characteristics		
Age at intake mother	31.9 (3.8)	29.4 (5.3)
Maternal educational level, % low/medium	33.7	22.6
Hours working, mother	28.8 (12.4)	22.5 (19.6)
Marital status, % single	4.3	6.8
Smoking during pregnancy, %	12.3	--
Alcohol during pregnancy, %	58.1	--
Breastfeeding at 6 months, %	30.4	51.8

Note. Unless indicated otherwise, values are Mean (SD). -- = Not measured.

Attachment genes

Using an additive genetic model, in both samples none of the genetic associations for attachment security and attachment disorganization reached significance (Table 2). Table 3 presents results of additional ANOVAs testing a recessive or co-dominant effect for DRD4 48 bp VNTR, DRD2, COMT, and 5-HTT VNTR. DRD4 associations were non-significant. For 5-HTT, short-allele carriers were more often securely attached and DRD2 A1 carriers showed higher disorganization scores, but only in Generation R. For COMT, no associations with attachment security emerged. However, COMT heterozygotes were more disorganized in both samples, see Table 3 (combined effect size $d = 0.20$, *95% CI* = 0.09; 0.32, $p = .001$).

Discussion

In both studies, no evidence emerged for additive effects of candidate genes putatively involved in attachment security and disorganization. Thus, the 'usual suspects' (Ebstein, Israel, Chew, Zhong, & Knafo, 2010) in the dopamine, serotonin, oxytocin and neuroplasticity systems were not related to attachment quality. Furthermore, proposed risk models for DRD4, DRD2, and 5-HTT failed to provide unequivocal results. No effects were found in either study for insecure or disorganized attachment in carriers of the DRD4 48 bp 7-repeat. And although DRD2 minor-T(A1)-allele carriers showed increased disorganization and 5-HTT short-allele carriers proved more securely attached in Generation R, neither finding was replicated in SECCYD.

However, a co-dominant effect of the COMT Val/Met proved replicable across studies (a small effect of $d = 0.20$). In carriers of the Val/Met genotype, disorganization scores were higher compared to both Val/Val and Met/Met carriers, a disadvantage also referred to as negative heterosis (Comings & MacMurray, 2000). Co-dominant effects for COMT Val/Met have been reported for neurobehavioral functioning (Gosso et al., 2008; Wahlstrom et al., 2010) and schizophrenia (for a meta-analysis, see Costas et al., 2010). However, these studies showed evidence of *positive* heterosis. Molecular heterosis is thought to be biologically plausible. Several studies (e.g. Tunbridge, Harrison, & Weinberger, 2006) suggest that there is an inverted U-shape with opposing gene expression occurring in heterozygotes compared to the homozygotes. Alternatively, a greater range of gene expression in heterozygotes compared to homozygotes could play a role. The range of expression of gene products could be greater in heterozygotes, providing a broader window for plasticity or response to stress (Comings & MacMurray, 2000).

Evidence from this inquiry might suggest the latter, with COMT Val/Met carriers possibly being more susceptible to environmental influences, which in turn may increase risk for attachment disorganization. Moreover, COMT Val158Met has been shown to be involved in regulation of emotional arousal (Drabant et

Table 2. *Minor allele frequencies and main effects of candidate genes on attachment security and attachment disorganization for both samples*

Gene	Marker	Minor allele	Generation R						NICHD SECCYD					
			MAF	N	*B*	95% *CI*	*r*	*p*	MAF	N	*B*	95% *CI*	*r*	*p*
Security														
Dopaminergic system														
DRD4	48bp VNTR	7+	19	647	0.11	-0.26; 0.47	.02	.57	12	478	0.19	-0.38; 0.76	.03	.52
DRD4	rs1800955	C	44	682	-0.06	-0.34; 0.22	-.02	.70	-	-	-	-	-	-
DRD2	rs1800497	T	18	641	-0.13	-0.50; 0.26	-.03	.48	19	512	-0.02	-0.54; 0.50	.00	.95
COMT	rs4680	G (val)	48	640	<0.01	-0.28; 0.28	.00	>.99	50	522	0.31	-0.06; 0.68	.07	.10
Serotonergic system														
5-HTT	44bp VNTR	short	44	677	0.21	-0.06; 0.49	.06	.13	59	512	0.05	-0.29; 0.38	.01	.77
Oxytonergic system														
OXTR	rs53576	A	34	687	-0.01	-0.31; 0.29	.00	.93	35	512	0.06	-0.35; 0.47	.01	.77
OXTR	rs2254298	A	12	690	0.16	-0.27; 0.59	.03	.47	11	503	0.13	-0.52; 0.78	.02	.70
Neuroplasticity														
BDNF	rs6265	A (met)	19	688	0.28	-0.08; 0.63	.06	.12	-	-	-	-	-	-
Disorganization														
Dopaminergic system														
DRD4	48bp VNTR	7+	19	647	-0.17	-0.44; 0.10	-.05	.22	12	478	0.28	-0.09; 0.64	.07	.14
DRD4	rs1800955	C	44	682	-0.02	-0.23; 0.18	-.01	.83	-	-	-	-	-	-
DRD2	rs1800497	T	18	641	0.25	-0.02; 0.52	.07	.07	19	512	0.19	-0.14; 0.52	.05	.26
COMT	rs4680	G (val)	48	640	0.13	-0.07; 0.34	.05	.20	50	522	0.10	-0.14; 0.34	.04	.42
Serotonergic system														
5-HTT	44bp VNTR	short	44	677	-0.05	-0.25; 0.15	-.02	.63	59	512	-0.05	-0.26; 0.17	-.02	.67
Oxytonergic system														
OXTR	rs53576	A	34	687	-0.20	-0.42; 0.26	-.07	.08	35	512	-0.11	-0.37; 0.15	-.04	.42
OXTR	rs2254298	A	12	690	0.15	-0.17; 0.47	.04	.35	11	503	0.10	-0.31; 0.52	.02	.63
Neuroplasticity														
BDNF	rs6265	A (met)	19	688	-0.03	-0.29; 0.22	-.01	.80	-	-	-	-	-	-

Note. Additive models are presented. *B* denotes change in security and disorganization scores per unit change in the predictor.

