CHILDREN'S EMOTIONAL FUNCTIONING IN THE PRESCHOOL PERIOD: EMOTION RECOGNITION, TEMPERAMENT, AND THEIR LINKS WITH EARLY RISK FACTORS

THE GENERATION R STUDY

Eszter Székely

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CONTENTS

CHAPTER 1

INTRODUCTION

Emotions are essential aspects of human nature, representing a significant part of our evolutionary-biological and cultural heritage as well as our adaptation to the physical and social environment (Darwin, 1872/1965; Ekman & Friesen, 1971; Izard, 1971). Emotions play a critical role in the evolution, ontogeny, and functioning of consciousness (Damasio, 1999; Izard, 1977); form the basis for conscience and moral behavior (Eisenberg & Fabes, 1998; Hoffman, 2000); define the quality of human experience; motivate adaptive thought and action; and facilitate prosocial behavior and creative problem solving (Isen, Daubmen, & Nowicki, 1987). Nevertheless, emotions can hurt as well as help. Inappropriate and uncontrolled emotional responses (in terms of frequency or intensity) are implicated in many forms of psychopathology (Cicchetti & Cohen, 1995), social difficulties (Eisenberg, Hofer, & Vaughan, 2007), and even in physical illness (Sapolsky, 2007). Although any emotion that runs out of control can have maladaptive effects, all emotions, even the negative ones, can have significantly beneficial effects on behavior, well-being, and adaptation (Izard & Ackerman, 2000).

Despite their prominence in human life, emotions did not gain a central place in science until the last quarter of the 20th century (Izard, 2002). At that time, a large proportion of researchers have still thought of emotions as epiphenomena of cognition. Although emotions can partially be viewed as any other type of information (Izard, 1993), they also carry subjectively experienced feeling and motivational states (Damasio, 1999) which provide them unique functional properties and relative independence of cognitive processes (Damasio, 1994; Izard, 1993; LeDoux, 1996). In addition, emotions can have a profound influence on the perceptual field, range of cognitive processes, and behavioral alternatives (Damasio, 1994; Lazarus, 1991).

According to the contemporary functionalist approach, emotions represent a person's attempt or readiness to establish, maintain, or change aspects of the person-environment transaction on matters of relevance to the person's goals (Campos, Frankel, & Camras, 2004). Whatever the goal, and whatever the source of the situational meaning to the individual, it is this meaning that gives rise to emotions (Gross & Thompson, 2007). The emotions generated as a result are multifaceted, whole-body phenomena, involving loosely coupled changes in subjective experience, central and peripheral physiology, and behavior (Mauss, Levenson, McCarter, Wilhelm, & Gross, 2005). Most emotion theories agree that emotions have motivational and regulatory functions and can be divided into basic emotions, which are prominent in infancy and early childhood, and more complex emotions, which often involve higher-order cognition (Izard, 2007). A basic emotion is a set of neural, bodily/expressive, and feeling/motivational components which are generated rapidly, automatically, and unconsciously when an ecologically meaningful stimulus is perceived (Izard, 2007). The resulting basic emotion preempts consciousness and tends to drive a rather narrow, stereotypical response (Panksepp, 2007). This stereotypical basic emotion response system changes with maturation and social learning (Izard, Stark, Trentacosta, & Schultz, 2008). Complex emotions involve dynamic and continual interactions between discrete emotions, appraisals, previously learned emotion-related cognitive content, and ongoing perceptual-cognitive processes. After early development, these emotions constitute by far the most

important source of human motivation and, because they typically involve complex affective-cognitive schemas, perceptual and cognitive processes become major tools in the regulation of complex emotions (Izard et al., 2008).

Two key aspects related to young children's emotional functioning are emotion recognition and temperament. *Emotion recognition* is the ability to accurately process and decode emotional information produced by the self and others (McClure, 2000). Accurate emotion recognition forms the basis of emotion knowledge: the understanding of expressive signals, labels, and functions of emotions (Izard et al., 2008). *Temperament* refers to constitutionally based individual differences in reactivity and self-regulation, in the domains of emotion, attention, and activity (Rothbart & Bates, 2006). Emotional states form the basis of temperament (Goldsmith & Campos, 1982) but they can also result, at least in part, from temperament, which sets emotion thresholds and shapes the characteristic patterns through which children experience and respond to emotional stimuli (Izard, 1993). Emotion recognition and temperament also influence each other reciprocally. The resultant processes play critical roles in the development of emotion regulation and emotion utilization in children (Izard et al., 2008). The dynamic interplay between maladaptive forms of emotion recognition and emotion regulation is a key factor in the development of psychopathology (Izard et al., 2008).

The present thesis focuses on children's emotion recognition and temperament in the early preschool years, a developmental period marked by remarkable advances in emotion regulation and social and language skills. Throughout the preschool period, maturation and emotion socialization contribute to substantial increases in emotion regulation, emotion vocabulary, and emotion-cognition connections, leading to significant increases in emotion knowledge and constructive emotion utilization (Denham, 1998). Due to the rapid growth of emotion-cognition connections, the preschool years also represent a sensitive period for developing a solid foundation for accurate perception and labeling of emotions of self and others (Izard, Fine, Mostow, Trentacosta, & Campbell, 2002). Furthermore, this is also the period when, after a phase of pronounced plasticity, the stability of temperamental characteristics starts to increase (Belsky, Fish, & Isabella, 1991; Caspi, 2000).

EMOTION RECOGNITION

Most of our social interactions involve perception of emotional information. Facial expressions are powerful nonverbal displays of such emotional information. The ability to accurately recognize them enables us to detect another person's emotional state and provides cues on how to respond in complex social situations (Grossmann & Johnson, 2007; Philippot & Feldman, 1990). This is especially true for very young children, who are verbally less able to express themselves (Widen & Russell, 2008) and rely to a greater extent on facial expressions than on situational cues (Hoffner & Badzinski, 1989). The discovery in the 1970s that facial expressions are universally recognized (Ekman, 1973; Izard, 1972), led to their widespread use as preferred indices of emotional states, and to the assessment

of facial expressions based on anatomical criteria and judgment of emotions by coders (Saarni, Campos, Camras, & Witherington, 2007).

Some basic aspects of facial expression recognition (FER) are already evident in the neonatal stages but the ability to swiftly and accurately decode facial expressions continues to develop over time (Herba & Phillips, 2004). There is evidence that infants are able to visually discriminate between certain basic expressions (Nelson & De Haan, 1997) and adapt their behavior in response to these expressions (Montague & Walker-Andrews, 2001). In older children, developmental studies have demonstrated that FER develops gradually and improves significantly throughout childhood and adolescence (Batty & Taylor, 2006; De Sonneville et al., 2002; Gao & Maurer, 2009; Herba, Landau, Russell, Ecker, & Phillips, 2006; Vicari, Reilly, Pasqualetti, & Vizzotto, 2000; Widen & Russell, 2008). Emotion categories are initially broad, including facial expressions that are similar in levels of pleasure and arousal (Bullock & Russell, 1986). For a 2-year-old, an "angry" facial expression may include the unpleasant states of anger, disgust, fear, and sadness (Widen & Russell, 2003). Over the years, emotion concepts gradually become more specific with happiness recognized first and most accurately, followed by sad or angry expressions, then by surprise or fear. Basic emotions (e.g., happiness, sadness) are recognized earlier than complex emotions (e.g., shame, guilt) and children typically find it difficult to recognize neutral facial expressions (De Sonneville et al., 2002; Gross & Ballif, 1991; Herba & Phillips, 2004). In summary, the development of FER follows a protracted time course, with more visible changes from age 3 and with highly advanced competency reached by age 10 (Batty & Taylor, 2006; Markham & Adams, 1992; Simonian, Beidel, Turner, Berkes, & Long, 2001). In more specific terms, the development of FER is characterised by a large increment in accuracy between 3 and 7 years of age, and an increase in processing speed between 7 and 10 years of age, particularly for negative emotions. However, adults still process facial expressions nearly twice as fast children (De Sonneville et al., 2002; Gao & Maurer, 2009).

Children's ability to label facial expressions is initially modest and improves with age (Markham & Adams, 1991). At approximately 2-3 years of age, children begin using emotional labels (Izard & Harris, 1995; Widen & Russell, 2003). In the beginning, these emotional labels are used very broadly, as they refer to different emotions of the same valence (Widen & Russell, 2003). Over the preschool period, emotional vocabulary expands, enabling children to identify more subtle emotional expressions (Camras & Allison, 1992). Thus, the preschool period provides an interesting time window to study children's developing FER and its correlates.

EMOTION RECOGNITION AND PSYCHOPATHOLOGY

Atypical FER patterns characterize various psychiatric conditions in both adult (Phillips, Drevets, Rauch, & Lane, 2003) and child populations (Guyer et al., 2007; Monk, 2008). Furthermore, the content of these biases is thought to be disorder specific (Hankin, Gibb, Abela, & Flory, 2010). For instance, symptoms of depression have been linked to biases in processing happy

and sad facial expressions (Leppänen, 2006; Surguladze et al., 2004), anxiety has been linked to a hypersensitivity to threatening stimuli such as anger or fearful expressions (Bar-Haim, Lamy, Pergamin, Bakermans-Kranenburg, & Van IJzendoorn, 2007; Hankin, et al., 2010; Lau & Pine, 2008; Mogg & Bradley, 2005), while psychopathy has been linked to an insensitivity to distress cues such as sad and fearful expressions (Marsh & Blair, 2008). Nevertheless, it is still unclear whether such emotion-specific biases in processing facial expressions are evident when considering more common symptoms of internalizing and externalizing behavior in the general population that may fall below a clinical threshold or diagnosis.

Biased processing of emotive facial expressions has also been implicated as a vulnerability marker of psychopathology, especially that of depression. A vulnerability marker is an enduring, trait-like biological or psychological factor that may predispose an individual to a disorder (Ingram & Luxton, 2005). Cognitive theorists have long hypothesized that negatively biased cognitive schemas, which influence how information is attended to, interpreted, and remembered, are a diathesis for depression (Beck, 1967; Ingram, Miranda, & Segal, 1998). More specifically, there is evidence for an increased sensitivity to negative emotional stimuli and a decreased sensitivity to positive emotional stimuli not only in patients with depression but also in high risk samples and after remission from a depressive disorder (Joormann & Gotlib, 2007; Kujawa et al., 2011; Leppänen, 2006; Reid, Salmon, & Lovibond, 2006; Scher, Ingram, & Segal, 2005). Together, these findings suggest that biased processing of emotional information may be an underlying trait marker rather than an associated state marker of depression. Consequently, examining what factors influence the ability to accurately recognize emotions, especially early in development when symptoms have not yet emerged, is of crucial importance to gain a better understanding of individual vulnerability.

TEMPERAMENT

Temperament was originally defined as largely heritable individual differences in emotional reactivity (Allport, 1961). Later definitions implied a broader approach and included individual differences in attention and activity level (Thomas & Chess, 1977). Contemporary perspectives recognize individual differences in all three areas, and define temperament as constitutionally based individual differences in reactivity and self-regulation, in the domains of affect, activity, and attention (Rothbart & Bates, 2006; Rothbart & Derryberry, 1981). Constitutional refers to the biological bases of temperament, which is influenced over time by heredity, maturation, and experience. Reactivity indicates responsiveness to change in the external and internal environment, and can be described in terms of latency, duration, and intensity of affective, attentional, and motor reactions. Self-regulation refers to processes that serve to modulate reactivity such as effortful control or orienting (Rothbart & Bates, 2006). Temperamental dispositions are not expressed continuously but require appropriate eliciting conditions (Rothbart & Bates, 2006). Basic biological processes of temperament appear to be similar across cultures, however, their outcomes may vary according to cultural values and individual experiences (Rothbart, 2007; Rothbart & Bates, 2006).

In infants and toddlers, there is converging evidence for three broad temperamental dimensions: surgency/extraversion, negative emotionality, and orienting/regulation (Gartstein & Rothbart, 2003; Rothbart & Bates, 2006). Importantly, these dimensions also correspond to individual differences observed in nonhuman animals (Gosling & John, 1999), emphasizing the importance of the biological underpinnings of temperament. As children's motor, cognitive, and verbal abilities develop, they express a larger variability in temperamental traits. In older children, developmental studies have identified a number of temperamental structures. The five-factor structure of temperament includes extraversion, neuroticism, conscientiousness, agreeableness, and openness to experience (Caspi & Shiner, 2006). Both the three-factor and the five-factor models of temperament show close similarities with personality factors such as the Big Three or the Big Five factors of personality (Rothbart & Bates, 2006). Other theories have focused on describing the underlying physical properties of temperamental approach and withdrawal such as Gray's (1991) behavioral activation system (BAS) and behavioral inhibition system (BIS). These frameworks explain individual behavioral differences in terms of underlying neural sensitivity to reward and punishment. Yet other studies have emphasized the importance of examining temperamental emotionality such as positive and negative emotionality (Clark, Watson, & Mineka, 1994; Rothbart & Bates, 1998) and temperamental fearfulness (Rothbart, Derryberry, & Posner, 1994), especially in relation to psychopathology (Clark & Watson, 1991; Rapee, 2002).

Temperamental traits show considerable stability over time, especially from age 3 years on (Caspi, 2000). Large increments in the development of executive attention and effortful control in the first 3 years of life may account for the relative instability of temperamental traits in infancy and toddlerhood (Rothbart & Bates, 2006). Temperamental stability increases in a relatively linear fashion through adolescence and young adulthood, and peaks some time after the age of 50 (Roberts & DelVecchio, 2000). However, expressions of specific temperamental traits may change over time and as attention systems stabilize, reactive tendencies are more readily controlled (Rothbart & Bates, 2006).

TEMPERAMENT AND PSYCHOPATHOLOGY

It has long been assumed that children's early individual differences could help set off a chain of transactions between the child and the environment that may lead to the development of clinical disorders (Nigg, 2006; Thomas, Chess, & Birch, 1968). The exact processes through which temperament and psychopathology are linked can vary substantially (Clark et al., 1994). In the *vulnerability association*, temperamental traits can predispose a child to develop specific forms of psychopathology, particularly under certain environmental conditions. In this association, the risk-inducing temperamental trait is viewed as distinct from the psychological disorder. In a *spectrum association*, psychopathology is rather considered as the extreme end of a continuously distributed temperamental trait. Thus, in this association psychopathology does not represent a categorically distinct condition, but rather an extreme expression of a dimensional trait. In the *maintenance association*,

temperamental characteristics may influence the manifestation, course, and prognosis of a disorder once it has already developed. In the *resilience association*, temperamental dispositions may protect against the development of psychopathology in the presence of stress and adversity by predisposing the child to deal adequately with challenging situations. In the *scarring association*, the experience of a disorder will produce enduring changes in children's characteristics (reviewed by Caspi & Shiner, 2006).

Numerous studies have examined the relationship between temperament and psychopathology, both concurrently and longitudinally. A number of major themes have emerged from this line of research. For instance, low positive emotionality seems to be a specific vulnerability marker of depression (Clark & Watson, 1991; Durbin, Klein, Hayden, Buckley, & Moerk, 2005; Dietz et al., 2008; Olino et al., 2011) and social anxiety (Brown, Chorpita, & Barlow, 1998; Chorpita, Plummer, & Moffitt, 2000), while behavioral inhibition or fearfulness is primarily implicated as a temperamental vulnerability for developing anxiety (Fox & Pine, 2012; Perez-Edgar & Fox, 2005; Rapee, 2002). High negative emotionality typically conveys a non-specific risk, predisposing for a wide variety of internalizing and externalizing difficulties (Rothbart & Bates, 2006), whereas low effortful control has been associated with childhood oppositional behavior, conduct problems, and ADHD (Caspi & Shiner, 2008; Nigg, 2006; Rothbart & Bates, 2006). High levels of positive emotionality and effortful control have further been implicated as resiliency factors against the development of internalizing problems (Muris & Ollendick, 2005; Silk, Shaw, Forbes, Lane, & Kovacs, 2006). Given the importance of temperament in adaptive and maladaptive psychological functioning, research to identify significant predictors and correlates of child temperament is necessary.

AIMS

Although there is now considerable body of data linking FER and temperamental characteristics to maladjustment and psychopathology, we know very little about the relations of FER and temperament to early risk factors. Therefore, the general aim of the present thesis is to provide greater insight into the relationship between preschoolers' FER/temperament and a number of important early risk factors of psychosocial maladjustment. One such risk factor is maternal depression. Maternal depression is the most common psychiatric disorder in the postpartum period (Brockington, 2004), affecting approximately 10-15% of childbearing women in Western societies (Gorman et al., 2004; O'Hara & Swain, 1996). Since mothers are usually the primary caregivers and spend considerable time with their offspring, many children are exposed to maternal depression. A large body of research has documented the adverse effects of maternal depression on child development from infancy through adolescence (Beardslee, Gladstone, & O'Connor, 2011; Downey & Coyne, 1990; Field, 1995; Hay, Pawlby, Waters, & Sharp, 2008; Murray et al., 2011). Most of these studies focused on outcomes such as cognitive and language development (Brennan et al., 2000; Hay & Kumar, 1995), academic achievement (Murray et al., 2010), social competence (Murray, Sinclair, Ducournau, & Turner, 1999), and psychopathology

(Goodman et al., 2011). Surprisingly few studies have examined the effect of maternal depression or depressive symptoms on offspring FER and temperament, and with regard to FER almost none has focused on the early preschool period. Therefore, the present thesis examines how maternal depressive symptoms influence young children's FER and temperamental traits such as positive emotionality and fearfulness.

Genetic vulnerability constitutes another risk for maladaptive emotional functioning. Historically, FER and temperament have had different relations to genetic research. Since temperament is considered to be constitutionally based, a lot of attention has been given to establishing the genetic basis of temperamental structures. Temperament is mainly considered an inherited emotional-cognitive-behavioral style of the child (Vaughn et al., 2008). Molecular genetic studies of child temperament most frequently examined genes involved in the serotonergic and dopaminergic pathways. However, in contrast to initial expectations molecular genetic research has failed to generate a replicable set of genes involved in the development of temperamental differences among children (Rothbart & Bates, 2006). The present thesis attempts to widen the search for relevant genetic differences in relation to child temperament beyond the usual suspects, using state of the art genetic tools such as genome wide association analysis and genetic pathway analysis. These techniques are now widely applied in medical research but still considered relatively unknown in the area of child development.

The genetic basis of FER is largely under-researched, although theoretical accounts provide a basis for expecting genetic influences on children's FER abilities (Munafo, Brown, & Hariri, 2007). Complex emotional behavior such as FER is likely affected by the interplay of both genetic and environmental influences (Rutter, 2003; Lau et al., 2009a). Previous research highlighted the significance of a functional polymorphism in the promoter region (5-HTTLPR) of the serotonin transporter gene in social-emotional behavior (Canli & Lesch, 2007). Surprisingly, only few developmental studies have looked at variations in the 5-HTTLPR with regard to facial emotion processing (Battaglia et al., 2005; Gibb, Benas, Grassia, & McGeary, 2009; Jacobs et al., 2011; Lau et al., 2009b; Perez-Edgar et al., 2009; Thomason et al., 2010). These studies mostly focused on children at high risk for affective psychopathology or somewhat older children; none has explored recognition accuracy. In the present thesis, normative data is provided on how the 5-HTTLPR polymorphism influences the accuracy for processing emotional stimuli early in life before affective problems typically emerge. Finally, this thesis also examines how FER and temperament are related to common emotional and behavioral problems in the preschool period.

SETTING

All studies included in the present thesis were conducted within the context of the Generation R Study, a population-based prospective child cohort from fetal life onward in Rotterdam, the Netherlands. The Generation R Study offers a unique opportunity to identify early environmental and genetic causes of normal and abnormal growth, development, and health (Jaddoe et al., 2010). Data collection in children and their parents ranges from

questionnaires to observations and biological measures. A genome wide association scan is also available in the participating children (Jaddoe et al., 2010). Results forthcoming from the Generation R Study will eventually contribute to the development of strategies for optimizing health and healthcare for pregnant women and their children.

The present series of investigation more specifically pertained to a subsample of the Generation R Study in which additional detailed behavioral observations were also available. This subsample is ethnically homogeneous (all children, their parents, and grandparents were born in the Netherlands) in order to exclude possible confounding or effect modification by ethnicity. Children participating in this subsample were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort (Jaddoe et al., 2008). Due to the eligibility criteria, the subsample mainly consists of two-parent families of middle-class background. Such ethnically homogeneous and low risk samples of the general population provide an excellent means to study non-clinical and biological determinants of child development.

THESIS OUTLINE

In Chapter 2, it is described how typically developing young children recognize emotional facial expressions (Chapter 2.1). Chapter 3 addresses the influence of an important early risk factor, maternal depression, on young children's FER (Chapter 3.1) and temperament (Chapter 3.2). Chapter 4 explores genetic influences on young children's FER and temperament. In particular, it deals with the influence of the serotonin transporter gene-linked (5-HTTLPR) polymorphism on children's FER (Chapter 4.1), and examines how this polymorphism interacts with environmental risk factors such as maternal anxiety and chronic difficulties in the development of childhood emotional problems (Chapter 4.2). In addition, amongst the first, results are presented from a genome wide association analysis and pathway analysis of young children's attachment relationship and temperamental fearfulness (Chapter 4.3). In Chapter 5, specific FER profiles are described with regard to common internalizing and externalizing problems in preschool children (Chapter 5.1), and the links between young children's positive emotionality, executive functioning, and internalizing problems are more closely examined (Chapter 5.2). In Chapter 6, the main findings are interpreted in a broader context and discussed in terms of methodological considerations, clinical relevance, and implications for future research.

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CHAPTER 2

EMOTION RECOGNITION IN THE EARLY PRESCHOOL YEARS

CHAPTER 2.1

RECOGNITION OF FACIAL EXPRESSIONS OF EMOTIONS BY 3-YEAR-OLDS

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ABSTRACT

Very few large-scale studies have focused on emotional facial expression recognition (FER) in three-year-olds, an age of rapid social and language development. We studied FER in 808 healthy three-year-olds using verbal and nonverbal computerized tasks for four emotion-categories (happiness, sadness, anger, and fear). Three-year-olds showed differential performance on the verbal and nonverbal FER tasks, especially with respect to fear. That is to say, fear was one of the most accurately recognized facial expressions as matched nonverbally and the least accurately recognized facial expression as labeled verbally. Sex did not influence emotion-matching nor emotion-labeling performance after adjusting for basic matching or labeling ability. Three-year-olds made systematic errors in emotion-labeling. Namely, happy expressions were often confused with fearful expressions whereas negative expressions were often confused with other negative expressions. Together, these findings suggest that three-year-olds' FER skills strongly depend on task specifications. Importantly, fear was the most sensitive facial expression in this regard. Finally, in line with previous studies we found that recognized emotion-categories are initially broad, including emotions of the same valence, as reflected in the non-random errors of three-year-olds.

INTRODUCTION

The ability to quickly and accurately recognize emotive facial expressions is critical for smooth social interaction and appropriate behavior modification. Deficits in recognizing emotions in young children can lead to extensive and long-lasting impairments in social functioning and may comprise a risk factor for psychopathology later in life (Batty & Taylor, 2006; Herba & Phillips, 2004; Izard, 1971). Facial expressions are of crucial importance for communicating emotions at all stages of development but may be especially significant early in life when children are verbally less able to express themselves (Markham & Adams, 1992; Widen & Russell, 2008).

In addition to studying emotion processing abnormalities in adults (for review, see Phillips, Drevets, Rauch, & Lane, 2003a) and in children (Guyer et al., 2007; for reviews, see Monk, 2008a; Phan, Wager, Taylor, & Liberzon, 2002), it is also important to investigate emotion processing in typically developing children (Durand, Gallay, Seigneuric, Robichon, & Baudouin, 2007; Widen & Russell, 2008; for reviews, see Gross & Ballif, 1991; Herba & Phillips, 2004) given its importance in psychosocial well-being throughout life and to provide normative data against which clinical studies can be compared.

Developmental studies have used very different paradigms to assess how accurately children can recognize facial expressions of emotions (Bruce et al., 2000; Gross & Ballif, 1991; Markham & Adams, 1992). When studying emotion processing or facial expression recognition (FER) in infants for example, alternative paradigms need to be applied such as habituation and preference, which makes comparison with older children problematic (McClure, 2000). Cross-sectional studies assessing FER during the preschool period, childhood, and adolescence have most frequently used (a) situation discrimination, (b) matching discrimination, (c) forced-choice labeling and (d) free labeling tasks. These tasks differ markedly according to the cognitive skills they require as well as their dependency on the development of emotion vocabulary and visual discriminatory skills (Markham & Adams, 1992). The relative difficulty of FER thus varies when the same stimulus is used with different procedures (Harrigan, 1984) and when similar procedures are used with different stimulus material and subjects (Camras & Allison, 1985). In order to gain a good understanding of emotion processing, one should include tasks tapping into both lexical and visuospatial emotion processing, since these are suggested to follow differential developmental pathways, while preferably accounting for children's basic verbal and visuospatial abilities (Gross & Ballif, 1991; Vicari, Reilly, Pasqualetti, Vizzotto, & Caltagirone, 2000). Using data from a cohort study of over 800 three-year-old children, we aim to examine emerging FER using tasks reliant on verbal and nonverbal abilities.

The majority of the developmental studies consistently demonstrate that FER emerges gradually and improves with age throughout childhood into (pre)adolescence (Batty & Taylor, 2006; De Sonneville et al., 2002; Durand et al., 2007; Gao & Maurer, 2009; Herba, Landau, Russell, Ecker, & Phillips, 2006; Vicari et al., 2000; Widen & Russell, 2008). Emotion concepts are initially broad, including emotions of the same valence, and

narrow gradually over the period of years (Thomas et al., 2001; Widen & Russell, 2008) with positive emotions such as happiness recognized first and most accurately, followed by sad or angry expressions, then by recognition of surprise or fear (Camras & Allison, 1985; Vicari et al., 2000). Basic emotions such as happiness, fear, and anger are recognized earlier than complex emotions such as jealousy and contempt (De Sonneville et al., 2002; Gross & Ballif, 1991; Herba & Phillips, 2004). In summary, the development of FER follows a long and continued time course, with more visible changes from age three and with adult-like competency reached by age ten (Batty & Taylor, 2006; Markham & Adams, 1992; Simonian et al., 2001). In more specific terms, the development of FER is characterized by a large increment in accuracy between three and seven years of age, and an increase in speed between seven and ten years of age (De Sonneville et al., 2002; Gao & Maurer, 2009). These studies tend to focus on slightly older children. We lack detailed knowledge on how younger children process emotions around time of rapid language development, emerging face processing-, and theory of mind skills (Baron-Cohen, Leslie, & Frith, 1985; de Heering, Houthuys, & Rossion, 2007; Durand et al., 2007; Igliori & Damasceno, 2006; Karmiloff & Karmiloff-Smith, 2002; Vicari et al., 2000).

In the present study we had the opportunity to reliably study FER in a large cohort of healthy three-year-old boys and girls. To our knowledge, this study is among the very few examining FER in (i) such a large sample of young children, and (ii) in a homogeneous age group, which is a powerful statistical tool to reduce possible confounding or effect modification by age. A further strength of the study is that we used verbal and nonverbal paradigms in a within-subjects design to assess how accurately three-year-olds identify facial expressions of four basic emotions: happiness, sadness, anger, and fear.

Based on previous research on emotion processing, we hypothesized the following:

1. Three-year-olds will demonstrate different patterns in performance on the verbal and nonverbal FER tasks, evident through their patterns in accuracy among emotion-categories for the two types of tasks. On the basis of previous literature, we expected the following rank order for emotions: (i) happiness, sadness, anger, and fear on the emotion-matching task (Camras & Allison, 1985; Vicari et al., 2000); (ii) happiness, sadness and anger, and finally fear on the emotion-labeling task (Vicari et al., 2000; Widen & Russell, 2003).
2. There will be no or only minor sex differences in favor of girls after adjusting for verbal and visuospatial abilities (Vicari et al., 2000). Although the meta-analysis of McClure (2000) reported consistent sex differences, a number of individual studies published since then have yielded mixed results regarding sex differences in the development of FER and its underlying neurobiology (DeSonneville et al., 2002; Guyer et al., 2008; Herba et al., 2006).
3. Children are more likely to confuse negative emotions such as sadness, anger, and fear systematically with each another (Camras & Allison, 1985; Thomas et al., 2001; Widen & Russell, 2003, 2008). Furthermore, we explored the hypothesis that children will confuse happy faces with fearful ones, as Widen and Russell (2003) found that two- and three-year-olds also used the emotion label 'happy' for fearful (and surprised) faces.

METHOD

Participants

The present study was embedded in the Generation R Study, a population-based prospective Dutch cohort from fetal life onward, which has been described in detail elsewhere (Jaddoe et al., 2007, 2008). A subsample of children and their parents, known as the Generation R Focus Cohort, has been followed through more detailed assessments. All children in this subsample were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort. Eligibility criteria for enrolment in the Generation R Focus Study included: (1) enrolment before a gestational age of 25 weeks in the Generation R Study, (2) Dutch ethnicity, defined as two parents and four grandparents born in the Netherlands to exclude possible confounding or effect modification by ethnicity (Jaddoe et al., 2007). The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center. Parental written informed consents were obtained for all participants.

The current study was conducted during the postnatal phase of the Generation R Focus Study on 1106 children. Participants and their parents were invited to the Generation R Research Center when children turned 36 months old. In total, 862 children visited the center with an accompanying parent. Fifteen mothers partook with twins, so we randomly excluded one of each twin-pair to avoid biases due to paired data. Emotion processing tasks were not administered in 29 children due to time constraints. We further excluded the first six children who were assessed in the center due to change in test procedure, and four others because they were older than 45 months at the time of visit. Consequently, the final study population consisted of 808 children with available data on FER tasks. Children's mean age was 37.5 months (SD = 1.45, range 34.7 – 44.9 months). There were 409 boys and 399 girls. The average age of mothers at the time of the study was 35.6 years (SD = 3.77, range 19.5 – 35.6 years). The majority of the mothers (n = 300; 37.5 %) belonged to the highest educational category. Of the total sample, 28 children (3.5%) were born preterm (i.e., before 37 weeks of pregnancy) and 30 children (3.7%) had low birth weight (i.e., lower than 2500g). All subjects had normal or corrected to normal vision. Furthermore, children underwent regular eye tests for vision from birth onwards at the Municipal Health Service Centers as part of the national program.

Response analysis

To examine whether non-response was selective, we compared basic characteristics of children with FER data (n=808) to children who participated postnatally in the Generation R Focus Study but did not have FER data (n =298). The non-response analysis showed that there were fewer preterm births among children with FER data compared to children without FER data ($\chi^2_{(1)} = 7.6$, $p = .006$). Children with FER data were also characterized by more highly educated mothers ($\chi^2_{(3)} = 16.4$, $p = .001$), who were on average 1.2 years older ($t_{(1104)} = -4.2$, $p < .001$) than mothers of non-participating children. There were no significant differences between participants and non-participants in terms of prevalence of low birth weight ($\chi^2_{(1)} = 3.2$, $p = .072$) and sex ($\chi^2_{(1)} = 1.5$, $p = .225$).

Materials and Procedure

FER tasks were specifically developed for this age group. Color images of four basic emotions (happiness, sadness, anger, and fear) were presented on a screen and children responded using a touch-sensitive monitor. Stimuli were selected from a widely used facial stimulus set, the NimStim, on the basis of which identities demonstrated the best recognized pose for a particular emotion-category (Tottenham et al., 2009). We chose open-mouth models for happy, angry, and fearful expressions, and closed-mouth models for sad expressions. Since all our participants were of Caucasian origin, we only included Caucasian models in the present study. Skin tone color and brightness were balanced within individuals' expression images to improve inter-stimulus consistency. Visual angles subtended by the faces were 14.17 degrees vertically and 11.67 degrees horizontally. Images were cropped to 510 x 620 pixels so that inter-pupillary distance was constant within the image. Faces were centered within the image for head height and horizontally on the nasion. Prior to the FER tasks, children were introduced to the idea of emotions and facial expressions. All computer tasks included a practice trial, which could be repeated up to 3 times in total if the child's answer was incorrect before the experimenter moved on to the test trials.

Matching tasks. In the *emotion-matching task*, children had to match the emotion of a target stimulus presented at the top of the computer screen with one of the two choices presented below (Figure 1). Sixteen trials of emotion-matching with two female and two male identity pairs and four basic emotions (happiness, sadness, anger, fear) were included. Stimuli presentation was properly balanced and randomized for emotion and identity. To ensure that children understood the concept of matching and to control for the effects of basic matching ability, a *shape-matching task* was included. This control task was administered prior to the emotion-matching task. Children were asked to match a target shape to one of two shapes. The shape-matching task had the same parameters and layout as the emotion-matching task. A practice trial and four test trials with different target shapes (a square, an oval, a diamond, and a cross) were included. Similar paradigms have been used previously by Hariri, Bookheimer, and Mazziotta (2000), and Herba et al. (2006).

Labeling tasks. In the *emotion-labeling task*, children heard a voice-bit asking in a neutral voice which person was feeling a specific emotion. Children were asked to point to the expression that matched this label (Figure 2). Sixteen trials were presented, four items for each of the four emotion-categories. In this task, all four emotion-categories were presented on each single trial (displayed by the same identity). A similar paradigm has been used previously by Fries and Pollak (2004). To control for the effects of labeling ability an *animal-labeling task* was included. This task was administered to children by their parents at age 30 months, six months preceding the FER assessments. Children were presented six black-and-white photos (4.5 x 4.5 cm) of animals (a horse, a fish, a ladybug, an owl, a hippopotamus, and a bear) on a white A4 sheet. Children indicated which one of six pictures matches the name of an animal read out by a parent. There were six trials in total. Similar paradigms have been previously used by Widen and Russell (2003).

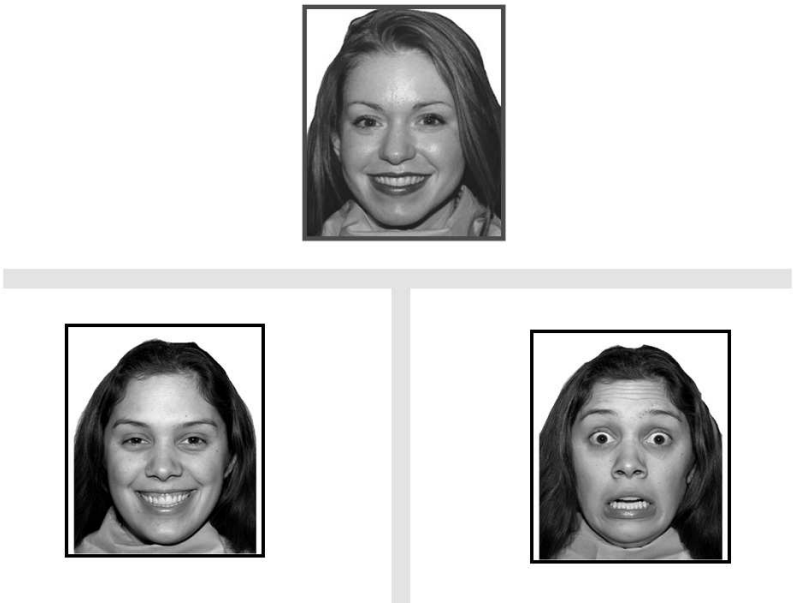

Figure 1. Emotion-matching task. Children were instructed to match 'who feels the same' by pointing to the face (bottom) that displayed the same emotion as the target face (top) on a touch-sensitive monitor.

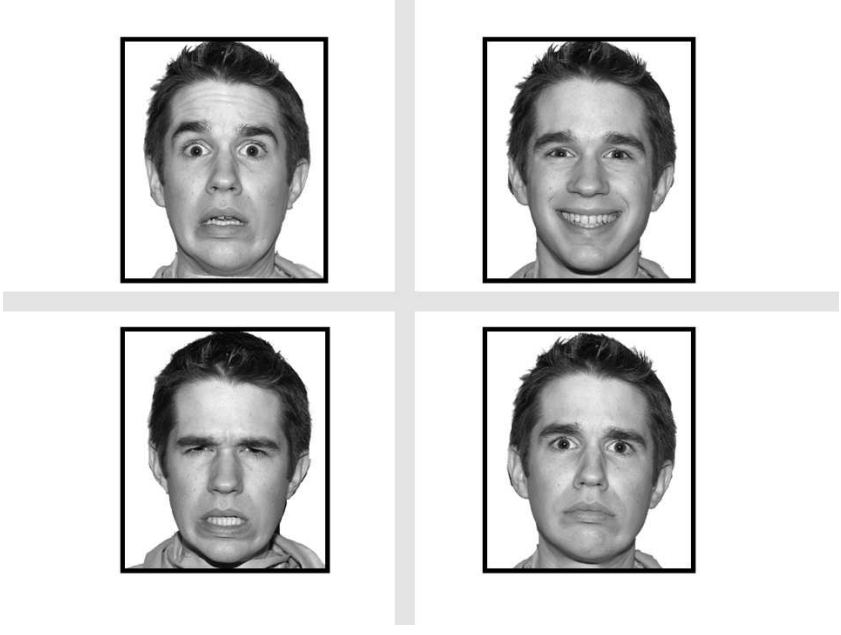

Figure 2. Emotion-labeling task. Children heard an emotion label (e.g., "Who feels happy?") and were required to point to the face whose affect best corresponded to the label.

Statistical Analyses

All statistical analyses were performed using Statistical Package for Social Sciences Version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

The main outcome variable for all the tasks was accuracy, i.e., the ratio of correct responses to the number of trials attempted. Accuracies per emotion-category were calculated for the emotion-matching and emotion-labeling tasks separately. For the shape-matching and animal-labeling control tasks, only overall accuracy scores were calculated. Separate ANOVA and multivariate ANCOVA models were conducted to assess FER in verbal (emotion-labeling task) and nonverbal (emotion-matching task) paradigms. In both cases, accuracy was related to the within-subjects factor of emotion-category, and for theoretical reasons was adjusted for the following factors: age, sex, and maternal education. Models were not adjusted for gestational age and birth weight of children, since they did not meaningfully change the effect estimates. For emotion-matching performance we further controlled for shape-matching accuracy, whereas for emotion-labeling performance we controlled for animal-labeling accuracy. Bonferroni correction was used to reduce errors due to multiple testing. Effect sizes (partial ε^2) for the main effects of covariates are also reported, although they have to be interpreted cautiously, as the covariates and the restricted age range were chosen to obtain the most valid effect estimates but not to explain maximal variance.

To investigate potential bias in children's errors, the emotion-labeling task was used in which all four target emotions (happiness, sadness, anger, fear) were presented in every trial. Each target emotion was presented four times, along with the three non-target emotions. For each target emotion, the total number of errors children committed was counted according to emotion-category. Subsequently, a mean error score was calculated for each non-target emotion-category by dividing the number of errors in that category by the number of trials per emotion-category (i.e., four for each emotion-category). This result represented the average number of incidents per target emotion of choosing a given non-target emotion when making a mistake. Error scores were exposed to a series of Chi-square tests while adjusting for multiple testing to see if any bias in children's errors could be detected.

Maternal education was defined as the highest education completed (highest – academic higher education, high – other postsecondary education, middle – secondary education, and low – primary education or no education). Main effects for emotion-category (happy, sad, angry, and fearful) and its interactions with the covariates (child's age, sex, maternal education level, shape-matching accuracy/animal-labeling accuracy) were investigated.

RESULTS

Mean accuracy scores and standard deviations (SDs) for the emotion-matching and emotion-labeling tasks are presented in Table 1 according to emotion-category. The level of chance performance was 0.50 on the emotion-matching task and 0.25 on the emotion-labeling task. Children's performances on these two tasks are thus not directly comparable.

Table 1. Mean accuracies and standard deviations on the nonverbal (emotion-matching) and verbal (emotion-labeling) FER tasks.

	Matching		Labeling	
Facial Expressions	Mean	*SD*	Mean	*SD*
Happy	0.66	0.29	0.53	0.34
Sad	0.59	0.29	0.51	0.33
Angry	0.62	0.29	0.55	0.36
Fearful	0.65	0.29	0.44	0.33

Note. There were two choice-stimuli available in the emotion-matching task and four choice-stimuli in the emotion-labeling task. Levels of difficulty on the two tasks were thus different.