Table 3. *ANOVAs for specific models in DRD4 VNTR, DRD2, COMT and 5-HTT VNTR for both samples*

Gene	Risk model	Genotype (*M*, *SD*)			Generation R			Genotype (*M*, *SD*)			NICHD SECCYD		
		aa *N*	Aa *N*	AA *N*	*F*	*r*	*p*	aa *N*	Aa *N*	AA *N*	*F*	*r*	*p*
Security													
DRD4 VNTR	7+	0.19 (2.59) 420	0.42 (2.40) 208	-0.14 (3.29) 18	0.75	.04	.39	1.11 (3.22) 377	1.66 (2.88) 85	0.67 (3.74) 16	1.19	.06	.28
DRD2 rs1800497	T (A1)	0.35 (2.60) 433	0.04 (2.54) 185	0.68 (2.53) 22	1.24	-.05	.27	1.19 (3.05) 333	1.29 (3.38) 167	0.58 (3.83) 12	0.02	.01	.88
COMT rs4680	homozygote	0.21 (2.53) 175	0.34 (2.63) 315	0.19 (2.54) 149	0.49	-.03	.49	0.77 (3.32) 140	1.31 (3.00) 245	1.39 (3.29) 137	0.74	-.04	.39
5-HTT VNTR	short	-0.10 (2.46) 215	0.52 (2.58) 323	0.23 (2.73) 139	6.32	.11	.01	1.03 (3.31) 135	1.34 (3.09) 147	1.17 (3.15) 230	0.40	.03	.53
Disorganization													
DRD4 VNTR	7+	3.45 (1.91) 421	3.34 (1.88) 208	2.81 (1.80) 18	0.88	-.04	.35	2.33 (1.97) 377	2.75 (2.27) 85	2.56 (2.45) 16	2.89	.09	.09
DRD2 rs1800497	T (A1)	3.29 (1.87) 434	3.62 (1.91) 185	3.50 (2.17) 22	3.99	.08	.05	2.31 (1.96) 333	2.56 (2.11) 167	2.33 (2.02) 12	1.68	.06	.20
COMT rs4680	homozygote	3.08 (1.97) 176	3.60 (1.83) 315	3.32 (1.92) 149	7.57	-.11	.01	2.12 (1.77) 140	2.58 (2.13) 245	2.31 (1.97) 137	4.20	-.09	.04
5-HTT VNTR	short	3.42 (1.90) 215	3.35 (1.86) 323	3.33 (2.00) 139	0.25	-.02	.62	2.47 (2.10) 135	2.27 (2.00) 147	2.35 (1.95) 230	0.53	-.04	.47

Note. df = 1. aa: homozygous for wildtype, Aa: heterozygous, AA: homozygous for minor allele. Models tested are all dominant (aa compared to Aa combined with AA), except for COMT, which is codominant (AA combined with aa compared to Aa). F, r, and p-values all refer to this two-group comparison.

al., 2006), which is considered central to disorganized attachment. Disorganized infants inability to regulate stress and emotions in arousing situations is striking, and their dysregulation is an early predictor of later psychopathology (Fearon, Bakermans-Kranenburg, Van IJzendoorn, Lapsley, & Roisman, 2010; Sroufe et al., 2005).

Genetic pathways are frequently indirect and subject to numerous biological and environmental influences (Ebstein et al., 2010; Kendler, 2005). Including environmental factors was beyond the scope of the current study, but gene-environment interactions may prove important. Several attachment GxE studies suggest that genetic effects may be contingent upon gene-environment co-action (Gervai et al., 2007; Spangler et al., 2009; Van IJzendoorn & Bakermans-Kranenburg, 2006; see also Rutter, 2006). Moreover, studies on GxE interaction in attachment could benefit from a shift from a conventional model of vulnerability genes, or 'risk alleles', to a focus on plasticity or susceptibility genes (Belsky et al., 2009). From this perspective, certain genes are thought to render individuals more responsive than others to both positive *and* negative environmental experiences (Belsky, Bakermans-Kranenburg, & Van IJzendoorn, 2007).

Previously reported associations for genes involved in attachment (DRD4 48 bp VNTR, 5-HTT) could not be replicated in the two cohorts. Current results thus confirm Burmeister and colleagues' (2008) conclusion that "testing plausible candidate genes for genetic association (…) has led to many false positives and irreproducible reports", something probably caused by a variety of factors (e.g., small samples, publication bias). Also population stratification, sufficient power and accurate assessment of the phenotype are crucial methodological aspects (Ebstein, 2006; Ioannidis, 2007; Little et al., 2009). Here the study populations were selected for Caucasian ethnicity only, securing an ethnically homogenous sample. Although only small single-gene effects were anticipated (i.e.,~1%; Plomin & Davis, 2009), power was sufficient to detect such small effects. Furthermore, the phenotype was assessed carefully, as the SSP is the gold standard for assessing attachment quality. Finally, direct replications were made possible by using the two largest attachment cohorts with molecular genetic data to date.

Genetic contributions to attachment may operate in ways not tested in here. For example, epistatic effects could play a role (e.g. Pezawas et al., 2008). Before evaluating these gene-gene interactions, more knowledge is needed about functionality and specific pathways of targeted genes. Also, effects of deletions or multiplications of larger DNA segments—copy number variations (CNVs)—are known to affect protein expression and gene function. These CNVs might act as vulnerability factors for neurodevelopmental phenotypes (Merikangas, Corvin, & Gallagher, 2009). Furthermore, epigenetic processes merit consideration, as these can modify gene expression and neural function without changing nucleotide sequence (Van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach, & Philibert, 2010; Zhang & Meaney, 2010).

Attachment is a developmental milestone and attachment disorganization a major risk factor for later-life psychopathology. Here we found evidence for negative heterosis, with carriers of the COMT Val/Met genotype showing more attachment disorganization than both Val/Val and Met/Met carriers; assuming it is not the result of Type 1 error, this could reflect greater vulnerability to a negative environment. Attachment is a complex behavioral phenotype in which polygenic effects might operate, in combination with environmental factors. The most important genetic effects on attachment might be hidden in interaction with environmental factors. The most promising avenue for future gene-oriented attachment studies is therefore the careful assessment of the interplay between (epi)genetic differences and child-rearing influences.

Appendix

Genotyping information Generation R. Genotyping of polymorphisms DRD4 -521C/T, DRD2, COMT, OXTR, and BDNF was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 min), then 40 cycles of 94° C (15 s) and 60° C (1 min)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97-99% of the samples. To confirm the accuracy of the genotyping results 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes.

Genotyping of the DRD4 48 bp VNTR was amplified using primers D4-F-GCGACTACGTGGTCTACTCG and D4-R-AGGACCCTCATGGCCTTG. Reactions were performed in a 384-wells format in a total reaction volume of 10 ul containing 10 ng DNA, 1 pmol/ul of each primer, 0,4 mM dNTPs, 1 M betaine, 1x GC buffer I (Takara Bio Inc.) and 0,5 U/ul LA Taq (Takara Bio Inc.). PCR cycling consisted of initial denaturation of 1 min at 94° C, and 34 cycles with denaturation of 30 seconds at 95°C, annealing of 30 seconds at 58°C and extension of 1 minute at 72°C. PCR fragments were size-separated on the Labchip GX (Caliper Life sciences) using a HT DNA 5K chip (Caliper Life sciences). The number of DRD4 repeats was determined using the size of the PCR-fragments. To assure genotyping accuracy 225 random samples were genotyped for a second time. Three samples (1.3%) gave different genotypes. These discrepancies were specific for the repeats longer than 7. The HT DNA 5K chip was unable to accurately distinguish the 7, 8, 9 and 10 repeat. As the frequency of the 8, 9 and 10 repeat is low; all samples with a 7 repeat or longer were analyzed as one group.

Genotyping of the 5-HTTLPR was performed using Taqman allelic discrimination. Primer sequences were taken from Hu et al. (2006). Reactions were performed in a 384-wells format in a total volume of 5 ul containing 2 ng DNA,

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

Genotyping information SECCYD. Extraction for all polymorphisms in the SECCYD was based on adaptations to Freeman et al. (2003). Specifically, buccal mucosa cells were collected with cotton swabs by the subject. The swabs were placed in 15-ml centrifuge tubes containing 2.5 mls of lysis buffer. The tubes were incubated in a water bath at 65°C for 2 hr to activate the proteinase K. After incubation the tubes were centrifuged at 300g for 4 min and the supernatant added to 4ml of isopropanol. Tubes were centrifuged again for 30 min. The supernatant was poured off, the pellet dried and 1 ml of lysis buffer without proteinase K was added. Pellets were resuspended by shaking overnight. The liquid was transferred to a 1,5 ml microfuge tube and 200 µl of an organic deproteinization reagent (ODPR) were added to each tube. The tubes were capped and shaken vigorously by hand. The denatured debris and remaining organic mix were then centrifuged at 5000g for10 min. Supernatant from the tube was transferred to a fresh 1.5-ml tube and 800 ul of isopropanol was added and mixed gently for approximately 1 min. The DNA was collected by centrifugation at 5000g for 10 min. The pellets were dried and washed with 1 ml ethanol 70% (v/v) by centrifugation at 5000g for 10 min. The ethanol wash was discarded, the tubes were inverted, and the pellets were dried for 60 min. The DNA was re-suspended in 250 ul of Tris EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) by rotation in an incubator at 37°C. The DNA was quantified by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer. Samples were aliquoted into storage vials and placed in a -80°C freezer.

The assay for genotyping DRD4 was based on methods developed Sander et al. (1997) and modified by Anchordoquy et al. (2003). The Genomics Core Facility modified it further as the following: 1 x Taq Gold Buffer, 2.25 mM final concentration of MgCl2, 10% DMSO, 0.2 mM dNTPs, 0.1 mM deazo GTP, 0.75 uM primers, 40 ng of DNA and 1 U of Taq Gold (Applied Biosystems, Foster City CA) in a volume of 12 microliters. The primer sequences are: 5'-6-FAM-GCGAC TACGTGGTCTACTCG-3' and reverse, 5'-AGGACCCTCATGGCCTTG-3'. The amplification procedure was as described by Anchordoquy et al. (2003). One microliter was removed and placed in a 96 well plate and 10 microliters of formamide containing LIZ-500 standard (Applied Biosystems, Foster City CA). The plate was run using a Fragment Analysis protocol in the 3730XL DNA Analyzer (Applied Biosystems, Foster City CA). Fragments were analyzed using

Genemapper software (Applied Biosystems, Foster City CA) with PCR products of (in bp): 379, 427, 475 (43), 523, 571, 619 (73), 667, 715, 763, and 811.

In order to genotype DRD2, Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.

For COMT, Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.

The assay for 5HTT was a modification of the method of Lesch et al. (1996) and Anchordoquy et al. (2003). The Genomics Core Facility modified it further as the following: 1 x Taq Gold Buffer, 1.8 mM final concentration of MgCl2, 10% DMSO, 0.2 mM dNTPs, 0.1 mM deazo GTP, 0.6 uM primers, 40 ng of DNA and 1 U of Taq Gold (Applied Biosystems, Foster City CA) in a volume of 15 microliters. The primer sequences were: forward, 5'-VIC- GGCGTTGCCGCTCTGAATGC-3' and reverse, 5'-GAGGGACTGAGCTGGACAACCAC-3'. The same amplification protocol as used for DRD4 was used for 5HTLL. One microliter was removed and placed ina 96 well plate and 10 microliters of formamide containing LIZ-500 standard (Applied Biosystems, Foster City CA). The plate was run using a Fragment Analysis protocol in the 3730XL DNA Analyzer (Applied Biosystems, Foster City CA). Fragments were analyzed using Genemapper software (Applied Biosystems, Foster City CA) with PCR products of 484 or 528 bp.

For OXTR rs53576, Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.

Finally, for OXTR rs2254298 Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters

with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.

6 Discussion: Vulnerability and plasticity

In the largest cohort study of attachment to date, the Generation R study, with carefully assessed biological markers and behavioral observations, we were able to investigate parental and genetic influences on infant attachment and stress regulation. In the current series of studies, infant attachment quality was related to cortisol stress reactivity, as assessed before and after the SSP. Insecure-resistant infants differed from all other groups, showing the largest increase in cortisol excretion after the SSP. Cortisol diurnal rhythm showed the expected diurnal pattern, but disorganized infants displayed a more flattened slope than non-disorganized infants. Maternal lifetime depression appeared to be a risk factor that further elevated cortisol reactivity in infants with an insecure-resistant attachment relationship. Also, the genetic make-up of the child was associated with cortisol reactivity; carriers of the risk genotype of FKBP5, a gene involved in the negative feedback loop of the HPA-axis, showed higher levels of cortisol reactivity. Furthermore, an interaction between insecure-resistant attachment and FKBP5 was found, representing a double risk for heightened cortisol reactivity levels in infants who carry the FKBP5 risk genotype and at the same time have an insecure-resistant attachment relationship with their mother.