Emotion-matching task

We hypothesized to find the following sequence order for emotions on the emotion-matching task: happiness, sadness, anger, and fear. Task performance was above chance level accuracy for all emotions as examined with one-sample t-tests ($p < .0125$, Bonferroni adjusted). Results indicated a significant main effect for emotion-category ($F_{(2.9, 2057.6)} = 18.0$, $p < .001$, $\varepsilon^2 = .025$; model with covariates $F_{(2.9, 2057.6)} = 15.1$, $p < .001$, $\varepsilon^2 = .004$). Three-year-olds were most accurate at matching happy faces (accuracy = 0.66) and fearful faces (accuracy = 0.65). Accuracies for matching angry faces (accuracy = 0.62) and sad faces (accuracy = 0.59) were significantly lower than for matching happy faces and fearful faces. The difference between matching angry and sad expressions were not significant. The marginal mean differences in accuracy between the different emotion-categories on the emotion-matching task are summarized in Table 2.

Multivariate ANCOVA tests revealed that maternal education ($F_{(1, 695)} = 3.5$, $p = .06$, $\varepsilon^2 = .005$) and sex ($F_{(1, 695)} = 0.1$, $p = .75$, $\varepsilon^2 = .001$) of the child were not significantly associated with the ability to accurately match emotions. Since our emotion-matching task mostly relied on visuospatial processing skills, in the main analyses we adjusted the results for basic matching ability. We found that better shape-matching ability predicted better emotion-matching accuracy ($F_{(1, 695)} = 27.4$, $p < .001$, $\varepsilon^2 = .038$). To test whether this could have contributed to the lack of a significant sex difference, we reanalyzed our data without adjusting for basic matching accuracy but it did not change the results. We found a main effect for age on emotion-matching performance despite the small age range ($F_{(1, 695)} = 17.8$, $p < .001$, $\varepsilon^2 = .025$). No significant interactions between emotion-category and the covariates were found. Results of the multivariate analyses for the emotion-matching task are summarized in Table 3.

Emotion-labeling task

We hypothesized to find the following sequence order for emotions on the emotion-labeling task: happiness, sadness and anger, and finally fear. Task performance was above chance level accuracy for all emotions as examined with one-sample t-tests ($p < .0125$, Bonferroni adjusted).

Table 2. Comparing mean accuracy scores on the nonverbal FER task across facial expressions

Facial Expressions			Mean Differences [a]	*p*
Happy	*vs.*	Fearful	0.01	1.0
		Angry	0.06 [+]	< .001
		Sad	0.09 [+]	< .001
Fearful	*vs.*	Angry	0.05 [+]	.004
		Sad	0.08 [+]	< .001
Angry	*vs.*	Sad	0.03	.14

Mean differences represent differences in marginal mean accuracies of one emotion-category over the other. For example, in the case of happy vs. fearful a mean difference score of 0.01 means that the marginal mean accuracy for matching happy expressions is 1% higher than the marginal mean accuracy for matching fearful expressions.
Note. Bonferroni adjustment was used to correct for multiple testing.
Note 2. The fully adjusted results are presented in the table; results for emotion comparisons were essentially identical when covariates were not included in the model.
[a] Differences in marginal mean accuracies, adjusted for child's age, sex, accuracy on shape-matching, and maternal education.
[+] Significant at $p < .008$.

Table 3. Multivariate analyses of mean accuracy scores on the nonverbal FER task

Source	*F*	*p*
Within-subjects effects (Greenhouse-Geisser) [b]		
Emotion	15.1	< .001
Emotion x Age	1.6	.18
Emotion x Maternal education	1.1	.33
Emotion x Shape-matching	3.7	.05
Emotion x Sex	1.2	.19
Between-subjects effects [c]		
Age	17.8	< .001
Maternal education	3.5	.06
Shape-matching	27.4	< .001
Sex	0.1	.75

[b] df1 = 2.9, df2 = 2016.13

[c] df1 = 1, df2 = 695

Repeated Measures ANOVA: emotion-category (happiness, sadness, anger, fear) as within-subjects factor; age, sex, shape-matching ability, and maternal education as covariates.

Results indicated a significant main effect for emotion-category ($F_{(3.0, 2395.0)} = 26.1$, $p < .001$, $\varepsilon^2 = .031$; model with covariates ($F_{(3.0, 2030.0)} = 17.8$, $p < .001$, $\varepsilon^2 = .003$). Three-year-olds labeled angry faces (accuracy = 0.55), happy faces (accuracy = 0.53), and sad faces (accuracy = 0.51) equally well (p values > .008, Bonferroni adjusted) while they were least accurate at labeling fearful faces (accuracy = 0.44) compared with all other emotion-categories ($p < .008$, Bonferroni adjusted). The marginal mean differences in accuracy between the different emotion-categories on the emotion-labeling task are summarized in Table 4.

Similar to the results of the emotion-matching task, multivariate ANCOVA tests revealed that maternal education ($F_{(1, 684)} = 3.8$, $p = .05$, $\varepsilon^2 = .003$) and sex ($F_{(1, 684)} = 4.9$, $p = .08$, $\varepsilon^2 = .001$) of the child did not influence children's emotion-labeling accuracies. To account for the influence of labeling skills on the verbal emotion-labeling task, we adjusted the results for basic labeling ability. We found that better animal-labeling ability predicted better emotion-labeling accuracy ($F_{(1, 684)} = 25.6$, $p < .001$, $\varepsilon^2 = .036$). To test whether this could have contributed to the lack of a significant sex difference, we reanalyzed our data without adjusting for basic labeling ability. This time, we found a main effect for sex ($F_{(1,795)} = 7.97$, $p = .005$, $\varepsilon^2 = .010$) indicating a female advantage in performance for all emotion-categories. We found a main effect for age on emotion-labeling performance despite the small age range ($F_{(1, 684)} = 16.4$, $p < .001$, $\varepsilon^2 = .023$). No interaction between emotion-category and the covariates turned out to be significant. Results of the multivariate analyses for the emotion-labeling task are summarized in Table 5.

Error-bias analysis

Consistent with our predictions, when children labeled happy expressions incorrectly (mean proportion incorrect 0.47), they most frequently selected fearful faces instead (141.75). This was followed by sad expressions (98.75) and consequently angry expressions (46.25; overall $\chi^2_{(2)} = 48.3$, $p < .001$). When the label 'sad' was improperly applied (mean proportion incorrect 0.49) three-year-olds incorrectly selected fearful faces most frequently (191.50) followed by angry faces (82.25). Selecting a happy expression in response to the label 'sad' was the least frequent event (28.75; overall $\chi^2_{(2)} = 136.9$, $p < .001$). Angry facial expressions (mean proportion incorrect 0.45) were most often confused with sad (143.25) expressions, to a lesser extent with fearful expressions (83.75). The least frequent event was to label happy expressions as angry ones (29.25; overall $\chi^2_{(2)} = 76.2$, $p < .001$). Finally, when children were not able to accurately identify fearful expressions (mean proportion incorrect 0.56), they selected sad (153.00) and angry expressions (152.75) far more often than happy ones (45; overall $\chi^2_{(2)} = 66.5$, $p < .001$). Consequently, three-year-olds were more likely to select a negative emotion when incorrect at labeling other negative emotions, thus choosing emotions with the same valence when confusing specific emotion-categories. Figure 3 depicts children's error biases for specific target emotions.

2.1

Table 4. Comparing mean accuracy scores on the verbal FER task across facial expressions

Facial Expressions			Mean Differences [a]	*p*
Fearful	*vs.*	Happy	-0.07 [+]	< .001
		Sad	-0.06 [+]	< .001
		Angry	-0.09 [+]	< .001
Happy	*vs.*	Sad	0.01	1.0
		Angry	-0.03	.35
Sad	*vs.*	Angry	-0.04	.08

Mean differences represent differences in marginal mean accuracies of one emotion-category over the other. For example, in the case of fearful vs. happy a mean difference score of -0.07 means that the marginal mean accuracy for matching happy expressions is 7% higher than the marginal mean accuracy for matching fearful expressions.
Note. Bonferroni adjustment was used to correct for multiple testing.
Note 2. The fully adjusted results are presented in the table; results for emotion comparisons were essentially identical when covariates were not included in the model.
[a] Differences in marginal mean accuracies, adjusted for child's age, sex, animal-labeling accuracy, maternal education.
[+] Significant at $p < .008$.

Table 5. Multivariate analyses of mean accuracy scores on the verbal FER task

Source	*F*	*p*
Within-subjects effects (Greenhouse-Geisser) [b]		
Emotion	17.8	< .001
Emotion x Age	0.9	.45
Emotion x Maternal education	0.5	.71
Emotion x Animal-labeling	2.1	.10
Emotion x Sex	0.9	.44
Between-subjects effects [c]		
Age	16.4	< .001
Maternal education	3.8	.05
Animal-labeling	25.6	< .001
Sex	4.9	.08

[b] df1 = 3.0, df2 = 2030.00

[c] df1 = 1.0, df2 = 684

Repeated Measures ANOVA: emotion-category (happiness, sadness, anger, fear) as within-subjects factor; age, sex, animal-labeling ability, and maternal education as covariates.

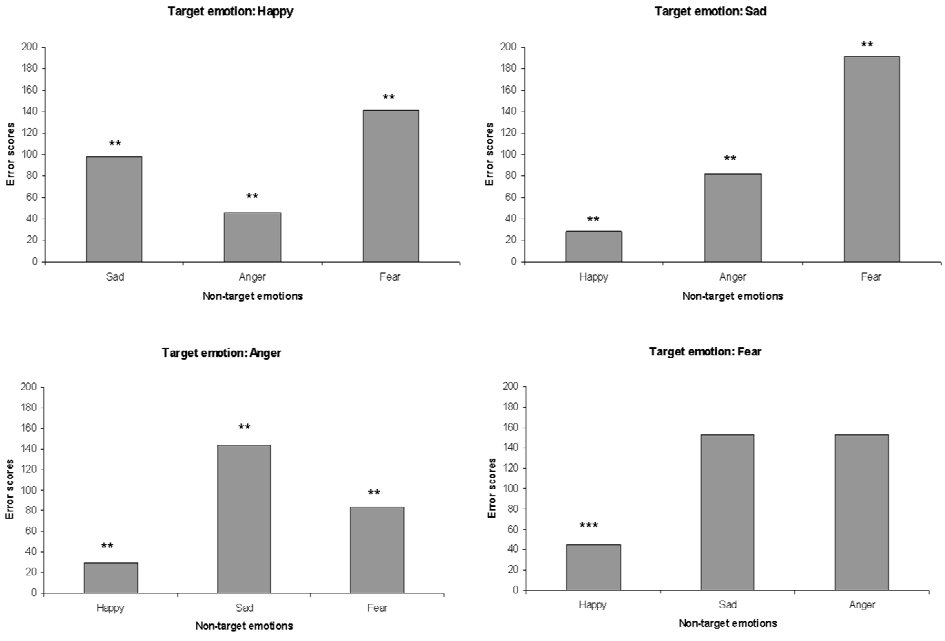

Figure 3. Errors in labeling happy, sad, angry, and fearful facial expressions. The errors indicate the number of wrong answers per non-target emotion-category averaged across the four trials of each target emotion.
Note. The observed range of the average error scores was 28.7 – 191.5. To enable better visual comparison across the target emotions we applied a scaling factor of 200, corresponding to the observed error maximum.
* $p < .05$, ** $p < .01$, *** $p < .001$.

DISCUSSION

Using data from a large-scale cohort study we investigated FER in three-year-old children, an age characterized by much cognitive, verbal, and socio-emotional change. To our knowledge, this is the first study to examine facial expression recognition using computerized paradigms reliant on verbal (i.e., labeling task) and nonverbal (i.e., matching task) abilities in a large sample of young children (N = 808). We were particularly interested in how these different task demands affect children's ability to recognize emotive facial expressions.

In line with our first prediction, three-year-olds showed differential performance on the verbal and nonverbal FER tasks reflected in their accuracy patterns among emotion-categories for the two types of tasks. These results are consistent with studies reporting an effect for task specifications on emotion-processing (for review, see Gross & Ballif, 1991). Vicari et al. (2000) proposed that the different performance patterns for FER observed with different types of tasks may be due to the recruitment of distinct cognitive processes, which may develop in different patterns. Markham and Adams (1992), and Camras and Allison (1985) also suggested that when considering the relative difficulty of various emotional expressions, the type of task should not

be ignored. It is noteworthy that the above studies supported this conclusion for some emotions (fear, surprise, and disgust) more than for others (happiness, sadness).

Our hypotheses on the rank order of emotion-categories were only partially confirmed on both tasks. On the nonverbal FER task, we expected that children would be most accurate at matching happy faces, followed by angry, sad and at last fearful faces. We found that three-year-olds most accurately matched happy and fearful faces, followed by sad and angry faces. This finding is inconsistent with that of Vicari et al. (2000) who found that fear was one of the least accurately matched facial expressions. There are, however, some methodological differences in the assessment procedure of the two studies. Firstly, Vicari et al. (2000) used a different stimulus set of facial expressions, they applied black and white photos, and a paper-pencil task version, while our task included color-pictures and was computerized. Other important differences were the number of alternatives available on each trial and the age group studied. The emotion-matching task of Vicari et al. (2000) had four alternatives while our emotion-matching task had only two alternatives to choose from on each trial. This was to make the task appropriate for three-year-olds by reducing the load on working memory. Finally, the child participants in the study by Vicari et al. (2000) were older (i.e., between the ages 5-10 years) than our three-year-olds. On the other hand, our findings are consistent with the study of Herba et al. (2006). They found the same rank order for emotions on their explicit emotion-matching task with a similar general computerized matching paradigm that we also used in the current study, but their design differed from the present one on the following levels: their participants were older (4-15 years), they applied different emotion intensities, and a different stimulus set.

As for the verbal FER task, we found that three-year-olds were approximately equally accurate at labeling happy, sad, and angry expressions and significantly less accurate at labeling fearful faces. This observation was in line with the findings of Widen and Russell (2003) who reported that around the ages of 3,5 – 4 years children generally master the use of three emotion labels (happy, angry, and sad). The label 'happy' stands for all kinds of positive emotions, whereas negative emotions can be divided into those with high arousal (e.g., angry) and with low arousal (e.g., sad). According to the authors, around 4 – 4,5 years of age the labels scared, disgusted, and surprised are added to the child's emotion-vocabulary, however, still with low accessibility at this age. Vicari et al. (2000) have also reported similar rank order for the emotion-categories in question (happiness, sadness, anger, fear) on their emotion-labeling tasks. The authors have stated that 5-10-year-olds perform at ceiling levels for happiness and sadness, while labeling of other emotion-categories such as fear improves with age. Gao and Maurer (2009) have investigated 5-, 7-, and 10-year-olds' abilities compared with adults to recognize happy, sad, and fearful expressions with the same face stimulus set as we used in our study. They have found that even the youngest children (5-year-olds) showed adult-like sensitivity for happy and sad facial expressions, whereas even 10-year-olds had inferior performance relative to adults in recognizing fearful expressions. He argued that this pattern might be due to relatively high exposure to happy and sad faces and low exposure to fearful faces in everyday life. Other studies have also found that the sensitivity to

fearful expressions is likely to show a distinct developmental trajectory (Camras & Allison, 1985; Durand et al., 2007; Vicari et al., 2000; Widen & Russell, 2003).

In accordance with our second prediction, sex did not influence performance on either FER task. This finding is consistent with other studies failing to report any sex differences in FER in childhood (De Sonneville et al., 2002; Herba et al., 2006, 2008; Vicari et al., 2000). Our data, however, were not consistent with McClure's (2000) meta-analysis, reporting a female advantage on FER in childhood. Perhaps methodological differences could explain the mixed results concerning sex effects on FER. Many of the frequently used FER tasks rely heavily on cognitive processes, which in turn need to be accounted for when investigating the influence of sex on emotion processing. Since our nonverbal emotion-matching task mostly relied on visuospatial processing skills, we adjusted the results for basic matching ability. Similarly, to account for the influence of labeling skills on the verbal emotion-labeling task, we adjusted the results for basic labeling ability. To test whether this could have contributed to the lack of a significant sex difference, we reanalyzed our data without adjusting for basic matching- or labeling abilities. For the emotion-matching task results remained unchanged, while on the emotion-labeling task we found a main effect for sex ($F_{1,795} = 7.97$, $p = .005$), indicating a female advantage in performance for all emotion-categories. Previously, we proposed that a female advantage may be more pronounced, if tasks rely to a greater extent on verbal rather than visuospatial ability (Herba et al., 2006).

Finally, as expected three-year-olds' errors on the emotion-labeling task were not random. Happy faces were most often mistaken for fearful faces, and emotions with a negative valence were confused with other negative emotions when misidentified on the verbal emotion-labeling task. The reason for using the verbal emotion-labeling task to explore any bias patterns in children's errors was that all emotion-categories were presented in each single trial while in the nonverbal emotion-matching task two alternatives were provided per trial.

Previous studies indicate that children's errors are based on the similarity of stimuli along underlying dimensions such as pleasure - displeasure (Gross & Ballif, 1991; Widen & Russell, 2008). The systematic pattern of children's mistakes is further supported by the study of Gao and Maurer (2009), which suggested that the pattern of confusion among facial expressions is systematic in childhood.

Only very few studies have looked at error biases in FER, especially in children. These studies all agree that emotions with a negative valence are frequently confused with one another, because discrimination between specific negative emotions develops slowly (Adolphs, Damasio, Tranel, & Damasio, 1996; Fox, 2004; Gao & Maurer, 2009; Gross & Ballif, 1991; Markham & Adams, 1992; Thomas et al., 2001). Our finding that children confused happy expressions with fearful ones is contradictory to the study of Calvo and Lundqvist (2008), who found no significant differences for happy expressions in adults between various types of incorrect responses. They argue that there are fewer distinct positive than negative emotions, which makes recognizing happiness a simpler task than recognizing specific negative emotions. Furthermore, most children from very early on are exposed to happy faces more often than to other emotional faces. Happiness can be relatively easily

distinguished by its unique mouth pattern, as all happy faces contain some form of a smile. In contrast, the identification of sadness, anger, and fear requires the integration of both the upper and lower face (Vicari et al., 2000). Neuroimaging and EEG studies suggest that the right hemisphere may be specialized to process negatively valenced emotional stimuli, while the left hemisphere processes positively valenced ones (Adolphs et al., 1996; Canli, Desmond, Zhao, Glover, & Gabrieli, 1998; Fox, Henderson, Rubin, Calkins, & Schmidt, 2001; Killgore & Yurgelun-Todd, 2007). Widen and Russell (2003) found using a free labeling paradigm that when only basic emotions were available, i.e., happiness, sadness, anger, fear, disgust, and surprise, children most often mislabeled surprised and fearful faces as happy ones. When the emotion-category 'surprise' was not available, children most often mislabeled fearful faces as happy ones. One possible explanation for our finding may be that in our study when children incorrectly identified happiness they could only select from negative alternatives (sadness, anger or fear). We used closed-mouth models for sad expressions, and open-mouth models for happy, angry, and fearful expressions. Since happy expressions can be relatively easily distinguished based on the mouth pattern this may have reduced the likelihood of incorrectly selecting sad expressions instead of happy ones. In displays of anger, bulging around the nose area is noticeable while this is absent for fear. Alterations in the upper face area are also apparent for anger and fear but we speculate that when children heard the question "who feels happy", it may have prompted them to concentrate on the lower face area, which is important in the recognition of happy expressions. This hypothesis could further be tested using eye-tracking methods to establish face-viewing patterns of children in response to particular emotion labels.

A very novel finding of our study is that when three-year-olds nonverbally matched facial expressions, fearful expressions were among the most accurately matched facial expressions along with happiness, whereas when 3-year-olds verbally labeled facial expressions, they were significantly the least accurate at labeling fearful faces. Prior studies have reported fear to be among the latest emerging and least accurately recognized primary emotions, which is likely to emerge along a different developmental trajectory than other emotions or have specific processing systems (Adolphs et al., 1996; Calvo & Lundqvist, 2008; Camras & Allison, 1985; Durand et al., 2007; Gao & Maurer, 2009; Gross & Ballif, 1991; Vicari et al., 2000; Widen & Russell, 2003).

Most studies on the neurobiology of emotion perception highlight the role of the amygdala in processing emotionally salient facial expressions, particularly fear (Guyer et al., 2008; Hare, Tottenham, Davidson, Glover, & Casey, 2005; LeDoux, 1994; Morris et al., 1996; Thomas et al., 2001). Adult and adolescent neuroimaging studies have shown that different neural pathways underlie FER depending on the paradigms used for assessment purposes. Amygdala activation has been consistently implied in unconscious emotion processing, and in emotion processing tasks with low cognitive demands such as passive viewing of emotional stimuli, viewing masked facial expressions, or implicit emotion processing (Herba et al., 2006; Lange et al., 2003; Monk, 2008a; Monk et al., 2003; Sheline et al., 2001; Whalen et al., 1998). Studies using task paradigms similar to our nonverbal FER task have consistently

reported increased bilateral amygdala activation, whereas labeling facial expressions has been associated with attenuated amygdala activation and increased prefrontal activation. Neocortical regions such as the prefrontal cortex and the anterior cingulate gyrus regulate emotional responses mediated by the amygdala through cognitive appraisal and labeling (Hariri et al., 2000; Hariri, Mattay, Tessitore, Fera, & Weinberger, 2003; Phillips et al., 2004).

Thus, subcortical structures interact with the prefrontal cortex in the various stages of emotion processing (Monk, 2008a; Phillips et al., 2003a, 2003b; Sprengelmeyer, Rausch, Eysel, & Przuntek, 1996), whereby prefrontal structures modulate emotional behavior through influencing attentional processes. Tasks with a greater reliance on attentional processes produce higher cortical activation and decreased subcortical activation in the brain (Guyer et al., 2008).

Given that the emotion-labeling task requires more explicit focus on the emotion such as the cognitive appraisal of stimuli and retrieval of specific emotion-categories, we propose that our emotion-labeling task will engage cortical structures more heavily than the emotion-matching task which may be more associated with the activation of subcortical structures involved in FER, in particular the amygdala. The amygdala is more responsive to fearful faces than other facial expressions (Leppänen, Moulson, Vogel-Farley, & Nelson, 2007; Morris et al, 1996; Vuilleumier, 2005) and more engaged during perceptual matching of facial expressions than during verbal labeling of facial expressions (Thomas et al., 2001). Consequently, our nonverbal emotion-matching task may have resulted in better performance for recognizing fearful facial expressions than the verbal emotion-labeling task. The association between the recognition of fearful facial expressions and the knowledge of the meaning of fear is acquired over development. Thus, it is possible that the emotion-labeling task required larger engagement of higher neocortical regions (Hariri et al., 2000, 2003) and cortical-subcortical interactions (Blair et al., 2007), which are less well developed in younger children (Killgore & Yurgelun-Todd, 2001). Batty and Taylor (2006) suggested that frontal brain areas continue to show large developmental changes, while the emotional activities in which they are implicated seem to be already in place early in life. It may be possible that the amygdala shortcuts the normal brain pathway for processing visual images and causes fearful faces to jump out more quickly (Fox, 2004). Kim, Somerville, Johnstone, Alexander, and Whalen (2003, 2004) have demonstrated inverse relationships between the amygdala and prefrontal regions. Given the evolutionary significance of fearful stimuli, it would make sense if the response to fearful stimuli would demonstrate earlier maturation than other emotional stimuli, despite the relatively undeveloped subcortical-cortical brain connections at this age, given that fear signals potential environmental threat (Thomas et al., 2001).

Limitations

The current study collected FER data of typically developing children at a large scale to provide sufficient statistical power to examine verbal and nonverbal processing of facial expressions of emotions at a very young age when FER abilities are suggested to go through marked changes. A further strength of the study is the use of specific control tasks for basic

matching and labeling ability prior the FER tasks. Similar control paradigms were previously used by others (Hariri et al., 2000 and Herba et al., 2006 for shape-matching; Widen & Russell, 2008 for animal-labeling), although no information on their reliability has been published. Despite the above strengths of the study, we were faced with the following limitations.

Firstly, the verbal and nonverbal emotion processing tasks applied in our study did not have equal number of alternatives available on each trial, which was to make the study age-appropriate by reducing the load on working memory; hence, the two tasks were not directly comparable. Secondly, we used the verbal emotion-labeling task to explore any bias patterns in children's errors as in this task all emotion-categories were presented in each single trial, while in the emotion-matching task not all emotion-categories were displayed in a single trial. Consequently, our findings on error-biases need to be restricted to verbal emotion processing. Moreover, the forced-choice design of our task has further limited the types of error that could occur, which may have influenced the pattern of children's incorrect responses. Future studies will hopefully address this question in diverse contexts of emotion processing. Thirdly, we used posed photographs of facial expressions in this study, which may be of less ecological validity than other emotional stimuli such as emotional vocalization, or dynamic display of emotional gestures. On the other hand, earlier research has demonstrated that basic emotions can be most reliably recognized from facial expressions, and that neural circuits involved in emotion processing are consistently engaged in response to pictures depicting facial expressions (Adolphs, 2002; Hariri et al, 2000, 2003). Thus, this way our results can be interpreted within the context of previous work on the development of FER measured behaviorally and neurally (Guyer et al., 2007).

Clinical Implications

Our use of a verbal and nonverbal task paradigm within a larger sample of young children adds to the growing literature on FER development. The study provides valuable normative data on emerging emotion processing skills of three-year-olds, an age that is considered to be critical in the development of FER. Greater understanding of early FER development will also inform clinical research on the subsequent identification of abnormal developmental patterns in emotion perception. Our findings may also guide future neuroimaging work on the underlying neurobiology of emerging emotion recognition.

Finding out more about the nature of children's early FER skills has additional advantages for child-rearing and education, as well as for designing earlier treatment interventions for children with atypical FER skills, as observed in a range of medical conditions such as Down syndrome and Williams syndrome (Plesa-Skwerer, Faja, Schofield, Verbalis, & Tager-Flusberg, 2006; Williams, Wishart, Pitcain, & Willis, 2005), neurodevelopmental disorders such as autism (Rump, Giovannelli, Minshew, & Strauss, 2009), problem behaviors such as internalizing and externalizing problems (Etkin & Wager, 2007; Hefter, Manoach, & Barton, 2005; Marsh & Blair, 2008; McClure, Pope, Hoberman, Pine, & Leibenluft, 2003; Monk et al., 2008b), and those exposed to social adversity such as parental maltreatment (Masten et al., 2008; Pollak, Cicchetti, Hornung, & Reed, 2000).

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CHAPTER 3

EMOTION RECOGNITION, TEMPERAMENT, AND THE INFLUENCE OF MATERNAL DEPRESSION

CHAPTER 3.1

MATERNAL DEPRESSIVE SYMPTOMS AND SENSITIVITY ARE RELATED TO YOUNG CHILDREN'S FACIAL EXPRESSION RECOGNITION

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ABSTRACT

A vast body of literature shows that maternal depression has long-term adverse consequences for children. However, only very few studies have documented the effect of maternal depression on children's ability to process emotional expressions, and even fewer incorporated measures of observed maternal sensitivity to further tease apart whether it is the symptoms per se or the associated impact via maternal sensitivity that affects children's developing emotion processing abilities. In a large community sample of Dutch preschoolers ($N = 770$), we examined independent and mediated effects of maternal depressive symptoms and sensitivity on children's ability to recognize emotional expressions using a nonverbal and a verbal task paradigm. Maternal depressive symptoms predicted less accurate emotion-labeling in children, while maternal sensitivity was associated with more accurate emotion-matching, especially for sadness and anger. Maternal sensitivity did not mediate the observed associations between mothers' depressive symptoms and children's emotion recognition, and effects were similar for boys and girls. Given that maternal depressive symptoms and sensitivity affected non-overlapping areas of young children's emotion recognition, prevention and intervention efforts should focus on both alleviating maternal depressive symptoms and improving maternal sensitivity at the same time in order to maximize benefit.

INTRODUCTION

A large body of research has documented the association between maternal depression and adverse child outcomes at various ages (Beardslee, Gladstone, & O'Connor, 2011; Field, 1995; Goodman & Gotlib, 1999; Hay, Pawlby, Waters, & Sharp, 2008; Murray et al., 2011). Even infants of a few months old react negatively to signs of maternal depression as reflected in cognitive and motor developmental delays, negative emotionality, and insecure attachment relationship (Field, 1995; Tronick & Reck, 2009; Wan & Green, 2009; Weinberg & Tronick, 1998). During childhood and adolescence, maternal depression is consistently associated with cognitive and language delays (Brennan et al., 2000; Hay & Kumar, 1995), decreased academic achievement (Murray et al., 2010), interpersonal difficulties (Murray, Sinclair, Ducournau, & Turner, 1999), and psychiatric disorders (Goodman et al., 2011). The above studies included both clinical and non-clinical samples of women. Although effects seem to be stronger for the clinically depressed, studies of community samples of women with self-reported symptoms of depression have also shown adverse outcomes in children (Downey & Coyne, 1990). For convenience, the term 'maternal depression' is used throughout the paper to refer to both types of studies. However, for our study we apply a more stringent terminology in reference to maternal depressive symptoms.

There are several mechanisms involved in the transmission of influences from maternal depression to child adjustment problems (for reviews, see Cummings & Davies, 1994; Goodman & Gotlib, 1999; Elgar, McGrath, Waschbusch, Stewart, & Curtis, 2004). According to Elgar et al. (2004), these mechanisms can be grouped into three sets of interrelated, mediating factors. The first one involves biological mechanisms such as genetic and in utero environmental influences which run unidirectionally from mother to child. The second factor includes psychosocial mechanisms such as exposure to negative cognitions and behaviors, interaction between mother and child, parenting, family functioning, and modeling. These mechanisms are considered bidirectional and, thus, have the capacity for mutual influences. The third group of mechanisms represents moderating, contextual factors such as social disadvantage and social resources which indirectly transmit mutual influences on maternal and child functioning. The present study focuses on the second group of mechanisms, more specifically on maternal sensitivity, which is an important determinant of the quality of mother-child interactions. It has been shown that depressed women are often less sensitive and responsive in their parenting skills than non-depressed women (Field, Healy, Goldstein, & Guthertz, 1990). They usually spend less time looking at their infants, touching them, and talking to them relative to non-depressed women (Cohn, Campbell, Matias, & Hopkins, 1990). In addition, they tend to exhibit less positive and more flat or negative emotions toward their children than non-depressed women (Campbell, Cohn, & Meyers, 1995; Hamilton, Jones, & Hammen, 1993). Even though depressed women can substantially vary in their interaction styles with their children (Field, Hernandez-Reif, & Diego, 2006), there is converging evidence that postpartum depression has adverse effects on mother-child interaction (see reviews by Beck, 1995; Lovejoy, Graczyk, O'Hare, & Neuman, 2000). Therefore, it is not surprising that some researchers hypothesized it is mothers' interaction

style rather than the exposure to maternal depression per se that carries the adverse effect on child functioning (Murray & Cooper, 1997a; Murray, Fiori-Cowley, Hooper, & Cooper, 1996), or in part accounts for this association (Goodman & Gotlib, 1999; NICHD ECCRN, 1999).

A crucial yet under-examined area is to investigate whether maternal depression and sensitivity also impacts on children's ability to process emotional facial expressions. Processing emotional cues such as facial expressions of others, is an important aspect of social functioning in humans (Haxby, Hoffman, & Gobbini, 2002; Philippot & Feldman, 1990). Younger children rely on facial expressions for information on the emotional state of others to a greater extent than on situational cues (Hoffner & Badzinski, 1989). Altered patterns in facial expression recognition (FER) have been reported in a wide range of child and adult clinical populations (for reviews, see Monk, 2008; Phillips, Drevets, Rauch, & Lane, 2003). Furthermore, biases in processing emotional stimuli are often regarded as an early marker of cognitive vulnerability for psychiatric disorders such as depression (Joormann & Gotlib, 2007; Kujawa et al., 2011; for review, see Leppänen, 2008). Studies have shown that both negative and positive early experiences have an important role in shaping children's ability to recognize facial expressions (De Haan, Belsky, Reid, Volein, & Johnson, 2004; Pollak, Cicchetti, Hornung, & Reed, 2000; Pollak, Messner, Kistler, & Cohn, 2009). Children of depressed mothers experience an atypical emotional environment characterized by a high exposure to sad, angry, or neutral expressions and a low exposure to happy expressions compared with children of non-depressed mothers (Dawson et al., 2003; Field, 1995). Maternal depression during the first years of a child's life occurs at a time when mothers have an important role in the socialization of emotion regulation and expression (Denham, 1998). Thus, maternal depression during the preschool years may play an important role in the development of children's emotion perception (De Haan et al., 2004; Montague & Walker-Andrews, 2002).

Only few studies have investigated the potential role of maternal depression and sensitivity on children's ability to recognize facial expressions. Prior studies showed that although infants of depressed mothers were less likely to look at facial expressions displayed by their mother or a stranger, their elevated cortisol levels after such experimental situations indicated that they may have found these situations more stressful than infants of non-depressed mothers (Diego et al., 2004; Pickens & Field, 1995). Other studies found that infants of depressed mothers discriminated sad from happy expressions but did not perceive sad expressions as novel (Hernandez-Reif, Field, Diego, Vera, & Pickens, 2006). In addition, they discriminated less well between neutral and happy expressions than infants of non-depressed women (Bornstein, Arterberry, Mash, & Manian, 2011). Diego et al. (2002) assessed the effect of interaction style of depressed mothers on infants' perception of facial expressions. They observed that infants of depressed mothers with an intrusive interaction style showed more differential responding to facial expressions of a stranger than infants of depressed mothers with a withdrawn interaction style. In childhood and early adolescence, following a negative mood induction daughters of depressed women exhibited attentional biases for negative emotions as well as experienced difficulties at

recognizing negative emotions relative to children of non-depressed women (Joormann, Gilbert, & Gotlib, 2009; Joormann, Talbot, & Gotlib, 2007; Kujawa et al., 2011). In contrast, without a mood prime, children of mothers with a history of depression showed attentional biases away from negative emotions compared to children of mothers with no history of depression (Gibb, Benas, Grassia, & McGeary, 2009).

There is some suggestion that boys may be particularly vulnerable for the effects of maternal depression (for reviews, see Downey & Coyne, 1990; Grace, et al., 2003; Murray & Cooper, 1997b). Much of this knowledge comes from studies that have tended to focus on outcomes such as children's emotional and behavioral problems, cognitive functioning, or language abilities. Among the few studies that have looked at children's ability to process emotional expressions in relation to maternal depression, two included only female offspring (Joormann et al., 2009; Joormann et al., 2007), one observed attentional biases for sad faces only in daughters and not in sons of depressed mothers (Kujawa et al., 2011), and one did not find that sex moderated the effect of maternal depression on attentional biases for sad faces in the offspring (Gibb et al., 2009). Of these studies, only the one by Gibb et al. (2009) did not deploy a negative mood induction.

The aim of the present study was to investigate the independent and mediated effects of mothers' depressive symptoms and observed sensitivity on young children's FER. Based on the literature, we hypothesized the following: i) higher levels of maternal depressive symptoms will be associated with lower levels of observed sensitivity; ii) children of mothers with high levels of depressive symptoms will recognize positive emotions (i.e., happiness) less accurately and negative emotions (i.e., sadness, anger, and fear) more accurately than children of mothers with no or low levels of depressive symptoms; iii) children of mothers with higher levels of observed sensitivity will recognize facial expressions, in general, more accurately than children of mothers with lower levels of observed sensitivity; iv) observed maternal sensitivity will partially mediate the association between maternal depressive symptoms and children's FER. Finally, we did not expect that sex will moderate the effect of maternal depression on children's FER, as no mood induction was used in the present study.

METHOD

Setting

The present investigation pertained to a subsample of children participating in the Generation R Study, a population-based prospective Dutch cohort from fetal life onward (Jaddoe et al., 2010). The subsample, known as the Generation R Focus Cohort, is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity. All children in this subsample were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort (Jaddoe et al., 2008). The study was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki and approved by the Medical Ethics Committee of the Erasmus Medical Center. Parental written informed consents were obtained for all participants.

Participants

The subgroup for the present study consisted of the 862 children and their mothers who attended the Focus Cohort assessments in our research centre when children were approximately three years old. In 838 mothers, we had information on maternal depressive symptoms at one or more time points: in 796 during the early postnatal period (2-6 months postpartum), and in 747 at the child's age of three years. Information on maternal sensitivity when the child was three years old was available in 820 of the 838 women. At age three years, 673 children had useable accuracy data for emotion-matching and 770 for emotion-labeling.

Maternal depressive symptoms

Information on maternal depressive symptoms was obtained by postal questionnaires. Depressive symptoms were assessed using the depression scale of the Brief Symptom Inventory (BSI), the short version of the SCL-90-R (Derogatis & Melisaratos, 1983). The BSI is a 53-item validated self-report inventory in which participants rate the extent to which they have been bothered (0="not at all" to 4="extremely") in the past week by various symptoms. The instrument is widely used to assess psychological distress (De Beurs, 2004; Derogatis, 1993). The BSI depression scale includes the following six items: "feeling suicidal", "feeling lonely", "feeling blue", "having no interest in anything anymore", "feeling hopeless about the future", and "feeling worthless". Summed scores were divided by the number of completed items with a maximum of one missing item allowed as recommended in the manual (De Beurs, 2004). Since we wanted to test the hypothesis that mothers' observed sensitivity would mediate the associations between maternal depressive symptoms and children's FER, we focused on assessing maternal depressive symptoms during the child's lifetime. Previous literature emphasized the importance of both postpartum and concurrent depressive symptoms in relation to adverse child outcomes (Brennan et al., 2000; Brockington, 2004; Josefsson & Sydsjö, 2007). Therefore, in the present study we included assessments of maternal depressive symptoms at both time points. To define postpartum depressive symptoms, we computed an average score based on BSI depression scores assessed at two and six months postpartum (the correlation between the two measures was $r = .48$, $p < .001$). To define maternal depressive symptoms during the preschool period, we used maternal BSI depression scores assessed when the child was three years old (correlations between mothers' BSI depression scores in the preschool period and at postpartum were $r = .37$, $p < .001$ at 2 months; and $r = .31$ $p < .001$ at 6 months). Correlations between maternal BSI depression scores across the different time points are similar to those reported by Josefsson, Berg, Nordin, & Sydsjö (2001). Internal consistencies of the depression scale for the present study were .80 at two months, .83 at six months, and .75 at three years. To test whether the BSI depression scale accurately tapped maternal depressive symptoms, we compared women's BSI depression score at two months postpartum to their score on the Edinburgh Postnatal Depression Scale (Cox, Holden, & Sagovsky, 1987), which was also administered to them at this time (Blom et al., 2010). The correlation between the two measures was $r = .67$, $p < .001$. In analyses of maternal depression, we focused on examining women with high and clinically significant

levels of symptoms compared to those with no or only mild depressive symptoms during their child's life. According to the available norms for Dutch female non-patient groups, a raw BSI depression score between 0.67 and 1.79 corresponds to 'high levels' and between 1.80 and 4.00 to 'very high levels' of depressive symptoms. Women with a score > 0.80 typically meet criteria for clinically significant depression (De Beurs, 2009). Therefore, women with BSI depression scores of ≥ 0.67 at postpartum and/or in the preschool period were regarded as 'ever' experiencing high or clinically significant depressive symptoms, whereas women with BSI depression scores below 0.67 both at postpartum and in the preschool period were considered as having none or steady low levels of depressive symptoms. According to this, 39 of the 770 women included in the analyses reported high or clinically significant levels of depressive symptoms either at postpartum (n = 24), in the preschool period (n = 12), or at both times (n = 3). To additionally examine the effect of subclinical symptoms of depression and the presence of a potential dose-response relationship between maternal depressive symptoms and other variables, we also analyzed continuous scores of maternal depressive symptoms. To this end, BSI depression scores in the postnatal and preschool period were combined into an average score (z-standardized) to reflect the general tendency of mothers to experience depressive symptoms during their child's preschool years.