In our collaborative effort with the NICHD Study of Early Child Care and Youth Development (SECCYD) to identify potential attachment genes, we found no evidence for additive effects of candidate genes putatively involved in attachment security and disorganization. Furthermore, proposed risk models for DRD4, DRD2, and 5-HTT failed to provide unequivocal results. However, a co-dominant effect of the COMT Val/Met proved replicable across both studies. In carriers of the heterozygous Val/Met genotype, disorganization scores were higher compared to both Val/Val and Met/Met carriers. Investigating the additional effect of maternal care on attachment quality, we found that genetic variation in the mineralocorticoid receptor gene (MR), which is involved in HPA-axis functioning, modulated infants' sensitivity to care. Infants carrying the minor allele of MR were more securely attached if their mothers showed more sensitive responsiveness, *and* less securely attached if their mothers showed more extremely insensitive behaviors, whereas these associations were not significant for carriers of the wildtype genotype of MR. The findings presented in this thesis provide attachment researchers with comprehensive results on vulnerability and plasticity factors in infant attachment and stress regulation. Moreover, the findings replicate and extend previous studies by making use of data from a large attachment cohort with physiological and genetic information.

Distribution of attachment in a large birth cohort
The assessment of attachment quality in a population based birth cohort provides the opportunity to compare the distribution of attachment classifications to meta-analytic findings. Outcomes from single well-powered studies are important, especially when heterogeneity plays a role in meta-analytic results. In the current study, the distribution of the attachment classifications was as follows: 58.6% secure (n = 486), 18.2% avoidant (n = 151), 22.4% resistant (n = 186). No classification could be assigned for n = 6 (0.7%) children. Of all children, 21.0% were classified as disorganized (n = 174), 79.0% were non-disorganized (n = 655). In Figure 1, the distribution of the current sample is presented together with the meta-analytic distribution of Van IJzendoorn et al. (1999), which represents a common distribution in non-clinical populations.

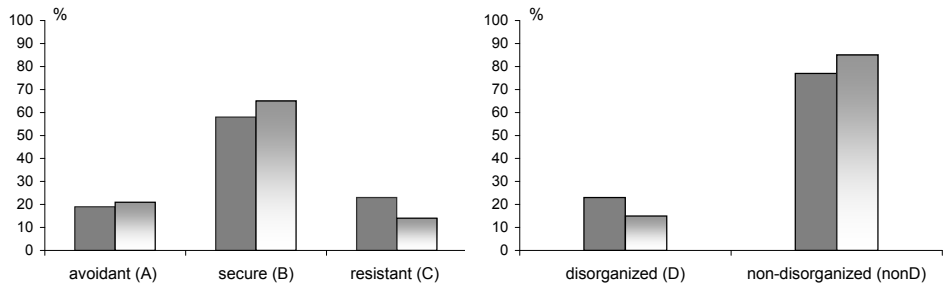

Figure 1. *Distribution of attachment classifications in the Generation R sample (solid bars) and from meta-analyses (shaded bars)*

In the current study, a slightly shortened version of the SSP was used, in order to make it fit into the schedule of the visit. This minimal procedural change did not appear to modify the stress of the SSP, since the number of infants for whom the situation appears to be most stressful (resistant and disorganized classifications) was not lower in the current study compared to the standard distribution.

Physiological vulnerability in attachment
Insecure-resistant infants showed the largest increase in cortisol levels from pre to post SSP; the effect was even stronger when they had depressive mothers. Disorganized children showed a more flattened diurnal cortisol pattern compared to non-disorganized children. These findings document the vulnerability of insecure-resistant infants in physiological stress regulation, especially in combination with care from mothers with a lifetime diagnosis of depression. It could be argued that heightened stress reactivity in the insecure-resistant group should be interpreted as supporting an arousal model, assuming associations between behavioral and physiological activation during stress (Spangler & Schieche, 1998). To test whether increases in cortisol were related to the amount of crying (i.e. an index

of behavioral and physiological arousal) during the SSP, we used a measure of observed crying. When adding crying to the model, it was a significant covariate, but insecure-resistant attachment remained a significant predictor, indicating an effect of insecure-resistant attachment on cortisol reactivity *independent* of the amount of crying during the SSP.

We also showed that disorganized infants differed from non-disorganized infants in their diurnal cortisol rhythm, as they displayed a more flattened daily curve. The relation between attachment and infant diurnal rhythm of cortisol excretion has been largely neglected, and was for the first time explored in the current thesis. Our findings stress the disturbed nature of disorganized attachments as one of the most important risks for developmental psychopathology. Overall, the findings suggest differential physiological concomitants of avoidant, resistant, and disorganized attachments.

Genetic vulnerability in attachment

Quality of the parent-infant attachment relationship influences physiological stress regulation in infants (Bowlby, 1969/1982; Hertsgaard, Gunnar, Erickson & Nachmias, 1995). To extend the findings from previous studies, we added a genetic component, as genetic factors also contribute to the stress regulatory HPA-axis (Bartels et al., 2003; Steptoe et al., 2009; Wüst et al., 2004a). We found a significant interaction effect for insecure-resistant attachment and a variant in the FKBP5 gene, a co-chaperone of the glucocorticoid receptor gene involved in the negative feedback loop of the HPA-axis. This indicates a double risk for heightened cortisol reactivity levels in infants who carry risk alleles of the FKBP5 SNP *and* have an insecure-resistant attachment relationship with their mother. Resistant attachment and FKBP5 predispose infants to increased cortisol reactivity both independently as well as in interaction. These outcomes provide support for a double-risk model (Belsky et al., 2007) as the combination of environmental (indexed by resistant attachment) and genetic (FKBP5) risks increased stress reactivity in an additive way.

In an effort to identify potential 'attachment genes', we investigated polymorphisms in two cohorts; The Generation R study and the NICHD Study of Early Child Care and Youth Development (SECCYD). In both studies, no evidence emerged for additive effects of candidate genes putatively involved in attachment security and disorganization. Thus, genes in the dopamine, serotonin, oxytocin and neuroplasticity systems were not related to attachment quality. Previously reported associations for genes involved in attachment (DRD4 48 bp VNTR, 5-HTT) could not be replicated in the two cohorts. However, a co-dominant effect of COMT Val/Met proved replicable across studies. In carriers of the heterozygous Val/Met genotype, disorganization scores were higher compared to both Val/Val and Met/Met carriers. Co-dominant effects for COMT Val/Met have been reported for neurobehavioral functioning (Gosso et al., 2008; Wahlstrom et al., 2010) and

schizophrenia (for a meta-analysis, see (Costas et al., 2010). A greater range of gene expression in heterozygotes compared to homozygotes could play a role, providing a broader window for plasticity or response to stress (Comings & MacMurray, 2000). Evidence from this inquiry might suggest the latter, with COMT Val/Met carriers possibly being more susceptible to environmental influences, which in turn may increase risk for attachment disorganization. Moreover, COMT Val158Met has been shown to be involved in regulation of emotional arousal (Drabant et al., 2006), which is considered central to disorganized attachment. Disorganized infants' inability to regulate stress and emotions in arousing situations is striking, and their dysregulation has been documented as an early predictor of later psychopathology (Fearon et al., 2010; Sroufe et al., 2005). Findings from these studies support the idea of interplay between genetic and environmental factors in explaining developmental outcomes (Rutter et al., 2006), and provide evidence for environment-dependent genetic vulnerabilities in attachment and stress regulation.