Maternal sensitivity

At 3 years postpartum maternal sensitivity was observed in our research center while mother and child dyads performed two tasks which were designed to be too difficult for the child: building a tower and an etch-a-sketch task. Mothers were instructed to help their child as they would usually do. Maternal sensitivity was coded from DVD recordings with the revised Erickson 7-point rating scales for Supportive Presence and Intrusiveness (Egeland, Erickson, Clemenhagen-Moon, Hiester, & Korfmacher, 1990). The subscales Supportive Presence and Intrusiveness were coded for each task. An overall sensitivity score was created by reversing the Intrusiveness scale, standardizing the scores on the subscales, and creating an average over both subscales and both tasks. A similar procedure was used by Alink et al. (2009). The two tasks were independently coded by 13 trained coders. Coders were blind to maternal reports of depressive symptoms and children's performance on the FER tasks. Coders were extensively trained and regularly supervised. Reliability of coding was assessed directly after the training and at the end of the coding process to detect possible rater drift. For the tower task, the intercoder reliability (intraclass correlation coefficients, ICC) for both subscales was .68 on average directly after the training (n = 20) and .80 on average at the end of the coding process (n = 33), resulting in an overall ICC of .75 (n = 53). For the etch-a-sketch task, the ICC for both subscales was .84 on average directly after the training (n = 15) and .77 on average at the end of the coding process (n = 40).

Facial expression recognition (FER)

At age three years, children's FER was assessed in our research center during the same visit when maternal sensitivity was also observed. A nonverbal emotion-matching task and a verbal emotion-labeling task were used to assess how accurately children recognize facial

expressions of basic emotions. Color images of four basic emotions (happiness, sadness, anger, and fear) were presented on a screen and children responded using a touch-sensitive monitor. Stimuli were selected from a widely used facial stimulus set, the NimStim, on the basis of which identities demonstrated the best recognized pose for a particular emotion-category (Tottenham et al., 2009). Prior to the FER tasks, children were introduced to the idea of emotions and facial expressions by a trained experimenter. Further specifications of the task and stimulus material can be found in Székely et al. (2011).

In the *emotion-matching task*, children had to match the emotion of the target face with one of two choices (displayed by the same identity, but different from the target face). Sixteen trials of emotion-matching with two female and two male identity pairs, and four basic emotions were included. Stimuli presentation was counterbalanced and randomized for emotion and identity. Prior to the emotion-matching task, a *shape-matching task* was presented in order to ensure that children understood the concept of matching and to control for the effects of basic matching ability. The shape-matching task had the same parameters and layout as the emotion-matching task, only children had to match geometrical shapes. A practice trial and four test trials with different target shapes were included in a fixed order. Similar paradigms have been used previously by Hariri, Bookheimer, and Mazziotta (2000), and Herba, Landau, Russell, Ecker, and Phillips (2006).

In the *emotion-labeling task*, all four emotions (displayed by the same identity) were shown in each trial. Children heard a voice-bit asking in a neutral voice which person was feeling happy, sad, angry, or scared. They were then asked to point at the photo that matched this label. Sixteen trials were presented, four items for each of the four emotion-categories with two female and two male identities. Stimuli presentation was counterbalanced and randomized for emotion and identity. A similar paradigm has been used previously by Fries and Pollak (2004). To control for the effects of labeling ability an *animal-labeling task* was included. This task was administered to children by their parents at age 30 months, six months preceding the FER assessments. Children were presented with six black-and-white photos of animals and had to indicate which one of six pictures matched the name of an animal read out by the parent. There were six trials in total with a fixed stimulus order. Similar paradigms have been previously used by Widen and Russell (2003).

Statistical analyses

Relations between maternal depressive symptoms and sensitivity were first explored using linear regression models. Then, independent effects of maternal depressive symptoms and sensitivity on children's FER were examined using repeated measures ANCOVAs. Outcome variables were accuracy scores (mean proportion correct) for matching and labeling happy, sad, angry, and fearful faces. Main predictors were maternal depressive symptoms and sensitivity. Dichotomous predictor variables were entered as between-subjects factors, continuous predictor variables were entered as covariates in the model. In case of a significant interaction effect between the predictor and emotion-category, separate linear regression analyses were run for each emotion to examine specificity of effect of the predictor. For these analyses we

applied a Bonferroni correction to reduce errors due to multiple testing ($\alpha = 0.05/4 = 0.01$). Interaction between the predictors and child sex was tested in all models. When this was not significant, we reported results from the model without the interaction term.

To examine whether the effect of maternal depressive symptoms on children's FER was mediated by sensitivity, we tested indirect effects of maternal depressive symptoms on children's FER via sensitivity using bootstrapping, a nonparametric resampling procedure. This method is preferred to alternative mediation tests (e.g., causal steps or normal theory approaches), as it respects the non-normality of the sampling distribution of the indirect effect (Preacher & Hayes, 2008; Shrout & Bolger, 2002). In addition, it has lower Type I error rates and greater power to detect indirect effects than alternative mediation tests (MacKinnon, Lockwood, Hoffman, West, & Sheets, 2002). In the present study, we investigated the significance of mediation using 95% bias-corrected bootstrap confidence intervals for indirect effects and contrasts of indirect effects applying 5000 bootstrap samples. Analyses were carried out in SPSS using a macro developed by Preacher and Hayes (2008). Confidence intervals were used to determine indirect relationships between maternal depressive symptoms and children's FER including maternal sensitivity as a potential mediator. Indirect effects are present when the confidence intervals do not include 0. In our case, this meant that the indirect effect was significant at $p < .05$.

Covariates

Analyses including children's FER as outcome were adjusted for child's age, sex, shape-matching/animal-labeling accuracy, gestational age at birth, maternal age at intake, maternal education, marital status, parity, and maternal smoking during pregnancy. Most covariates were selected based on previous literature (Brennan et al., 2000; Campbell, Matestic, Stauffenberg, Mohan, & Kirchner, 2007). Shape-matching and animal-labeling accuracies were included to control for children's basic matching and labeling ability. Information on child's sex and gestational age at birth was obtained from midwives and obstetricians. Information on maternal education, marital status, parity, and maternal smoking during pregnancy was collected via questionnaires. Child's age and shape-matching accuracy were assessed when the outcome, FER, was measured. Animal-labeling accuracy was assessed by the parent at age 30 months. Maternal education was coded as 'low' (primary education or no education), 'medium' (secondary education), or 'high' (college or university degree). Since there were only eight mothers (1%) in the low category, low and medium categories were collapsed for further analyses. Marital status was dichotomized into 'married or living with a partner' and 'living alone'. Parity was dichotomized into 'primiparous' and 'multiparous'. Maternal smoking was assessed in the first, second, and third trimester and summarized as 'yes, at least sometime during pregnancy' and 'never during pregnancy'. Percentages of missing data on the covariates ranged from 0.1% to 2.5%.

Non-response analysis

To examine patterns of non-response, we compared basic characteristics of children in the present sample ($N = 770$) to children who visited our research centre at three years but did

not have any (useable) FER data (N = 92). This latter group included 29 children to whom FER tasks were not administered due to time constraints, 7 children with incomplete FER data, and 56 children who had available FER data but were not included in analyses due to the following reasons: 6 children were tested at the beginning of the assessments using a different test procedure; 1 child was older than 45 months at the time of visit; 7 twins were randomly excluded from each participating twin pair to avoid biases due to paired data; 24 children without available information on maternal depressive symptoms; and 18 children without available information on maternal sensitivity. Children without useable FER data were on average 0.48 months older (t (860) = 2.92, p = .004) and were born 0.63 weeks earlier (t (105.17 – corrected for unequal group variances) = -2.77, p = .007) than children who had useable FER data. Furthermore, their mothers were slightly younger (mean difference = 0.84 years, t (860) = -2.02, p = .044) and less highly educated (54.5% vs. 67.7%, χ^2 (1) = 5.87, p = .015) than mothers of children included in the analyses. The two groups were comparable in terms of distribution of boys and girls, marital status, parity, and smoking during pregnancy (all p values > .05). Importantly, depressive symptoms and maternal sensitivity did not affect non-response (all p values > .05).

RESULTS

Descriptive statistics

Sample characteristics are presented in Table 1 separately for mothers with high and low levels of depressive symptoms. Mothers with high levels of depressive symptoms were more often living alone (χ^2 (1) = 24.45, p < .001) and smoking during pregnancy (χ^2 (1) = 4.86, p = .027) than mothers with lower levels of depressive symptoms. Given that the animal-labeling task was mostly administered to children by their mothers at home, we examined whether maternal depressive symptoms (as assessed in the present study) were associated with children's scores on the animal-labeling task. Results indicated that children's accuracy for animal-labeling were comparable between those children whose mothers experienced high levels of depressive symptoms (at postpartum and/or in the preschool period) and children whose mothers reported no or only mild depressive symptoms (mean difference = 0.01, t (761) = 0.632, p = .528).

As shown in Table 1, children's performances on the shape-matching and animal-labeling tasks were close to ceiling level. In the present study, a ceiling level performance on the control tasks was desired, as it indicated that most children were able to understand and perform basic matching and labeling. This was necessary to be able to complete our FER tasks. Importantly, it also meant that a lower score on the FER tasks was not due to children's inability to match or label.

Maternal depressive symptoms and sensitivity

Mother's depressive symptoms predicted their observed sensitivity. Women with high levels of depressive symptoms were less sensitive than women with no or mild depressive

Table 1. Sample characteristics according to high and low levels of maternal depressive symptoms

	Maternal depressive symptoms	
	High (n = 39) % or M (SD)	Low (n = 731) % or M (SD)
Child characteristics		
Sex (boys)	33.3	51.4
Age (months)	37.66 (1.57)	37.49 (1.44)
Gestational age at birth (weeks)	39.39 (2.79)	40.12 (1.60)
Control tasks accuracy		
Animal labeling	.93 (.11)	.95 (.12)
Shape matching	.85 (.28)	.90 (.20)
Emotion-matching accuracy		
Happiness	.68 (.29)	.67 (.29)
Sadness	.62 (.30)	.59 (.29)
Anger	.53 (.33)	.62 (.29)
Fear	.61 (.33)	.65 (.29)
Emotion-labeling accuracy		
Happiness	.47 (.39)	.53 (.33)
Sadness	.40 (.32)	.52 (.33)
Anger	.42 (.38)	.56 (.36)
Fear	.36 (.35)	.45 (.33)
Maternal characteristics		
Age at intake (years)	32.17 (4.96)	32.06 (3.63)
Educational level (high)	53.8	68.2
Marital status (living alone)	17.9	2.8
Parity (primiparous)	61.5	62.1
Smoked during pregnancy (yes)	33.3	18.9

symptoms (β = -.246; *95% CI*: -.465, -.028; p = .027). In addition, there was also a dose-response relationship between mothers' depressive symptoms and observed sensitivity, as higher levels of depressive symptoms also continuously predicted lower observed sensitivity (β = -.065; *95% CI*: -.123, -.007; p = .027).

Maternal depressive symptoms and FER

Maternal depressive symptoms did not predict children's emotion-matching accuracy. However, maternal depressive symptoms significantly predicted children's emotion-labeling accuracy (Table 2). Children of mothers with high levels of depressive symptoms were less accurate at labeling emotions than children of mothers with no or only mild symptoms. Analyses using continuous scores of maternal depressive symptoms confirmed this finding

(Table 2). There were no significant interaction effects between maternal depressive symptoms and emotion-category on children's emotion-matching or emotion-labeling accuracies (all *p* values > .05). Likewise, there was no significant interaction effect between mothers' depressive symptoms and child sex for emotion-matching or emotion-labeling accuracies (all *p* values > .05).

Table 2. Main effects of maternal depressive symptoms and maternal sensitivity on children's facial expression recognition

	Emotion-matching task Main effects		Emotion-labeling task Main effects	
	F (df1, df2)	*p*	*F* (df1, df2)	*p*
Maternal depressive symptoms				
Dichotomous (high vs. low)	0.040 (1,657)	.841	5.742 (1, 744)	.017
Continuous (*z*-score)	0.019 (1, 657)	.891	5.018 (1, 744)	.025
Maternal sensitivity (*z*-score)	8.664 (1, 661)	.003	0.355 (1, 749)	.551

Note. Main effects are between-subjects effects of predictors examined separately using repeated measures ANCOVAs. All models were adjusted for child's age, sex, basic matching/labeling ability, gestational age at birth, maternal age, education, marital status, parity, and prenatal smoking

Next, we also ran separate regression models to determine whether the observed association between maternal depressive symptoms and children's emotion-labeling accuracy was driven by mothers' postnatal or concurrent depressive symptoms. Outcomes were children's accuracy scores for emotion-labeling, predictors were either postpartum or concurrent maternal depressive symptoms. Models were adjusted for the same covariates as analyses including the composite maternal depression measure. Results indicated that the observed link between the composite maternal depression score and children's overall emotion-labeling accuracy was mainly driven by mothers' concurrent depressive symptoms (dichotomous BSI depression scores: F (1, 670) = 4.436, p = .036; continuous BSI depression scores: F (1, 670) = 5.976, p = .015). The effect of maternal postpartum depressive symptoms on children's emotion-labeling accuracy did not reach statistical significance (dichotomous BSI depression scores: F (1, 708) = 2.709, p = .100; continuous BSI depressions cores: F (1, 708) = 1.969, p = .161).

Maternal sensitivity and FER

Observed maternal sensitivity had a significant main effect on children's emotion-matching accuracy (Table 2). Children whose mothers were more sensitive generally matched emotions more accurately. In addition, the interaction effect of maternal sensitivity by emotion-category was also significant for emotion-matching accuracy (F Greenhouse-Geisser (2.9, 1912.6) = 8.648, p = .003). To specify the nature of the effect of maternal sensitivity on the different emotions, separate linear regressions were run for each emotion. Results indicated that children with more sensitive mothers especially matched sad faces (β = .046;

95% CI: .012, .081; $p = .008$) and angry faces ($\beta = .061$; *95% CI*: .028, .094; $p < .001$) more accurately. Maternal sensitivity was not associated with children's emotion-labeling accuracy (all p values > .05). There was no significant interaction effect for maternal sensitivity by child sex on emotion-matching or emotion-labeling accuracy (all p values > .05).

Maternal depressive symptoms, maternal sensitivity, and FER

Results of the bootstrap analyses for emotion-matching accuracy are included in Appendix A and for emotion-labeling accuracy in Appendix B. Since all confidence intervals included 0, there was no evidence in the present sample that maternal sensitivity mediated the relationship between mothers' depressive symptoms and children's FER.

DISCUSSION

Despite the prevalence of maternal depression and its known long-term significant impact on the offspring, surprisingly few studies have examined the associations between maternal depression and children's early emotional functioning such as their ability to recognize salient emotional cues. Furthermore, even fewer studies have incorporated measures of maternal sensitivity to further tease apart whether it is the depressive symptoms per se or the associated impact via maternal sensitivity that affects children's developing emotion processing abilities. Thus, using data from a large-scale longitudinal prospective cohort study of preschoolers we examined associations between maternal depressive symptoms, observed maternal sensitivity, and children's ability to process emotional facial expressions.

In line with our first hypothesis, higher levels of maternal depressive symptoms were associated with lower levels of observed sensitivity. Results indicated that even subclinical levels of depressive symptoms were associated with decreased sensitivity as observed in a laboratory setting. This finding is in accordance with results from other large-scale population-based studies namely, that mothers' depressive symptoms are associated with lower sensitivity in their interaction with their preschool-aged children (Campbell et al., 2004; Campbell, et al., 2007; NICHD ECCRN, 1999). Thus, symptoms of maternal depression, whether or not they reach diagnosable levels, may underlie unresponsive, unavailable, or less sensitive care of the child (Cummings, Davies, & Campbell, 2000).

Our second hypothesis that maternal depressive symptoms would be associated with lower recognition accuracy for positive emotions and higher recognition accuracy for negative emotions was not supported by the results. Importantly, we found that maternal depressive symptoms predicted reduced general recognition accuracy on the verbal FER task even after taking into account basic labeling ability. Most developmental studies have examined attentional biases in viewing emotional stimuli in relation to maternal depression. These studies all supported the presence of attentional biases for negative emotions, although there is still debate concerning the direction of this bias. Studies that used a negative mood induction found biased attention toward sad faces (Joormann et al., 2007; Kujawa et al., 2011), while those without a mood induction reported attentional biases away from sad

faces (Gibb et al., 2009). These studies typically included two categories of emotion (i.e., happiness and sadness) except for the study by Gibb et al. (2009), which also included anger. In addition, these studies examined school-aged children and young adolescents, while our participants were all of preschool age. One study which compared daughters of depressed mothers to daughters of never-depressed mothers in their ability to correctly identify happy, sad, and angry expressions of varying intensities, reported that daughters of depressed women required higher intensities to correctly identify sad faces and made more errors in identifying angry faces than daughters of never-depressed women (Joormann et al., 2009). In the present study, we used full intensity expressions given the young age of our participants. These important differences in study design make it difficult to compare our findings with the above studies. However, our observation of a reduced overall accuracy for labeling facial expressions in general fits well with the results of a meta-analysis which reported robust global deficits in FER among adult patients with major depression relative to healthy controls (Demenescu, Kortekaas, Den Boer, & Aleman, 2010).

Noteworthy is that effects of maternal depressive symptoms emerged only on the verbal FER task. There is evidence that verbal and visuospatial FER abilities may develop along different trajectories in childhood (Vicari, Reilly, Pasqualetti, Vizzotto, & Caltagirone, 2000). One potential explanation for this differential development lies in the underlying neurobiology of verbal and non-verbal FER. Studies using task paradigms similar to our non-verbal FER task have consistently reported increased bilateral amygdala activation (Hariri et al., 2000; Hariri, Mattay, Tessitore, Fera, & Weinberger, 2003; Phillips et al., 2004), whereas paradigms similar to our verbal FER task have been associated with increased prefrontal activation (Guyer et al., 2008; Phan, Wager, Taylor, & Liberzon, 2002). Prefrontal brain structures modulate emotional behavior through shaping the activation of the amygdala (Herba & Phillips, 2004). One study found that maternal depression was related to lower frontal brain activation in the offspring, which partly mediated the link between maternal depression and child behavior problems at age three years (Dawson et al., 2003). Although we did not examine brain activation patterns in the present study, it is plausible that maternal depressive symptoms lead to lower levels of prefrontal activation in children, and our verbal FER task was more sensitive to such differences than our non-verbal FER task. This hypothesis should be tested in future studies. Alternatively, it could be that children of mothers with depressive symptoms had difficulties connecting appropriate verbal labels to facial displays of emotions. Murray, Kempton, Woolgar, and Hooper (1993) found that the speech of depressed mothers was less focused on infant experience than that of well mothers. This may make it difficult for the developing child to create links between emotional experiences and appropriate verbal labels.

In the present study, we did not find evidence for a differential effect of maternal depressive symptoms on boys and girls in relation to their FER. Although gender differences have not been observed in rates of major depression during childhood, by adolescence girls are twice as likely to develop depressive disorders as boys (Lewinsohn, Clarke, Seeley, & Rhode, 1994). In parallel, a view emerging from previous reports on the effect of maternal depression on child outcome, is that boys may be particularly vulnerable

to the adverse effects of maternal depression from infancy through adolescence in terms of cognitive development (Hay, Pawlby, Waters, & Sharp, 2008; Kurstjens & Wolke, 2001; Murray, Arteche, Fearon, Halligan, & Cooper, 2010), their reaction to less efficient maternal interactions associated with the disorder (Murray et al., 1993), as well as school drop-out (Ensminger, Hanson, Riley, & Juon, 2003). However, a recent review suggests that maternal depression may signal differential risk for boys and girls depending on the outcome studied (Beardslee et al., 2011). As discussed in the introduction, gender differences in children's emotion processing in relation to maternal depression are quite inconsistent. Two studies focused only on girls (Joormann et al., 2009, 2007), one study found an effect of maternal depression only for girls and not for boys (Kujawa et al., 2011), and one did not find sex differences at all (Gibb et al., 2009). Our study is consistent with the most recent, which was incidentally the only study that, similar to ours, did not deploy negative mood induction.

Our third expectation that observed maternal sensitivity would be associated with more accurate FER in children was partially supported by the results. Children of more sensitive mothers performed better on our non-verbal FER task, especially at identifying sad and angry expressions. The NICHD ECCRN study (1999) also reported beneficial effects for maternal sensitivity on children's cognitive and social outcomes. Campbell et al. (2007) identified maternal sensitivity as an important, independent predictor of child outcome. Our finding that maternal sensitivity had an especially positive effect on identifying sad and angry faces may seem surprising at first glance. However, in one of our previous studies using the same sample (Székely et al., 2011) we reported that children found sad and angry faces the most difficult to identify on our non-verbal FER task. Thus, it may be that sensitive maternal behavior provides a more stimulating emotional environment, which in turn may further stimulate the development of non-verbal FER in children (De Haan et al., 2004).

Fourthly, although we had expected that, to some extent, maternal sensitivity would explain the link between maternal depressive symptoms and children's FER, our results did not provide evidence for this hypothesis. This is in contrast with earlier studies which reported a mediating role for maternal sensitivity in the association between maternal depression and poor child outcome (Campbell et al., 2007). Nevertheless, our observation is in accordance with other studies which did not find that maternal sensitivity explained the link between maternal depression and child outcome (Murray et al., 1999; NICHD ECCRN, 1999). Importantly, risk from depressed mother to child can also be transmitted via alternative pathways. Since the present study included a relatively low-risk sample and analyses were adjusted for contextual risk factors, we cannot comment on the role of contextual adversity in the transmission mechanism. Environmental factors, however, do not fully account for the risk posed to children of depressed mothers. Twin and adoption studies have suggested that genetics explain approximately 30% to 40% of the variance in adult major depression (Beardslee et al., 2011). Risk for depression is significantly higher among first-degree relatives and the highest among the offspring of depressed parents (Rice, Harold, & Thaper, 2002). In addition to the notion that children of depressed parents inherit the likelihood for depression per se, heritability also contributes

significantly to vulnerabilities to depression (Goodman & Gotlib, 1999; Goodman, 2007). Biased processing of emotional expressions is often implicated as a vulnerability marker of depression (Leppänen, 2006), and is subserved by the same neural circuitry in the brain which is thought to be dysregulated in depression (Ressler & Mayberg, 2007).

In summary, maternal depressive symptoms and sensitivity both affected children's developing FER, but in different ways: maternal depressive symptoms had an overall negative effect on children's accuracy to label emotions, whereas sensitivity exerted more positive emotion-specific effects that were seen for the nonverbal FER task. The ability to accurately recognize emotional expressions is important for smooth social interaction and general well-being throughout life. For instance, in the same sample we found that children who labeled angry expressions more accurately at age three were rated by their parents as more prosocial on the corresponding scale of the Strengths and Difficulties Questionnaire (Goodman, 1997) at age five (unpublished data). Identifying early predictors and correlates of young children's FER is important to increase our understanding of normal and pathological socio-emotional development.

Limitations

There are several strengths of the current study: it includes a large group of very young, typically developing children; maternal depressive symptoms were assessed prospectively from birth until the child's age of three years when the outcome, children's FER, was measured; maternal sensitivity was observed in our laboratory using structured interaction tasks; children's FER was assessed using computerized tasks specifically developed for this age group. However, despite these strengths we were faced with a number of limitations. Firstly, high or clinically relevant depressive symptoms in the current, low-risk sample were generally rare (n = 39). Therefore, it is difficult to speculate about the influence of truly severe or chronic maternal depression on children's FER. However, the fact that we found negative associations even between sub-clinical levels of maternal depressive symptoms and children's ability to label emotions emphasizes the importance to study the link between maternal depression and children's emotion processing. Secondly, we relied on maternal self-reports of depressive symptoms. There are indications that self-report measures in community samples are more reflective of general "distress" rather than "true depression" (Atkinson et al., 2000). However, self-report measures are commonly used in epidemiological studies and the fact that maternal sensitivity and our outcome measures were independently assessed or observed minimizes any problems due to shared methods variance. Thirdly, we lacked information on maternal depressive symptoms between the first and the third year of the child's life. Given the episodic nature of depression, we may have missed some women who experienced elevated levels of depressive symptoms only during this time period (Brennan et al., 2000). Nevertheless, in the current study we had information on postpartum and concurrent symptoms of depressions, both of which have been shown to have important implications for poor child outcomes (Bayer et al., 2008; Beck, 1998; Brennan et al., 2000; Grace et al., 2003; Murray et al., 1999; Radke-Yarrow, Cummings, Kuczynski, & Chapman,

1985, Teti et al., 1995; Trapolini et al., 2007). Fourthly, no information was available on children's verbal ability, therefore, analyses including emotion-labeling accuracy were not adjusted for this variable. Previous studies found that maternal depression is associated with lower vocabulary scores in children (Brennan et al., 2000), which, in turn, may affect the identification of more subtle emotional expressions (Camras & Allison, 1985). However, the present study assessed the recognition of basic emotional expressions and prior to the emotion-labeling task experimenters made sure that children understood the emotion labels corresponding to the facial expressions they had to recognize throughout the task. Finally, we had only cross-sectional observations of maternal sensitivity. Ideally, the mediating variable is assessed before the outcome. In the present study, maternal sensitivity and children's FER were observed independently but during the same laboratory visit. However, possible reverse causality is generally less of a problem in negative studies.

Conclusion

Maternal depressive symptoms negatively impact young children's ability to verbally identify emotional expressions, while maternal sensitivity exerts a beneficial effect on children's ability to nonverbally identify emotional expressions. Given that maternal depressive symptoms and sensitivity affected non-overlapping areas of children's FER, and that maternal sensitivity did not explain the association between mothers' depressive symptoms and children's FER, prevention and intervention efforts should focus on both alleviating maternal depressive symptoms and improving maternal sensitivity at the same time in order to maximize benefit.

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APPENDIX A

Mediation analyses: Testing indirect effects of maternal depressive symptoms on children's emotion-matching accuracy including maternal sensitivity as a potential mediator

	Indirect effects of maternal depressive symptoms on emotion-matching via maternal sensitivity				
	TOTAL	HAPPY	SAD	ANGRY	FEARFUL
	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)
Maternal depressive symptoms					
Dichotomous (high vs. low)	-.0042 (-.0152, .0011)	-.0047 (-.0196, .0013)	-.0061 (-.0230, .0029)	-.0054 (-.0215, .0069)	.0016 (-.0026, .0125)
Continuous (*z*-scores)	-.0006 (-.0031, .0009)	-.0006 (-.0043, .0011)	-.0008 (-.0051, .0018)	-.0001 (-.0037, .0037)	-.0002 (-.0006, .0031)

Note. Models were adjusted for child's age, sex, shape-matching accuracy, gestational age at birth, maternal age, education, marital status, parity, and prenatal smoking.

APPENDIX B

Mediation analyses: Testing indirect effects of maternal depressive symptoms on children's emotion-labeling accuracy including maternal sensitivity as a potential mediator

	Indirect effects of maternal depressive symptoms on emotion-labeling via maternal sensitivity				
	TOTAL	HAPPY	SAD	ANGRY	FEARFUL
	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)	Bootstrap estimate (95% *CI*)
Maternal depressive symptoms					
Dichotomous (high vs. low)	-.0019 (-.0119, .0022)	-.0028 (-.0157, .0029)	-.0017 (-.0146, .0041)	.0012 (-.0051, .0129)	-.0047 (-.0182, .0011)
Continuous (*z*-scores)	-.0004 (-.0033, .0004)	-.0006 (-.0041, .0006)	-.0004 (-.0038, .0006)	.0002 (-.0011, .0034)	-.0010 (-.0049, .0004)

Note. Models were adjusted for child's age, sex, animal-labeling accuracy, gestational age at birth, maternal age, education, marital status, parity, and prenatal smoking.

CHAPTER 3.2

MATERNAL DEPRESSIVE SYMPTOMS ARE ASSOCIATED WITH LOW POSITIVE EMOTIONALITY AND LOW FEARFULNESS IN YOUNG CHILDREN

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Revision submitted

ABSTRACT

Objective: To examine whether maternal depressive symptoms predict low positive emotionality and high temperamental fearfulness in young children. **Method:** Maternal depressive symptoms were modeled from pregnancy to the early preschool years of the child using a semi-parametric mixture model. Positive emotionality and temperamental fearfulness were assessed using laboratory observations in a large cohort of typically developing Dutch preschoolers (N = 661, 338 boys), all approximately 36 months old (M = 37.55, SD = 1.49). In addition to demographic and socioeconomic background, models were adjusted for maternal harsh parenting. **Results:** After controlling for background risk, more severe and chronic symptoms of maternal depression were associated with lower positive emotionality in a socially engaging context and with lower fearfulness in a novel context that primarily elicited a startle response from children. **Conclusions:** Findings provide important insight into the relatedness of two significant early risk factors of psychopathology: maternal depressive symptoms and temperamental vulnerability. Findings emphasize the necessity of early screening for maternal depressive symptoms in the general population.

INTRODUCTION

A large body of research has documented the adverse effects of maternal depression on child development from infancy through adolescence (Beardslee, Gladstone, & O'Connor, 2011; Downey & Coyne, 1990; Field, 1995; Hay, Pawlby, Waters, & Sharp, 2008; Murray et al., 2011). Most of these studies focused on outcomes such as cognitive and language development (Brennan et al., 2000; Hay & Kumar, 1995), academic achievement (Murray et al., 2010), social competence (Murray, Sinclair, Ducournau, & Turner, 1999), and psychopathology (Goodman et al., 2011). Few studies have examined the effect of maternal depression on child temperament.

Temperament generally encompasses individual differences in affect, activity, attention, and self-regulation (Rothbart & Bates, 2006). Although these early emerging traits have a strong biological basis, temperament is shaped by the interplay of heredity and experience (Caspi & Shiner, 2008; Rothbart & Posner, 2006). Since temperament systems guide the adaptation to environmental circumstances (Clark & Watson, 1999), they are often included in risk models of psychopathology (Caspi & Shiner, 2008; Caspi & Shiner, 2006; Rothbart & Bates, 2006; Nigg, 2006; Thomas & Chess, 1977). Among others, temperamental vulnerability has been implicated as a diathesis in the intergenerational transmission of depression (Silberg & Rutter, 2000). According to Clark and Watson's (1991, 1999) tripartite model, the most cited temperament model of depressive disorders, low positive emotionality and high negative emotionality predispose to risk for depression. In particular, the model posits that low positive emotionality has a relatively specific association to depression, while high negative emotionality constitutes a non-specific risk factor that plays a role in numerous other disorders such as anxiety, substance use, and conduct disorders (Clark, 2005). In addition, a third temperamental construct, behavioral inhibition (Kagan, 1989), may also be related to risk for developing depression (Fox, Henderson, Marshall, Nichols, & Ghera, 2005; Hirshfeld-Becker et al., 2008), although behavioral inhibition is currently primarily viewed as a risk factor for anxiety disorders (Perez-Edgar & Fox, 2005). Behavioral inhibition shares low approach and exploration with low positive emotionality, but this similarity is limited to novel, fear-eliciting contexts (Laptook et al., 2008). The overlap between negative emotionality and behavioral inhibition is that they both share a substantial fear component (Muris & Dietvorst, 2006). There is evidence that such lower-order components can contribute unique predictive information (Reynolds & Clark, 2001), and that fear-related (as opposed to anxiety-related) disorders do play a role in the intergenerational transmission of major depression (Warner, Wickramaratne, & Weissman, 2008). Despite, very few studies have examined the relations between temperamental fearfulness, this common facet of both negative emotionality and behavioral inhibition, and the risk for depression.

Laboratory observations of child behavior are particularly useful when examining the influence of maternal depression on child outcome, especially when maternal depression is assessed using self-reports. If women report on both their own depressive symptoms and their offspring's behavior, associations may be inflated due to shared method variance. In addition, depressed women may show biases in rating their own children's behavior, thereby leading to

potential dysphoria-related reporting biases (Fergusson, Lynskey, & Horwood, 1993). Importantly, laboratory assessments are also ideally suited to study behaviors (e.g., fearful reactions) which occur at a low base rate in naturalistic settings (Durbin, Hayden, Klein, & Olino, 2007).

The literature concerning the links between parental mood disorders and observed early child temperament is quite limited. In a sample of two- and three-year olds, Kochanska (1991) observed that parents with bipolar disorder were more likely to have behaviorally inhibited children than non-depressed parents, whereas children of parents with major depression did not differ from any of the two groups. Rosenbaum et al. (2000) reported that children (aged two to six years) whose parents had both panic disorder and major depression had significantly higher rates of behavioral inhibition than control subjects, whereas children whose parents had either disorder alone had intermediate rates of behavioral inhibition, which did not significantly differ from rates observed in the control group or among children of parents with both disorders. In a large sample of three-year-olds, Durbin et al. (2005) described a link between low positive emotionality and lifetime history of maternal mood disorders (major depression and dysthymia), even after controlling for current maternal depressive symptoms. The observed association was relatively specific, as no such links were found between maternal mood disorder and child negative emotionality or behavioral inhibition, furthermore, low positive emotionality was not related to paternal mood disorder or other forms of parental psychopathology. Olino et al. (2010) reported that parental depressive disorders were related to high negative emotionality and behavioral inhibition in a large community sample of three-year-olds, especially at high and moderate levels of child positive emotionality. Finally, Olino et al. (2011) modeled developmental trajectories of observed positive and negative emotionality from infancy through age nine years, and found that offspring of mothers with a history of childhood-onset depression had consistently lower levels of positive emotionality. Variability across children in the course of negative emotionality was not sufficient to examine associations between maternal depression and child negative emotionality. In two of these studies, exploratory analyses were also conducted at the lower-order trait level. Result showed that, while child fearfulness was not associated with parental depression in the smaller sample (Durbin et al., 2005), fear had a significant effect in the larger sample, indicating that higher levels of child fearfulness were associated with a greater probability of parental depressive disorder (Olino et al., 2010).

All the above cited studies included children of parents with a current or past history of depressive disorder. Little is known about whether sub-clinical levels of maternal depressive symptoms, which are more common in the general population, are also associated with temperamental vulnerability in the offspring. In addition, studying the effect of maternal depressive symptoms in a large longitudinal cohort has several strengths. The most important one is that depressive symptoms can be assessed in more detail using repeated measurements. Statistical techniques are now available that allow modeling the course and severity of depressive symptoms simultaneously on the basis of repeated assessments of symptoms by identifying groups of individuals who report similar symptom patterns (Muthén, 2002; Nagin, 2005). Such a study design also allows examining the effect of

maternal depressive symptoms which may not reach clinically significant levels but are more prevalent in the general population.

More recent studies of child temperament have indicated the importance of parsing out temperament variables into finer grained constructs, and taking into account the specificity of the context in which a temperamental trait is observed (Carver, 2004; Dyson, Klein, Olino, Dougherty, & Durbin, 2011). Researchers have particularly suggested differentiating between social and non-social forms of fear and behavioral inhibition (Dyson et al., 2011; Majdandžić & Van den Boom, 2007). This is in line with research on anxiety disorders that demonstrates differences between social and non-social phobias at the phenotypic (Spence, Rapee, McDonald, & Ingram, 2001), genotypic (Eley, Rijsdijk, Perrin, O'Connor, & Bolton, 2008), and neurobiological levels (Goldin, Manber, Hakimi, Canli, & Gross, 2009). Moreover, laboratory indices of fear/behavioral inhibition in social and non-social contexts are not significantly related (Dyson et al., 2011; Kochanska, 1991; Majdandžić & Van den Boom, 2007; Rubin, Hastings, Stewart, Henderson, & Chen, 1997), and fearfulness observed in social and non-social situations may have different parent-rated temperamental and psychopathological correlates (Dyson et al., 2011). In addition, social and specific fears exhibit different developmental trajectories (Gullone & King, 1991).

In a large-scale longitudinal population-based cohort, we investigated whether maternal depressive symptoms, modeled longitudinally from before birth until the child's age of three years, predict positive emotionality and fearfulness in young children. We chose to focus on the early preschool years, as temperament begins to stabilize during this period (Caspi, 2000), while depressive disorders are extremely rare before middle childhood (Lavigne, LeBailly, Hopkins, Gouze, & Binns, 2009). We expected that more severe and chronic symptoms of maternal depression, as represented by women in the higher depression trajectories, will be associated with lower positive emotionality and increased fearfulness in the offspring. In addition, we examined the exploratory hypothesis that associations between maternal depressive symptoms and children's positive emotionality and fearfulness will show distinct patterns in social and non-social contexts.

METHOD

Setting

The present investigation pertained to a subsample of children participating in the Generation R Study, a population-based prospective Dutch cohort from fetal life onward (Jaddoe et al., 2010). The subsample, known as the Generation R Focus Cohort, is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity. All children were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort (Jaddoe et al., 2008). The study was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki and approved by the Medical Ethics Committee of the Erasmus Medical Center. Parental written informed consents were obtained for all participants.

Participants

The subgroup for the present study consisted of the 862 mother-child dyads who visited our research center when the child was approximately 36 months old (M = 37.55, SD = 1.49). Information on maternal depressive symptoms was available in 860 women. Of these, 722 women qualified for the analyses on calculating depressive symptoms trajectories, meaning that they had data on maternal depressive symptoms on at least two of the four assessments (assessments were conducted prenatally, and at two-, six-, and 36 months postnatally). Child temperament was assessed using four episodes of laboratory observation: 636 children completed the Stranger Approach episode, 623 the Jumping Spider episode, 643 the Popping Bubbles episode, and 650 the Puppet Game episode. The total number of children with sufficient maternal data on depression and available temperament assessment in at least one episode was 661.

Maternal depressive symptoms

Information on maternal depressive symptoms was obtained by postal questionnaires at four time points: at 20 weeks of gestation, and postpartum at two months, six months, and three years. Depressive symptoms were assessed using the depression scale of the Brief Symptom Inventory (BSI), the short version of the SCL-90-R (Derogatis & Melisaratos, 1983). The BSI is a 53-item validated self-report inventory in which participants rate the extent to which they have been bothered (0="not at all" to 4="extremely") in the past week by various symptoms. The instrument is widely used to assess psychological distress (De Beurs, 2004; Derogatis, 1993). The BSI depression scale includes the following six items: "feeling suicidal", "feeling lonely", "feeling blue", "having no interest in anything anymore", "feeling hopeless about the future", and "feeling worthless". Summed scores were divided by the number of completed items with a maximum of one missing item allowed as recommended in the manual (De Beurs, 2004). Internal consistencies of the depression scale for the present study ranged from .74 to .82. To test whether the BSI depression scale accurately tapped maternal depressive symptoms, we compared women's BSI depression score at two months postpartum to their scores on the Edinburgh Postnatal Depression Scale, which was also administered to them at this time. The correlation between the two measures was r = .65, p < .001.

Child temperament

Child temperament was assessed at three years of age using four episodes from the Laboratory Temperament Assessment Battery (Lab-TAB; Goldsmith, Reilly, Lemery, Longley, & Prescott, 1999; Goldsmith & Rothbart, 1999). The Lab-TAB is a widely used, standardized instrument for the observational assessment of early temperament. The Stranger Approach and Jumping Spider episodes (from the Preschool Version) were selected to assess fearfulness. The Stranger Approach episode evokes distress in the presence of a stranger, while the Jumping Spider episode elicits a reaction in response to an unfamiliar object. Thus, these two episodes are thought to tap different (i.e., social and non-social) aspects of fear (Majdandžić & Van den Boom, 2007). To assess positive emotionality, we

selected the Popping Bubbles episode (from the Preschool Version) and the Puppet Game episode (from the Locomotor Version) from the Lab-TAB. The Popping Bubbles episode elicits joy in a structured play setting, while the Puppet Game episode elicits joy in a social interaction situation with two puppets. Episodes were coded from DVD recordings according to the original coding systems described in the Lab-TAB manual. We conducted regular checks to make sure that episodes closely followed the procedure as described in the manual. Coders received extensive training and their reliability was established on a set of 25 DVDs before data were coded. Coders were not involved in assessment of the Lab-TAB and were blind to information regarding maternal depressive symptoms. As suggested in the manual, episodes were ordered to minimize differential effects. Below, we describe them in the order in which they were conducted.