Plasticity in attachment and stress regulation

Originally, GxE studies have focused mainly on double risk models (or: diathesis stress models; Rutter, 2006). Nevertheless, not all children are equally susceptible to risk factors, and studies on GxE interaction in attachment could benefit from a shift from a conventional model of vulnerability genes, or 'risk alleles', to a focus on plasticity or susceptibility (Belsky et al., 2009). From this perspective, certain genes are thought to render individuals more responsive than others to both positive *and* negative environmental experiences (Bakermans-Kranenburg & Van IJzendoorn, 2007; Belsky, Bakermans-Kranenburg & Van IJzendoorn, 2007). Applying the concept of differential susceptibility to the study of attachment, we found that infants carrying the minor allele of the mineralocorticoid receptor gene (MR) were more securely attached if their mothers showed more sensitive responsiveness, *and* less securely attached if their mothers showed more extremely insensitive behaviors, whereas these associations were not significant for carriers of the wildtype genotype of MR. Genetic variation in MR thus seems to modulate infants' sensitivity to care, both in a positive (maternal sensitive responsiveness), as well as in a negative environment (maternal extreme insensitivity). As MR is involved in the fast onset of responses and associated with processing of stressful information (DeRijk & De Kloet, 2008), infants who are faster and better in processing information on maternal behaviors in stressful circumstances might be more susceptible to the effects of both positive (sensitive responsiveness) and negative parenting (extreme insensitivity), for better *and* for worse. This supports the differential susceptibility hypothesis (Belsky et al., 2007; Ellis, Boyce, Belsky, Bakermans-Kranenburg, & Van IJzendoorn, in press). When testing this hypothesis, careful assessment of the environment is essential. Defining the mere absence of adversity as a positive environment may lead to the under-detection

of differential susceptibility findings and an overrepresentation of vulnerability findings (Belsky et al., 2009). The use of observations of both negative and positive environmental factors makes it possible to accurately assess GxE processes in the present study.

Limitations

Some limitations of the current thesis need to be discussed. First, the Generation R Focus Study is a relatively homogeneous sample. However, the use of a homogeneous sample may have only led to an underestimation of effects, and not to an overestimation of the effects. Second, cortisol was sampled at 14 months of age, and cortisol levels at this age do show some intra-individual instability (De Weerth & Van Geert, 2002). However, data on the development of cortisol secretion throughout infancy and childhood are scarce, and we did find evidence for an established pattern. Again, instability may have led to an underestimation of the differences among attachment groups. Third, a relatively large part of the participants could not be included in cortisol analyses, due to various reasons. Clearly informing parents about sampling could help to gain more and better saliva samples, however, sampling might remain difficult in 14-month-olds. Fourth, a slightly shortened version of the SSP was used, in order to make it fit into the schedule of the visit. This minimal procedural change did not appear to modify the stress of the SSP, since the number of infants for whom the situation appears to be most stressful (resistant and disorganized classifications) was not lower in the current study compared to the standard distribution. Fifth, maternal sensitive responsiveness and extreme insensitivity might be thought to reflect two extremes on a caregiving continuum. However, conceptually as well as statistically they indicate different, weakly related dimensions of parenting. Furthermore, quality of maternal care was not associated with attachment security. Generally, maternal care is only weakly to moderately associated with attachment, and null findings have also been reported (Barry et al., 2008). Sixth, we did not include maternal genotype in the present study, which could be associated with quality of maternal care (Bakermans-Kranenburg & Van IJzendoorn, 2008; Kaitz et al., 2010). This should be incorporated in future GxE investigations. When conducting GxE research, the environment and outcome should be assessed as carefully as the genotypes. Recently two meta-analyses have been published that failed to find a significant interaction effect between 5-HTTLPR genotype and stressful life events on depression (Munafo, Durrant, Lewis, & Flint, 2009; Risch et al., 2009). The authors of these meta-analyses conclude that the field had been too eager to accept GxE studies in the absence of genetic main effects, and that genome-wide association studies should be given priority (Risch et al., 2009). It should however be noted, as others have done (Bakermans-Kranenburg & Van IJzendoorn, 2010), that the selection of studies for inclusion in these meta-analyses was somewhat particular, and that the quality of the studies varied substantially, including

sometimes weak measures for life events (the environmental factor). In a narrative review on the same topic Uher and McGuffin (2008; 2010) reviewed all pertinent studies, showing that the method of assessment of environmental adversity was an important predictor of the outcome of the study. Detailed interview-based and observational approaches were associated with positive GxE findings, whereas all non-replications used self-report questionnaires. High-quality GxE studies with careful measurement of the environment and the outcome variables are needed, as well as explicit hypotheses about how a *specific* gene and a *specific* environmental condition interact to predict a specific outcome (Bakermans-Kranenburg & Van IJzendoorn, 2010). In the current study, we were able to apply these methods, providing robust results on GxE interplay in infant attachment and stress regulation.

Finally, genetic contributions to attachment may operate in ways not tested in here. For example, epistatic effects could play a role (e.g. Pezawas et al., 2008). Before evaluating these gene-gene interactions, more knowledge is needed about functionality and specific pathways of targeted genes. Also, effects of deletions or multiplications of larger DNA segments—copy number variations (CNVs)—are known to affect protein expression and gene function. These CNVs might act as vulnerability factors for neurodevelopmental phenotypes (Merikangas, Corvin & Gallagher, 2009). Furthermore, epigenetic processes merit consideration, as these can modify gene expression and neural function without changing nucleotide sequence (McGowan et al., 2009; Van IJzendoorn et al., 2010; Zhang & Meaney, 2010).

Clinical implications and future directions

Because infant attachment patterns have been shown to be relatively stable in stable environments (Fraley, 2002) insecure attachments may have long-term consequences for mental health, in particular in combination with other risk factors such as low quality of maternal care, maternal depression or genetic risk. From a biological perspective (Sapolsky, 2004) adverse early experiences can make humans and other animals more prone to stress and stress-related diseases, and attachment relationships may mediate the intergenerational transmission (Meaney, 2001) of this elevated vulnerability to emotional dysregulation.

From a differential susceptibility view, our study shows that genetic make-up can modulate infants' openness to maternal care in both a negative and a positive way. A similar effect was found in a study of children with externalizing behavior problems (Bakermans-Kranenburg & Van IJzendoorn, 2006); children with the 7-repeat allele of the dopamine D4 receptor gene (DRD4) who were reared by insensitive mothers displayed more problem behaviors than children without the genetic variant. Carriers of the 7-repeat who were reared by sensitive mothers showed however the lowest levels of externalizing behavior. In the case of behavior problems, DRD4 seemed to moderate children's susceptibility to

parenting. The significance of viewing infants as susceptible instead of merely vulnerable provides major possibilities for intervention studies, and may help in explaining differential effectiveness of interventions. Recently, a moderating effect of the DRD4 gene was found on the effectiveness of an attachment based intervention (Video-feedback Intervention to promote Positive Parenting – Sensitive Discipline, VIPP-SD; Juffer, Bakermans-Kranenburg, & Van IJzendoorn, 2008). A larger intervention effect was found in children with the 7-repeat allele of the DRD4 gene (Bakermans-Kranenburg, Van IJzendoorn, Pijlman, Mesman, & Juffer, 2008). The plasticity of young children, for better or for worse, may provide behavioral scientists and clinicians with a framework that helps interpretation of seemingly confusing child developmental outcomes. Furthermore, future studies could benefit from incorporating both negative and positive environments in their designs, to fully capture the range of environmental influences in children's lives. As attachment is a complex behavioral phenotype in which polygenic effects might operate in combination with environmental factors, the most important effects might be hidden in gene-environment interactions. Promising avenues for future attachment studies are therefore the careful assessment of the interplay between (epi)genetic differences and child-rearing influences.

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Nederlandse samenvatting (Summary in Dutch)

Ervaringen in de vroege kindertijd zijn van grote invloed op de ontwikkeling van de regulatie van emoties en stress. Onderzoek bij dieren en bij kinderen in kindertehuizen wijst uit dat een gebrek aan warmte en genegenheid veel invloed heeft op de manier waarop kinderen met stress en stressvolle situaties omgaan. Omdat kinderen in de eerste levensjaren afhankelijk zijn van hun opvoeders, bepaalt de manier waarop ouders reageren op signalen van het kind in grote mate hoe kinderen later zelf met stress omgaan. Ervaringen gedurende het eerste levensjaar zijn tevens van groot belang voor de vorming van een gehechtheidsrelatie. De gehechtheidsrelatie is een belangrijke ontwikkelingsmijlpaal die een rol speelt in het reguleren van emoties en stress. De aanwezigheid van een sensitieve, responsieve ouder bevordert de kans op het ontwikkelen van een veilige gehechtheidsrelatie en kan daarmee helpen de stressreacties van het kind te reguleren. Gedurende het eerste levensjaar leren kinderen in welke mate hun ouders emotioneel beschikbaar zijn in tijden van stress. Variatie in de emotionele beschikbaarheid van ouders (bijvoorbeeld consequente sensitiviteit, inconsequente sensitiviteit, of consequente insensitiviteit) draagt bij aan verschillen in de kwaliteit van gehechtheid, en daardoor mogelijk ook aan verschillen in stressregulatie.

De kwaliteit van gehechtheid kan worden gemeten met de Vreemde Situatie-procedure (*Strange Situation Procedure*). Moeder en kind bevinden zich samen in een spelkamer, waar na een paar minuten een onbekende persoon binnenkomt. In de loop van ongeveer twintig minuten verlaat de moeder tweemaal kort de spelkamer, waarbij ze het kind achterlaat (éénmaal met de onbekende persoon, éénmaal alleen). Dit is voor het een kind een vreemde en stressvolle situatie die gehechtheidsgedrag oproept. Aan de hand van gedragsobservatie kunnen verschillen in de kwaliteit van de gehechtheidsrelatie worden geclassificeerd. In een stressvolle situatie zoeken veilig gehechte kinderen troost bij hun moeder. De veilige haven die de moeder biedt, zorgt ervoor dat het kind effectief getroost wordt en het de omgeving verder kan verkennen. Het evenwicht tussen contact met de ouder en exploratie is bij deze kinderen in balans. Onveilig-vermijdend gehechte kinderen laten (bijna) niet zien dat ze overstuur zijn en vermijden de moeder bij de hereniging. Onveilig-ambivalent gehechte kinderen zijn daarentegen boos en overstuur, maar ook ambivalent in hun contact met de moeder. Hierdoor is het voor deze kinderen moeilijk om troost te vinden en weer verder te gaan met spel en exploratie. Beide onveilige groepen zijn niet in staat de juiste balans te vinden tussen contact met de moeder en exploratie. De meest onveilige vorm van gehechtheid,

gedesorganiseerde gehechtheid, kenmerkt zich door gedragingen als plotseling stilvallen (*stilling* of *freezing*), afwisselingen tussen toenadering en vermijden van moeder, onverklaarbare wisselingen in affect, en gezichtsuitdrukkingen van angst of desoriëntatie wanneer moeder terugkomt na de korte scheiding.

De kwaliteit van gehechtheid heeft een belangrijke invloed op de verdere ontwikkeling van het kind. Kinderen die een veilige gehechtheidsrelatie met de opvoeder hebben opgebouwd, hebben meer vriendjes, zijn gemiddeld sociaal vaardiger en meer veerkrachtig. Onveilig en gedesorganiseerd gehechte kinderen hebben daarentegen vaker emotionele en gedragsproblemen. Gezien de belangrijke invloed van de gehechtheidsrelatie op de verdere ontwikkeling van kinderen, is het van groot belang te onderzoeken hoe verschillen in kwaliteit van gehechtheid ontstaan. In de huidige studie onderzochten we verschillende neurobiologische aspecten van gehechtheid.

Gehechtheid en stressregulatie

Dit onderzoek is uitgevoerd binnen de Generation R studie; een grootschalig prospectief cohortonderzoek onder Rotterdamse kinderen. In dit geboortecohort worden groei, ontwikkeling en gezondheid bestudeerd, vanaf de zwangerschap tot in de jongvolwassenheid. In een subgroep binnen dit cohort, bestaande uit bijna 1000 ouders en hun kinderen van Nederlandse nationaliteit, werden gedetailleerde metingen verricht waarop het huidige onderzoek is gebaseerd. In het eerste deel van het onderzoek hebben we ons gericht op de stresshuishouding van jonge kinderen. We waren geïnteresseerd in het verband tussen de kwaliteit van de gehechtheidsrelatie en de mate van stress die kinderen ervaren. Voor en na de Vreemde Situatie Procedure werd speeksel bij de kinderen afgenomen. In het speeksel is vervolgens de concentratie van het stresshormoon cortisol gemeten. Een hogere waarde van dit hormoon geeft weer dat het stresssysteem van het kind is geactiveerd. Uit het onderzoek bleek dat de groep kinderen met een onveilig-ambivalente gehechtheidsrelatie de hoogste waardes had, vergeleken met de andere groepen. Deze kinderen maximaliseren hun gehechtheidsgedrag en zijn tegelijkertijd niet in staat de ouder als bron van troost te gebruiken. Dit leidt tot hoge cortisolwaardes en laat de kwetsbaarheid en stressgevoeligheid van onveilig-ambivalent gehechte kinderen zien. Zelfs wanneer we controleerden voor het relatief meer huilen van de kinderen in deze groep, bleef het effect significant.