Stranger Approach. In the *Stranger Approach* episode the child deals with social fear when a novel, slightly threatening stranger approaches. The situation is modeled after real-life events. During the observation the child was left alone in a room. After some time, a female stranger disguised as a male entered the room and asked standard questions from the child in a neutral tone of voice while slowly approaching the child. The episode was divided into nine epochs for scoring. Intensities of fear expressions, distress vocalizations, activity decrease, approach, avoidance, gaze aversion, verbal hesitancy, and nervous fidgeting were scored in each epoch as specified in the manual. Averages were computed for each child response or parameter across epochs. Interrater reliabilities (ICCs, single measures) for these averages ranged between .71 - .97. In the next step, a fear composite for this episode was formed by first reversing the approach average, then converting all averages into *z*-scores, and finally combining them into one overall composite by taking the mean of all averages (*z*-scores).

Jumping Spider. The *Jumping Spider* episode is designed to elicit a startle/fear reaction based on an unexpected event, which is generally a predominant source of fear among preschool children. In this episode, the child and the experimenter were seated across from each other at a child-sized table on which there was a toy spider in a cage. The experimenter introduced a toy spider as a soft, fuzzy animal which does not bite and prompted the child to pet it. When the child's hand was approximately 5cm away from the spider, the experimenter made it "jump". This process was repeated four times in total. After the four trials, or when the child did not want to pet the spider anymore, the experimenter explained to the child that it was only a toy, and allowed the child to make the spider jump if he wanted to. The episode was divided into five epochs for scoring. Intensity of fear expressions, vocal distress, bodily fear, approach, withdrawal, gaze aversion, and startle response were scored in each epoch. Averages were computed for each child response or parameter across epochs. Interrater reliabilities (ICCs, single measures) for these averages ranged between .66 - .95. In the next step, a fear composite for this episode was formed by converting all averages into *z*-scores, and combining them into one overall composite by taking the mean of all averages (*z*-scores).

Popping Bubbles. In the *Popping Bubbles* episode the child engages in the pleasurable activity of blowing and popping bubbles. This episode is divided into a low and a high pleasure

phase. In the low pleasure phase, the experimenter demonstrated how to blow bubbles and asked the child to do the same. In the high pleasure phase, the experimenter encouraged the child to engage in chasing and popping a series of bubbles first with the hands (3x), then with the feet (3x), and finally with both the hands and feet (3x). In total, this episode included eleven epochs for scoring. Intensity of smiling, presence of laughter, and vigor of approach were scored in each epoch. Interrater reliabilities (ICCs, single measures) for these averages ranged between .71-.75. In the next step, a positive emotionality composite for this episode was formed by converting all averages into *z*-scores, and combining them into one overall composite by taking the mean of all averages (*z*-scores).

Puppet Game. The *Puppet Game* episode is designed to measure enjoyment in response to social stimulation. In this episode the experimenter performed a standard dialogue in an animated, lively fashion with the help of two hand puppets. The experimenter played the role of both puppets and used different 'voices' for the two puppets. During the dialogue, the experimenter tickled the child first with one hand puppet, then with the other hand puppet, and finally with both puppets. Following the dialogue, the experimenter allowed the child to play with the puppets for 30 seconds. The episode was divided into five epochs for scoring. Intensity of smiling, presence of laughter, positive vocalizations, and positive motor acts were scored in each epoch. Averages were computed for each child response or parameter across epochs. Interrater reliabilities (ICCs, single measures) for these averages ranged between .66 - .95. In the next step, a positive emotionality composite for this episode was formed by converting all averages into *z*-scores, and combining them into one overall composite by taking the mean of all averages (*z*-scores).

Covariates

Covariates for the present study were selected based on previous studies which included large longitudinal cohorts and focused on specific aspects (e.g., severity and chronicity) of maternal depressive symptoms (e.g., Brennan et al., 2000; Cents et al., 2012). Furthermore, in our covariate selection we tried to stay as close as possible to the study by Cents et al. (2012), as those parameters defined the probability of trajectory membership in our cohort. Covariates included child's age, sex, gestational age at birth, mother's age at intake, maternal education, marital status, parity, family income, maternal smoking and alcohol consumption during pregnancy. Information on child's sex and gestational age at birth was obtained from midwives and obstetricians. Information on maternal age, education, parity, maternal smoking and alcohol consumption during pregnancy was collected via questionnaires. Child's age was assessed when temperament was observed in our research center. Maternal education was dichotomized into 'low and medium' (primary or secondary education), and 'high' (college or university degree). Maternal marital status was dichotomized into 'being married or cohabiting' and 'being single'. Parity was dichotomized into 'primiparous' and 'multiparous'. Net family income was categorized based on the social security level for a 2-person household into 'below modal income' (≤ 2000 € a month) and above 'modal income' (> 2000 € a month). Maternal smoking and alcohol consumption during pregnancy

were used as dichotomous variables (yes/now). Finally, analyses were also adjusted for a parenting measure in order to examine the effect of maternal depressive symptom course on child temperament, independent of maternal parenting style. A measure of maternal harsh parenting was chosen to this end, as there are indications that harsh discipline may be a stronger mediator of the link between maternal depression trajectories and child outcome than sensitive maternal behavior (Campbell et al., 2007; Harnish et al., 1995). Information on maternal harsh discipline was obtained by postal questionnaires when the child was three years old using an adapted version of the Parent-Child Conflict Tactics Scale (Straus, Hamby, Finkelhor, Moore, & Runyan, 1998). Based on the questionnaire items a 'harsh parenting' scale was constructed using exploratory and confirmatory factor analyses (M*plus* Version 5, Muthén & Muthén, 1998-2007). The harsh parenting scale included six of the 14 original items (factor loadings > .05) and matched previous definitions and assessments of the construct 'harsh parenting' very well (Chang, Schwartz, Dodge, McBride-Chang, 2003). The full analytic procedure of the scale construction is described in detail elsewhere (Jansen et al., 2010). A harsh discipline composite was calculated by adding the scores on the six items. This yielded a composite score ranging from 0 to 12, with higher scores reflecting higher incidence of harsh discipline. Composite scores were dichotomized for further analyses using the 80th percentile score as cut-off in line with previous publications on this cohort (Jansen et al., 2012). Percentages of missing data on the covariates ranged from 0.2% to 2.4%.

Statistical analyses

Trajectories of maternal depressive symptoms

Using data from the larger total Generation R cohort, Cents et al. (2012) calculated trajectories of mothers' depressive symptoms using a semi-parametric mixture model (SAS procedure Proc Traj; Jones, Nagin, & Roeder, 2001; Nagin & Tremblay, 2001). Detailed information on the trajectories and exact modeling procedure can be found in Cents et al. (2012) but briefly, models with three-six trajectory groups were estimated in the total cohort. The BIC value continued to increase, as more groups were added. Trajectories in the four-group model (in the total cohort) were conceptually interesting and average posterior probabilities (ranging from 0.70 to 0.92, mean = .84) indicated a good to very good model fit. In the five-group model, the smallest trajectory estimated in the four-group model (1.5%) was further divided into even smaller groups (1.2% and 0.4%). Thus, we considered the four-group model as the most optimal model for describing the total cohort (Cents et al., 2012). Women's group membership in the present study was assigned based on the underlying solution for the total cohort, as, when modeling trajectories, it makes sense to use all information that is available on the underlying population from which the sample was pooled. Three of the four trajectory groups were represented with sufficient numbers for analyses in the present subsample. These three trajectory groups are described below. The first trajectory consisted of mothers (43.8%) who reported no or very few depressive symptoms throughout all four assessments. The second and largest trajectory (51.2%) included mothers who reported to have mild depressive symptoms. Mothers in this

trajectory reported significantly higher depressive symptoms in the early postnatal period than in the prenatal and third year assessment. The third trajectory (4.9%) was named 'moderate' depressive symptoms. Mothers assigned to this trajectory reported depressive symptoms around the score of 0.80, which signals clinically significant symptoms (De Beurs, 2009). These mothers also reported significantly higher depressive symptoms in the early postnatal period than in the prenatal and third year assessment.

Main analyses

Separate linear regression models were run to examine independent effects of the different trajectories of maternal depressive symptoms on child temperament. Predictors were trajectory groups of maternal depressive symptoms (i.e., no symptoms, mild symptoms, or moderate symptoms). Outcomes were continuous scores in the two positive emotionality and two fearfulness episodes. All analyses were adjusted for covariates described earlier. Differential effects of maternal depressive symptoms for boys and girls were examined by adding an interaction term including the predictor and the child's sex to all models. When this was not significant, we reported results from the model without this interaction term.

Response analysis

Participants who attended the three-year assessments in our research center but did not have any Lab-TAB episodes available ($n = 201$) were compared along baseline characteristics with participants who were included in at least one Lab-TAB analysis ($n = 661$). Children without Lab-TAB data were on average 0.51 months older (t (285.27-corrected for unequal variances) = 3.82, $p < .001$), had mothers who were less highly educated ($\chi 2$ (1) = 4.49, $p = .034$) and more often multiparous ($\chi 2$ (1) = 13.14, $p < .001$) than children participating in our analyses. Importantly, the two groups did not differ in terms of distribution of class memberships of maternal depressive symptoms and in terms of prevalence of harsh parenting ($p > .05$).

RESULTS

Sample characteristics

Sample characteristics are presented in Table 1 separately for the three trajectory-groups of maternal depression. Women with no depressive symptoms tended to be more highly educated than women with mild or moderate levels of depressive symptoms ($\chi 2$ (2) = 6.62, $p = .04$). Women in the moderate depressive symptoms trajectory had a lower family income ($\chi 2$ (2) = 26.39, $p < .001$) and were more often single ($\chi 2$ (2) = 37.16 $p < .001$) than women with no or only mild symptoms of depression. Additionally, women in the higher trajectories were on average younger (F (2, 658) = 4.64, $p = .01$) and more likely to use harsh discipline ($\chi 2$ (2) = 10.06, $p = .007$). Mothers in the three depression trajectories were comparable in terms of parity, and smoking and drinking habits during pregnancy ($p > .05$). Furthermore, children in the three groups were similar in terms of gestational age at birth, age at visit, and distribution of boys and girls ($p > .05$).

Table 1. Sample characteristics

	Trajectories of maternal depressive symptoms		
	No	Mild	Moderate
Total (*N* = 661)	(*n* = 291)	(*n* = 338)	(*n* = 32)
Child characteristics			
Age (months), *Mean (SD)*	37.3 (1.4)	37.5 (1.4)	37.4 (1.6)
Sex (% boys)	48.5	54.1	43.8
Gestational age at birth (weeks), *Mean (SD)*	40.1 (1.6)	40.0 (1.8)	39.5 (2.6)
Maternal characteristics			
Age at intake (years), *Mean (SD)*	32.5 (3.5)	31.8 (3.7)	30.9 (5.3)
Education (% low, intermediate)	26.7	36.3	34.4
Family income (% below modal)	8.0	11.4	38.7
Marital status (% single)	1.4	3.3	21.9
Parity (% primiparous)	63.6	65.4	78.1
Smoking during pregnancy (% yes)	16.9	21.6	28.1
Drinking during pregnancy (% yes)	74.4	70.3	69.0
Harsh discipline style (% yes)	18.9	28.9	35.5

Correlations between scores in the four Lab-TAB episodes are presented in Table 2. Scores in those episodes assessing positive emotionality with and without a social component, the Puppet Game and Popping Bubbles episodes, were moderately correlated. Scores in those episodes assessing fearfulness with and without a social component, the Stranger Approach and Jumping Spider episodes, were not correlated. Correlations between scores in those episodes assessing positive emotionality and fearfulness with a social component, the Puppet Game and Stranger Approach episodes were low, whereas between those episodes assessing positive emotionality and fearfulness without a social component, the Popping Bubbles and Jumping Spider episodes were very low. There were no differences between boys and girls with regard to their scores on the four LAB-Tab episodes (Stranger Approach: t (634) = 1.14, p = .26; Jumping Spider: t (621) = -1.21, p = .23; Popping Bubbles: t (641) = 1.14, p = .25; Puppet Game: t (648) = 0.23, p = .82).

Table 2. Correlations between the four Lab-TAB episodes

Lab-TAB episodes (*N* = 661)	1	2	3	4
1. Stranger Approach	-			
2. Jumping Spider	.04	-		
3. Popping Bubbles	-.04	.09*	-	
4. Puppet Game	-.15***	.10*	.33***	-

* $p < .05$, *** $p < .001$.

Main results

Results of the unadjusted separate regression analyses are presented in Table 3, results of the fully adjusted regression models are presented in Table 4. In the unadjusted analyses, maternal depressive symptoms predicted only children's fearfulness in the Jumping Spider episode significantly; a higher trajectory class membership of mothers was associated with lower fearfulness in the Jumping Spider episode (linear trend: $N = 623$, $B = -0.11$, $SE = 0.04$, $p = .005$).

Table 3. Unadjusted associations between trajectories of maternal depressive symptoms, positive emotionality, and temperamental fearfulness in children

	Positive emotionality episodes						Fear episodes					
	Popping Bubbles ($N = 643$)			Puppet Game ($N = 650$)			Stranger Approach ($N = 636$)			Jumping Spider ($N = 623$)		
	B	*SE*	*p*	*B*	*SE*	*p*	*B*	*SE*	*p*	*B*	*SE*	*p*
Trajectories of maternal depression												
No	0 (reference)			0 (reference)			0 (reference)			0 (reference)		
Mild	.03	.03	.45	-.07	.06	.23	.03	.03	.29	-.12	.05	.008
Moderate	-.17	.10	.09	-.14	.13	.27	.03	.08	.72	-.18	.11	.099
p for trend			.69			.14			.34			.005

Note. Models were unadjusted for covariates.

In the adjusted regression models, maternal depressive symptoms did not predict children's positive emotionality in the Popping Bubbles episode (linear trend: $N = 595$, $B = -0.03$, $SE = 0.04$, $p = .39$). However, maternal depressive symptoms were predictive of children's positive emotionality in the Puppet Game episode, which elicited joy response in a socially interactive situation. Children of mothers with more severe and chronic depressive symptoms had lower levels of positive emotionality in this episode (linear trend: $N = 602$, $B = -0.12$, $SE = 0.05$, $p = .02$).

Maternal depressive symptoms were not predictive of children's fearfulness in the Stranger Approach episode (linear trend: $N = 589$, $B = 0.02$, $SE = 0.03$, $p = .45$). However, they predicted children's fearfulness in the Jumping Spider episode, an episode eliciting startle reaction in an unfamiliar situation. In particular, children of mothers with more severe and chronic depressive symptoms were less fearful in this episode (linear trend: $N = 575$, $B = -0.13$, $SE = 0.04$, $p = .002$).

In order to make sure that the distinct trajectory groups were not merely reflecting differences in severity of maternal depressive symptoms, we examined the additional predictive value of trajectories over a general index of symptom severity. Analyses were limited to those episodes where associations between maternal depressive symptom trajectories and children's temperament scores were significant. Severity of maternal depressive symptoms was indicated by the average of the standardized BSI depression

Table 4. Adjusted associations between trajectories of maternal depressive symptoms, positive emotionality, and temperamental fearfulness in children

	Positive emotionality episodes						Fear episodes					
	Popping Bubbles (*N* = 595)			Puppet Game (*N* = 602)			Stranger Approach (*N* = 589)			Jumping Spider (*N* = 575)		
	B	*SE*	*p*	*B*	*SE*	*p*	*B*	*SE*	*p*	*B*	*SE*	*p*
Trajectories of maternal depression												
No	0 (reference)			0 (reference)			0 (reference)			0 (reference)		
Mild	.003	.05	.95	-.11	.06	.06	.03	.04	.33	-.14	.05	.005
Moderate	-.19	.11	.09	-.27	.14	.06	.007	.09	.94	-.22	.12	.06
p for trend			.64			.02			.45			.002

Note. Models were adjusted for child's age, sex, gestational age at birth, maternal age, educational level, parity, marital status, smoking and alcohol consumption during pregnancy, maternal harsh discipline. Effect estimates did not meaningfully change when family income was added to the model.

scores across the four assessment waves. Hierarchical linear regression models were run by adding the variable representing severity of maternal depressive symptoms to the basic model and adding trajectories of maternal depressive symptoms (categorical variable) in the next step. In case of children's positive emotionality score in the Puppet Game episode, trajectories did not significantly add to the variance explained by severity of maternal depressive symptoms alone (N = 572, B = -0.08, SE = 0.07, ΔR^2 = .002, ΔF =1.05, p =.30). However, in case of children's fearfulness score in the Jumping Spider episode, trajectories had a significant independent contribution to the model that explained individual variance in children's fearfulness by severity of maternal symptoms alone (N = 547, B = -0.14, SE = 0.06, ΔR^2 = .009, ΔF =5.31, p =.02). Given that trajectory modeling seemed to provide a significant advantage over a predefined measure of symptom severity with regard to one of our findings, we thought it is important to use the trajectories for modeling maternal depressive symptoms in the present study relative to a simple index of symptom severity.

None of the interaction terms including trajectories of maternal depressive symptoms and child sex was significant (p > .05), suggesting that the influence of maternal depressive symptoms on positive emotionality and temperamental fearfulness was similar for boys and girls.

DISCUSSION

The present study aimed at examining relations between two important risk factors of child psychopathology namely, maternal depression and child temperament. There are several strengths of this study: the large sample size and homogeneous age range of our young participants; the prospective assessment of maternal depressive symptoms from pregnancy until the child's age of three years, when the outcome, children's temperament, was observed in our laboratory; and the modeling technique used to identify common underlying patterns in women's depressive symptomatology.

Our first hypothesis that more severe and chronic symptoms of maternal depression would be associated with lower positive emotionality in children was partially supported. We did not find evidence for this link in the unadjusted analyses, however, once adjusting for important demographic and socioeconomic factors which may confound the underlying association between maternal depressive symptoms and child positive emotionality, the association was present in the more socially engaging context of interacting with puppets, but was absent in the socially less demanding situation of chasing and popping bubbles. Our finding supports earlier observations that maternal depression is related to low positive emotionality in the offspring (Dietz et al., 2008; Durbin et al., 2005; Olino et al., 2011), but also calls for its examination in specific contexts rather than averaging data across various situations. Interestingly, when Durbin et al. (2005) examined relations between maternal depression and child positive emotionality at the subtrait level, maternal depression was correlated with low positive affect and engagement but not with sociability. Our findings of lower positive emotionality in children of mothers with higher levels of depressive symptoms pertained to positive emotionality observed in a social context. However, our positive emotionality composite in the socially more relevant episode also included measures of affect and engagement. Thus, it is difficult to directly compare our findings to those of Durbin et al. (2005). Related to our findings, an earlier study of almost 700 five-year-olds showed that maternal depression did not impact children's play at school with structured material, whereas it did have a negative effect on children's social interactions during school play and with their mothers (Murray, et al., 1999).

Indirectly relevant to our findings, Silk, Shaw, Forbes, Lane, and Kovacs (2006) observed that if children (aged four to seven years) were able to generate positive emotionality despite being exposed to maternal depression, they were less likely to develop internalizing problems. In summary, our findings provided further support for a link between maternal depressive symptoms and low positive emotionality in children, but also showed that there may be important differences between the contexts in which these are observed. This finding is important because low positive emotionality in children is suggested to be a vulnerability marker for depression (Dietz et al., 2008).

Contrary to our second hypothesis, more severe and chronic symptoms of maternal depression were associated with decreased fearfulness in the offspring. This observation was limited to the episode that assessed fear of unfamiliar situations by provoking a startle reaction. Previous studies have most commonly researched fearfulness or behavioral inhibition in relation to parental anxiety disorders. These observations supported the hypothesis that parental anxiety disorders are associated with behavioral inhibition in children (Biederman et al., 1991; Rosenbaum et al., 2000). Behavioral inhibition has been proposed to be an early temperamental precursor of later anxiety disorders (Kagan, 1997; Kagan & Snidman, 1999). Some studies reported high levels of behavioral inhibition and less effective fear regulatory strategies in children of depressed parents (Biederman et al., 2001; Feldman et al., 2009). However, others authors argued that parental depression in the absence of comorbid anxiety does not place children at risk for anxiety problems

(Biederman et al., 1991; Hirshfeld-Becker et al., 2004). It seems more likely that maternal/parental depression provides a non-specific risk for a wide range of internalizing and externalizing problems in childhood (Biederman et al., 2001). The link, for instance, between maternal depression and disruptive behavior problems in childhood and adolescence is well known (Hay, Pawlby, Angold, Harold, & Sharp, 2003; Kim-Cohen, Moffitt, Taylor, Pawlby, & Caspi, 2005). In line with our findings, a large epidemiological study found that maternal psychopathology such as depression was associated with fearless temperament in toddlers, which was a significant predictor of conduct problems with callous unemotional traits in childhood and early adolescence (Barker, Oliver, Viding, Salekin, & Maughan, 2011). Low levels of fear were both concurrently and prospectively implicated in conduct problems, and fearless temperament has consistently been proposed as a precursor for later conduct problems with callous unemotional traits (for review, see Frick & Morris, 2004). Depression is heterogeneous in its manifestation and etiology. This may account for the observed inconsistencies across studies, reporting elevated or decreased rates of inhibition in the offspring in relation to maternal depression. Hypersensitivity of the fear network is thought to be more pronounced in familial than in non-familial depression, as reflected in increased activity in the amygdala (Rosenberg, MacMaster, Mirza, & Easter, 2006) and associations with fear-related (anxiety) disorders (Warner, et al., 2008). Studies that mostly include mothers with a recurrent form of depression, may observe high levels of behavioral inhibition in the offspring, while others do not. In summary, our results suggest that maternal depressive symptoms were associated with low temperamental fearfulness in young children as measured in a novel, non-social context. Although this association was not particularly strong, small effects in low-risk, community samples may be more pronounced and meaningful in clinical samples.

In case of children's fearfulness in the Jumping Spider episode, we found empirical support for the use of trajectories relative to a pre-defined measure of severity of maternal depressive symptoms, as trajectories contributed additional explained variance to the model including severity of depressive symptoms alone. Given the heterogeneity of depressive symptoms and the course of depression, it is likely that there are distinct trajectories of maternal depressive symptoms in the general population. Furthermore, we identified the trajectories of maternal depressive symptoms in a larger sample that also included the present subsample. In summary, we view this approach as more appropriate than modeling maternal depressive symptoms using a traditional growth model.

Potential mechanisms by which maternal depression influences child temperament may include genetic vulnerabilities, exposure to depressive symptoms of the mother, impaired interactions between the depressed mother and her offspring, and through contextual risk factors, as depressed mothers tend to experience accumulated psychosocial adversities (Murray & Cooper, 1997). Importantly, our observation of a link between maternal depressive symptoms and child temperament was independent of general contextual risk factors and measures of harsh parenting.

Limitations

Despite the strengths outlined earlier, the present study also had some limitations which need to be discussed here. In particular, we relied on maternal self-reports of depressive symptoms. There are indications that self-report measures in community samples are more reflective of general "distress" rather than "true depression" (Atkinson et al., 2000). However, self-report measures are commonly used in epidemiological studies and the fact that child temperament was independently observed minimizes any problems due to shared methods variance. On a related note, all assessments of maternal depressive symptoms were based on whether symptoms were present during the child's lifetime. Consequently, it was not possible to make distinctions about environmentally and genetically mediated transmission. Second, we did not observe child temperament at an earlier age, hence, causality cannot be inferred easily. Consistent with a transactional perspective, associations among the caregiving environment and child characteristics are likely to be bidirectional (Bell & Harper, 1977; Sameroff & MacKenzie, 2003). Children's temperamental traits and maternal depressive symptoms could exert mutual influence, with each potentially serving to exacerbate the other (Forbes et al., 2008). Nevertheless, the fact that we observed longitudinal relations between two significant risk factors for psychopathology highlights the need for further investigation of the underlying mechanisms linking maternal depression and child temperament. Third, our assessment of positive emotionality and fearfulness was limited, as both temperamental traits were assessed using two episodes, each of which measured the particular trait in a specific context (i.e., social vs. non-social). Reliability of behavioral responses to a single task is generally lower than to multiple tasks. Nevertheless, the large sample size in the present study may have improved our ability to detect robust associations, despite the small number of observations. Fourth, high or clinically relevant depressive symptoms in the current, low-risk sample were generally rare (n = 32). Therefore, it is difficult to speculate about the influence of particularly severe or chronic maternal depression on child temperament. However, the fact that we found associations even between high but still sub-clinical levels of maternal depressive symptoms and child temperament emphasizes the importance to study the link between maternal depression and temperamental traits in children. Fifth, we did not examine the influence of paternal depressive symptoms on offspring positive emotionality and temperamental fearfulness. Consequently, we need to be cautious with generalizing our results to parental depressive symptoms at large. Similar to maternal depression, paternal depression is also related to elevated rates of psychopathology in the offspring (Hammen, 2009). Nevertheless, there is some indication that maternal depression may be more strongly related to risk for psychopathology in the offspring than paternal depression, particularly in younger children (Connell & Goodman, 2002; Tully, Iacono, McGue, 2008). Temperamental vulnerabilities in young children may be differently linked to maternal and paternal depression (Durbin et al., 2005; Olino et al., 2010). Future studies incorporating information on fathers' depression or depressive symptoms are necessary to further clarify this question.

Clinical implications

Our findings emphasize the importance of early screening and, if necessary, treatment of maternal depressive symptoms. Simultaneously, they also encourage the use of personalized prevention programs which focus on the offspring of depressed women. These programs should prevent the occurrence of specific disorders in children of depressed women by taking their temperamental characteristics into account.

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CHAPTER 4

EMOTION RECOGNITION, TEMPERAMENT, AND THE INFLUENCE OF GENES

CHAPTER 4.1

RECOGNITION OF SCARED FACES AND THE SEROTONIN TRANSPORTER GENE IN YOUNG CHILDREN

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ABSTRACT

Background: Previous research highlights the significance of a functional polymorphism located in the promoter region (5-HTTLPR) of the serotonin transporter gene in emotional behavior. This study examined the effect of the 5-HTTLPR polymorphism on emotion processing in a large sample of healthy preschoolers. **Methods:** The 5-HTTLPR genotype was classified in 605 children as homozygous for the short allele (SS), homozygous for the long allele (LL), or heterozygous (LS). Emotion processing was assessed using age-appropriate computer tasks in which children matched happy, sad, angry, and fearful facial expressions preceded by a shape-matching task to assess basic matching ability. **Results:** We found that young children could differentiate between emotion-categories (F = 12.1, p < .001). The effect of 5-HTTLPR genotype depended on the emotion-category presented (F = 2.3, p = .031). This effect was explained by the finding that SS children were less accurate at recognizing fearful faces than LL or LS children (F = 5.3, p = .005). We did not find any significant differences as a result of 5-HTTLPR genotype for happy, sad, or angry expressions (p > .05). **Conclusions:** Results indicate that 5-HTTLPR allele status selectively impacts the processing of fearful faces, and that this pattern is already apparent in very young typically developing children. Results may signal an early vulnerability for affective problems before disorders emerge.

INTRODUCTION

The ability to accurately recognize facial expressions of emotions is critical for optimal psychosocial development. This ability is probably even more significant in children, particularly the very young, who use the skills of facial expression recognition (FER) in fomenting relationships with their parents, siblings, and peers. Deficits in facial expression recognition are already evident from childhood in a wide range of disorders such as anxiety, depression, and autism spectrum disorder (Monk, 2008). Developing a pathophysiological explanation for why some children can recognize facial expressions better than others is an important first step to understand why children with psychopathology have these deficits.

One promising avenue is in the area of candidate gene studies for which pre-existing information is available on gene-brain-behavior relations. Previous research highlighted the significance of a functional polymorphism in the promoter region (5-HTTLPR) of the serotonin transporter gene (SLC6A4) in social-emotional behavior (Canli & Lesch, 2007). The polymorphism has further been implicated in anxiety-related traits (Holmes, Lit, Murphy, Gold, & Crawley, 2003; Lesch et al., 1996) and depression (Caspi et al., 2003; Karg, Burmeister, Shedden, & Sen, 2011; Uher & McGuffin, 2010). The 5-HTTLPR has two common variants: the short (S) and the long (L) allele. The presence of the S allele is associated with lower SLC6A4 transcription and decreased serotonin reuptake from the synaptic clefts compared with the presence of two L alleles (Heinz et al., 2000).

Evidence for a link between the 5-HTTLPR polymorphism and overt behavior such as anxiety-related traits and biased mood is rather inconsistent (Munafó et al., 2009; Uher & McGuffin, 2008). However, this inconsistency might reflect inadequate control for non-genetic factors (e.g., age, sex, and population stratification), insufficient power, or heterogeneity in the methods applied (Hariri, Drabant, & Weinberger, 2006).

An important yet poorly understood aspect of the function of 5-HTTLPR is how it changes over development. The serotonin system is sensitive to individual experiences and the integrity of the nervous system (Hariri et al., 2006). Only few developmental studies have looked at variation in the 5-HTTLPR and facial emotion processing (Battaglia et al., 2005; Gibb, Benas, Grassia, & McGeary, 2009; Jacobs et al., 2011; Lau et al., 2009; Pérez-Edgar et al., 2009; Thomason et al., 2010). These studies mostly focused on children at high risk for affective psychopathology or somewhat older children; none has explored processing accuracy. Consequently, we lack normative data on how the 5-HTTLPR polymorphism influences the accuracy for processing emotional stimuli early in life before affective problems emerge.

Within the context of a large-scale cohort study of typically developing preschoolers ($N = 605$), we investigated whether the 5-HTTLPR polymorphism is associated with children's FER accuracy. Results will add to our knowledge on the developmental aspect of the influence of the 5-HTTLPR on processing emotional cues. Studying this question in typically developing young children is critical in elucidating the effects of the 5-HTTLPR polymorphism on biased emotional behavior, since results may signal an early vulnerability for affective disorders.

Neuroimaging studies have consistently reported increased amygdala reactivity (Munafó, Brown, & Hariri, 2008) and decreased amygdala prefrontal functional coupling (Heinz et al., 2005; Pezawas et al., 2005) in response to fearful stimuli in S allele carriers relative to individuals homozygous for the L allele. The amygdala is central to the processing of emotionally salient stimuli, particularly fear (Guyer et al., 2008; Thomas et al., 2001), while the prefrontal cortical structures are important in the regulation of emotional responses through shaping the activation of the amygdala (Hariri, Mattay, Tessitore, Fera, & Weinberger, 2003; Lange et al., 2003). In addition, the amygdala is involved in both conditioned and unconditioned fear responses (Davis & Whalen, 2001). Bilateral lesions in this structure can lead to selective impairment in recognizing fear but not other emotions (Adolphs et al., 2005; Vuilleumier, 2005).

Consequently, we hypothesized that the S allele of the 5-HTTLPR will affect the recognition of fearful expressions but not other types of emotional expressions. We tested this with an emotion-matching task utilizing facial expressions, which has proven to be effective at consistently engaging the amygdala across various populations (Hariri et al., 2006).

METHOD

Setting

The current study was conducted in a subsample of children participating in the Generation R Study, a population-based prospective Dutch cohort from fetal life onward. The study has been described previously (Jaddoe et al., 2007, 2010). The subsample, known as the Generation R Focus Cohort, is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity. All children were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort (Jaddoe et al., 2008).

The study was in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Center. Parental written informed consents were obtained for all participants.

Participants

Postnatally, 1106 children participated in the Generation R Focus Study. In 675 (61.0%) of these children, we had cord blood taken during delivery. Non-response in cord-blood samples was largely due to logistical reasons related to the high percentage (31.6%) of home births in the Netherlands at the time (National Office for Statistics, period 2004/2006). 5-HTTLPR allele status was successfully determined in 657 children. Since 605 (92%) of the 657 children completed the FER tasks at age three years, our final study sample comprised 605 children. Genotype was classified as homozygous for the short allele (SS), homozygous for the long allele (LL), or heterozygous (SL). There were no significant differences in terms of genotype between those children who did and who did not complete the FER tasks (X^2 (2) = 0.59, p = .75). The final sample included 294 boys (48.6%) and 311 girls (51.4 %), with a mean age of 37.5 months (Range: 34.7 - 44.9 months).

Genotyping

The 43-base pair insertion/deletion in the promoter region of the SCL6A4 gene was genotyped using Taqman allelic discrimination. The forward primer was 5'-GGCGTTGCCG CTCTGAATGC-3', and the reverse primer was 5'-GAGGGACTGAGCTGGACAACCAC-3'. We used the same primer sequences as Hu et al. (2006). Reactions were performed in a 384-well 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.

Emotion processing

Emotion processing was assessed using a computerized *emotion-matching task*, which was specifically developed for this age group (see Székely et al., 2011). Color images depicting four basic emotions (happiness, sadness, anger, and fear) were presented on a screen and children responded using a touch-sensitive monitor. Children were required to match the emotion of a target face presented at the top of the computer screen with one of the two choices presented below (Figure 1). In total, there were 16 trials with two female and two male identity pairs and four basic emotions (happiness, sadness, anger, fear). To control for the effects of basic matching ability a *shape-matching task* was included prior the emotion-matching task. Children were asked to match a target shape to one of two shapes. The shape-matching task had the same layout as the emotion-matching task, only children had to match geometrical shapes. Similar paradigms have been used previously by Hariri, Bookheimer, and Mazziotta (2000), and Herba, Landau, Russell, Ecker, and Phillips (2006). Accuracy on both tasks was calculated as the ratio of correct responses to the number of trials attempted.

Prior to the emotion-matching task, children were introduced to the topic of emotions and facial expressions through a brief dialogue with the interviewer. Computerized tasks began with a practice trial, which could be repeated up to the times in total if the child's answer was incorrect before the experimenter moved on to the test trials.

Statistical Analysis

All statistical analyses were performed using SPSS Version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). We investigated the association between the 5-HTTLPR polymorphism and FER with a 4 x 3 mixed-factor analysis of variance (ANOVA) with emotion-category (happy, sad, angry, and fearful) as the within-subjects factor, and 5-HTTLPR genotype (LL, SL, SS) as the between-subjects factor. Since population genotype distribution is assumed to be unrelated to covariates (Davey Smith & Ebrahim, 2005), and the effect estimates were not affected by adjusting the analyses for child's age, basic shape-matching ability, maternal age,

Figure 1. Emotion-matching task. Children were instructed to match 'who feels the same' by pointing to the face (bottom) that displayed the same emotion as the target face (top) on a touch-sensitive monitor.

family-, and socioeconomic status, models presented here are unadjusted for covariates. We specifically examined the effect of sex on the association between 5-HTTLPR genotype and FER, but, because neither main effects for sex nor interaction effects including sex were significant, it was not included in the final analyses. Lastly, we tested whether the association between the 5-HTTLPR genotype and FER is affected by current anxiety/depressive symptoms of children. To this end, we used mother-rated information ($N = 600$) of children's anxious/depressive symptoms assessed using the Anxious/Depressed syndrome scale of the Child Behavior Checklist 1½– 5 (CBCL; Achenbach & Rescorla, 2000) at age three years, when the outcome, FER, was also measured. Anxious/Depressed syndrome scale scores of children were *z*-transformed for further analyses.

RESULTS

Sample Characteristics

Of the 605 participants, 192 (31.7%) were homozygous for the L allele, 291 (48.1%) were heterozygous, and 122 (20.2%) were homozygous for the S allele. Genotypic distribution was in Hardy-Weinberg equilibrium, $\chi^2(2) = 0.38$, $p = 0.83$.

Sample characteristics are shown in Table 1 stratified for 5-HTTLPR genotype. There were no significant differences in sample characteristics across the three genotype groups ($p > .05$). Exact test statistics are reported in the footnote to Table 1.

Table 1. Sample characteristics by 5-HTTLPR genotype

	LL (*n* = 192)	SL (*n* = 291)	SS (*n* = 122)
Child characteristics			
Age (months), mean (*SD*)	37.4 (0.09)	37.6 (0.08)	37.7 (0.13)
Gender, *n* (%)			
boy	105 (54.7)	143 (49.1)	63 (51.6)
girl	87 (45.3)	148 (50.9)	59 (48.4)
Basic matching ability, mean (*SD*)	0.91 (0.18)	0.92 (0.18)	0.91 (0.18)
Maternal characteristics			
Age (years), mean (*SD*)	32.3 (0.28)	32.1 (0.22)	32.2 (0.35)
Education level, *n* (%)			
primary	3 (1.6)	2 (0.7)	0 (0.0)
secondary	58 (30.2)	86 (29.6)	38 (31.1)
postsecondary education	59 (30.7)	83 (28.5)	35 (28.7)
university degree or higher	70 (36.5)	119 (40.9)	48 (39.3)
missing	2 (1.0)	1 (0.3)	1 (0.8)
Net family income, *n* (%)			
≤ 2200 €	37 (19.3)	48 (16.5)	20 (16.4)
> 2200 €	146 (76.0)	226 (77.7)	97 (79.5)
missing	9 (4.7)	17 (5.8)	5 (4.1)
Family status (mother), *n* (%)			
have a spouse	183 (95.3)	269 (92.4)	116 (95.1)
single	6 (3.1)	13 (4.5)	4 (3.3)
missing	3 (1.6)	9 (3.1)	2 (1.6)

Test statistics for differences in sample characteristics across genotype groups: children's age at visit (F (2, 602) = 0.85, p = .43); sex (χ^2 (2) = 1.43, p = .49); maternal age at intake (F (2, 605) = 0.25, p = .78); level of maternal education (χ^2 (8) = 4.11, p = .85); net family income (χ^2 (4) = 1.33, p = 0.86); and family status of the mother (χ^2 (4) = 2.25, p = .69). Importantly, we did not find any 5-HTTLPR driven differences in basic shape-matching ability (F (2, 602) = 0.12, p = .89).

Main Results

The main effect of emotion-category was significant ($F_{\text{Greenhouse-Geisser}}$ (2.9, 1687.1) = 12.1, p < .001), indicating that young children could differentiate between the different emotion-categories. The main effect of genotype was not significant (p > .05), meaning that 5-HTTLPR genotype did not predict differences in recognition patterns across all emotion-categories. Importantly, the effect of genotype by emotion-category was significant ($F_{\text{Greenhouse-Geisser}}$ (5.8, 1618.0) = 2.3, p = .031), pertaining to a significant difference in matching fearful faces (F (2, 602) = 5.3, p = .005). Children homozygous for the S allele were less accurate at matching fearful faces (mean accuracy = 0.58, *SD* = 0.33) than those homozygous for the L allele (mean accuracy = 0.68, *SD* = 0.28, p = .009), or heterozygous children (mean accuracy

= 0.67, *SD* = 0.28, *p* = .009; Figure 2). We did not find any significant differences as a result of 5-HTTLPR genotype for matching happy, sad, or angry faces (Table 2).

Having adjusted for shape-matching accuracy, the emotion-category by genotype effect was essentially unchanged (*F* (5.86, 1681.23) = 2.3, *p* = .032) and pertaining to the effect for matching fearful faces (*F* (1, 574) = 4.5, *p* = .011). The mean difference in accuracy for matching fearful faces between the LL and SS group was 0.10, *p* = .009; and between the LS and SS groups was 0.09, *p* = .01. Accuracies for matching fearful faces in the LL and LS group did not differ significantly (*p* > .05).

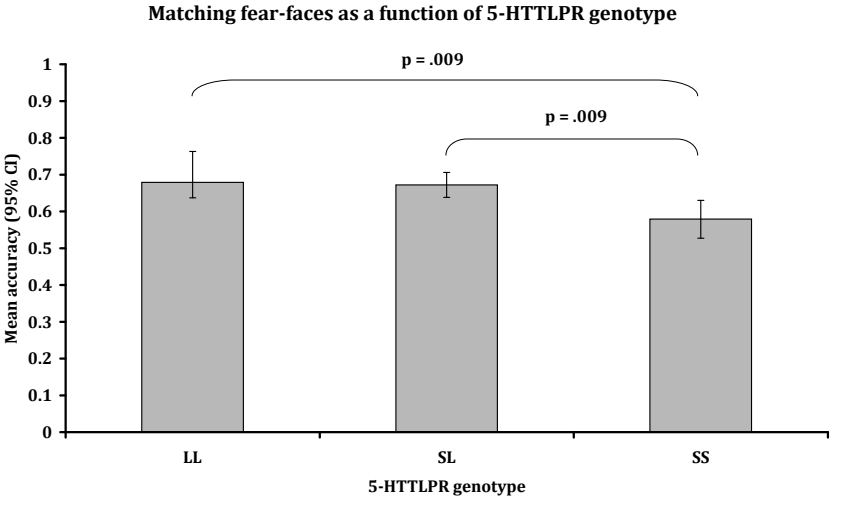

Figure 2. Recognizing fearful expressions as a function of 5-HTTLPR genotype. Bars represent mean accuracy scores (with 95% *CI*) in recognizing fearful facial expressions for children in the LL, SL, and SS genotype groups of the 5-HTTLPR.
Bonferroni adjusted p values = .027.
Additive model: $\beta_{5\text{-HTTLPR}}$ = - 0.05 (95% CI: -0.08; -0.01), p = .007.

Table 2. Mean differences in accuracy scores to recognize emotional facial expressions according to 5-HTTLPR genotype

Facial expressions	LL (reference)			LS				SS			
	Mean	Diff.	95% *CI*	Mean	Diff.	95% *CI*	*p*	Mean	Diff.	95% *CI*	*p*
Happy	0.67	-	-	0.70	- 0.03	- 0.09; 0.04	1.00	0.66	0.01	- 0.05; 0.11	0.89
Sad	0.57	-	-	0.59	- 0.02	- 0.08; 0.05	1.00	0.61	- 0.04	- 0.13; 0.04	0.62
Angry	0.62	-	-	0.61	0.01	- 0.06; 0.08	1.00	0.64	- 0.02	- 0.10; 0.07	1.00
Fearful	0.68	-	-	0.69	0.01	- 0.06; 0.07	1.00	0.58	0.10	0.02; 0.18	0.009**

Mean = mean recognition accuracy; Diff. = difference in mean recognition accuracy score relative to the LL (reference) group; *CI* = confidence interval.
** *p* < .01.

Mean Anxious/Depressed scores did not differ significantly across genotype groups (F (2, 597) = 0.7, p = .50). Importantly, the emotion-category by genotype effect remained significant after adjusting for current anxiety/depressive symptoms of children (F (5.86, 1539.51) = 2.3, p = .036). Similar to the original analysis, this effect pertained to matching fearful faces (F (2, 528) = 4.8, p = .009) but not other emotions (p > .05). Children with SS genotype were less accurate at matching fearful faces than both LS children (mean difference: 0.083, p = .038) and LL children (mean difference = 0.095, p = .023). There was no significant difference in matching fearful faces between LS and LL children (mean difference: 0.013, p = .99).

Next, we also checked whether the significant genotype effect for matching fearful faces would still hold when assuming an additive or a dominant nature of effect for the 5-HTTLPR. We found that the effect of genotype for matching fearful faces was significant using an additive model ($ß$ = -0.05; 95% *CI*: -0.08; -0.01, p = .007) but not using a dominant model (F (1, 595) = 1.8, p = .18).

DISCUSSION

In the present study we investigated the association between the 5-HTTLPR polymorphism and FER accuracy in a large group of typically developing preschoolers. Findings confirmed our hypothesis that the S allele in young children affects the processing of fearful expressions but not other emotional expressions (ie., happy, sad, or angry). More specifically, we found that children homozygous for the S allele were less accurate at matching fearful faces than LS or LL children. This association was independent of basic matching ability or current anxiety/depressive symptoms.

Previous studies reported associations between the S allele and various aspects of fear-related processes in children such as increased amygdala reactivity (Lau et al., 2009), biased attention to fearful faces (Thomason et al., 2010), more fearful temperament (Hayden et al., 2007; Pauli-Pott, Friedl, Hinney, & Hebebrand, 2009), and increased biological stress reactivity (Gotlib, Joormann, Minor, & Hallmayer, 2008). Results of these studies cannot be used to derive specific hypothesis about the direction of effect, as we focused on detection accuracy for emotional faces.

There is some empirical support for an increased sensitivity to negative or threatening emotional stimuli in depression and anxiety (Gotlib, Krasnoperova, Yue, & Joormann, 2004; MacLeod, Mathews, & Tata, 1986). Accordingly, this may also be reflected in higher accuracy scores for processing such emotions (Bhagwagar et al., 2004; Surcinelli, Codispoti, Montebarocci, Rossi, & Baldaro, 2006). If so, our finding of a decreased accuracy for processing fearful faces by SS children were unexpected. However, there is considerable debate on whether variability in the 5-HTTLPR conveys vulnerability for affective disorders or conveys differential susceptibility. In the latter case, the direction of the genetic effect also depends on the environment (Belsky et al., 2009). This implies that we must be very careful when speculating about the possible clinical implications of our findings.

One potential explanation for our results is that serotonergic neurons are involved in the modulation of both physiological and behavioral responses to fearful or stressful situations (Gardner, Thrivikraman, Lightman, Plotsky, & Lowry, 2005). Poorer recognition of fearful faces may reduce children's ability to accurately identify and avoid such situations. In turn, these incidents may serve as stressful life events which together with differences in serotonergic activity may lead to affective problems later in life. Replication is necessary to confirm these findings, particularly regarding the direction of effect of the S allele on fear processing.

The literature on whether the S allele has a recessive or dominant effect is quite inconsistent (Uher & McGuffin, 2008). The preferred model of action for the S allele may also depend on the allele groupings that different studies have used to achieve greater power. Our finding of a recessive effect for the S allele is in line with other developmental studies of young children (Gotlib et al., 2008; Hayden et al., 2007; Jacobs et al., 2011; Pauli-Pott et al., 2009).

It is important to note that, in addition to its function as a neurotransmitter, serotonin also plays a role as a trophic factor in the formation of the central nervous system during embryogenesis (Buznikov, Shmukler, & Lauder, 1996). Animal studies suggest that serotonin transporters are already present in the rodent embryo at E11 (Schroeter & Blackely, 1996) and take part in the accumulation of maternal serotonin to act as a morphogen (Buznikov et al., 1996). In humans, serotonin is amongst the earliest appearing neurotransmitters at approximately 5 weeks of gestation (Bennett et al., 2002). Thus, inappropriate serotonin transporter expression during embryonic and fetal brain development could lead to disruptions in the morphogenic serotonin homeostasis which may result in anatomical, functional, and behavioral anomalies later in life (MacKenzie & Quinn, 1999; Nordquist & Oreland, 2010). The S allele of the 5-HTTLPR has been associated with reduced volumes in the amygdala and perigenual anterior cingulate cortex, as well as with lower functional connectivity and reduced white matter connections between these structures (Pacheco et al., 2009; Pezawas et al., 2005). These corticolimbic structures comprise a significant part of the neural circuitry implicated in facial expression recognition and emotion processing (Hariri & Holmes, 2006; Phillips, Drevets, Rauch, & Lane, 2003). As such, the activity of the corticolimbic circuitry, which mediates emotional behavior, might be sensitive to alterations in serotonergic neurotransmission and contribute to differences in emotional behavior and in the processing of environmental threat (Paton, Belova, Morrison, & Salzman, 2006).

Despite the strengths of this study such as the large number of participants, healthy children of young age, and objective assessment of the phenotype, we were faced with some limitations. Firstly, we did not screen for previously identified rare variants in the 5-HTTLPR and exonic regions of the SLC6A4 gene, which could have potentially affected our results. However, given the small minor allele frequency of these variants (all < 5%, many < 1%), especially in healthy populations (Glatt et al., 2001; Kraft, Slager, McGrath, & Hamilton, 2005), we do not expect that the inclusion of any of these rare variants would significantly alter our results. Secondly, we did not examine the interaction of the 5-HTTLPR with environmental factors such as negative life events, which may also moderate developmental effects of the

5-HTTLPR on emotional behavior (Brown & Harris, 2008). As our study is longitudinal in nature, we will be able to test these gene-environment interactions as our sample ages.

Conclusion

In conclusion, our findings indicate that 5-HTTLPR genotypic effects on accuracy to recognize fearful facial expressions exist already in very young children. These data may help understand how this one aspect of neurophysiology and function may combine with other risk factors in the development of later psychopathology. We aim to follow our sample forward to determine if the children with deficits in fearful facial recognition are more likely to develop psychopathology in the future.

Such findings may also open the door for new forms of cognitive behavioral therapy, by helping children who have deficits in fearful facial recognition develop these skills through the use of cognitive training. Such an approach may diminish risk to young children by helping them identify situations that may be high risk and fear inducing in the truest sense.

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CHAPTER 4.2

A STUDY OF THE 5-HTTLRP BY ENVIRONMENTAL INTERACTION FROM FETAL LIFE ONWARD

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ABSTRACT

Objective: We examined within the Generation R Study whether the functional polymorphism (5-HTTLPR) in the promoter of the serotonin transporter gene interacts with prenatal maternal chronic difficulties, prenatal maternal anxiety, or postnatal maternal anxiety to influence child emotional development. **Method:** 2136 Northern European children were genotyped for 5-HTTLPR and rs25531. Mothers reported chronic difficulties and anxiety symptoms at 20 weeks pregnancy and when the child was three years old. Child emotion recognition accuracy was observed in our laboratory at age three years; child emotional problems were rated by parents using the CBCL/1½-5 at age five years. **Results:** There were consistent main effects of maternal chronic difficulties and anxiety on child emotional problems, but no main effect of the 5-HTTLPR. Moreover, children with the s allele were at increased risk for emotional problems if their mothers reported prenatal anxiety symptoms ($B = 2.02$, $p < 0.001$) or postnatal anxiety symptoms ($B = 1.64$, $p < 0.001$). Additionally, in children of mothers with prenatal anxiety symptoms, the s allele was associated with less accurate emotion-matching ($B = -0.11$, $p = 0.004$). **Conclusions:** This population-based study shows that vulnerability due to the 5-HTTLPR genotype is not specific for certain adverse exposures or severe events, but suggests that the small effects of gene-environment interaction on emotional development become manifest early in life.

INTRODUCTION

A decade ago, Caspi et al. (2003) reported that carriers of the short (s) allele of the common functional polymorphism (5-HTTLPR) in the promoter region of the serotonin transporter gene (*5-HTT, SLC6A4*) are at higher risk to develop depression following stressful life-events (SLE) or childhood maltreatment than adults homozygous for the long (l) allele. This finding represented an important epidemiological support for gene-environment interaction (GxE), and was followed by numerous replication attempts. The first two meta-analyses did not show evidence for a significant interaction effect of the 5-HTTLPR and SLE on depression (Munafo, Durrant, Lewis, & Flint, 2009; Risch et al., 2009; $\kappa = 5$ and $\kappa = 14$, respectively). Karg, Burmeister, Shedden, and Sen (2011) used a different statistical approach for their meta-analysis ($\kappa = 54$) and allowed for chronic diseases as indicators of stress. Unlike the former, this latter meta-analysis found evidence for a moderation by the 5-HTTLPR on the relation between stress and the development of depression.

This inconsistency fuelled a debate about the validity of GxEs (Duncan & Keller, 2011). Variation in inclusion criteria, different statistical methods, additional allelic variation (rs25531), heterogeneity of subjects, and choice of phenotype may explain the divergent results (Rutter, Thapar, & Pickles, 2009; Uher & McGuffin, 2009). It was suggested that the moderation by the 5-HTTLPR was specific to certain stressors such as childhood maltreatment or severe illness, and to a lesser extent to compilation of stressful life-events (Caspi, Hariri, Holmes, Uher, & Moffitt, 2010). It was also posited that the interaction effect between the 5-HTTLPR and childhood maltreatment would be specific to persistent major depression (Uher et al., 2011). Furthermore, it was reported that studies using observational data to assess the environment more often replicated the GxE than studies using self-report measures (Caspi et al., 2010). The current study further explores early moderation of environmental effects by the 5-HTTLPR on child development within the context of this debate.

The 5-HTTLPR polymorphism plays an important role in stress-sensitivity across species. The s allele has been associated with lower transcriptional efficiency of the promoter and consequently reduced serotonin transporter availability (Lesch et al., 1996). Neuroimaging studies showed that the s allele is associated with increased amygdala reactivity and decreased amygdala-prefrontal functional coupling when viewing threatening stimuli (Caspi et al., 2010; Hariri et al., 2002). The findings by Lau et al. (2009) suggest that the effect of the 5-HTTLPR on amygdala reactivity may differ in healthy individuals compared to patients. Healthy s-allele carriers showed increased amygdala reactivity in response to fearful faces, whereas the l-allele was associated with increased amygdala response to fearful and happy faces in patients.

Serotonin is one of the earliest emerging neurotransmitters, appearing at approximately 5 weeks of gestation (Bennett et al., 2002). Consequently, the 5-HTTLPR may influence early neurodevelopment and brain function (Brown & Harris, 2008; Caspi et al., 2010). It has been shown that stress during pregnancy affects the development of the brain areas involved in emotional development, i.e., the hippocampus, the frontal lobe, and the amygdala (Lupien, McEwen, Gunnar, & Heim, 2009). These prenatal programming effects may lead to changes

in amygdala volume and could modify the trajectories of connections. Possibly, these effects are further moderated by the 5-HTTLPR. To better understand early moderation by the 5-HTTLPR and its consequences, it is essential to study GxE during the fetal period and early childhood (Caspi et al., 2010). We previously showed that the 5-HTTLPR interacts with prenatal maternal anxiety to affect child negative emotionality in infants (Pluess et al., 2011). Here, we further explored the effect of the 5-HTTLPR in a broader context during early development, and performed additional analyses to test the validity of the results. Firstly, we explored whether the 5-HTTLPR moderates the effects of prenatal maternal chronic difficulties and maternal anxiety symptoms on child emotional problems. We contrasted the interaction effects observed for maternal and paternal prenatal anxiety to examine direct intrauterine effects. Secondly, we studied whether the 5-HTTLPR interacts with postnatal maternal anxiety symptoms on the risk of child emotional problems. Thirdly, we used an observational measure of emotion recognition to explore the interaction of the 5-HTTLPR and maternal anxiety on children's ability to recognize emotional faces. Phillips, Drevets, Rauch, and Lane (2003) postulate that emotion perception involves three interrelated processes: the recognition of emotionally salient cues; emotional behavioral responses; and the regulation of affect. Here, we focused on the first process, utilizing facial expressions as emotionally salient cues. This follows a common approach in emotion processing research of typically developing children (McClure, 2000). Moreover, deficits in these abilities have repeatedly been linked to psychiatric problems at different ages (Monk, 2008). Genetic factors contribute to influence facial expression recognition (Lau et al., 2009; McClure, 2000), possibly through the alteration of key neurotransmitter systems such as the serotonin system (Canli & Lesch, 2007). Finally, we tested whether any observed GxEs in Northern European children were consistent across ethnicities in subgroups of children of Turkish, Moroccan, and Surinamese descent.

METHOD

Design

This study was embedded in the Generation R Study, a population-based Dutch cohort from fetal life forward. The Generation R Study has previously been described in detail (Jaddoe et al., 2007, 2008). All children were born between April 2002 and January 2006. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center. Written informed consent was obtained from parents of the participating children.

Population of analysis

In genetic analyses, population stratification can increase the rate of false positive findings in heterogeneous samples like the Generation R Cohort. The GWAS data in our multi-ethnic cohort made it possible to compare and complement the self reported ethnicity of participants with genetic information about descent. We firstly selected children of Northern European descent, which was determined by principle component analyses

of genome wide association data, as described previously (Jaddoe et al., 2007). Principle component analyses yield factors that can be interpreted as the direction which maximizes the variance of the sample while being uncorrelated to previous components. From the group of 3410 children with a self-reported North-European ethnicity, 2841 (83%) children of Northern European descent were identified. Within these children, information about the 5-HTTLPR polymorphism was available in 2589 children.

In 2136 (82.5% of the 2589 eligible children), information was available on maternal chronic difficulties, maternal anxiety, and child emotional problems. To test for generalizability, we included 581 Turkish, Moroccan, and Surinamese children. Additional analyses were performed in a smaller sample of children, participating in the Generation R Focus Study (Jaddoe et al., 2008), in which observational data are available. Information on 5-HTTLPR allele status and prenatal maternal anxiety symptoms was available for 617 children. At age three years, 570 of these children also had data on emotion-matching accuracy.

Thus, the study population comprised 2136 children of Northern European descent, of whom 570 children also participated in the Focus Study. The generalizability of our findings was tested in 228 Turkish, 146 Moroccan, and 207 Surinamese children.

Genotyping

DNA was derived from cord blood samples at birth. The 43-base pair insertion/deletion in 5-HTTLPR and rs25531 were genotyped using Taqman allelic discrimination. The forward primer was 5'-GGCGTTGCCGCTCTGAATGC-3', and the reverse primer was 5'-GAGGGACTGAGCTGGACAACCAC-3' (Hu et al., 2006). Upstream of the *5*-HTTLPR, rs25531 results in two functional variants of the l-allele; l_A and l_G (Wendland, Martin, Kruse, Lesch, & Murphy, 2006). The l_G variant may be associated with lower *5-HTT* expression (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. To check for potential contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases. To evaluate genotyping accuracy, 225 random samples were genotyped twice. No discrepancies were found. Genotyping information about DRD4 and MAO-A are presented in the supplementary material S6.

Maternal Chronic Difficulties and Anxiety Symptoms

Prenatal chronic difficulties of the mother were assessed at 20 weeks pregnancy using a validated self-report questionnaire (Hendriks & van der Willige, 1990). In this 13-item questionnaire, long-lasting situational and relational difficulties which occurred in the preceding year were measured on a five-point scale (e.g., "Have you had any financial problems in the past year?").

Prenatal and postnatal anxiety symptoms of parents were assessed at 20 weeks pregnancy and three years after birth using the Brief Symptom Inventory (BSI). The BSI is a 53-item validated self-report inventory in which participants rate the extent to which they have been bothered (0="not at all" to 4="extremely") in the past week by various symptoms (De Beurs, 2004; Derogatis, 1993). In the current study, we employed the anxiety scale of the BSI, which consists of six items (e.g., "nervousness or shaking inside"). Prenatally, internal consistencies (Cronbach's alphas) were .59 for the anxiety scale and .92 for the total BSI. Postnatally, internal consistencies were .65 for the anxiety scale and .84 for the total BSI.

Child Emotional Problems and Emotion Recognition

Firstly, we studied the effect of gene-environment interaction on child emotional problems measured with the Child Behavior Checklist/1½-5 (CBCL/1½-5; Achenbach & Rescorla, 2000). The 99 items included in the checklist are scored on a three-point scale, ranging from 0="not true" to 2="very true". We used the Internalizing scale to define emotional problems at age five years. The Internalizing scale (36 items) covers the Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn syndrome scales.

Secondly, we studied the effect of gene-environment interaction on child emotion recognition. Emotion recognition was assessed using a computerized *emotion-matching task* (Székely et al., 2011). Color images depicting four basic emotions (happiness, sadness, anger, and fear) were presented on a touch-sensitive screen and children were required to match the emotion of a target face presented at the top of the computer screen with one of two faces presented below. In total, there were 16 trials with two female and two male identity pairs and four basic emotions. To control for the effects of basic matching ability a *shape-matching task* was included prior to the emotion-matching task. Similar paradigms have been used previously (Hariri, Bookheimer, & Mazziotta, 2000). Accuracy on both tasks was calculated as the ratio of correct responses to the number of trials attempted.

Covariates

Gestational age was established by fetal ultrasound examinations. Gestational age, Apgar score, birth weight, and gender were obtained from midwife and hospital registries at birth; information about maternal age, parity, educational level and child age at the time of the assessment of behavior was obtained by questionnaire.

Statistical analysis

We examined selective non-response by comparing characteristics between mothers and children who were included and those excluded from the present study using chi-square statistics, independent t-tests, and Mann Whitney U-tests. Using the same tests, we also compared characteristics of mothers and children in this study according to 5-HTTLPR genotype. Correlation between the predictor variables was calculated using the Spearman's correlation coefficient for non-parametric variables (two-tailed). Power calculations were performed in Quanto (Gauderman, 2006). These calculations were based on continuous measurements in 2136 independent individuals, assuming an additive genetic model.

Gene-environment interaction

Before computing product terms for the interactions, predictor variables were centered. We studied main effects of the 5-HTTLPR polymorphism, prenatal maternal chronic difficulties, and prenatal and postnatal maternal anxiety symptoms on child emotional problems. To test our hypothesis, we firstly tested the interaction of the 5-HTTLPR with i) prenatal maternal chronic difficulties and ii) prenatal maternal anxiety symptoms on child emotional problems. In these analyses, we controlled for the interaction between postnatal maternal anxiety symptoms and 5-HTTLPR to examine the specificity of any intrauterine exposure effect. To evaluate possible confounding, we also tested for the presence of any interaction effect of the 5-HTTLPR by prenatal *paternal* anxiety symptoms on child emotional problems.

Secondly, we tested for interaction between the 5-HTTLPR and postnatal maternal anxiety symptoms on child emotional problems. Again, to test specificity, we controlled for prenatal exposure to maternal anxiety symptoms.

Thirdly, we examined the specificity of the GxE including the 5-HTTLPR by examining whether the associations between maternal chronic difficulties/anxiety symptoms and child emotional problems are moderated by i) the dopamine D4 receptor gene repeat (DRD4 48bp VNTR) and ii) the polymorphism in the promoter of the monoamine oxidase A gene (MAO-A). Based on a review of previous studies, we expected to find that, compared to non-carriers, carriers of the DRD4 7 repeat (DRD4 7R) were more likely to develop emotional problems if their mothers experienced anxiety symptoms (Bakermans-Kranenburg, Van IJzendoorn, 2011), although the reviewed studies typically focused on externalizing problems. With respect to the MAO-A, we expected to find a decreased risk of emotional problems in carriers of the high activity MAO-A allele in the presence of maternal anxiety symptoms compared to carriers of the low activity MAO-A allele (Kim-Cohen et al., 2006).

Fourthly, we examined the interaction effect of the 5-HTTLPR by prenatal maternal anxiety symptoms on child emotion recognition accuracy. Linear regression analyses were run under the assumption of an additive genetic model.

Logistic regression analyses were run to present results for a three-categorical genetic model with the l/l genotype as reference category. We repeated the analyses including the rs25531, for which we assigned individuals to three groups based on functional similarity of the s-allele and the l_g allele; high expression (l_a/l_a), intermediate expression (l_a/l_g or s/l_a), and low expression (l_g/s or s/s).

Fifthly, we tested the generalizability of our findings by performing a meta-analysis including both Northern European and non-European children, using the inverse Z score method assuming random effects (R 2.14.0).

Missing data

All models were adjusted for maternal age, educational level, smoking during pregnancy, parity, gestational age, gender, Apgar score, and child age. To test for independent effects, the analyses were also adjusted for the interaction term of other predictors. Missing data occurred in this longitudinal project due to attrition and failure to complete all assessments

as follows: maternal education (0.02%), smoking during pregnancy (0.004%), Apgar score (0.1%), parity (0.002%), prenatal maternal anxiety (11%), postnatal maternal anxiety (19%), prenatal paternal anxiety (17%), and postnatal paternal anxiety (26%). Missing values were imputed using the multiple imputation method in SPSS (SPSS, 2008). Results were averaged across five imputed data sets. The level of significance for all analyses was set at $\alpha = .05$.

Non-response analysis

Children (n = 453) excluded from our study had younger (29.9 vs. 31.9 years, t = 8.37, $p < 0.001$) and less highly educated mothers (28.7 vs. 39.8%, $\chi 2 = 18.7(1df)$, $p < 0.001$), who more often smoked during pregnancy (33.9 vs. 20.0%, $\chi 2 = 40.4(1df)$, $p < 0.001$) than mothers of our participants (n = 2136). The distribution of gender and parity did not differ between the two groups.

RESULTS

Of the 2136 children of Northern European descent, 719 (33.7%) were homozygous for the l-allele, 1029 (48.2%) were heterozygous, and 388 (18.2%) were homozygous for the s-allele. Genotypic distribution conformed to Hardy-Weinberg equilibrium (HWE), $\chi^2(2) = 0.55$, $p = 0.76$.

Maternal and child characteristics are presented in Tables S1 and S5. The distribution of the characteristics did not differ according to 5-HTTLPR genotype, which suggests that the s-allele is not significantly correlated to these characteristics. The correlation between the environmental risk factors ranged from 0.18 (prenatal maternal chronic difficulties and postnatal anxiety symptoms) to 0.48 (prenatal maternal chronic difficulties and prenatal maternal anxiety symptoms). Child 5-HTTLPR genotype was not significantly correlated with prenatal chronic difficulties, prenatal anxiety, or postnatal anxiety symptoms of the mother. Child emotional problems scores ranged from 0 to 49 (M = 5.2, SD = 5.3). The study of emotional problems had 0.89 power to detect gene-environment interaction with an explained variance of 1% (Uher et al., 2010). The power was 0.86 to detect a GxE effect explaining 0.4% of the variance. The high power is likely the result of our sample size and the high frequency of the s-allele (Luan, Wong, Day, & Wareham, 2001). Due to the smaller sample size, the study on emotion recognition accuracy was not sufficiently powered (0.70) to find a significant GxE with an explained variance of 0.01.

We first tested the interaction effect of the 5-HTTLPR by prenatal maternal stressors on child emotional problems (Table 1). Regression analyses indicated no genetic main effect of the 5-HTTLPR on child emotional problems. Prenatal maternal chronic difficulties and anxiety symptoms both predicted child emotional problems. The 5-HTTLPR moderated the association between maternal chronic difficulties and child emotional problems, but this effect was not independent of prenatal maternal anxiety symptoms (B = 0.07, SE = 0.09, p = 0.44). Also, the 5-HTTLPR moderated the effect of prenatal maternal anxiety symptoms on emotional problems (B = 2.02, SE = 0.55, p = 0.001). Compared to l/l carriers, l/s carriers

(B = 2.06, SE = 0.87, p = 0.018) and s/s carriers (B = 3.32, SE = 1.11, p = 0.003) were at an increased risk for child emotional problems. To further evaluate the possibility of an intrauterine effect and the role of confounding, we also examined the effect of the interaction between the 5-HTTLPR and prenatal *paternal* anxiety symptoms on child emotional problems. We found no significant interaction between the 5-HTTLPR and prenatal anxiety symptoms of the father (B = 0.54, SE = 0.82, p = 0.51) (see Table S2).

Table 1. Results of the interactions between 5-HTTLPR genotype and maternal prenatal chronic difficulties and prenatal and postnatal anxiety symptoms on children's risk to develop emotional problems

		Emotional problems at 5 years (N = 2136) [a]					
		B	*SE*	*p*	R^2	ΔR^2	ΔF
Prenatal maternal chronic difficulties							
step 1	prenatal chronic difficulties	0.19	0.06	0.003	0.099	-	-
step 2	*5-HTTLPR*	0.05	0.16	0.730	0.099	0.000	0.69
step 3	*5-HTTLPR* x chronic difficulties	0.06	0.08	0.434	0.099	0.000	0.369
per genotype							
l/l		Ref					
l/s		0.10	0.12	0.407			
s/s		0.20	0.16	0.207			
Prenatal maternal anxiety symptoms							
step 1	prenatal maternal anxiety (pre anx)	3.11	0.38	<0.001	0.098	-	-
step 2	*5-HTTLPR*	0.11	0.16	0.494	0.099	0.000	0.42
step 3	*5-HTTLPR* x pre anx	2.02	0.55	0.001	0.109	0.010	24.71
per genotype							
l/l		Ref					
l/s		2.06	0.87	0.018			
s/s		3.32	1.11	0.003			
Postnatal maternal anxiety symptoms							
step 1	postnatal maternal anxiety (post anx)	2.21	0.60	0.001	0.107	-	-
step 2	*5-HTTLPR*	0.07	0.16	0.656	0.107	0.000	0.23
step 3	*5-HTTLPR* x post anx	1.64	0.71	0.023	0.109	0.002	5.15
per genotype							
l/l		Ref					
l/s		3.47	1.15	0.004			
s/s		3.12	1.50	0.040			

l = l allele; s = s allele; Ref = reference.
[a] Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age child at behavioral assessment, 5-HTTLPR x prenatal maternal anxiety symptoms, or 5-HTTLPR x postnatal maternal anxiety symptoms.

Next, we tested for interaction between the 5-HTTLPR and postnatal maternal anxiety symptoms on child emotional problems. The 5-HTTLPR moderated the association between postnatal anxiety symptoms and emotional problems (B = 1.64, SE = 0.71, p= 0.023). Compared to l/l carriers, l/s carriers (B = 3.47, SE = 1.15, p = 0.004) and s/s carriers (B = 3.12, SE = 1.50, p = 0.04) were at an increased risk for child emotional problems. The effect was independent of the interaction between the 5-HTTLPR and prenatal maternal anxiety symptoms. Importantly, the reverse was also true; the interaction of prenatal maternal anxiety symptoms and 5-HTTLPR was independent of the interaction between 5-HTTLPR and postnatal anxiety symptoms. The regression slopes are plotted in Figure 1.

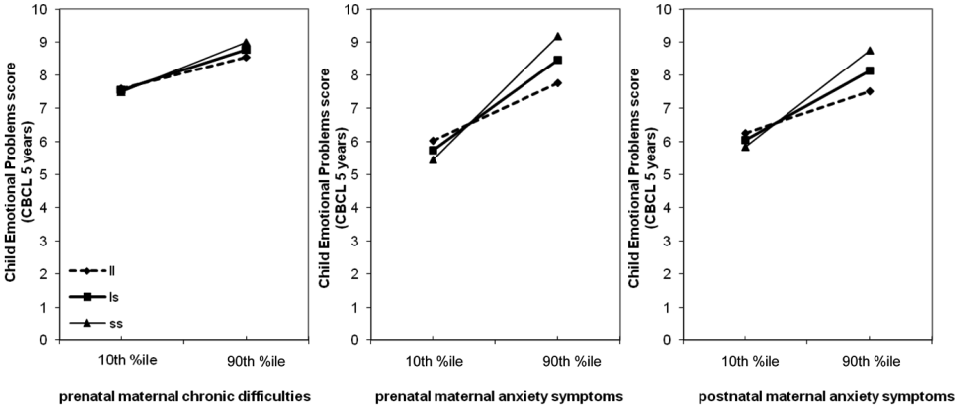

Figure 1. The associations between prenatal maternal chronic difficulties, prenatal maternal anxiety symptoms, postnatal maternal anxiety symptoms, and child emotional problems according to 5-HTTLPR genotype. The 5-HTTLPR does not moderate the relation between prenatal maternal chronic difficulties and child emotional problems (B = 0.06, SE = 0.08, p = .434). However, the 5-HTTLPR moderates the association between prenatal maternal anxiety symptoms and child emotional problems (B = 2.02, SE = 0.55, p = .001) as well as between postnatal maternal anxiety symptoms and child emotional problems (B = 1.64, SE = 0.71, p = .023). Coefficients come from a multiple regression model adjusted for child age, sex, gestational age at birth, maternal age, education, parity, smoking during pregnancy, 5-HTTLPR x prenatal maternal anxiety symptoms or 5-HTTLPR x postnatal maternal anxiety symptoms (centered).
CBCL = Child Behavior Checklist.

To further study the specificity of this interaction including the 5-HTTLPR, we also ran the analyses studying genetic variants of the DRD4 gene and the MAO-A gene. MAO-A interacted with prenatal maternal anxiety symptoms resulting in a decreased risk of child emotional problems (B = -2.98, SE = 0.95, p = 0.002) (see Table S3). The significant interaction effect of the DRD4 7R by prenatal maternal anxiety symptoms on child emotional problems was not in the hypothesized direction (B = -1.44, SE = 0.67, p = 0.03). Neither of these two genetic variations modified the effect of maternal chronic difficulties or postnatal anxiety on child emotional problems.

Then, we studied the interaction effect of the 5-HTTLPR by prenatal maternal anxiety symptoms on children's emotion-matching accuracy. There were no main effects of the 5-HTTLPR (B = -0.01, SE = 0.01, p = 0.36) or prenatal maternal anxiety symptoms (B = -0.02, SE = 0.04, p = 0.66) on children's emotion-matching accuracy. However, the 5-HTTLPR significantly interacted with prenatal maternal anxiety symptoms to influence child emotion-matching accuracy (B = -0.12, SE = 0.05, p = 0.011). Compared to l/l carriers, s/s carriers matched emotions less accurately if exposed to higher levels of maternal anxiety before birth. This effect was independent of the interaction effect between the 5-HTTLPR and postnatal maternal anxiety symptoms. In Figure 2, regression slopes are plotted. Allelic variance at rs25531 did not change the results nor improved the model fit (see Table S4).

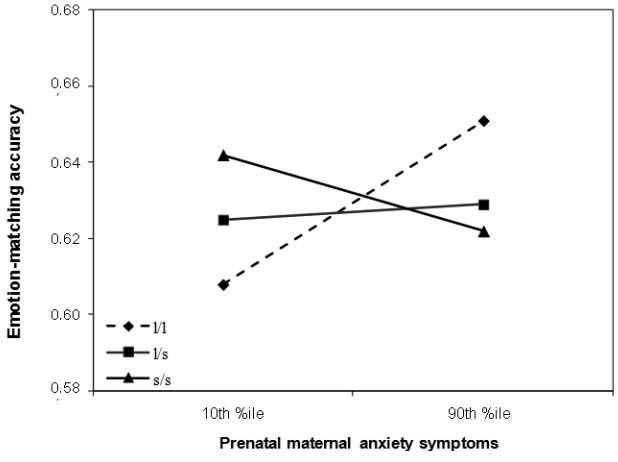

Figure 2. The relation between prenatal maternal anxiety symptoms and children's emotion-matching accuracy according to 5-HTTLPR genotype. Children's 5-HTTLPR genotype moderates the association between mothers' prenatal anxiety symptoms and children's emotion-matching accuracy (B = -0.12, SE = 0.05, p = .011). Coefficients come from a multiple linear regression model adjusted for child age, sex, gestational age at birth, shape-matching accuracy, maternal age, education, parity, smoking during pregnancy, and 5-HTTLPR x postnatal maternal anxiety symptoms. Values on the x-axis represent the 10th and 90th percentile score for prenatal maternal anxiety symptoms (centered).

We then examined the effect of these interactions in the three largest ethnic minorities in the Generation R cohort. Genotype distributions in Turkish and Moroccan children conformed to HWE (Turkish children $\chi^2_{(1)} = 0.45$, $p > 0.05$, Moroccan children $\chi^2_{(1)} = 0.01$, $p > 0.05$), but deviated in Surinamese children ($\chi^2_{(1)} = 5.33$, $p < 0.05$). This deviation is likely due to large heterogeneity in this population. Hence, we limited the analyses to Turkish (n = 228) and Moroccan children (n = 146). In Turkish and Moroccan children, the interaction effects were not-significant. In Turkish children, the interaction between the 5-HTTLPR and postnatal maternal anxiety symptoms went in the same direction and within the confidence intervals of effects in the Northern European children (Table 2).

Table 2. Results of the interactions between 5-HTTLPR genotype and maternal prenatal chronic difficulties and prenatal and postnatal anxiety symptoms on the risk to develop emotional problems in Turkish, Moroccan, and Northern European children

		Emotional problems at 5 years								
		Turkish children [a] *n* = 228			Moroccan children [a] *n* = 146			Meta-analysis Northern European, Turkish, and Moroccan children [a] *n* = 2510		
		B	*SE*	*p*	*B*	*SE*	*p*	*B*	*SE*	*p*
Prenatal maternal chronic difficulties										
step 1	prenatal chronic difficulties	-0.06	0.72	0.78	0.01	0.17	0.96			
step 2	*5-HTTLPR*	-0.11	0.72	0.88	-2.09	0.80	0.01			
step 3	*5-HTTLPR* x chronic difficulties	0.4	0.27	0.62	0.06	0.23	0.79	0.07	0.07	0.37
Prenatal maternal anxiety symptoms										
step 1	prenatal maternal anxiety (pre anx)	1.19	0.96	0.21	1.55	1.00	0.12			
step 2	*5-HTTLPR*	-0.09	0.71	0.90	-1.88	0.81	0.02			
step 3	*5-HTTLPR* x pre anx	1.73	1.46	0.24	-2.82	1.74	0.12	0.67	0.07	0.36
Postnatal maternal anxiety symptoms										
step 1	postnatal maternal anxiety (post anx)	2.36	1.67	0.16	0.93	1.82	0.61			
step 2	*5-HTTLPR*	-0.11	0.71	0.88	-2.01	0.79	0.01			
step 3	*5-HTTLPR* x post anx	2.23	2.84	0.44	1.90	3.30	0.57	1.68	0.67	0.01

[a] Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age child at behavioral assessment, 5-HTTLPR x prenatal maternal anxiety symptoms, or 5-HTTLPR x postnatal maternal anxiety symptoms.

DISCUSSION

We studied whether children with the s allele of the 5-HTTLPR were more vulnerable to prenatal maternal chronic difficulties, prenatal maternal anxiety symptoms, or postnatal maternal anxiety symptoms than children with the l allele. Our results suggest that during fetal life and early childhood, the effect of maternal anxiety is moderated by child 5-HTTLPR genotype, which places children at increased risk to develop emotional problems and affects their ability to accurately recognize emotions.

Because of the considerable controversy surrounding how to best understand GxE studies of the 5-HTTLPR, it is important to place our findings in the context of the debate around the importance of 'main effects'. Others have posited that the study of gene-environment interaction should be conditional on the presence of a genetic main effect on the phenotype (Risch et al., 2009). We do not agree. In our study, like others, we did not find a main effect of the 5-HTTLPR on child emotional problems or emotion recognition accuracy. If the genetic effect is only apparent in the high range of the environmental stressor and not in

the low range, gene-environment interaction may be present without a genetic main effect (Rutter et al., 2009). An indication for this effect can be seen in the figures illustrating the results of our study, which shows a cross-point in the range of variance in the environmental predictor. A number of potential biological mechanisms may explain our findings as well as our rational. In the future, we intend to test whether the 5-HTT is liable to methylation under environmental stress, which also influences the efficiency of the gene (Van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach, & Philibert, 2010). For this reason, we argue that the lack of a main effect is not an obstacle to understanding GxE relations in our study.

This study examined moderation of exposure to maternal anxiety by the 5-HTTLPR during pregnancy and early childhood. In line with our hypothesis, we found that the 5-HTTLPR interacts with prenatal maternal anxiety symptoms to influence the risk for child emotional problems. The interaction effect of the 5-HTTLPR by chronic difficulties was accounted for by that of the 5-HTTLPR by prenatal anxiety symptoms. These two measures are correlated, and it may be that maternal anxiety impacts to a greater degree on maternal physiology and the fetal environment than chronic difficulties. Further support for a direct intrauterine interaction effect of the 5-HTTLPR by maternal anxiety symptoms during pregnancy comes from the absence of any interaction effect of the 5-HTTLPR by fathers' prenatal anxiety symptoms.

The 5-HTTLPR also moderated the effect of postnatal maternal anxiety symptoms on child emotional problems. Plausibly, different mechanisms account for independent prenatal and postnatal interaction effects. In rats, it has been shown that the *5-HTT* interacts with prenatal maternal stress to specifically affect the offspring's glucocorticoid receptor gene mRNA and corticosterone response, whereas the interaction between the *5-HTT* and postnatal stress specifically influenced behavioral responses of the offspring (Belay et al., 2011). Likewise, differential effects of prenatal and postnatal adversity on the developing brain have been recognized in humans (Lupien et al., 2009). During pregnancy, maternal stress hormone levels may affect the development of the amygdala, the hippocampus, and the prefrontal cortex, which are involved in regulating the HPA-axis. We found an interaction effect of the 5-HTTLPR by prenatal maternal anxiety on children's ability to recognize emotional expressions. This finding provides indirect evidence for the potential influence of the 5-HTTLPR on the development of specific brain networks underlying emotion perception.