De stressregulatie kan niet alleen bekeken worden aan de hand van reacties op stressvolle situaties. De concentratie van cortisol volgt een ritme gedurende de dag, met hoge waardes bij het ontwaken en een afname over de loop van de dag. Hierbij waren we geïnteresseerd in verschillen in het verloop van dit dagritme tussen de verschillende groepen kinderen. Tot op heden is hier nog geen onderzoek naar gedaan, en om dit te kunnen meten hebben de ouders thuis gedurende een normale dag meerdere speekselmonsters afgenomen bij hun kind. Uit de resultaten bleek dat het dagritme van kinderen met een gedesorganiseerde gehechtheidsrelatie

verschilde van dat van alle andere kinderen. De gedesorganiseerde kinderen hadden een meer afgevlakt dagritme, wat wil zeggen dat de cortisolwaardes in deze groep aan het begin van de dag minder hoog waren en in de loop van de dag minder sterk daalden. Een afgevlakt ritme komt vaak voor bij kinderen die opgroeien in extreem gedepriveerde omstandigheden, zoals kindertehuizen. De gedesorganiseerde kinderen in het huidige onderzoek lieten eenzelfde patroon zien, en deze bevindingen onderstrepen het belang van gedesorganiseerde gehechtheid als een belangrijke voorspeller van latere ontwikkelingsproblematiek.

De conclusies over de rol van depressie van de moeder in de ontwikkeling van de gehechtheidsrelatie lopen uiteen. Sommige onderzoeken laten een duidelijk negatief effect zien, waarbij depressieve moeders minder vaak een veilige gehechtheidsrelatie met het kind opbouwen. Dit zijn vaak onderzoeken bij moeders met zware depressieve klachten. Andere onderzoeken rapporteren geen effect van depressie op de kwaliteit van de gehechtheidsrelatie. Verder zijn er verschillende onderzoekers die een effect rapporteren van depressie van moeder op de stressregulatie van het kind, waarbij meer depressieve klachten zouden leiden tot meer negatieve uitkomsten. In het huidige onderzoek vonden we geen direct bewijs voor een negatieve invloed van depressieve klachten van moeder op de stressregulatie van het kind. Wanneer we echter specifiek keken naar de stressreactie van onveilig-ambivalent gehechte kinderen (die als groep al verhoogde cortisol waardes hadden), bleek dat de combinatie met een depressieve moeder de kans op hoge cortisolwaardes nog verder deed toenemen. Deze kinderen hadden dus een dubbel risico op een verslechterde stressregulatie.

Niet alleen de omgeving (in dit geval: ouders of opvoeders) van het kind kan een rol spelen in de manier waarop kinderen omgaan met stress, steeds meer onderzoekers besteden aandacht aan de rol van genen. Genen die op basis van hun werking in de hersenen een mogelijke associatie hebben met een bepaalde ziekte of bepaald gedrag kunnen een logische 'kandidaat' zijn voor verder onderzoek. In het onderzoek naar de stressregulatie van kinderen zijn vooral de 'kandidaat-genen' van belang die de werking van het stresssysteem beïnvloeden. Het FKBP5-gen is één van de genen die hierbij een rol speelt; en is van belang bij het bepalen of een stressreactie (bijvoorbeeld huilen of wegkruipen) moet worden gestopt of voortgezet. In onze studie vonden we een gezamenlijk effect van een specifieke variant van het FKBP5-gen en onveilig-ambivalente gehechtheid. Uit het onderzoek was al duidelijk dat de onveilig-ambivalent gehechte kinderen een verhoogd risico hadden op een toename in cortisolwaardes na een stressvolle situatie. Wanneer zij ook de risico-variant van het FKBP5-gen droegen, was er een nog sterkere toename in hun cortisolwaardes te zien. Deze bevindingen onderschrijven het 'dubbel-risico-model', waarbij meerdere risicofactoren bijdragen aan een toegenomen kans op negatieve uitkomsten.

Genen en gehechtheid
De kwaliteit van een gehechtheidsrelatie wordt vooral beïnvloed door de kwaliteit van de interactie met de opvoeder gedurende het eerste levensjaar. Het afgelopen decennium is er desalniettemin steeds meer aandacht voor een genetische factor in verschillen in de kwaliteit van gehechtheid. Het onderzoek naar kandidaatgenen voor gehechtheid is tot nu toe vooral uitgevoerd binnen steekproeven van bescheiden grootte. In een exploratief onderzoek naar het bestaan van mogelijke 'gehechtheidsgenen' hebben we in samenwerking met de *NICHD Study of Early Child Care and Youth Development (SECCYD)* de associatie tussen verschillende kandidaatgenen en gehechtheid onderzocht in een groep van ruim 1100 kinderen. In beide onderzoeken vonden we weinig consistente uitkomsten. Alleen wanneer we naar specifieke risicomodellen keken, vonden we in beide studies een effect van het COMT-gen. Dit gen is werkzaam in het dopaminesysteem en is van belang is bij processen van aandacht, motivatie en beloning. Kinderen met een specifieke variant van dit gen hadden in beide steekproeven een verhoogde kans op gedesorganiseerde gehechtheid. Het precieze mechanisme achter dit verband blijft nog onduidelijk, maar het is mogelijk dat deze genetische variant meer ruimte laat voor omgevingsinvloeden. Kinderen die deze variant dragen zouden daardoor meer beïnvloedbaar kunnen zijn door de omgeving. Genetische invloeden zijn in dat geval niet zozeer een vaststaand risico, maar eerder afhankelijk van interactie met de omgeving. Deze 'gen-omgevingsinteracties' worden in veel studies onderzocht. Vaak richten onderzoekers zich daarbij op het identificeren van 'risico-genen', maar onderzoek naar interacties tussen genen en omgeving zou juist kunnen profiteren van een verschuiving van de focus op risico-genen naar een focus op ontvankelijkheid en plasticiteit. Vanuit dit standpunt kunnen genen niet alleen zorgen voor een risico, maar, afhankelijk van de omgeving, ook voor bescherming. Dit mechanisme, waarbij kinderen met een bepaald gen gevoeliger zijn *for better and for worse*, wordt ook wel differentiële ontvankelijkheid (*differential susceptibility*) genoemd.