Despite the regular contact most fathers have with their children during the preschool years, the 5-HTTLPR did not significantly moderate the effect of paternal anxiety symptoms during the early preschool years on child emotional problems. Compared with mothers, fathers scored lower on anxiety symptoms in this period, which may explain the absence of an interaction effect in fathers. It might also be that fathers spend less time with their children, and thus may have less impact on their child's development.

The DRD4 7R and the high activity MAO-A gene variants also moderated the effect of prenatal maternal anxiety symptoms on child emotional problems. However, the results for the DRD4 were not in line with our hypothesis. The results for the MAO-A were less consistent than our findings with the 5-HTTLPR, which, as hypothesized, also interacted with postnatal maternal anxiety symptoms on the risk for developing emotional problems.

To limit the possible bias due to population heterogeneity, we only included children of Northern European descent based on a principle component analysis of GWAS data. The analyses in Turkish and Moroccan children suggest that the observed moderation of postnatal anxiety effects by the 5-HTTLPR can –to some extent– be generalized to other ethnicities.

Last, we did not find evidence to suggest that variance in rs25531 had a large impact on our results. Clearly, the impact of rs25531 also depends on the frequency of the G allele in a specific sample or population. Furthermore, there is no consensus about the genetic model underlying the effect of the 5-HTTLPR. Overall, our findings indicate an additive effect of the s allele, suggesting that heterozygosity is also associated with an increased risk for child emotional problems.

Although our study has notable strengths, including the large sample size and prospective assessments, there are some limitations which need to be considered. Firstly, both maternal and paternal anxiety symptoms were assessed using self-reports. Furthermore, these symptom scores yield a relatively crude measure which cannot be translated into a clinical diagnosis of anxiety disorder. However, our results show that even subclinical symptoms of anxiety influence child emotional functioning. Secondly, we did not explore the interaction effect between maternal 5-HTTLPR status and the environment on child emotional development. Kistner-Griffin et al. (2011) found that the l-allele of the 5-HTTLPR in mothers was associated with autism spectrum disorder in the offspring. Possibly, maternal 5-HTT influences fetal brain development through metabolic pathways in the placenta (Bonnin & Levitt, 2011). In order to gain further insight into the underlying mechanisms, it would be of interest to further explore the influence of maternal 5-HTTLPR child emotional development.

Brown and Harris (2008) recently hypothesized that the interaction between the 5-HTTLPR and early childhood adversity may affect neurodevelopment and brain function, which could result in a life-time increased risk to develop psychopathology. Moreover, the authors suggested that these early effects on neurodevelopment may even account for the risk of depression associated with the interaction between the 5-HTTLPR and SLE during adulthood. This suggests that gene-environment interaction during adulthood is observed particularly in those s-allele carriers who were exposed to environmental adversity during childhood. Our findings support the first part of this hypothesis by reporting significant interaction effects of the 5-HTTLPR by environment both in utero and the preschool period on child emotional functioning.

In complex traits, such as emotional problems, large effects of one polymorphism in interaction with the environment are rare. Moreover, the small effects of the 5-HTTLPR may be plausible from an evolutionary perspective. In rhesus monkeys, s allele carriers showed delayed early neurobiological development, impaired serotonergic functioning and HPA-axis reactivity, and aggression, but only if they were reared by peers. If rhesus monkeys were reared by their mothers, the s allele carriers showed better behavioral outcomes than l allele carriers (Suomi, 2006). Next to an increased sensitivity to stress, the s allele has been associated with improved cognitive function such as decision making and social cognition

(Canli & Lesch, 2007; Homberg & Lesch, 2011). Hence, s allele carriers seem very attentive to the environment, capable to process information under stress, which likely contributed to the adaptive success and survival of the species (Homberg & Lesch, 2011; Suomi, 2006). It has been suggested that the 5-HTTLPR acts as a plasticity gene, influencing the individual´s susceptibility to the environment (Belsky & Pluess, 2009; Ellis, Boyce, Belsky, Bakermans-Kranenburg, & Van IJzendoorn, 2011). We explored moderation by the 5-HTTLPR within the diathesis-stress framework. Future research should further explore possible differential effects of the 5-HTTLPR according to the quality of the environment. However, this would need to include the assessment of positive environmental exposures rather than just the absence of environmental adversity (e.g., maternal anxiety symptoms in our study).

In sum, our findings suggest that the *5-HTTLPR* moderates the impact of the environment on the fetus and the young child, influencing their ability to accurately recognize emotions and their risk for developing emotional problems later in life. The impact of moderation by the *5-HTTLPR* does not lie in the magnitude of the effect, but in the non-specificity and its generalizability across ethnicities. Future studies are necessary to examine whether the small effects observed in large samples are replicable or further converge to the null (Hardy & Low, 2011).

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SM Table 1. Sample characteristics according to 5-HTTLPR genotype (N = 2136)

	Child 5-HTTLPR				
	l/l (*n* = 719)	l/s (*n* = 1029)	s/s (*n* = 388)	Test statistic [a, c]	*p*
Mother					
Age at intake (years)	32.2(3.9)	31.8(4.0)	31.8(4.0)	3.01	0.05
Education					
higher education (%)	39.7	41.0	36.7	2.02	0.35
Marital status					
married/living together (%)	96.4	95.8	96.3	0.56	0.76
Smoking during pregnancy					
never (%)	79.5	80.0	80.9	0.31	0.81
Child					
Gestational age (weeks) [b]	40.4 (37.4-42.1)	40.4 (37.6-42.1)	40.3 (37.7-42.1)	3.05	0.08
Birth order					
first child (%)	58.1	60.9	61.3	1.75	0.42
Gender					
boys (%)	50.1	52.1	49.2	1.22	0.55

l = l allele; s = s allele.
[a] M (SD) unless otherwise indicated
[b] median (100% range)
[c] with the chi-square statistic for categorical variables, one-way ANOVA for normally distributed continuous variables, and the Kruskal-Wallis test for non-normally distributed continuous variables.

SM Table 2. Interaction effects of the 5-HTTLPR by prenatal and postnatal paternal anxiety symptoms on child emotional problems (N = 2136)

	Emotional problems at 5 years [a]		
	B	*SE*	*p*
5HTTLPR x prenatal paternal anxiety symptoms [b]			
additive genetic model	0.54	0.82	0.51
per genotype			
l/l	Ref		
l/s	0.48	1.14	0.67
s/s	1.26	1.75	0.47
5HTTLPR x postnatal paternal anxiety symptoms			
additive genetic model	-0.53	0.99	0.59
per genotype			
l/l	Ref		
l/s	0.08	1.38	0.95
s/s	-1.27	2.07	0.54

l = l allele; s = s allele; Ref = reference.
[a] Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, age of child at behavioral assessment, 5-HTTLPR x prenatal paternal anxiety symptoms, or 5-HTTLPR x postnatal paternal anxiety symptoms.
[b] centered.

SM Table 3. Interaction effects of the MAO-A and DRD4 48bp VNTR by prenatal maternal chronic difficulties and prenatal and postnatal maternal anxiety symptoms on children's risk to develop emotional problems

	Emotional problems at 5 years		
	B	*SE*	*p*
MAO-A [a] *N* = 1000			
MAO-A x Prenatal maternal chronic difficulties	-0.24	0.14	0.080
MAO-A x Prenatal maternal anxiety symptoms	-2.98	0.95	.002
MAO-A x Postnatal maternal anxiety symptoms	1.30	1.41	0.359
DRD4 7R [b] *N* = 2136			
DRD4 x Prenatal maternal chronic difficulties	-0.11	0.09	0.207
DRD4 x Prenatal maternal anxiety symptoms	-1.44	0.67	0.029
DRD4 x Postnatal maternal anxiety symptoms	-0.48	0.90	0.597

Note. Models are adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, child age at behavioral assessment, and prenatal or postnatal maternal anxiety symptoms.
[a] Analyses were performed in boys only; beta = effect estimate for the high activity MAO-A variant (4, *n* = 642) compared to the low activity MAO-A (3, *n* = 358).
[b] Assuming an additive genetic model; homozygous without the 7 repeat (*n* = 1394), heterozygous (*n* = 679), and homozygous for the 7 repeat (*n* = 63).

SM Table 4. The moderating effect of the variance in rs25531 on the association between prenatal and postnatal maternal anxiety symptoms and child emotional problems (N = 2033)

	Emotional problems at 5 years		
	B	*SE*	*p*
rs25531 x prenatal maternal chronic difficulties			
additive[a]	0.04	0.08	0.642
rs25531 x prenatal maternal anxiety symptoms			
additive[a]	1.90	0.53	0.001
rs25531 x postnatal maternal anxiety symptoms			
additive[a]	2.05	0.76	0.009

Note. Adjusted for maternal age, maternal education, Apgar score, gender, gestational age, birth order, maternal smoking during pregnancy, child age at behavioral assessment, prenatal or postnatal maternal anxiety symptoms.
[a] The distribution of the variation at rs25531 was as follows: s/l$_a$ 42.6%; s/l$_g$ 5.6%; l$_a$/l$_a$ 25%; l$_a$/l$_g$ 8.1%; l$_g$/l$_g$ 0.3%; s/s 18.4%. Additive genetic model: l$_a$ (n = 505) vs. l$_a$/l$_g$ or s (n = 1029) vs. l$_g$ and/or s (n = 499). Hardy-Weinberg Equilibrium (HWE): χ^2 (2) = 0.23, p = .84.

SM Table 5. Subsample characteristics according to 5-HTTLPR genotype

	Child 5-HTTLPR				
	l/l (n= 183)	l/s (n = 270)	s/s (n = 117)	test [a]	p
Mother					
Age at intake (years), Mean (*SD*)	32.3 (3.9)	32.12 (3.7)	32.3 (3.9)	0.06	0.94
Educational level (% high)	66.9	69.5	68.6	0.37	0.83
Parity (% primiparous)	61.7	58.1	61.5	0.73	0.69
Smoked during pregnancy (% yes)	24.0	19.3	15.4	3.52	0.17
Child					
Age (months), Mean (*SD*)	37.5 (1.3)	37.6 (1.5)	37.6 (1.5)	0.59	0.55
Gender (% boys)	45.9	50.0	47.0	0.80	0.67
Gestational age at birth (weeks), Mean (*SD*)	40.3 (1.3)	40.3 (1.3)	40.2 (1.3)	0.42	0.66
Shape-matching accuracy, Mean (*SD*)	0.91 (0.2)	0.92 (0.2)	0.91 (0.2)	0.12	0.89

l = l allele; s = s allele.
[a] with the chi-square statistic for categorical variables and one-way ANOVA for continuous variables.

SM 6 GENOTYPING DRD4 48BP VNTR AND MAO-A VNTR

Genotyping of the VNTR polymorphism in the DRD4 gene. Genotyping of the DRD4 48bp VNTR was amplified using primers D4-F-GCGACTACGTGGTCTACTCG and D4-R-AGGACCCTCAGGCCTTG. Reactions were performed in a 384-wells format in a total reaction volume of 10ul containing 10ng 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 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 VNTR polymorphism in exon 3 of the MAOA gene. The VNTR polymorphism in exon 3 of the MAOA gene was amplified using primers F-

ACAGCCTGACCGTGGAGAAG and R-GAACGGACGCTCCATTCGGA. Reactions were performed in a 384-wells format in a total reaction volume of 20 ul containing 10 ng DNA, 0,5 pmol/ul of each primer, 0,2 mM dNTPs, 1,5 mM MgSO4, PCR buffer (1x), PCRx enhancer (1x) and 2,5 U/ul Platinum Taq. PCR cycling consisted of initial denaturation of 2 min at 95° C, and 36 cycles with denaturation of 30 seconds at 95°C, annealing of 30 seconds at 55°C and extension of 1 minute at 68°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 MAOA repeats was determined using the size of the PCR-fragments. To assure genotyping accuracy 250 random samples were genotyped for a second time. No discrepancies were found.

CHAPTER 4.3

BEYOND THE USUAL SUSPECTS: IN SEARCH OF THE GENETIC BASIS OF ATTACHMENT AND TEMPERAMENTAL FEARFULNESS USING A COMBINED GENOME WIDE ASSOCIATION AND PATHWAY ANALYSIS APPROACH

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Manuscript in preparation for submission

ABSTRACT

We present findings of a combined genome wide- and pathway analysis study on attachment and temperamental fearfulness in a racially homogeneous sample (N = 641). Infant attachment was observed in the Strange Situation Procedure at 14 months, fearfulness was observed using the Stranger Approach episode of the Laboratory Temperament Assessment Battery at 36 months. As expected, no significant results were found for attachment security. For attachment disorganization, we found one nearly genome wide significant association located in the CACNA2D3 gene, and evidence for the role of the dopamine receptor mediated signaling pathway. For temperamental fearfulness, a significant association with the asparagine and aspartate biosynthesis pathway was found. Findings go beyond the usual genetic suspects in attachment and temperament, and should be replicated in larger samples.

INTRODUCTION

The idea that the environment plays a major part in explaining individual differences in quality of the infant-parent attachment relationship is central to attachment theory (Ainsworth, Blehar, Waters, & Wall, 1978; Bokhorst et al., 2000; Roisman & Fraley, 2008; Sroufe, 2005). In contrast, temperament theory and research have documented the rather large role of genetic influences on the emergence and development of temperamental traits such as reactivity and inhibition (Bouchard, & Loehlin, 2001; Emde et al., 1992; Goldsmith, Lemery, Buss, & Campos, 1999; Kagan, 2003). Here we present the results of a genome wide association study (GWAS) combined with a pathway analysis on observed infant attachment security and disorganization, and preschool temperamental fearfulness in the largest, racially homogeneous infant attachment sample (N = 641) available to date.

Our first aim is to test the hypothesis that temperamental fearfulness is associated with genetic pathways related to the serotonergic and dopaminergic systems, but that attachment security is not associated with any genetic pathway. Attachment disorganization might partly be constitutionally determined as neurobiological deficits may play a role in its assessment or in the vulnerability of children to become disorganized in the face of certain parenting behaviors. One of the candidate pathways for disorganization is the dopaminergic pathway related to attention deficits. The second aim of the current study is to go beyond 'the usual suspects' (Ebstein, Israel, Chew, Zhong, & Knafo, 2010) of a small selection of the dopaminergic and serotonergic system genes and explore a large range of potentially important genes within and outside these pathways implicated in the development of attachment and temperamental fearfulness.

Attachment security

In the eighties and nineties, twin studies seemed to show that most human traits and characteristics are largely determined by genetic differences and for a smaller part by the unique environment (Rowe, 1995). Therefore, Harris (1998) argued that parenting does not play an important role in child development compared to the contributions of the child's genes and group socialization processes. In stark contrast, attachment theory proposes that individual differences in security are explained by parental caregiving behavior rather than genetic differences (Cassidy & Shaver, 2008; Sroufe, Egeland, Carlson, & Collins, 2005; Van IJzendoorn et al., 2000). Inspired by Ainsworth's seminal work on attachment and childrearing in her Uganda and Baltimore samples (Ainsworth, 1967; Ainsworth et al., 1978), attachment researchers have considered parental sensitivity the most important determinant of individual differences in attachment security (Bakermans-Kranenburg & Van IJzendoorn, 2010; Main, 1990).

Observational and experimental studies of attachment have generally confirmed this hypothesis, although the combined effect size for the association between parental sensitivity and attachment security is relatively modest (in De Wolff and Van IJzendoorn's, 1997, meta-analysis, the combined effect was r = .24; see also Bakermans-Kranenburg, Van IJzendoorn, & Juffer, 2003 for a meta-analysis on experimental evidence). Moreover,

numerous studies demonstrated that parental sensitivity to their infant's attachment signals is partly determined by parents' own secure or insecure mental representation of childhood attachment experiences (Van IJzendoorn, 1995). However, maternal sensitivity accounts for only a third of the association between parental attachment representation and infant attachment, leaving a *transmission gap* of unexplained variance in infant attachment security (Van IJzendoorn, 1995). Genetic and temperamental factors influencing attachment in parents and in their children have been proposed to bridge this intergenerational transmission gap.

Twin studies, however, have failed to find convincing evidence for a large role of constitutional factors –genetic or temperamental- in determining attachment differences (Bakermans-Kranenburg & Van IJzendoorn, 2010). Of the five twin studies on child-mother attachment security that have been published thus far, four document a minor role for genetic influences on differences in child-mother attachment security and a rather substantial role for shared environment (making siblings within the same family more similar to each other and less similar to children in other families) (Bokhorst et al., 2003; O'Connor & Croft, 2001; Ricciuti, 1992; Roisman & Fraley, 2008). The fifth study, the Louisville Twin Study (Finkel & Matheny, 2000) investigated the quality of attachment in twin pairs with an adapted separation-reunion procedure originally designed for assessing temperament, which might have increased the still modest estimate (25%) of heritability of attachment.

In the Bokhorst et al. (2003) study, attachment and temperament showed contrasting pictures; shared and non-shared environmental factors were predominant for attachment security, whereas for temperament more heritability was found. Genetic factors explained 77% of the variance in temperamental reactivity, and measurement error and unique environmental factors (making siblings within the same family different from each other) accounted for 23%. Temperamental reactivity of twins with similar attachments to their parent was not more similar than reactivity of twins with divergent attachments to their parent. Thus, differences or similarities in temperamental reactivity were not associated with attachment concordance or discordance within twin pairs.

Parents may interact (in-)sensitively with their twin children depending on the genetic make-up of the children. Fearon et al. (2006) however found that correlations for parental sensitivity to each of the twins as rated from 1.5 hours of home observations by independent coders were high in both monozygotic and dizygotic twins (between .64 and .69). Genetic modeling indicated that shared environmental factors explained 66% of the variance in sensitivity as experienced by the child, and non-shared environmental factors explained 34%. Thus, genetic differences between children do not seem important for the kind of parenting they receive, at least in terms of sensitivity, and there is no reason to believe that temperamental or other constitutional differences would have more influence. Roisman and Fraley (2008) basically replicated these findings using home observations of parental sensitivity and attachment security (with an abbreviated Attachment Q-Sort) in a much larger sample. They found low heritability estimates for attachment security, and large heritability for temperamental dependency in the same toddlers.

ATTACHMENT DISORGANIZATION

Secure, insecure-avoidant, and insecure-ambivalent attachment patterns are considered organized attachment behavior strategies, that is, functional adaptations to sub-optimal caregiving environments with parents who are insensitive (but not abusive or neglecting). Some children display in addition to their organized attachment behavior conflicted, contradictory, or fearful behaviors in the presence of their attachment figure, indicating that their otherwise organized attachment strategy may fall apart in the face of a stressor. Frightening parental behavior has been found to be one of the causes of disorganized attachment, and indeed many maltreated children display disorganized behavior (Cyr, Euser, Bakermans-Kranenburg, & Van IJzendoorn, 2010; Hesse & Main, 2006; Main & Solomon, 1990). Disorganized attachment is considered the most anxious type of attachment, and has been shown to constitute a risk factor for externalizing problem behavior in childhood (Fearon, Bakermans-Kranenburg, Van IJzendoorn, Lapsley, & Roisman, 2010) and psychopathology in adolescence (Carlson, 1998; Sroufe et al., 2005).

In a meta-analysis on the determinants of infant attachment disorganization, Madigan et al. (2006) found robust and moderate associations between maternal unresolved loss or trauma, maternal anomalous behavior, and disorganized attachment in 12 studies including more than 800 families. However, less than half of the association between unresolved loss or trauma and disorganized attachment could be explained by mediation of anomalous parenting. As was the case for attachment security, again an intergenerational transmission gap was found, and it was hypothesized that genetic or other constitutional factors including temperament might bridge the gap.

Interestingly, in the only twin study on disorganized attachment that has been conducted to date disorganization did not show genetic or shared environmental effects. Only unique factors (non-shared environment and error of measurement) explained the individual differences in disorganization (Bokhorst et al., 2003). Conducting a meta-analysis of 12 samples including 1,877 participants, Van IJzendoorn, Schuengel, and Bakermans-Kranenburg (1999) found no association between infant disorganized attachment and constitutional or temperamental factors. In the eight studies (N = 1,639) that assessed difficult or reactive temperament, again no association was found with disorganized attachment.

Temperamental fearfulness

Over the past 20 years Mary Rothbart, Hill Goldsmith, and their associates have unified various theories of temperament under the same umbrella of a psychobiological theory (see also Kagan, 2003). Derryberry and Rothbart (1997) defined temperament as affective, motivational, and cognitive (attentional) adaptations that are firmly based on an inherited neurobiological infrastructure but also partly shaped by experience. Individual differences in reactivity to new (social or physical) stimuli and differences in emotion regulation are among the most prominent features of temperament (Rothbart & Derryberry, 1981). Temperamental fearfulness to strangers is one of the most widely studied dimensions of the broad temperament phenotype (see, e.g., Kochanska's research program, Kochanska, Aksan, & Carlson, 2005), and is supposed to be most comparable --although in no way identical -- to attachment.

In contrast to attachment that supposedly emerges from the early interactions between parent and infant, the psychobiological temperament theory assumes that reactivity and regulation of affect are determined primarily by neurobiological structures internal to the child (Vaughn, Bost, & Van IJzendoorn, 2008). Rothbart and Bates (2006) suggest that children's reactivity is also shaped by experiences in the (social) environment, and reactive children might learn to be more open to new experiences. However, temperament is considered mainly an inherited behavioral-cognitive style of the individual child (Vaughn et al., 2008). For that reason we predict that individual differences in temperamental traits are much more associated with genetic differences than attachment security and disorganization.

4.3

Although attachment and temperament have different developmental origins they might influence one another. For instance, Kagan and Fox (2003) argued that the patterns of secure and insecure attachment behavior as displayed in the stressful Strange Situation Procedure (SSP) were in fact the expression of underlying temperamental differences in inhibition or fearfulness. However, in several studies using observational assessments of temperament no evidence for *main* effects of temperament on security have been found (e.g., Burgess, Marshall, Rubin, & Fox, 2003; Pauli-Pott, Haverkock, Pott, & Beckmann, 2007; Kochanska et al., 2005; for a review, see Vaughn et al., 2008). It should be noted that this does not imply that attachment and temperament are completely dissociated phenotypes in child development. On the contrary, in the differential susceptibility paradigm (Belsky, Bakermans-Kranenburg, & Van IJzendoorn, 2007) temperament is considered to be one of the most important susceptibility factors, moderating the relation between (child rearing) environment and a broad range of child developmental outcomes (Ellis, Boyce, Belsky, Bakermans-Kranenburg, & Van IJzendoorn, 2011).

Usual suspects for attachment and temperament: DRD4 and 5HTTLPR

So far, molecular genetic studies on attachment have focused on candidate genes from dopaminergic and serotonergic pathways. The first studies on the genetic basis of secure and disorganized attachment, for example, involved genetic polymorphisms related to the dopamine system (in particular the DRD4; Gervai et al., 2005; Lakatos et al., 2000, 2002) with promising preliminary results. However, in a secondary analysis of a combined sample of 542 infant-mother dyads it was impossible to replicate these original findings (Bakermans-Kranenburg & Van IJzendoorn, 2007).

Later, Barry, Kochanska and Philibert (2008) tested main and interaction effects of the serotonergic system gene 5HTTLPR in relation to attachment security and parental sensitivity. They found in a rather small sample (N = 87) that the continuous measure of security was only marginally associated with child genotype. A significant interaction effect showed that among infants homozygous for the long allele (*ll*), there was no association between parental sensitivity and attachment, whereas for carriers of the short allele insensitive parenting elevated the risk of insecure attachment. In a recent study on attachment in about 100 Italian infants, a larger number of genes were genotyped. After Bonferonni corrections no significant associations were found between attachment security and the 5-HTTLPR, COMT, GABRA6, DRD4, and DRD4/-521 polymorphisms (Frigerio et al., 2009).

Similarly few candidate genes in the serotonergic and dopaminergic pathways have been examined in molecular genetic studies of temperamental fearfulness and other dimensions of temperament. With respect to fearfulness, it is expected that children with the short allele of the 5HTTLPR would be rated as more fearful, inhibited, or shy than children with at least one copy of the long allele, but the evidence is conflicting (Arbelle et al., 2003; Auerbach, Faroy, Ebstein, Kahana, & Levine, 2001; Battaglia et al., 2005; Schmidt, Fox, Robin, Hu, & Hamer, 2002). Recently Hayden and colleagues (2007) tested associations between the 5-HTTLPR and indices of fearful child temperament, derived from maternal reports and standardized laboratory observations (Lab-TAB) in a community sample of 95 preschool-aged children. The authors found that children homozygous for the short allele of the 5-HTTLPR were rated as significantly more nervous during standardized laboratory procedures than children with at least one copy of the long allele. The mothers of these children also rated them as more shy than those with at least one copy of the long allele.

Rothbart and Bates (2006) reviewed an extensive literature concerning some of the molecular genetics of the reactive and regulatory facets of temperament. Much of this research is recent, and relatively few of the molecular results have been replicated. Nevertheless, the authors speculate that alleles contributing to differences in serotonin transport and dopamine reception are implicated in temperamental fearfulness in human children and adults (see also Rihmer et al., 2010), as well as in other primates. Candidate genes for temperamental executive attention are usually suggested to be located in the dopaminergic system (Posner, Rothbart, & Sheese, 2007). However, the molecular genetics of temperament did not yet generate a replicated set of genes involved in the development of temperamental differences in fearfulness or other dimensions of temperament.

Here we widen the search for relevant genetic differences studied in association with attachment and temperament beyond the usual suspects, using genome wide association and pathway analyses in a large, non-clinical, Caucasian sample of about 700 infants who have been observed in the Strange Situation Procedure at 14 months of age, and who were observed using the Lab-TAB at 36 months of age. The sample is part of the Generation R Study, a prospective Dutch cohort study investigating growth, development, and health from fetal life onward, which has been described in detail elsewhere (Jaddoe et al., 2007; 2008).

GWAS and pathway analysis

Genome wide association studies (GWAS) are conducted to identify novel genetic variants that are associated with a specific phenotype. One of the reasons for GWAS is that across domains candidate gene and linkage studies have often failed to deliver definitive or even replicable results. Indeed, most reported candidate gene associations in medical and behavioral research are not robust: of the many putative associations which have been studied three or more times, less than 20 have been consistently replicated (Hirschhorn, Lohmueller, Byrne, & Hirschhorn, 2002). Candidate gene association studies are susceptible to false positive findings due to population structure, because there is no way to assess differences in the genetic background of cases and controls. Other possible causes for non-

replication are population-specific gene–gene or gene– environment interactions. Further, publication bias is often made responsible for inconsistencies through putative selective publication of initially positive results while the numerous negative studies would remain unpublished. However, in the absence of a massive publication bias, this is unlikely to explain all of the initial positive reports. A central problem remains that candidate gene studies are a shot in the dark: They are limited to specific variants in biological candidate genes, each with a tiny *a priori* probability of being causally related to the phenotype (Altschuler, Daly, & Lander, 2008).

4.3

Contrary to candidate gene approaches, GWAS are hypothesis free, because the genetic markers that are studied throughout the genome are merely used to identify loci associated with the phenotype and are not selected based on their assumed biological function (Hirschhorn & Daly, 2005). In GWAS, a dense map of common (frequency > 1%) single nucleotide polymorphisms (SNPs) are tested for association. SNPs are the most abundant form of DNA variation in the human genome, and to date 12 million SNPs have been identified. In GWAS, subsets of common SNPs are analyzed, and thus the results rely on the expectation that a relevant allele is likely to be correlated with an allele of an assayed SNP. Such non-random association of alleles at nearby loci (linkage disequilibrium or LD) is an important and widespread feature of the genome. To date, the GWAS approach has been successfully applied to identify novel disease genes underlying type 1 diabetes, type 2 diabetes, rheumatoid arthritis, Crohn's disease, bipolar disorder, and hypertension (Wellcome Trust Case Control Consortium, 2007).

To our knowledge Generation R includes the largest, racially homogeneous infant attachment sample to date. Nevertheless, its size still is rather limited for a GWAS, with risks for false positives as a result. A major challenge in using the GWA approach is that, because of the need to rigorously correct for the large number of genotype-phenotype tests, it tends to focus exclusively on individual genes or regions that have the strongest statistical evidence for association. However, individuals can be at risk for attachment disorganization or temperamental fearfulness through multiple genetic variants acting on different proteins in the same functional network. When the evidence for a significant contribution of genetic variation in a region comes only from a subset of individuals, the signal strength for a single gene may be small, even in large samples. These effects are likely to be pruned out in GWAS by the need to adjust for multiple testing.

Pathway-based approaches have been suggested as an alternative strategy to use GWA data. These approaches can yield insights that are not obtained by the focus on individual genes that have the strongest statistical evidence for association. A pathway-based approach identifies whether SNPs in genes predefined to belong to a certain pathway show more significant association results than expected by chance. Thus, the grouping of genes in pathway-based approaches is based on existing knowledge of gene function. Pathway analysis can confirm *a priori* hypothesized causal pathways, but can also be used to detect novel pathways and mechanisms. This approach has been successfully applied to mental disorders, for example schizophrenia (Walsh et al., 2008).

Aims of the current study

In the current study, we apply GWAS and pathway analysis to observed attachment and temperament. Our first aim is to identify possible new candidate genes associated with attachment and temperament. In order to go beyond the "usual suspects", we use GWA data to explore hypothesis-free which of 2.5 million SNPs are implicated in the development of attachment and temperamental fearfulness. The second aim is to test a wide range of potentially important gene pathways. We test the hypothesis that temperamental fearfulness is associated with genetic pathways related to the serotonergic and dopaminergic systems, and that attachment security is not associated with any genetic pathway. One of the candidate pathways for attachment disorganization is the dopaminergic pathway related to attention deficits. Yet, despite these hypotheses, we test all 165 pathways using the same a priori possibility. This is because, as evident from the review above, in fact still little is known about the molecular genetic basis of attachment and temperament.

METHOD

Setting

The present study was conducted in a subsample of children participating in the Generation R Study, a population-based prospective Dutch cohort from fetal life onward (Jaddoe et al., 2007, 2008). The subsample is ethnically homogeneous (Caucasian) to exclude possible confounding (e.g., population stratification) or effect modification by ethnicity. All children were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort (Jaddoe et al., 2007).

The study is in accordance with the guideline proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus Medical Center. Parental written informed consents were obtained for all participants.

Study Population

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. At age 14 months (M = 14.7, SD = 0.9), infants and their mothers visited the research center where they participated in the Strange Situation Procedure (SSP). For N = 641 infants (330 boys), information on both genotype and attachment was available. At age 36 months (M = 37.5, SD = 1.5), children visited our research center again, and fearful temperament was assessed using the Stranger Approach (SA) episode from the Laboratory Temperament Assessment Battery (Lab-TAB). For N = 596 children (307 boys), information on both genotype and fearfulness was available.

Procedures and Measures

Genotyping. The genome-wide genotyping was performed with Illumina 610 Quad Array (Illumina, San Diego, CA, USA) (sample call rate ≥97.5%). Individuals with excess of

autosomal heterozygosity, mismatch between genotypic and phenotypic gender, and outliers identified by the identity-by-state (IBS) clustering analysis were excluded. This genotype data was used to impute 2.5 million autosomal SNPs described in HapMap's Phase II European population panel with the imputation software MACH (http://www.sph.umich.edu/csg/abecasis/MACH). Such SNP imputation infers genotypes probabilistically according to shared haplotype stretches between the study samples and HapMap release 22 build 36 data. Genotyping took place in the Genetic Laboratory of the Department of Internal Medicine, Erasmus Medical Center Rotterdam. For the present study SNPs were excluded if the minor allele frequency (MAF) was 5% or less, the Hardy-Weinberg equilibrium (HWE) p-value was less than 10E-5, or the SNP call rate was equal to or less than 90%.

Strange Situation Procedure. Parent-infant dyads were observed in the Strange Situation Procedure (SSP). The SSP is a well-validated, widely used procedure to measure the attachment quality. The procedure consists of seven episodes of three 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 two (pre-) separation episodes with one minute, keeping the critical reunion episodes intact (see Luijk et al., 2010).

Attachment behaviors are 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 with the parent, which does not effectively comfort the child and allow it to return to play. Examples of disorganized/disoriented 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 recordings according to established coding systems (Ainsworth et al., 1978; Main & Solomon, 1990) by reliable coders trained at the University of Minnesota (Alan Sroufe's lab). Using discriminant analysis on a large set of SSPs, Richters, Waters, and Vaughn (1988) developed an algorithm to transform the SSP behavioral ratings into a continuous attachment security score that was highly predictive of the secure versus insecure classifications. Van IJzendoorn and Kroonenberg (1990) adapted their algorithm to be able to leave out the laborious coding of crying behaviors, producing a valid Attachment Security Scale based on the interactive scales (Ainsworth et al., 1978), which has been widely used because of the advantages of conducting statistical analyses on continuous instead of categorical variables. 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 (Main & Solomon, 1990).

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

Attachment security and disorganization scores were significantly correlated, r = -.27 (N = 720, p < .01), indicating that more securely attached children were less disorganized.

Temperamental fearfulness. Fearful temperament was measured using the Stranger Approach (SA) episode of the Laboratory Temperament Assessment Battery (Lab-TAB, Preschool Version; Goldsmith, Reilly, Lemery, Longley, & Prescott, 1999). The Lab-TAB is a widely used standardized instrument for the laboratory assessment of early temperament. In the SA episode, the child deals with social fear when a novel, slightly threatening stranger approaches. The situation is modeled after real-life events. The child is left alone in a room. After 10 seconds a stranger enters the room and asks standard questions from the child in a neutral voice. The episode is divided into nine epochs for coding. Episodes were coded from DVD recordings according to the original coding system provided in the Lab-TAB manual. Peak intensity of fearful facial expressions, distress vocalizations, activity decrease, approach, avoidance, gaze aversion, verbal hesitancy, and nervous fidgeting were coded in each epoch by trained coders, who were blind for genetic information and children's attachment. Scores for these various observed parameters were subsequently averaged across all epochs. ICCs for these averages ranged from .71 and .97. For analyses, a fearfulness composite was calculated by taking the mean of the standardized averages. Composite scores ranged from 0 to 1, with higher scores indicating a more fearful temperament. Temperamental fearfulness was not significantly correlated with the attachment security scale or the disorganized scale, r = .04 (n = 592, p = .35) for security, and r < .001 (n = 582, p = .93) for disorganization.

Statistical Analysis

Genome wide association studies (GWAS) on attachment disorganization and security were performed under an additive model using linear regression implemented in GRIMP (Estrada et al., 2009). Since population genotype distribution is assumed to be unrelated to covariates (Davey Smith & Ebrahim, 2005), models were unadjusted for covariates. Genome wide significance was pre-specified as $p < 1\times10^{-8}$, and indicative SNPs as $p < 1\text{x}10^{-5}$. Quantile-quantile (Q-Q) plots of p values were generated by plotting observed $-\log_{10} p$ values that were ranked in order from smallest to largest on the y-axis against the $-\log_{10} p$ value distribution that would be expected under the null hypothesis of no association on the x-axis. The genomic inflation factor (λ) was estimated using the estlambda option in GenABEL version 1.5-6 (Aulchenko et al., 2007).

The analysis of gene pathways was based on the GWAS data. Pathway analysis reflects the idea that a limited number of biological pathways contribute to the etiology of complex traits (Carlborg & Haley, 2004), implying that large proportions of the genes identified by GWAS are functionally related and/or interact with one another in biological pathways (e.g., in neurogenesis or signal transduction). In Generation R, we determined 550.000 SNPs per individual using an Illumina array and imputed an additional 2 million SNPs for GWA analyses. With this data genetic pathways were examined.

There are several publicly accessible bioinformatics tools which enable assessing the enrichment of pathways in the GWAS results (Elbers et al., 2009). Although the development of these tools is still at an early stage, they provide an additional approach

for the assessment and biological interpretation of the GWAS results. In the current study, pathway analysis was performed using the ANalysis THrough Evolutionary Relationships (PANTHER) 6.1 classification system (Thomas et al., 2003). A list of all genes containing SNPs with association *p* values lower than $1x10^{-3}$ was generated. Subsequently, this gene list was statistically compared using PANTHER to a reference list of all known genes to identify under- and over-represented pathways.

RESULTS

Attachment

Quantile-quantile plots for both the attachment disorganization (genomic control $\lambda = 0.994$) and attachment security (genomic control $\lambda = 0.984$) ratings displayed only modest inflation of the χ^2-test statistic, indicating no genome-wide significant associations (Figures 1 and 2). Nevertheless, for attachment disorganization one nearly genome wide significant association with rs4928027 ($p = 7.4x10^{-8}$) was observed. This SNP is located in the CACNA2D3 gene on chromosome 3 (3p21.1). Five other independent indicative SNPs ($p < 1x10^{-5}$) of the GWAS for disorganized attachment are presented in Table 1. No GWAS significant findings could be identified for attachment security (Table 2). Here, the SNP with the lowest *p* value was rs17465169 ($p = 2.8x10^{-6}$) located in the TRIB2 gene on chromosome 2 (2p24.3).

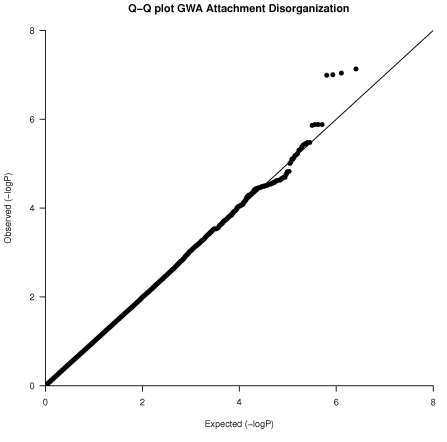

Figure 1. Quantile – Quantile plot of GWAS results for attachment disorganization. Quantile-quantile (Q-Q) plots of *p* values were generated by plotting observed $-\log_{10}$ *p* values that were ranked in order from smallest to largest on the y-axis against the $-\log_{10}$ *p* value distribution that would be expected under the null hypothesis of no association on the x-axis.

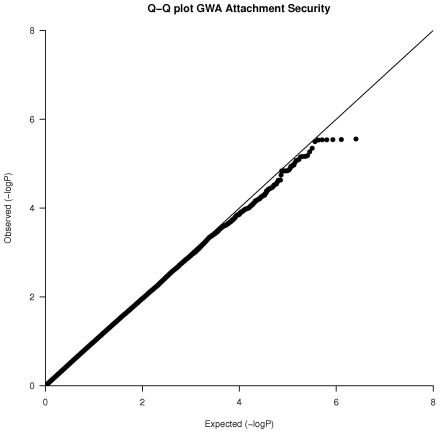

Figure 2. Quantile – Quantile plot of GWAS results for attachment security. Quantile-quantile (Q-Q) plots of *p* values were generated by plotting observed $-\log_{10}$ *p* values that were ranked in order from smallest to largest on the y-axis against the $-\log_{10}$ *p* value distribution that would be expected under the null hypothesis of no association on the x-axis.

Table 1. Independent top SNPs of GWA for disorganized attachment (N = 641)

		Allele									
nr	rs-name	A1	A2	MAF	chr	position	# SNPs	*B*	*SE*	*p*	Closest Gene
1	rs4928027	A	G	.3358	3	54275651	14	.5980	.1110	7.4E-08	CACNA2D3
2	rs6953895	G	C	.3413	7	56555803	1	.5380	.1180	5.0E-06	CHCHD2
3	rs4760820	G	C	.4167	12	70683263	1	.5410	.1190	5.9E-06	TPH2
4	rs6463452	G	A	.4377	7	47865561	3	-.4930	.1090	6.6E-06	FLJ21075
5	rs816229	T	C	.2226	7	56180794	1	.5830	.1300	7.3E-06	PHKG1
6	rs7972837	A	G	.3848	12	1,00E+08	1	.4820	.1090	9.2E-06	MED13L

Rs-name=name of SNP; MAF=minor allele frequency; chr=chromosome; SNP=single nucleotide polymorphism; *SE*=standard error.
Note. SNPs are genome wide indicative, defined as an association with a $p < 1.0E-05$.

Table 2. Independent top SNPs of GWA for attachment security (N = 641)

		Allele									
nr	rs-name	A1	A2	MAF	chr	position	# SNPs	*B*	*SE*	*p*	Closest Gene
1	rs17465169	A	G	.1420	2	12449984	1	-1.1270	.2400	2.8E-06	TRIB2
2	rs2346286	A	G	.2195	3	16070981	7	.8090	.1730	2.9E-06	GALNTL2
3	rs12702179	T	C	.5023	7	45880438	4	.6690	.1470	5.4E-06	IGFBP1
4	rs17405552	A	G	.1376	2	2,00E+08	1	-.9830	.2190	6.9E-06	ITGAV
5	rs9363340	C	G	.0851	6	66052728	1	1.1310	.2540	8.1E-06	EGFL11
6	rs1861567	A	T	.1746	16	4,76E+07	2	-.8140	.1840	9.4E-06	CBLN1

Rs-name=name of SNP; MAF=minor allele frequency; chr=chromosome; SNP=single nucleotide polymorphism; *SE*=standard error.
Note. SNPs are genome wide indicative, defined as an association with a $p < 1.0E-05$.

Next, we evaluated whether among the top associations pathways relevant for attachment disorganization and security were enriched in the GWAS results. For this purpose, two gene lists were compiled of genes that contained SNPs with *p* values lower than $1x10^{-3}$ (resulting in $N = 445$ genes for attachment disorganization; $N = 404$ genes for attachment security). The pathway analysis for attachment disorganization revealed that five pathways were significantly enriched after Bonferroni adjustment (Table 3). One of these five pathways was the dopamine receptor mediated signaling pathway (DRMSP; $p = .016$). Eight out of the 84 DRMSP genes (only one expected based on chance) were associated with attachment disorganization, none of which were the traditionally implicated dopamine candidate genes (e.g., DRD4). The associated genes were CLIC5, FRMD3, TPH2, EPB41L5, PPP1CB, EPB41L3, PTPN3, and FRMD4A. For attachment security, only two pathways were significantly enriched after Bonferroni adjustment (Table 3). These pathways overlapped with pathways that were found for attachment disorganization.

Table 3. Significant PANTHER categories for attachment disorganization, security, and temperamental fearfulness

Pathway	No. of genes in the pathway	No. of genes observed	No. of genes expected	Over/under	*p* value unadjusted	*p* value adjusted*
Attachment disorganization						
Wnt signaling pathway	348	21	5.76	+	.00000059	.000098
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	179	12	2.96	+	.000058	.0097
Dopamine receptor mediated signaling pathway	84	8	1.39	+	.000097	.016
Cadherin signaling pathway	168	11	2.78	+	.00014	.024
Angiogenesis	229	13	3.79	+	.00015	.025
Attachment security						
Wnt signaling pathway	348	19	5.34	+	.0000028	.00046
Cadherin signaling pathway	168	12	2.58	+	.000015	.0025
Temperamental fearfulness						
Asparagine and aspartate biosynthesis	5	3	0.09	+	.00010	.017

* Bonferroni adjusted *p* values.
Note. Inclusion criteria for candidate genes in the pathway analysis was a $p < .001$ in the GWAS.
Note 2. Mapped vs. unmapped IDs for the reference list = 25431:0; for attachment disorganization = 421:24; for security = 390:14; and for temperamental fearfulness = 446:2.

Temperamental fearfulness

The quantile – quantile plot for temperamental fearfulness (genomic control $\lambda = 1.001$) displayed no inflation of the χ^2-test statistic either, indicating no genome-wide significant associations (Figure 3). While no GWAS significant findings could be identified for temperamental fearfulness, we found six independent indicative SNPs (Table 4). The SNP with the lowest p value was rs11776617 ($p = 9.1\text{x}10^{-7}$) located in the LOC13788 gene on chromosome 8. The function of this gene is unknown, but it lies within 60 Kb from the gene UBXN2 (8q12.1). The UBX domain-containing protein 2B gene is involved in cellular-level processes such as mitosis.

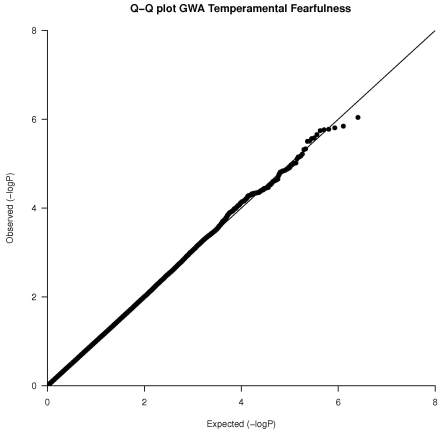

Figure 3. Quantile – Quantile plot of GWAS results for temperamental fearfulness. Quantile-quantile (Q-Q) plots of p values were generated by plotting observed $-\log_{10} p$ values that were ranked in order from smallest to largest on the y-axis against the $-\log_{10} p$ value distribution that would be expected under the null hypothesis of no association on the x-axis.

Table 4. Independent top SNPs of GWA for temperamental fearfulness (N = 596)

		Allele									
nr	rs-name	A1	A2	MAF	chr	position	# SNPs	*B*	*SE*	*p*	Closest Gene
1	rs11776617	A	G	.2073	8	59482761	4	-.0260	.0050	9.1E-07	LOC13788
2	rs7126622	G	C	.4426	11	1,00E+08	3	-.0200	.0040	1.7E-06	OPCML
3	rs11989326	C	T	.1998	8	59505836	4	-.0250	.0050	3.1E-06	CYP7A1
4	rs4919273	C	G	.1844	10	1,00E+08	2	.0230	.0050	7.0E-06	HPSE2
5	rs16935969	G	A	.0528	10	79890802	2	.0500	.0110	9.6E-06	RPS24
6	rs3785634	G	C	.2693	17	1,55E+07	1	.0210	.0050	9.9E-06	TRIM16

Rs-name=name of SNP; MAF=minor allele frequency; chr=chromosome; SNP=single nucleotide polymorphism; *SE*=standard error.
Note. SNPs are genome wide indicative, defined as an association with a $p < 1.0\text{E-}05$.

For temperamental fearfulness, only one pathway was significantly enriched after Bonferroni adjustment (Table 4). This was the asparagine and aspartate biosynthesis pathway.

DISCUSSION

GWAS and pathway approaches can direct the attention of researchers to new groups of genes relevant for a phenotype (McCarthy & Hirschhorn, 2008). In the present pathway analyses, the arguably most exciting finding is the evidence for the role of the dopamine receptor mediated signaling pathway in the development of attachment disorganization. Furthermore, for attachment disorganization one nearly genome wide significant association was observed with a SNP located in the CACNA2D3 gene for which no pathway information is available in PANTHER 6.1. The lack of significant GWAS or pathway analysis results for attachment security was as expected. Lastly, we found remarkably few significant GWAS or pathway analysis results for temperamental fearfulness.

Although the current sample is rather small from a GWAS perspective (N = 641), it is, to our knowledge, the largest infant sample with *observed* attachment and temperament. Furthermore, the sample is not only racially homogeneous but also from a socio-economically similar, middle-class background. Twin studies have demonstrated that genetic effects emerge most powerfully in environmentally homogeneous and middle-class samples (e.g., Ouellet-Morin et al., 2008). We did not examine genetic pathway-by-environment interaction effects which might have uncovered the interplay between the genome and the environment (Caspi et al., 2002, 2003; Rutter, 2006). We also had to ignore epigenetic changes of the genome, although the behavioral consequences of structural DNA may change quite drastically due to methylation, for instance (Meaney, 2010; Van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach, & Philibert, 2010).

Dopamine receptor mediated signaling pathway

The evidence for the role of the dopamine receptor mediated signaling pathway in attachment disorganization supports the hypothesis that alterations in dopamine metabolism partly underlie variations in attachment disorganization, a hypothesis on which several candidate gene studies have been based that primarily focused on the DRD4 (Gervai et al., 2005; Lakatos et al., 2000, 2002). However, the main effect of the DRD4 on attachment disorganization has not been replicated and could not be confirmed in a secondary analysis (Bakermans-Kranenburg & Van IJzendoorn, 2007). Nevertheless, DRD4 plays a central role in the differential susceptibility model as a moderator of environmental influences on attachment disorganization in infants as well as adults (Bakermans-Kranenburg & Van IJzendoorn, 2011; Ellis et al., 2011).

Here we found that eight dopamine-system related genes (only 1.39 was expected based on chance) were associated with attachment disorganization, none of which were the traditionally implicated dopamine candidate genes (e.g., DRD4). Several of these genes do not have a well-described biological function in the dopamine pathway, while others

are involved in basic cell-level processes such as protein phosphorylation, anion transport, or amino acid metabolization necessary for dopamine synthesis. The two most interesting candidates for further research are TPH2 and PPP1CB. The gene TPH2 on chromosome 12 (12q21.1) is predominantly expressed in the brainstem, and encodes a protein involved in the biosynthesis of serotonin. Mutations in this gene may be associated with psychiatric diseases such as bipolar affective disorder and major depression (Invernizzi, 2007; Russo, Kema, Bosker, Haavik, & Korf, 2009). The gene PPP1CB encodes a protein, PP1, which is suggested to function as a suppressor of learning and memory in mouse studies (Gräff, Koshibu, Jouvenceau, Dutar, & Mansuy, 2010; Haege et al., 2010).

CACNA2D3

Quantile – quantile plots for attachment disorganization and security indicated almost no genome wide significant associations. Nevertheless, for attachment disorganization one nearly genome wide significant association with rs4928027($p = 7.4x10^{-8}$) was observed. This SNP is located in the CACNA2D3 gene on chromosome 3 (3p21.1). Note that the top four points in the plot were associated as they are all CACNA2D SNPs in linkage disequilibrium. The voltage-dependent calcium channel subunit alpha-2/delta-3 gene encodes a protein, which in mice is only expressed in the brain. In humans, by contrast, it is also detected in the heart and skeletal muscle (Gong, Hang, Kohler, Li, & Su, 2001). In the rodent brain CACNA2D3 proteins are strongly expressed in the cerebral cortex, caudate-putamen, and hippocampus (Cole et al., 2005). Amongst others, it is involved in neurological system processes such as neuronal action potential propagation, neurotransmitter secretion, and sensory perception.

Wnt and Cadherin pathways

For both attachment security and disorganization, the Wnt- and the Cadherin signaling pathways were significantly enriched. Both pathways serve basic functions in neural development. For instance, the Wnt signaling pathway is involved in neural tube development, while the Cadherin signaling pathway is believed to play a role in neurogenesis (Wheelock & Johnson, 2003). Possibly, the overlap in the Wnt- and Cadherin pathways, if not chance, is caused by the moderate interdependence of attachment security and disorganization. Alternatively, it may also reflect a methodological problem with large genes. Large genes with many haplotype blocks (many independent SNPs in one gene) increase the risk of false positive findings (Elbers et al., 2009). In this study, the Wnt- and the Cadherin pathways include some large genes, thus, their significance should be interpreted with caution.

Few significant genes and pathways for temperament

As for temperamental fearfulness, among the indicative SNPs there was only one SNP, rs7126622, that is linked with neural functioning. This SNP is located in the OPCML gene on chromosome 11 (11q25). The protein encoded by this gene is located in the plasma membrane, and may have an accessory role in opioid receptor function. In rats, this protein binds opioid alkaloids in the presence of acidic lipids and expresses selectivity for mu ligands.

The encoded protein has been highly conserved in species during the evolution, thus, it may have a fundamental role in mammalian systems (Rose, Behm, Drgon, Johnson, & Uhl, 2010).

For temperamental fearfulness, the only significantly enriched pathway was the asparagine and aspartate biosynthesis pathway. This pathway is involved in the biosynthesis of the aminoacids asparagine and aspartate. Aspartate has been proposed as a glutamate-like neurotransmitter in the central nervous system, as both glutamate and aspartate use the same reuptake mechanisms and have similar postsynaptic effects (for a review, see Johnson, 1978). The aminoacid asparagine was found to be a possible metabolic pathway to synthesize aspartate (Reubi, Toggenburger, & Cuénod, 1980).

4.3

In the present study, fewer pathways were identified for observed fearful temperament than for disorganized attachment. However, this cannot be interpreted as evidence for a stronger genetic underpinning of disorganized attachment. Firstly, we did not statistically compare the pathway analyses. Secondly, the difference in the number of significant findings only suggests that the SNPs with the most significant associations with disorganized attachment clustered in predefined groups, whereas the SNPs showing the strongest associations with temperamental fearfulness did not cluster in these groups. Yet, a random distribution of top associations is, of course, much more likely to result in no or few identified pathways given the same number of observations. In this respect, the finding for temperamental fearfulness is perhaps surprising. However, before we assume that fearful temperament is relatively less well explained by SNPs, we must consider the assessment of the phenotype. Fearful temperament was observed in one of the settings of the Lab-TAB procedure (Goldsmith et al., 1999), during the interaction of the child with a stranger. A broader and thus stronger observational assessment of temperamental fearfulness might show divergent results, and is advisable for future research.

Limitations

The current study is not well powered. Currently, samples exceeding 100,000 persons with GWA data are being published. Typically, these large studies from many countries investigate outcomes assessed using self-report questionnaires such as smoking or simple measures such as height or blood pressure. In contrast, one of the early GWAS, and perhaps the first to examine a behavioral or cognitive phenotype, had fewer than 500 participants (Papassotiropoulos et al., 2006). Arguably, standardized tests or observational measures such as the SSP or Lab-TAB may substantially reduce the numbers of participants needed (Ebstein, 2006; Rutter, 2006), as does the use of continuous measures instead of categorical disease measures derived from case control designs.

The current study lacked a replication sample. Most GWAS, in contrast to candidate gene studies, test the initial findings in an independent replication. Yet, many of the published studies only present data from a meta-analysis or a discovery sample without formal replication (Franke, Neal, & Faraone, 2009). In contrast, Weiss et al. (2008) was able to replicate findings with regard to autism both in an independent clinical and a population-

based sample. This underscores the need to collect standardized observational data across laboratories and countries and conduct GWAS in large age-restricted samples.

The effect sizes for common genetic variants studied in behavior or disease are typically modest. Only in a few instances, common genetic variants have been observed with effects sizes above a relative risk of 2 per allele (e.g., for APOE4 in Alzheimer's disease; Goedert & Spillantini, 2006, or CFH in age-related macular degeneration; Klein et al., 2005). However, most estimated effects are much smaller; increases in risk by a factor of 1.1 to 1.3 per associated allele are more typical (Altshuler et al., 2008). However, these effects are difficult to detect in smaller observational studies with fewer participants. It should be noted that in GWAS much less emphasis is given to effect sizes. Instead, *p* values are regarded as more informative. This is due to the fact that association signals identify small regions but not necessarily the functional SNP or even the causal gene.

Furthermore, the results of pathway analyses may depend on the *p* value cut-off chosen for the selection of genes related to the outcome. We used the most common cut-off ($<1x10^{-3}$), but less stringent cut-offs ($<5x10^{-3}$) have been used as well (Vink et al., 2009). It is worthy of note that when we applied a more stringent $p < 1x10^{-4}$ threshold for the SNPs of the GWAS to qualify for the gene-pathway analyses in PANTHER, none of the pathways were significantly enriched anymore. This holds equally for attachment disorganization, security, and temperament.

Lastly, a potential disadvantage of the pathway approach is that prior knowledge in correctly assigning genes to a certain pathway is crucial. The majority of genes in the genome are still relatively unknown; their biological function needs to be established and tied to a specific pathway. For example, the CACNA2D3 gene, which showed the strongest observed association, has no pathway information available in PANTHER 6.1. This implies that none of the observed pathway findings was driven by this single association. Because pathway tools make use of functional information from gene and protein databases, they are biased toward well-studied genes, interactions, and pathways. Moreover, genes may have diverse functions and this might not always be reflected in pathway assignment. The knowledge of gene function changes by the day, and hence pathway databases are updated regularly. The updates and further specifications of pathways provide more information, but at the same time make pathway analyses incomparable if they are conducted with different databases or different versions of the same software.

In summary, we conducted a combined genome wide and pathway analysis study of attachment and temperament in the largest racially and socio-economically homogeneous attachment and temperament sample of children available to date. This study represents the first step in an effort to use state of the art gene analysis tools in child attachment and temperament research, and goes beyond the usual genetic suspects to expand our knowledge on the underlying genetics of individual differences in attachment and temperament. We hope that our approach invites researchers in the field of child development to pool their samples in an attempt to replicate and extend our preliminary findings.

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CHAPTER 5

EMOTION RECOGNITION, TEMPERAMENT, AND BEHAVIOR PROBLEMS IN PRESCHOOL CHILDREN

CHAPTER 5.1

THE ASSOCIATIONS OF INTERNALIZING AND EXTERNALIZING PROBLEMS WITH FACIAL EXPRESSION RECOGNITION IN PRESCHOOLERS

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ABSTRACT

Altered patterns of facial expression recognition (FER) have been linked to internalizing and externalizing problems in school children and adolescents. In a large sample of preschoolers (N = 727), we explored concurrent and prospective associations between internalizing/externalizing problems and FER. Internalizing/externalizing problems were rated by parents at 18 and 36 months using the Child Behavior Checklist. FER was assessed at 36 months using age-appropriate computerized tasks of emotion-matching and emotion-labeling. Internalizing problems were associated with emotion-specific differences at both ages: at 18 months they predicted more accurate labeling of sadness; at 36 months they were associated with less accurate labeling of happiness and anger. Externalizing problems at both ages were associated with general FER deficits, particularly with regard to matching emotions. Findings suggest that in young children, internalizing problems contribute to emotion-specific differences in FER, while externalizing problems tend to be associated with more general FER deficits. Knowledge of the specific FER patterns associated with internalizing/externalizing behavior can prove useful in the refinement of emotion-centered preventive interventions.

INTRODUCTION

The ability to accurately recognize facial expressions of emotion is important for human social interaction. It is particularly significant in very young children, who particularly rely on facial expression recognition (FER) when communicating with parents, siblings, and peers. Deficits in FER are already evident in childhood for a wide range of disorders such as anxiety, depression, autism spectrum disorder, bipolar disorder, and severe mood dysregulation (Guyer et al., 2007; Monk, 2008). Emotion-specific deficits in FER have been associated with particular symptoms of psychiatric disorder in adults (Phillips, Drevets, Rauch, & Lane, 2003) and children (Guyer et al., 2007). For instance, symptoms of depression have been linked to biases in processing happy and sad facial expressions (Leppänen, 2006; Surguladze et al., 2004), anxiety has been linked to a hypersensitivity to threatening stimuli such as anger or fearful expressions (Bar-Haim et al., 2007; Waters, Henry, Mogg, Bradley, & Pine, 2010), and psychopathy has been linked to an insensitivity to sad and fearful expressions (Blair, Colledge, Murray, & Mitchell, 2001; Marsh & Blair, 2008). Nevertheless, it is still unclear whether such emotion-specific biases in processing facial expressions are evident when considering more common internalizing and externalizing behavior in the general population that may fall below a clinical threshold or diagnosis.

Earlier studies have provided some evidence for general FER deficits in relation to more common internalizing and externalizing problems in middle childhood and adolescence. In a community sample of seven-year olds, poor emotion knowledge (a combined measure of FER and emotion situation knowledge) was related to teacher-reported concurrent depressive symptoms and social withdrawal (Schultz, Izard, Ackerman, & Youngstrom, 2001). In addition, poor emotion knowledge at age seven was also found to be predictive of self-reported internalizing problems at age 11 years in an economically disadvantaged sample (Fine, Izard, Mostow, Trentacosta, & Ackerman, 2003). In a community sample of three- to four-year olds, general emotion knowledge deficits predicted subsequent years' aggressive behavior (Denham et al., 2002). In a large cohort of school-age children, antisocial behavior problems were related to less accurate decoding of happy and sad faces at low intensities and to the misidentification of fear as sadness (Bowen & Dixon, 2010). In an adolescent community sample, Blair and Coles (2000) reported inverse relationships between psychopathic traits (manifested as conduct problems or callous-unemotional traits) and the recognition of sad and fearful facial expressions. Finally, there are also studies that failed to show relations between children's emotion knowledge and internalizing problems (Lancelot & Nowicki, 1997) or externalizing problems (Egan, Brown, Goonan, Goonan, & Celano, 1998; Izard et al., 2001).

However, the studies examining FER in relation to internalizing and externalizing behavior problems presented above tend to differ on a number of levels, which likely contributes to the lack of coherence or clarity in terms of whether there is evidence for deficits in FER in relation to internalizing or externalizing behavior problems, and whether any deficits or biases that emerge are general or emotion-specific. Many of these investigations have focused on middle childhood and adolescence. However, effects may vary according to

sample composition, developmental period, and rater (for review, see Trentacosta & Fine, 2010). In addition, divergence in task paradigms or stimulus material may also explain heterogeneous results (Dalgleish et al., 2003; Easter et al., 2005; Egan et al., 1998; Guyer et al., 2007). Previous research has shown that FER tasks which rely to a greater or lesser extent on verbal or visuospatial abilities may yield different results, which may in part reflect the differing underlying development of these processes (Vicari, Reilly, Pascualetti, Vizzotto, & Caltagirone, 2000). Thus, the question remains how specific aspects of FER are related to childhood internalizing and externalizing problems. Finally, some earlier studies have tended to collapse the recognition of individual emotions into a global emotion knowledge measure. Therefore, it is still unclear whether specific internalizing or externalizing syndromes are associated with recognition biases for particular emotions (Trentacosta & Fine, 2010).

Most previous studies in this area, with rare exceptions, rely on cross-sectional designs, which are not suitable to establish temporal relations. In one study, depressive symptoms in girls between ages 9 and 12 years were associated with poorer memory for emotional faces at age 12 years (Guyer, Choate, Grimm, Pine, & Keenan, 2010). No previous study to our knowledge has examined whether internalizing and externalizing problems evident in early childhood may influence the development of FER in the preschool period. Studies that apply a prospective design to investigate whether early internalizing and externalizing problems can exert long-term effects on FER are warranted to unravel the complex temporal nature of this relationship.

Our current study extends the literature by: i) examining whether internalizing and externalizing behavior problems relate to FER in a large sample of very young children; ii) exploring whether particular syndrome subscales of internalizing and externalizing problems are associated with any emotion-specific differences in FER abilities of preschoolers; and iii) testing within a longitudinal framework whether early internalizing and externalizing problems can predict later FER patterns. Internalizing and externalizing problems were rated by parents at 18 and 36 months using the Child Behavior Checklist 1½-5 (CBCL; Achenbach & Rescorla, 2000). In order to examine specificity of associations, we also studied links between FER and the following three syndrome subscales: anxious/depressed, emotionally reactive, and aggressive behavior. FER was assessed at 36 months using a verbal emotion-labeling and a nonverbal emotion-matching task.

We hypothesized that at 36 months both internalizing and externalizing behavior problems would be associated with poorer overall accuracy on both FER tasks. Furthermore, based on previous literature, we expected that specific syndromes would be associated with distinct emotion-specific FER patterns. More specifically, in line with adult and adolescent literature on depression and anxiety, we anticipated that anxious/depressed children would demonstrate increased accuracy on negative emotions such as sadness and fear, whereas we expected reduced accuracy on recognizing happy expressions. Furthermore, we expected that children with aggressive behavior would likely demonstrate more general deficits in FER, since previous literature linking emotion-specific deficits to externalizing problems have focused on callous-unemotional traits, which represents a very specific subgroup of children

with externalizing behavior problems. Finally, we explored whether specific internalizing and externalizing problems very early in life (age 18 months) can predict FER accuracy at a later age (36 months). Although this hypothesis is exploratory given the lack of previous studies in this area, we anticipated that higher levels of internalizing and externalizing behavior problems at 18 months would be associated with less accurate FER ability at 36 months.

METHOD

Setting

The present investigation was conducted in a subsample of children participating in the Generation R Study, a population-based prospective Dutch cohort from fetal life onward (Jaddoe et al., 2010). The subsample, known as the Generation R Focus Cohort, is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity. All children were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort (Jaddoe et al., 2008). The study was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki and approved by the Medical Ethics Committee of the Erasmus Medical Center. Parental written informed consents were obtained for all participants.

Participants

We included data from the participants at two time points: at 18 and 36 months of age. Of the 1106 postnatally participating children, 908 had mother-rated CBCL data at 18 months. Of these children, 863 had mother-rated CBCL data and 819 had partner-rated (mainly fathers) CBCL data at 36 months. Of these, 740 children completed at least one emotion recognition task at 36 months in our research center. The final sample for analyses included 727 children who also had information on the covariates.

Predictors: internalizing and externalizing problems

The Child Behavior Checklist (CBCL) 1½-5 for toddlers (Achenbach & Rescorla, 2000) was used to obtain standardized parent reports of children's emotional and behavior problems. The checklist includes 99 items that are scored by the parent based on the previous 6 months on a three-point scale (not true, somewhat / sometimes true, and very true / often true). The reliability and the validity of the Dutch translation have been demonstrated (Tick, Van der Ende, & Verhulst, 2007). The CBCL was completed when children were 18 months of age by the mother and at 36 months by both parents. For subsequent analyses mother and father ratings at 36 months were averaged to reduce problems due to multiple testing and potential reporter bias. The CBCL Internalizing and Externalizing scales and three CBCL syndrome subscales were used in analyses. The Internalizing score was calculated by summing the following CBCL subscales: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn. The Externalizing score was calculated by summing the Attention Problems and Aggressive Behavior subscales. The three CBCL syndrome subscales included in analyses were Anxious/Depressed and Emotionally Reactive from the internalizing category

and Aggressive Behavior from the externalizing category. We focused on these specific syndromes, since earlier studies showed that they can be detected at clinically significant levels as early as 18 months of age (Côté et al., 2009; Côté et al., 2007; Tremblay et al., 2004).

Outcome: facial expression recognition (FER)

FER was assessed using a nonverbal emotion-matching task and a verbal emotion-labeling task. Color images of four basic emotions (happiness, sadness, anger, and fear) displayed by adult models were presented on a screen and children responded using a touch-sensitive monitor. Stimuli were selected from a widely used facial stimulus set, the NimStim, on the basis of which identities demonstrated the best recognized pose for a particular emotion-category (Tottenham et al., 2009). We chose open-mouth models for happy, angry and fearful expressions, and closed-mouth models for sad expressions. Since all our participants were of Caucasian origin, we only included Caucasian models in the present study. The FER tasks were carried out during a one hour visit to our research center when children were approximately 36 months old. Prior to the FER tasks, children were introduced to the idea of emotions and facial expressions by a trained experimenter. Children were seated comfortably at a child-size table in front of a computer screen. All children started with the emotion-matching task and continued with the emotion-labeling task. All computer tasks included a practice trial, which could be repeated up to 3 times in total if the child's answer was incorrect before the experimenter moved on to the test trials.

In the *emotion-matching task*, a target stimulus appeared first at the top of the screen for one second. This was followed by the appearance of the two choice stimuli at the bottom half of the screen, below the target stimulus. Children had to match the emotion of the target stimulus with one of the two choices (displayed by the same identity but different from the target stimulus). After the child's response the screen went blank for one second to clearly indicate the beginning of a new trial. If the child did not respond within ten seconds, the program automatically proceeded with the next trial. Sixteen trials of emotion-matching with two female and two male identity pairs, and four basic emotions were included. Stimuli presentation was counterbalanced and randomized for emotion and identity. Prior to the emotion-matching task, a *shape-matching task* was included in order to ensure that children understood the concept of matching and to control for the effects of basic matching ability. The shape-matching task had the same parameters and layout as the emotion-matching task, only children had to match geometrical shapes. A practice trial and four test trials with different target shapes were included in a fixed order. Similar paradigms have been used previously (Hariri, Bookheimer, & Mazziotta, 2000; Herba, Landau, Russell, Ecker, & Phillips, 2006).

In the *emotion-labeling task,* all four emotions (displayed by the same identity) appeared on the screen at the same time. After one second, children heard a voice-bit asking in a neutral voice which person was feeling happy, sad, angry or scared. Children were asked to point at the expression that matched this label. After the child's response the screen went blank for one second to clearly indicate the beginning of a new trial. If the child did not respond within ten seconds, the program automatically presented the next trial. Sixteen trials

were presented, four items for each of the four emotion-categories, using two female and two male identities. Stimuli presentation was counterbalanced and randomized for emotion and identity. A similar paradigm has previously been used by Fries and Pollak (2004). To control for the effects of general labeling ability, an *animal-labeling task* was included. This task was administered to children by their parent at age 30 months, six months preceding the FER assessments. Children were presented six black-and-white photos (4.5 x 4.5 cm) of animals on a white A4 sheet, and indicated which one of six pictures matched the name of an animal read out by a parent. There were six trials in total with a fixed stimulus order. Similar paradigms have been previously used by Widen and Russell (2003). All FER tasks and test procedures were described in detail elsewhere (Székely et al., 2011).

Statistical analysis

Descriptive data of the study population are presented in Table 1. CBCL scores of boys and girls at 18 and 36 months were compared. Effects of age, sex, and control tasks on emotion-labeling and emotion-matching accuracies were examined. All hypotheses were tested using repeated measures ANCOVAs. Separate analyses were run for the emotion-labeling and emotion-matching tasks. Main outcomes were accuracy scores (mean proportion correct) for labeling and matching happy, sad, angry, and fearful expressions. Predictors for our hypotheses on broader dimensions of internalizing and externalizing problems and FER were Internalizing and Externalizing scores at 36 months (for cross-sectional analyses) and at 18 months (for longitudinal analyses). Predictors for our hypotheses on the specific syndromes and FER were Anxious/Depressed, Emotionally Reactive, and Aggressive Behavior scores at 36 months and at 18 months. Analyses including the three syndrome subscales were seen as follow-up analyses to further specify the effect of the broader Internalizing and Externalizing scales. Therefore, results from these analyses were interpreted using Bonferroni adjustment for multiple testing ($\alpha = .05/3 = .017$). Although repeated measures models are primarily used to test equality of means of repeatedly measured or related outcomes across groups of a factor variable, they also provide effect estimates for continuous independent variables, which are entered as covariates. Thus, our continuous predictor variables were entered as covariates. The advantage of this, as opposed to running separate linear regressions, is that repeated measures models do take into account the relation between the outcomes. Between-subjects effects of the predictor variables on emotion-labeling and emotion-matching accuracies are reported in Table 2. Within-subjects effects of the predictor variables by emotion-category are reported in the text.

The interaction between the predictors and child sex was tested in all models. When this was not significant, we reported results from the model without the interaction term.

Covariates

All models were adjusted for child's age, sex, gestational age at birth, birth weight, maternal smoking during pregnancy (yes/no), and maternal education. Maternal education was coded as 'low' (primary education or no education), 'medium' (secondary education), or 'high' (college or university degree). Information on child's sex, gestational age at birth,

and birth weight was obtained from midwives and obstetricians. Information on maternal smoking during pregnancy and maternal education were collected via questionnaires. Furthermore, in order to ensure that children understood the basic requirements of the task, we adjusted for basic labeling ability (animal-labeling accuracy) when predicting emotion-labeling accuracy, and basic matching ability (shape-matching accuracy) when predicting emotion-matching accuracy. Basic labeling ability was assessed via a parent-completed questionnaire when children were 30 months of age, and children's age and basic matching ability were assessed during the lab visit at 36 months of age.

Response analysis

Children in the final sample (N = 727) were 0.46 weeks older (t (1104) = -3.41, p= .001) and 155.5 grams heavier (t (1104) = -4.10, p < .001) at birth than children not included in analyses (n = 379). In addition, mothers of our study participants were more highly educated (χ^2 (2) = 15.99, p < .001) and fewer of them smoked during pregnancy than mothers of nonparticipating children (χ^2 (1) = 7.21, p = .007). The two groups did not differ in terms of age and distribution of boys and girls (p > .05). At 18 months, participating children had lower Internalizing scores (t (956) = 2.25, p= .025) and lower Anxious/Depressed scores (t (959) = 2.77, p = .006) than nonparticipating children. At 36 months, participating and nonparticipating children had similar CBCL scores (p > .05).

RESULTS

Study population

Descriptive data are presented in Table 1. Boys tended to have higher Externalizing scores (at 18 months: t (725) = 2.70, p = .007; at 36 months: t (725) = 1.89, p = .058) and higher Aggressive Behavior scores than girls (at 18 months: t (725) = 2.46, p= .006; at 36 months: t (725) = 1.70, p= .090). Boys and girls did not differ in their Internalizing, Anxious/Depressed or Emotionally Reactive scores.

Mean accuracy scores and standard deviations on the emotion-labeling and emotion-matching tasks are presented in Table 1. As expected, more accurate animal-labeling predicted more accurate emotion-labeling (F (1, 723) = 21.84, p < .001) and more accurate shape-matching predicted more accurate emotion-matching (F (1, 705) = 31.32, p < .001). Despite the small age range, older children were more accurate at both labeling emotions (F (1, 723) = 20.85, p < .001) and matching emotions (F (1, 705) = 20.59, p < .001). Girls labeled emotions more accurately than boys (F (1, 725) = 7.59, p = .006), but they did not significantly differ from boys in matching emotions (F (1, 707) = 1.84, p = .175).

Main results

Main effects (between-subjects effects) of CBCL scores on overall emotion-labeling and emotion-matching accuracy are shown in Table 2. Emotion-specific effects of CBCL scores (within-subjects effects of CBCL scores by emotion-category) on emotion-labeling and emotion-matching accuracies are presented in the text.

Table 1. Sample characteristics

Sample characteristics (N = 727)	N	% or $M(SD)$
Sex (boys)	727	50.8
Age (months)	727	37.5 (1.45)
Gestational age at birth (weeks)	727	40.1 (1.69)
Birth weight (grams)	727	3523.9 (539.3)
Maternal education (high)	727	66.8
Mother smoked during pregnancy (yes)	726	20.2
Emotion-labeling accuracy		
Happiness	727	0.53 (0.34)
Sadness	727	0.51 (0.33)
Anger	727	0.55 (0.36)
Fear	727	0.44 (0.33)
Emotion-matching accuracy		
Happiness	727	0.66 (0.29)
Sadness	718	0.59 (0.29)
Anger	709	0.62 (0.29)
Fear	727	0.65 (0.29)
Control task accuracy		
Animal-labeling	727	0.94 (0.11)
Shape-matching	727	0.90 (0.21)

Internalizing/externalizing problems at 36 months and FER: cross-sectional analyses

Broadband scales. There was a significant main effect of Internalizing scores on both emotion-labeling and emotion-matching (Table 2). Higher Internalizing scores were associated with lower overall FER accuracy. In addition, we found a significant interaction effect between Internalizing scores and emotion-category on the emotion-labeling task (F (2.96, 2123.38) = 3.14, p = .025). Higher Internalizing scores were specifically associated with less accurate labeling of happy faces (B = -0.03, SE = 0.01, p= .022) and angry faces (B = -0.06, SE = 0.01, p < .001).

The association of Externalizing scores with emotion-matching accuracy was also significant, whereas with emotion-labeling accuracy it showed a statistical trend (Table 2). In general, higher Externalizing scores were associated with lower overall FER accuracy. Externalizing scores had no emotion-specific effects on FER accuracy (p > .05).

Syndrome scales. Anxious/Depressed scores were associated with both emotion-labeling and emotion-matching accuracy (Table 2). Higher Anxious/Depressed scores were related to lower overall accuracy on both tasks. In addition, Anxious/Depressed scores had an emotion-specific effect on emotion-labeling (F (2.96, 2122.91) = 3.28, p = .021), although this association was slightly above significance threshold after applying Bonferroni correction for

Table 2. Concurrent and longitudinal associations between behavior problems and emotion-labeling and emotion-matching accuracies of preschoolers

	Emotion-labeling		Emotion-matching	
	F (df1, df2)	*p*	*F* (df1, df2)	*p*
Behavior problems				
Age 36 months				
Broadband scales				
Internalizing	9.77 (1, 717)	.002	7.33 (1, 630)	.007
Externalizing	3.08 (1, 717)	.080	6.96 (1, 630)	.009
Syndrome scales				
Anxious/Depressed	9.16 (1, 717)	.003	8.73 (1, 630)	.003
Emotionally Reactive	3.31 (1, 717)	.069	6.31 (1, 630)	.012
Aggressive Behavior	3.97 (1, 717)	.047	6.07 (1, 630)	.014
Age 18 months				
Broadband scales				
Internalizing	0.62 (1, 718)	.431	3.04 (1, 634)	.081
Externalizing	1.94 (1, 721)	.164	4.46 (1, 637)	.035
Syndrome scales				
Anxious/Depressed	2.19 (1, 721)	.139	0.23 (1, 637)	.633
Emotionally Reactive	0.26 (1, 719)	.613	3.62 (1, 635)	.057
Aggressive Behavior	1.25 (1, 721)	.264	5.26 (1, 637)	.022

Note. Models were adjusted for child's age, sex, basic labeling/matching ability, gestational age at birth, birth weight, maternal smoking during pregnancy, and maternal education.

multiple testing ($p < .017$). Similar to Internalizing scores, children with higher Anxious/Depressed scores were less accurate at labeling happy faces ($B = -0.04$, $SE = 0.01$, $p = .014$) and angry faces ($B = -0.06$, $SE = 0.02$, $p < .001$). Regression coefficients (*B*s) of Anxious/Depressed scores on emotion-labeling accuracy at both ages are depicted in Figure 1. This figure is also representative of the relations between Internalizing scores and emotion-labeling accuracy, since the same pattern was observed for Internalizing scores at both ages.

There was a significant association between Emotionally Reactive scores and emotion-matching accuracy, but Emotionally Reactive scores were not significantly related to emotion-labeling accuracy ($p > .017$)). Children with higher Emotionally Reactive scores generally matched emotions less accurately. There were no emotion-specific effects of this syndrome scale on FER accuracy ($p > .017$).

Finally, Aggressive Behavior scores were associated with overall emotion-matching accuracy, but not emotion-labeling accuracy (Table 2). Children with higher Aggressive Behavior scores matched emotions overall less accurately. There were no emotion-specific effects of this syndrome scale for emotion-labeling or emotion-matching ($p > .017$).

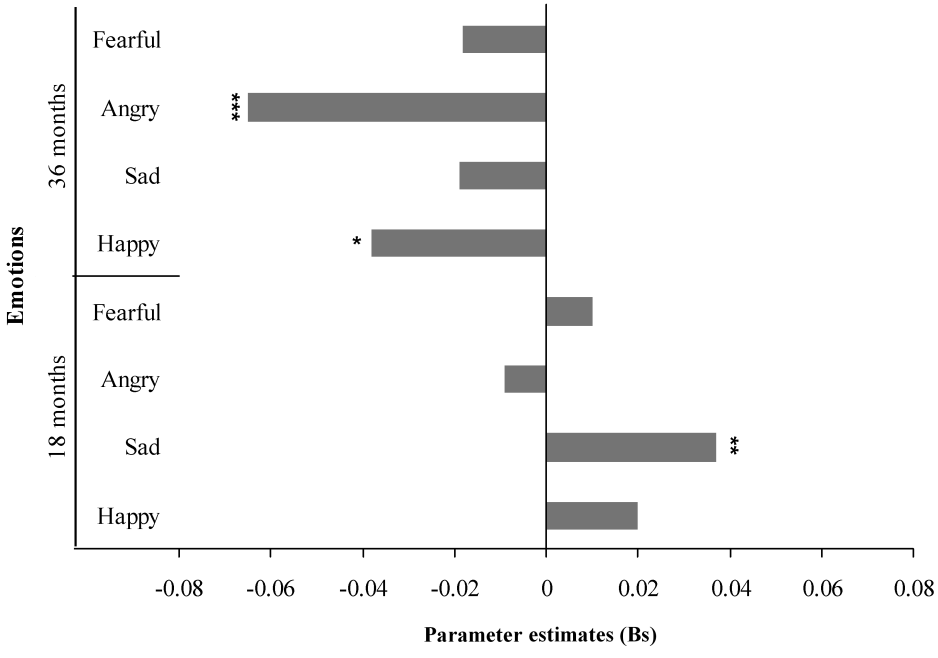

Figure 1. Concurrent and longitudinal associations between anxious/depressed problems and labeling different emotions. Regression coefficients are parameter estimates (*B*s) from repeated measures ANCOVAs. Models were adjusted for child's age, sex, basic labeling ability, gestational age at birth, birth weight, maternal smoking during pregnancy, and maternal education.
* $p < .05$, ** $p < .01$, *** $p < .001$.