Binnen ons onderzoek hebben we het kader van differentiële ontvankelijkheid toegepast op de associatie tussen sensitief opvoedingsgedrag en kwaliteit van gehechtheid. Hierbij werd het mineralocorticoid receptor gen (afgekort MR) als ontvankelijkheidsgen bestudeerd. Kinderen die een specifieke variant van dit gen hadden, waren veiliger gehecht als hun moeders meer sensitief responsief gedrag lieten zien (zoals het geven van complimentjes). Dragers van dezelfde genetische variant waren echter minder veilig gehecht wanneer hun moeders extreem insensitief gedrag lieten zien (zoals hardhandig aanpakken). Bij kinderen die de genetische variant niet droegen, waren deze associaties niet significant. Genetische variatie binnen het MR gen blijkt de gevoeligheid van kinderen voor zowel positieve als negatieve omgevingen te beïnvloeden. Het MR gen is betrokken bij het verwerken van stressvolle informatie, en het zou kunnen zijn dat kinderen die beter en sneller informatie kunnen verwerken over het gedrag van hun moeder

in stressvolle situaties, meer ontvankelijk zijn voor de effecten van zowel positief (sensitieve responsiviteit) als negatief (extreme insensitiviteit) opvoedgedrag.

Suggesties voor toekomstig onderzoek en klinische implicaties
In onderzoek naar de invloed van genetische factoren is het van belang dat zowel genen als omgeving zorgvuldig worden gemeten. In het huidige grootschalige onderzoek konden we deze aanpak toepassen, wat leidde tot robuuste resultaten over het samenspel tussen genen en omgeving in de ontwikkeling van gehechtheid en stressregulatie. Desalniettemin kunnen genen op andere manieren het gedrag beïnvloeden dan in de huidige studie is onderzocht. Hierbij is het van belang zich te realiseren dat genen op zich zelf geen gedrag veroorzaken. Ze coderen slechts voor eiwitten, die leiden tot neurologische processen, die op hun beurt van invloed kunnen zijn op gedrag. Eén van de effecten die mogelijk een rol zou kunnen spelen, is de interactie tussen genen onderling, epistasie. In onderzoek naar deze gen-gen interacties is voldoende kennis over de functies van specifieke genen van belang. Daarnaast kunnen *copy number variations* (CNV's), het ontbreken of te veel aanwezig zijn van stukken DNA, van belang zijn, omdat deze variaties de functie van het gen kunnen veranderen. Ten slotte kan ook de omgeving de werking van genen beïnvloeden; bij deze epigenetische effecten kunnen omgevingsinvloeden de gen-expressie veranderen, zonder dat het gen zelf verandert.

Uit eerder onderzoek blijkt dat onveilige gehechtheid consequenties kan hebben voor de ontwikkeling op lange termijn, vooral in combinatie met risicofactoren. Op basis van de huidige resultaten is het aannemelijk dat insensitief gedrag, depressie van moeder, stressreactiviteit en genetische risico's hierin een belangrijke rol spelen. Door de longitudinale opzet van de Generation R studie zal het op korte termijn mogelijk zijn de invloed van gehechtheid en risicofactoren op de verdere ontwikkeling te onderzoeken. Wanneer we de gevonden resultaten vanuit het kader van differentiële ontvankelijkheid bekijken, laat het huidige onderzoek zien dat neurobiologische kenmerken (genen, stresshuishouding) een kind gevoeliger kunnen maken voor de omgeving (de kwaliteit van zorg die de moeder biedt), zowel in positieve als in negatieve zin. Deze plasticiteit, *for better or for worse*, kan een raamwerk bieden aan pedagogen voor de interpretatie van soms verwarrende bevindingen in onderzoek naar gehechtheid en de ontwikkeling van kinderen. De ontwikkeling van gehechtheid is complex, en wellicht zijn de belangrijkste invloeden het best te identificeren in het samenspel tussen neurobiologische aspecten en de opvoedingsomgeving.

Dankwoord (Acknowledgements)

Tot slot: dit proefschrift had niet tot stand kunnen komen zonder de hulp, steun en inzet van velen. Daarom wil ik graag iedereen bedanken die hieraan heeft bijgedragen; om te beginnen de kinderen en ouders van het Generation R onderzoek. Zonder jullie medewerking was dit onderzoek niet mogelijk geweest. Ook de studenten die hebben geholpen bij het coderen van de gedragsobservaties; ik had deze klus niet kunnen klaren zonder jullie hulp. Verder wil ik graag mijn collega's in Leiden en in Rotterdam bedanken; slechts weinig aio's kunnen zich gelukkig prijzen met zoveel mede-promovendi. Bedankt allemaal, voor jullie advies, koffiepauzes en humor. Hierbij mag ik de F-side zeker niet ongenoemd laten, ook al is het voorzitterschap van deze bijzondere club niet makkelijk! Lieve vrienden en familie, op deze plek is niet genoeg ruimte om iedereen persoonlijk te bedanken, maar jullie hebben me steeds enorm geholpen met jullie belangstelling, advies en afleiding. Dankjulliewel! Lieve Arnold, zonder jou had ik dit zeker weten niet kunnen doen. Dank je voor je geduld en vertrouwen.

Curriculum Vitae

Maartje Luijk werd geboren op 12 februari 1983 in Spijkenisse. In 2000 behaalde zij haar HAVO diploma aan het PENTA college te Spijkenisse. Nadat ze haar propedeuse had behaald aan de PABO Thomas More in Rotterdam, begon zij aan de opleiding Algemene en Gezinspedagogiek. In 2006 is zij afgestudeerd op een meta-analyse naar het IQ van kinderen in kindertehuizen, waarvoor zij de Emile Scriptieprijs ontving. Sinds haar afstuderen werkte Maartje als promovenda bij de afdeling Algemene en Gezinspedagogiek (AGP), waar zij onderzoek deed naar neurobiologische factoren van gehechtheid bij jonge kinderen. Dit onderzoek voerde zij uit binnen de Generation R studie aan het Erasmus MC te Rotterdam. Naast haar aanstelling als promovenda was Maartje ook één dag in de week aangesteld als docent bij AGP, deze aanstelling is nu verder uitgebreid.