Internalizing/externalizing problems at 18 months and FER: longitudinal analyses

Broadband scales. Internalizing scores at 18 months did not predict overall emotion-labeling or emotion-matching accuracy (Table 2). Rather, Internalizing scores were associated with emotion-specific differences in labeling accuracy (F (3, 716) = 4.83, p = .002). In particular, higher Internalizing scores predicted more accurate labeling of sad faces (B = 0.03, SE = 0.01, p = .005). This association remained significant even after adjusting for concurrent Internalizing scores, i.e. Internalizing scores at 36 months.

Externalizing scores at 18 months were not predictive of emotion-labeling accuracy, but higher Externalizing scores at 18 months predicted lower overall emotion-matching accuracy at 36 months (Table 2). Contrary to findings for Internalizing scores, Externalizing scores did not significantly predict any emotion-specific patterns in FER accuracy ($p > .05$).

Syndrome scales. Anxious/Depressed scores did not predict overall emotion-labeling or emotion-matching accuracy ($p > .017$). However, the interaction of Anxious/Depressed scores by emotion was significant for emotion-labeling (F (3, 719) = 3.31, p = .002). Higher Anxious/Depressed scores at 18 months predicted better labeling of sad faces at 36 months (B = 0.04, SE = 0.01, p = .004; Figure 1). This association remained significant even after

adjusting for Anxious/Depressed scores at 36 months. Figure 1 illustrates the effect of Anxious/Depressed scores at 18 and 36 months on labeling accuracy for each emotion.

Emotionally Reactive scores at 18 months did not predict overall emotion-labeling or emotion-matching accuracy, neither they predicted any emotion-specific differences in labeling or matching accuracy ($p > .017$).

Aggressive Behavior scores at 18 months did not predict emotion-labeling accuracy but did predict emotion-matching accuracy. Higher Aggressive Behavior scores were related to lower overall accuracy for matching emotions (Table 2), however, this association was not significant anymore after applying Bonferroni-correction for multiple testing ($p > .017$). Aggressive Behavior scores at 18 months did not predict emotion-specific differences on either FER task ($p > .017$).

5.1

DISCUSSION

This study sought to examine both concurrent and longitudinal associations between internalizing and externalizing problems and facial expression recognition in a large community-based sample of preschool children.

Internalizing problems and FER

Children with higher scores for internalizing problems at 36 months, in particular those with anxious/depressed problems, demonstrated a lower overall accuracy for both labeling and matching facial expressions at 36 months of age. Most interestingly, and consistent with our hypotheses, concurrent and longitudinal investigations revealed that more general internalizing problems and the more specific syndrome of anxious/depressed problems were associated with emotion-specific differences in FER. Higher Internalizing and Anxious/Depressed scores at 18 months predicted increased accuracy in labeling sad faces at 36 months. Higher Internalizing and Anxious/Depressed scores at 36 months were associated with reduced accuracy for labeling happy and angry faces.

The literature on emotion knowledge and internalizing problems is quite inconsistent. Some studies reported overall deficits (Walker, 1981), while others reported emotion-specific deficits (Lenti, Giacobbe, & Pegna, 2000) or no association between emotion knowledge and internalizing problems (Lancelot & Nowicki, 1997). These studies included very different internalizing problems such as depression and dysthymia (Lenti et al., 2000), social phobia (Simonian, Beidel, Turner, Berkes, & Long, 2001), and social withdrawal (Eisenberg et al., 2001). In addition, they represented a mix of community and clinical samples of mainly older children and adolescents (Trentacosta & Fine, 2010).

Our findings indicated that at both 18 and 36 months, emotion-specific effects of internalizing problems were mostly explained by anxious/depressed problems. This finding is not surprising in the context of relevant literature on information processing biases in anxiety and depression. According to this literature, anxiety and depression are characterized by specific information processing biases for certain emotions. For instance, heightened sensitivity toward negative emotional stimuli (e.g., sad faces) is well documented

in depression (Hankin, Gibb, Abela, & Flory, 2010). Ellis et al. (1997) found that children (7-16 years) with mood disorders recognized sad faces better than children without mood disorders, and this was even more pronounced in younger participants. Joormann, Talbot, and Gotlib (2007) found that daughters at elevated risk for depression selectively attended to sad faces, while control daughters selectively attended to happy faces. There is also evidence for difficulties in identifying positive (e.g., happy) facial expressions in depression (Surguladze et al., 2004; Joormann & Gotlib, 2006). Attention biases associated with the processing of threat-related information (e.g., anger) are typical in anxiety (for review, see Lau & Pine, 2008). Most studies in anxious youth found elevated attention toward threatening stimuli (Telzer et al., 2008; Ehrenreich & Gross, 2002), but attentional avoidance of threat was also reported (Stirling, Eley, & Clark, 2006). Within our community sample of very young children, we found consistent FER patterns which were detectable very early in life. More specifically, we showed that anxious/depressed problems were associated with increased labeling accuracy for negative (sad) expressions and decreased labeling accuracy for positive (happy) and threatening (angry) expressions. This pattern was consistent across ages and it suggests the existence of altered emotion-specific recognition patterns in children with anxious/depressed problems. Although the effect of anxious/depressed problems on a particular emotion was not always significant at both ages, it is important to note the relative similarities in patterns of labeling accuracy across emotions at 18 and 36 months. These similarities especially concern the relations between labeling sadness and anger. Children with increasing Anxious/Depressed scores at 18 months labeled sadness the best and anger the worst. Children with increasing Anxious/Depressed scores at 36 months still labeled sadness relatively well and anger the worst. Thus, the relative difference between labeling sadness and anger was the same at both ages, only, children with concurrent anxious/depressed problems performed slightly worse for all emotions.

Externalizing problems and FER

Concurrent externalizing problems and aggressive behavior were associated with less accurate emotion-labeling and emotion-matching, although associations were weaker for emotion-labeling. Longitudinally, externalizing problems and aggressive behavior predicted less accurate emotion-matching. Although the effect of aggressive behavior on emotion-matching was not significant anymore after applying Bonferroni correction for multiple testing, its direction was consistent with that observed for externalizing problems.

These findings are in accordance with studies observing general emotion knowledge deficits in children with externalizing problems (Trentacosta & Fine, 2010) and aggressive behavior (Denham et al., 2002), and extend the literature by showing that externalizing problems are also predictive of later FER deficits, even after adjusting for concurrent externalizing behavior. A handful of studies have demonstrated emotion-specific FER deficits in children with externalizing problems. For instance, in a nonclinical sample of adolescents antisocial behavioral traits were inversely related to recognizing distress cues such as sadness and fear, but not other emotions (Blair & Coles, 2000). In community samples of children and

adolescents, Dadds et al. (2006) demonstrated that psychopathic traits were related to specific deficits in the recognition of fear. However, studies demonstrating associations between externalizing problems and emotion-specific deficits have tended to select a very specific subgroup of children: those who scored high on psychopathic traits, in particular callous-unemotional traits. Callous-unemotional traits are important in the diagnosis of psychopathy (Frick & Hare, 2001; Frick & Viding, 2009), and children high on these specific traits form only a very small subsample of individuals with antisocial behavior or those with elevated externalizing behavior problems. Thus, our lack of emotion-specific effects in the context of our present study is not inconsistent with this literature. In clinical samples which included children with more diverse externalizing problems, FER deficits were typically observed on a more general scale. For instance, Walker and Leister (1994) reported that adolescents with a clinical diagnosis of externalizing behavior (mainly conduct disorders, oppositional disorders, and impulse control problems) recognized a wide range of emotional expressions (i.e., happy, sad, angry, scared, and surprised) less accurately than non-disordered adolescents. The only emotion which was equally well recognized by the two groups was disgust. According to the authors, adolescents with externalizing problems may have learnt to become more aware of disgust cues as a result of repeated exposure from disapproving peers and adults.

It is also plausible that children who score more highly on externalizing problems may experience more difficulties with attending to the task at hand, thus, demonstrating general deficits in performance. Although we cannot completely rule out this possibility, it is important to bear in mind that early externalizing problems were also predictive of overall FER deficits, even after adjusting for concurrent externalizing behavior. In addition, all analyses were adjusted for basic matching or labeling ability. These measures were specifically included in analyses to ensure that children understood the basis concepts of the task and were able to perform the basic requirements of the task. Particularly in the case of emotion-matching (where we found the strongest evidence for externalizing problems to be associated with decreased accuracy for matching emotions), the shape-matching task was administered on the computer immediately prior to the emotion-matching task. If children were unable to attend to this kind of computer task, or unable to understand the concept of matching, then we would expect a low performance on this control task, which was not the case.

It is also important to note that children's performance differed on the emotion-labeling and emotion-matching tasks. Current internalizing problems were associated with global FER deficits on both tasks, whereas emotion-specific differences related to early and current internalizing problems consistently emerged for emotion-labeling. The emotion-matching task emerged as more sensitive than emotion-labeling for capturing global FER deficits in relation to externalizing problems. Task paradigms similar to our emotion-matching task consistently engaged the amygdala across various populations and experimental designs (Hariri, Drabant, & Weinberger, 2006). The amygdala facilitates identification and detection of emotionally salient stimuli such as facial expressions (Thomas et al., 2001). The emotion-labeling task has a greater reliance on attention processes due to its elements of cognitive appraisal and labeling, and therefore produces higher prefrontal cortical activation in the

presence of dampened amygdala activation (Blair et al., 2007; Hariri, et al., 2000; Hariri, Mattay, Tessitore, Fera, & Weinberger, 2003; Phillips et al., 2004). Prefrontal structures modulate emotional behavior, which is mediated by the amygdala, through influencing attention processes (Monk et al., 2008; Sprengelmeyer, Rausch, Eysel, & Przuntek, 1996). Since emotion-specific attention biases are consistently implicated in internalizing problems such as anxiety and depression, we believe that our emotion-labeling task was more sensitive to detect any resulting emotion-specific differences in recognition accuracy than our nonverbal emotion-matching task.

Our study is the first attempt to explore the long-term effects of early internalizing and externalizing problems (at 18 months and 36 months) on FER (at 36 months) using a large-scale longitudinal sample. Despite the strengths, we were faced with some methodological limitations. The main limitation concerns the lack of available FER data at 18 months. At that age, children would have been too young to complete our FER tasks, and using a different paradigm at 18 months would have complicated the comparison of our results. This limitation prevented us from studying reverse causality. Consequently, we can not exclude the possibility that internalizing and externalizing problems at 18 months resulted from FER differences which were already present at that age. Nevertheless, this study succeeded in exploring the long-term effects of internalizing and externalizing problems assessed as early as 18 months on FER at 36 months even after adjusting for concurrent internalizing and externalizing problems (at 36 months). Another limitation is that FER accuracy was assessed for full intensity expressions, and we did not present children with more subtle displays of emotions (i.e., lower intensities of facial expressions). In real life, facial expressions are rarely displayed at their maximum intensity. Using stimuli of varying emotional intensity may be an important and more realistic method of examining developing FER abilities (Herba & Phillips, 2004). However, previous studies have demonstrated that neural circuits involved in emotion processing are consistently engaged in response to photos depicting full intensity facial expressions (Adolphs, 2002; Hariri et al., 2000). Furthermore, the use of lower emotional intensities likely becomes more important in case of older children, who may show ceiling-level performance at recognizing full intensity facial expressions. This was not the case with our young, preschool participants.

Conclusion

Our results suggest a qualitatively different relationship between internalizing and externalizing problems and children's FER. In the case of internalizing problems, emotion-specific patterns emerged, particularly for children scoring high on anxious-depressed symptoms such that these children tended to be more accurate at recognizing sadness, and less accurate at recognizing happiness or anger. Externalizing behavior problems on the other hand, were associated with more general (i.e., less emotion-specific) FER deficits. Furthermore, longitudinal links were evident such that internalizing and externalizing problems at age 18 months were predictive of later FER accuracy, even after adjusting for concurrent internalizing or externalizing behavior problems. A better understanding

of the associations between internalizing and externalizing problems and FER early on is important for identifying subtypes of children who might be particularly at risk for developing psychiatric disorders.

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CHAPTER 5.2

POSITIVE EMOTIONALITY, EXECUTIVE FUNCTIONING, AND INTERNALIZING PROBLEMS IN YOUNG CHILDREN

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ABSTRACT

Objectives: Temperament and psychopathology are intimately related; however, findings on the relation between positive emotionality, defined as the child's pleasure or excitement in social interactions, and psychopathology are inconclusive. We aimed to examine the longitudinal relation between low positive emotionality and internalizing problems in young children from the general population. Furthermore, we explored whether executive functioning mediates any observed association. **Methods:** Within the Generation R Study, a Dutch population-based prospective birth cohort, we observed positive emotionality in 802 children using the Laboratory Temperament Assessment Battery at age three years. Internalizing problems (Emotionally Reactive, Anxious/Depressed, and Withdrawn) were assessed at age six years using the Child Behavior Checklist. Parents rated executive functioning at age four using the Behavior Rating Inventory of Executive Function. Association analyses between positive emotionality and internalizing problems were explored using multiple logistic regressions. We performed mediation analyses using bootstrap methods. **Results:** Children with a higher observed positive emotionality at age three had a lower risk for Withdrawn problems at age six years (*Odds ratio* = 0.82 per standard deviation increase in positive emotionality score, 95%*CI*: 0.69, 0.98). This effect was not explained by preexisting internalizing problems. Part of this association was mediated by fewer problems in the shifting domain of executive functioning ($p < 0.001$). We did not find any relation between positive emotionality and Emotionally Reactive or Anxious/Depressed scales. **Conclusion:** In the present study, we demonstrated a link between low positive emotionality in preschoolers and Withdrawn problems at a later age. Our findings indicate a partial role for executive functioning, particularly the shifting domain, in this association. In sum, low positive emotionality in the preschool period may lead to inflexibility and rigidity under new circumstances, which in turn will likely affect the child's desire to engage with the environment, leading to withdrawn behavior.

INTRODUCTION

Temperament is defined as biologically based individual differences in affect, activity, attention, and self-regulation (Caspi & Shiner, 2010). These individual differences can be influenced by genetic factors, maturation, and experience, but are relatively stable over time (Rothbart & Bates, 2006). During early childhood, three dimensions are identified as core constructs in most temperament models: *surgency/extraversion*, *negative affectivity*, and *effortful control* (Kagan, 1997; Rothbart & Bates, 2006). Extraversion or positive emotionality refers to differences between individuals to express joy and exuberance in the form of positive facial expressions, vocalizations, or motor acts, and represents the child's willingness to engage in social interactions. Children high on negative emotionality or neuroticism are described as fearful, sad, vulnerable, or anxious. Effortful control taps children's individual differences to sustain attention, and to regulate and plan behavior.

Much of the interest has focused on the interplay between temperament and concurrent and prospective psychopathology. Existing literature suggests a relation between temperament and psychopathology; however, the nature of this relation is unclear (Caspi & Shiner, 2010). Temperamental traits may predispose individuals to psychopathology (vulnerability hypothesis), psychopathology may be the extreme presentation of a temperamental trait that exist across a continuum (spectrum hypothesis), or they may share a common cause (Caspi & Shiner, 2010; Widiger & Smith, 2008). Temperament may also change the presentation and course of an existing psychopathology (Caspi & Shiner, 2010). There is clear evidence that children with high negative emotionality are at higher risk for developing internalizing problems such as depression, anxiety, or social withdrawal (Baker, Baibazarova, Ktistaki, Shelton, & Van Goozen, 2012; Crawford, Schrock, & Woodruff-Borden, 2011; Dougherty et al., 2011; Eisenberg et al., 2001). Similar findings have been reported in children low on effortful control (Crawford et al., 2011; Eisenberg et al., 2001; Oldehinkel, Hartman, Ferdinand, Verhulst, & Ormel, 2007). In contrast, findings on the relation between low positive emotionality and risk for future psychopathology are inconclusive. Some studies suggest that positive emotionality is specifically implicated in depression (Clark, Watson, & Mineka, 1994; Lonigan, Hooe, David, & Kistner, 1999; Shankman et al., 2005). Young children with low positive emotionality show more symptoms of depression (Dougherty et al., 2011; Oldehinkel, Hartman, De Winter, Veenstra, & Ormel, 2004). However, the link between positive emotionality and other types of psychopathology in childhood is unclear (Healey, Brodzinsky, Bernstein, Rabinovitz, & Halperin, 2010).

Many factors influence the complex relation between temperament and psychopathology, among those are parenting and stressful life events (Durbin, Klein, Hayden, Buckley, & Moerk, 2005; Feldman et al., 2009). The quality of parenting, in particular insensitive parenting, is associated with both temperament and psychopathology in the offspring. Some researchers postulated that higher order cognitive processes, known as executive functioning, might also underlie the link between temperament and development of psychopathology (Dragan, Dragan, Kononowicz, & Wells, 2011; Healey et al., 2010). Previous studies established a relation between early childhood temperament and executive functioning, mainly working

memory and inhibition, using evidence from brain electrical activity, parental reports, and children's performance in a laboratory setting (Blair, 2002; Wolfe & Bell, 2007). Similarly, Hongwanishkul, Happaney, Lee, and Zelazo (2005) reported a negative association between positive emotionality and working memory, but not with other domains of executive function. In 74 preschool children, Healey el al. (2010) showed that the relation between negative emotionality and child's internalizing/externalizing problems was mainly mediated by verbal executive functioning (i.e., language and memory). Although, in general, executive functioning is a process of higher order cognition, domains of executive functioning may act differently in the association between temperament and psychopathology.

Establishing a single one to one relation between a temperamental trait and a certain psychopathology is likely inadequate to explain the complex relation between the two; however, exploring the longitudinal relation between positive emotionality and psychopathology with consideration of the possible intermediate mechanisms, would contribute to a better understanding of the nature of this association. Based on this background, we thought to extend the literature by examining the longitudinal relation between positive emotionality and internalizing problems in a large sample of young children from the general population. Furthermore, we aimed to explore whether specific aspects of executive function may play a role in this relation. We hypothesized that children's positive emotionality at a young age could predict later internalizing problems, independent of preexisting internalizing problems. Moreover, we anticipated that executive functioning is one possible pathway through which positive emotionality predisposes the child to developing internalizing problems. To minimize shared-method bias common to studies relying solely on parental reports, we measured positive emotionality at age three years using laboratory-based observations. Parents reported on children's internalizing problems at six years and executive functioning at age four years.

METHOD

Participants

The present investigation pertained to a subsample of children participating in the Generation R Study, a population-based prospective Dutch cohort from fetal life onward (Jaddoe et al., 2010). The subsample, known as the Generation R Focus Cohort, is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity. All children were born between February 2003 and August 2005 and form a prenatally enrolled birth cohort (Jaddoe et al., 2008). The study was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki and approved by the Medical Ethics Committee of the Erasmus Medical Center. Parental written informed consents were obtained for all participants, and confidentiality was guaranteed.

Of the 1106 postnatally participating children, 862 visited our research center with an accompanying parent at age three years (mean age = 38±2 months, age range = 35 - 49 months). Positive emotionality was successfully observed in our laboratory in 802 children.

In total, 304 children were excluded from all analyses either because they dropped out of the study before the lab visits took place, or because their laboratory assessments were not useable. These children were more likely to have younger mothers (mean difference: -0.9, t = -3.5 p = 0.001) or lower educated mothers (21.0% versus 7.6% lower educational levels, x^2 = 39.8 (2), p < 0.001). At age six years, information on internalizing problems was available in 721 children (90 % of 802). At age four years, information on executive functioning was available in 714 children (89 % of 802).

Positive Emotionality

Positive emotionality was assessed using the Popping Bubbles (Preschool Version) and the Puppet Game (Locomotor Version) episodes from the Laboratory Temperament Assessment Battery (Lab-TAB; Goldsmith Reilly, Lemery, Longely, & Prescott, 1995; Goldsmith & Rothbart, 1999). The Lab-TAB is a widely used, standardized instrument for observational assessment of early temperament.

In the *Popping Bubbles* episode, the child engages in the pleasurable activity of blowing and popping bubbles. This episode is divided into a low pleasure phase (blowing bubbles) and a high pleasure phase (popping bubbles), and consists of 11 epochs for coding. Peak intensity of smiling, presence of laughter, and vigor of approach were coded in each epoch.

The *Puppet Game* episode measures enjoyment in response to social stimulation. The experimenter performs a standard dialogue in an animated, lively fashion with two hand puppets. During the dialogue, the child is tickled by the puppets at midsection. If the child becomes distressed, tickling takes place further away from the child, or the puppets pretend to tickle each other. In the end, the child is allowed to play with the puppets for 30 seconds. The episode is divided into 5 epochs for coding. Peak intensity of smiling, presence of laughter, positive vocalizations, and positive motor acts were coded in each epoch.

Episodes were coded according to the Lab-TAB manual. For each parameter, average scores were calculated by dividing the summed score for that parameter across the number of epochs in the episode. Regular checks were conducted to ensure that episodes followed the procedure described in the manual. Coders were extensively trained and reliability was established before data were coded. The mean Intraclass Correlation Coefficients (ICCs, single measures) for the average scores were 0.74 (range 0.71 - 0.75, n=25) for the Popping Bubbles episode, and 0.84 (range 0.66 - 0.95, n=25) for the Puppet Game episode. For each episode, a positive emotionality composite was created by taking the mean of the standardized average scores of all parameters in the episode. The two positive emotionality composites were moderately correlated (r = 0.33, p < 0.001). Therefore, two composite scores were combined into one overall '*positive emotionality*' composite, ranging from 0 to 1 with higher scores indicating higher positive emotionality.

Internalizing Problems

When children were six years old (mean age = 70±2 months, range 61 - 86 months), the Child Behavior Checklist/1½-5 (CBCL/1½-5) was used to obtain a standardized parental-rating of children's emotional and behavior problems (Achenbach & Rescorla, 2000).

Although some children were older than 6 years at the time of the assessment (23%), we used the version CBCL/1½-5 in all children for the reason of comparability. The CBCL/1½-5 contains 99 problem items, scored on seven empirically based syndromes which were derived by factor analyses: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems, and Aggressive Behavior. The broadband scale *Internalizing* is the summed score of items in the first four syndrome scales. We chose to study three syndrome scales of the internalizing broadband scale: *Emotionally Reactive*, *Anxious/Depressed*, and *Withdrawn*. Higher scores indicate more problems. The reliability and validity of the Dutch version of the CBCL/1½-5 has been demonstrated previously (Tick, van der Ende, Koot, & Verhulst, 2007).

Children's internalizing problems were also assessed at 1½ years using the CBCL/1½-5 (mean age = 18±1, range 17 - 24 months). To rule out preexisting internalizing problems accounted for any observed association between positive emotionality at age three and internalizing problems at age six (reverse causality), all analyses were adjusted for the internalizing scores at 1½ years.

Executive Function

We used the Behavior Rating Inventory of Executive Function-Preschool Version (BRIEF-P; Gioia, Espy, & Isquích, 2003) to measure executive functioning at age four years (mean age = 49±1 months, range 47 - 60 months). The BRIEF-P is a parent-completed questionnaire with 63 items which assesses child's ability in five related but non-overlapping theoretically and empirically derived clinical scales: *inhibition* (16 items), to stop his/her own behavior; *shifting* (10 items), to change focus from one mindset to another; *emotional control* (10 items), to modulate emotional responses; *working memory* (17 items), to hold information in mind for the purpose of completing a task; *planning/organization* (10 items) to manage current and future-oriented task demands within the situational context. The clinical raw scores yield *T* scores based on gender and age. Higher scores indicate more problems.

The BRIEF-P measures executive functioning within everyday life setting, and does not have the limitations of performance-based tests and environmental effect during the administration. The subscales of BRIEF-P show adequate to high test-retest reliability and high content validity indicating suitability for research purposes (Ormel et al., 2005).

Covariates

The choice of potential confounders was determined a priori and based on background knowledge about the causal structure of the study question (Caspi & Shiner, 2010; Ormel et al., 2005; Rothbart & Bates, 2006). Information on date of birth, birth weight, and gender was obtained from midwives and hospital registries. Gestational age at birth was established using the ultrasound examination during pregnancy. Birth order, maternal age, and education were assessed by questionnaires at enrollment. Maternal education was defined by the highest completed education and classified as primary (no or only primary education), secondary (lower or intermediate vocational education), and higher education (higher vocational education or university). Maternal smoking was assessed at enrollment and in mid and late pregnancy to define whether the mother had never smoked during

pregnancy, stopped smoking when pregnant, or continued to smoke during pregnancy. We used the depression subscale of the Brief Symptom Inventory (BSI) to assess maternal depressive symptoms when children were three years old (Derogatis, 1993). The BSI is a validated self-report questionnaire, including a six-item depression scale (e.g., "feeling suicidal" and "feeling lonely"). High validity and reliability have been reported for the Dutch translation of the instrument (De Beurs, 2004).

Statistical Analysis

All children with information on temperament were included in the analyses. Missing values of the covariates, internalizing problems, or executive function scores were imputed using multiple imputations available in SPSS-version 17.0.2 for Windows (SPSS Inc, Chicago, IL, USA). The percentages of missing for the outcome variables and the mediators were between 10% and 12%. Ten independent datasets were generated using information on temperament, covariates, outcomes, and the mediators. Pooled estimates for ten datasets were calculated. We used independent sample *t*-test and chi-square statistics to explore whether the non-response was selective.

We first explored the correlations between positive emotionality, the three scales of the CBCL/1½-5, and executive function scores. We report the Pearson correlation coefficients (*r*).

In the main analyses, the determinant was positive emotionality. To facilitate the interpretation of the findings, the scores on positive emotionality were divided by the standard deviation (*SD*) and the effect estimates were reported as effect per *SD* increase in the determinant. Outcomes were Emotionally Reactive, Anxious/Depressed, and Withdrawn, as measured by the CBCL/1½-5. CBCL scale scores were dichotomized using the 80th percentile scores as cut-off to classify children as having problems, in line with previous publications on this cohort (Cents et al., 2010; Velders et al., 2011). We also tested an alternative cut-off using the 93rd percentile score, as recommended by the CBCL/1½-5 manual to examine consistency and to test if any effect was observed only due to the cut-off choice (Achenbach & Rescorla, 2000). The association between positive emotionality and the CBCL/1½-5 scales was examined using multiple logistic regressions. To rule the possibility of reverse causality, we controlled the analyses for children's Internalizing scores at age 1½ years.

Next, we examined whether any observed association was mediated by executive functioning. In the first step of the mediation analysis, multiple linear regressions were used to explore the association between positive emotionality and executive functioning. Executive functioning scores were log-transformed to satisfy the assumption of normality. We considered an executive function scale to be a possible mediator if the association was significant at $p < 0.10$. In the second step, we tested the indirect effect of positive emotionality on internalizing problems via executive functioning using 99% bias-corrected bootstrap confidence intervals applying 5000 bootstrap samples. Analyses were carried out in SPSS using a macro developed by Preacher and Hayes (2008). In these models, an indirect effect is present if the confidence intervals do not include 0. In our case, this meant that the indirect effect was significant at $p < 0.001$.

Since positive emotionality may predict psychopathology differently in boys and girls (Caspi & Silva, 1995), we also explored the interaction between positive emotionality and gender. The final models were adjusted for gender, gestational age at birth, and age at the time of assessments, internalizing problems at age 1½ years, maternal age, maternal education, maternal history of cigarette smoking, and maternal depressive symptoms.

RESULTS

In our study sample, the mean age of mothers at enrollment was 32.0±3.8, and 37% of the mothers had higher educational levels. About 80% of the mothers reported no history of smoking. Table 1 represents the baseline characteristics separately for boys and girls. There were no significant differences between boys and girls in the baseline characteristics.

Table 1. Sample characteristics

	Total Valid Observation (*n*=802)	Boys (*n*=405)	Girls (*n*=397)
Child			
Gestational age at birth, wk	802	40.0±1.8	40.0±1.7
Birth weight, g	801	3544±548	3464±547
Being firstborn, %	801	61.7	62.6
Internalizing problem score at 1½ yr	733	3.9±3.4	4.0±3.4
Mother			
Age, yr	802	31.7±3.9	32.2±3.7
Depressive symptoms	724	0.19±0.26	0.16±0.17
Education, %	793		
Primary		7.4	7.6
Secondary		56.3	53.7
High		36.3	38.7
Smoking, %	800		
Never		80.0	79.5
Until pregnancy was known		6.4	9.9
Continued during pregnancy		13.6	10.6

Note. Numbers are means and standard deviations (*M*±*SD*) for continuous variables.
Note 2. Maternal age, education, and smoking refer to the assessments at enrollment. Maternal depressive symptoms were assessed when children were three years old.

Means, standard deviations, and bivariate correlations among positive emotionality at three years, Emotionally Reactive, Anxious/Depressed, and Withdrawn scores at six years, and executive function scores at four years are presented in Table 2. Positive emotionality

was correlated with Withdrawn scores (r = -0.11, p < 0.01), but not with other CBCL scores. There were significant negative correlations between positive emotionality and shifting, emotional control, and working memory problem scores on the BRIEF-P. There was no significant correlation between observed positive emotionality at age 3 years and internalizing problem score at 1½ years (r = -0.04, p = 0.31).

Table 2. Means, standard deviations, and bivariate correlations for positive emotionality, executive functioning, and internalizing problems

	1	2	3	4	5	6	7	8	Mean (*SD*)	Range
1. Positive Emotionality	-								0.49 (0.15)	0.00-0.92
Internalizing problems										
2. Emotionally-Reactive	-0.05	-							1.5 (2.0)	0.0-15.0
3. Anxious/Depressed	-0.05	0.62**	-						1.1 (1.5)	0.0-10.0
4. Withdrawn	-0.11**	0.52**	0.47**	-					1.0 (1.2)	0.0-11.0
Executive Function										
5. Inhibition	-0.05	0.42**	0.24**	0.36**	-				22.1 (5.1)	16.0-46.0
6. Shifting	-0.15**	0.41**	0.35**	0.41**	0.37**	-			13.6 (3.3)	10.0-29.0
7. Emotional Control	-0.10**	0.49**	0.35**	0.37**	0.56**	0.56**	-		14.1 (3.5)	10.0-29.0
8. Working Memory	-0.08*	0.39**	0.25**	0.41**	0.72**	0.39**	0.46**	-	21.5 (4.8)	17.00-49.0
9. Planning/Organization	-0.05	0.32**	0.20**	0.36**	0.67**	0.37**	0.49**	0.76**	13.5 (2.9)	10.0-30.0

SD: Standard deviation; * p < 0.05 (two-tailed); ** p < 0.01 (two-tailed).

Table 3 summarizes the association between positive emotionality and internalizing problems. Positive emotionality did not predict CBCL scores in the top 15th percentile for Emotionally Reactive problems (Odds ratio [*OR*] = 0.93 per *SD* increase in positive emotionality score, 95%Confidence interval [*CI*]: 0.77, 1.14, p = 0.50). Similarly, we did not find any significant association between positive emotionality and Anxious/Depressed problems (*OR*=0.92 per *SD* increase in positive emotionality score, 95%*CI*: 0.77, 1.10, p = 0.37). Higher positive emotionality at three years reduced the odds of Withdrawn problems by about 20% (*OR* = 0.82 per *SD* increase in positive emotionality score, 95%*CI*: 0.67, 0.98, p = 0.03). There was no significant interaction between gender and positive emotionality in predicting internalizing problems (data not shown here).

Table 3. The associations between observed positive emotionality and internalizing problems

	Internalizing problems					
	Emotionally Reactive (*n*=150)		Anxious/Depressed (*n*=199)		Withdrawn (*n*=201)	
Observed Positive Emotionality	*OR* (95%*CI*)	*p*	*OR* (95%*CI*)	*p*	*OR* (95%*CI*)	*p*
Unadjusted (per *SD*)	0.90 (0.75, 1.09)	0.29	0.91 (0.77, 1.07)	0.25	0.81 (0.68, 0.96)	0.01
Adjusted (per *SD*)	0.93 (0.77, 1.14)	0.50	0.92 (0.77, 1.10)	0.37	0.82 (0.69, 0.98)	0.03

Note. Models were adjusted for child's gender, age, gestational age at birth, internalizing at 1½ years, maternal age, educational level, history of cigarette smoking during pregnancy, and maternal psychopathology at the child's age of three years.
OR: odds ratio; *CI*: confidence interval; *SD*: standard deviation.

5.2

Next, we examined the association between positive emotionality and executive functioning (Table 4). In the present sample, there was no significant association between positive emotionality and inhibition. However, children with lower positive emotionality scores had higher problem scores on shifting (*B* = -0.02 per *SD* increase in positive emotionality score, 95%*CI*: -0.03, -0.002, *p* = 0.03). Children's positive emotionality was not associated with problem scores on emotional control, working memory, and planning/organization.

Table 4. The associations between observed positive emotionality and executive function problem

	Executive Function Problem					
	B (95%*CI*)	*p*	*B* (95%*CI*)	*p*	*B* (95%*CI*)	*p*
Observed Positive Emotionality	Inhibition		Shifting		Emotional Control	
Unadjusted (per SD)	-0.01 (-0.03, 0.01)	0.47	-0.02 (-0.04, -0.01)	0.01	-0.02 (-0.04, 0.001)	0.06
Adjusted (per SD)	-0.01 (-0.03, 0.01)	0.53	-0.02 (-0.03, -0.002)	0.03	-0.02(-0.03, 0.004)	0.12
Observed Positive Emotionality	Working Memory		Planning/Organization			
Unadjusted (per SD)	-0.01 (-0.03, 0.004)	0.13	-0.01 (-0.02, 0.01)	0.31		
Adjusted (per SD)	-0.01 (-0.03, 0.004)	0.15	-0.01 (-0.02, 0.01)	0.36		

CI: confidence interval; *SD*: standard deviation.
Note. Models were adjusted for child's gender, age, gestational age at birth, internalizing scores at 1½ years, maternal age, educational level, history of cigarette smoking during pregnancy, and maternal psychopathology at the child's age of three years. Executive function problem scores were log-transformed. *Note 2*. The B's are not interpretable since the mathematically transformed scores were used in the analyses.

Finally, we tested whether shifting problems mediated the association between observed positive emotionality and Withdrawn problems in this sample. When the shifting problem score was included in the association model between positive emotionality and Withdrawn, a 16% change in the effect estimate was observed from *OR* of 0.82, 95%*CI*: 0.69, 0.98 to an

OR of 0.85, 95%*CI*: 0.71, 1.02, $p = 0.09$. Using the bootstrapping method, we showed that the indirect effect of positive emotionality on Withdrawn through shifting was significantly different from zero ($p < 0.001$).

When we repeated analyses using the alternative 93rd % percentile cut-off score, results remained essentially unchanged.

DISCUSSION

This population-based study aimed to characterize the longitudinal relation between observed positive emotionality and parent reported internalizing problems: Emotionally Reactive, Anxious/Depressed, and Withdrawn. Further, we explored the role of executive functioning in this association. Children with lower levels of positive emotionality had a higher risk of having Withdrawn problems, independent of confounders. This effect was not explained by preexisting internalizing problems. Our hypothesis regarding the mediating role of executive function in the association between positive emotionality and internalizing problems was partially confirmed, since shifting problems but not other executive function problems mediated the relation between positive emotionality and Withdrawn problems.

In the present study of children from the general population, we found a longitudinal relation between lower levels of observed positive emotionality at age three years and a higher risk of being withdrawn at age six as reported by the parents. The Withdrawn scale of the CBCL/1½-5 addresses behavioral problems in young children such as lack of involvement with others, unresponsiveness to affection, and showing little interest in things around him/her. Withdrawn problems do not explicitly tap an emotion, but were shown to be associated with long-term developmental difficulties such as pervasive developmental problems, anxiety, or depression (Boivin, Hymel, & Bukowski, 1995; Strauss, Forehand, Smith, & Frame, 1986). Many researchers have defined two distinct groups of withdrawn children: children who withdraw because they lack the desire for social interaction, and shy and reserved children, who want to interact with the environment, but withdraw because of fear and anxiety (Asendorpf, 1990). Different temperamental traits may be the predisposing factor in these two groups of withdrawn children; as shown by Laptook et al. (2008), the children with little desire for social interaction are those who present low positive emotionality. Our findings suggest that low positive emotionality increases the risk for socially withdrawn behavior later in life. This finding is consistent with the vulnerability hypothesis behind the association between temperament and psychopathology. Although positive emotionality was not related to preexisting internalizing problems in young children, we cannot entirely rule out that low positive emotionality, observed within a laboratory setting, and Withdrawn problems assessed by the CBCL/1½-5, are the extremes of the same continuum (spectrum hypothesis).

In our study, we found that positive emotionality in preschoolers was associated with shifting problems. However, positive emotionality was not related to other aspects of parent-reported executive functioning. As opposed to adults, executive functioning is hard to

tease apart in young children because of ongoing developmental processes (Hughes, 2002). However, the neuropsychological assessment of individuals with frontal lobe abnormalities suggests two distinct dimensions of executive functioning: behavioral and cognitive dimensions. Inhibition, emotional control, and shifting aspects fall into the behavioral dimension, whereas working memory and planning/organization are considered as cognitive aspects (Anderson, Anderson, Northam, Jacobs, & Mikiewicz, 2002). Previously, Healey et al. (2010) showed that negative emotions were associated with poor working memory. However, they failed to demonstrate a relation between positive emotionality and working memory. Similarly, we found no link between positive emotionality and cognitive executive functioning (i.e., working memory and planning/organization), but with shifting problems only. Shifting problems, as assessed by the BRIEP-P, tap problem behavior in young children such as being stubborn, not creative in problem solving, and acting upset by change in plan. The specific neurobiological characteristics of shifting (i.e., the ability to make transition and change the focus from one mindset to another) are less well known than those of other aspects of executive functioning (Gioia et al., 2002). Our findings suggest that children who engage less in pleasurable activity and are little responsive to social stimulation are less flexible in their social interaction with others and show difficulties in adapting to the changing circumstances. In our large sample, the relation between lower levels of positive emotionality at age three years and being withdrawn at age six years was mediated by shifting problems. These findings support the hypothesis that higher order cognitive processes, in particular attentional shifting, are one of the important factors playing a role in children's vulnerability to develop withdrawn behavior.

Our study did not provide evidence for the hypothesis that positive emotionality in preschoolers predicts Emotionally Reactive or Anxious/Depressed problems at age six. There are several possible explanations for these negative findings. First, our study was conducted in a population-based sample with relatively healthy children. Other community-based studies reported similar negative results (Hayden, Klein, Durbin, & Olino, 2006), whereas studies in referred samples suggest predisposition of children with certain temperamental traits to internalizing problems such as anxiety (Ruiz-Caballero & Bermúdez, 1997). Second, we assessed internalizing problems at age six years. Many vulnerable children develop symptoms of anxiety or depression after the preschool period (Brent & Weersing, 2010; Pine & Klein, 2010). Follow-up of these children and assessment of internalizing problems at a later age, particularly during adolescence, will help to further characterize the longitudinal relation between temperament and anxiety/depression.

In the present study, we used structured laboratory observations to measure child temperament. Executive functioning and internalizing problems, however, were assessed using parental ratings of child behavior. Each of these approaches has advantages and limitations (Caspi & Shiner, 2010; Rothbart & Bates, 2006; Verhulst & van der Ende, 2010). Using observational methods the researcher can precisely control the setting for an observation of a certain behavior. These types of assessment provide an "outsider's view" of child behavior (Caspi & Shiner, 2010). However, lab tasks often limit the type of behavior

that can be observed, mainly because of the short time available for assessment. Parental reports, on the other hand, are based on observations of child behavior in naturalistic settings over a long period of time. Using parents, as a source of information, is an inexpensive and easy-to-administer method for research. Nevertheless, parental reports of child behavior may be affected by several factors which introduce bias (Seifer, 2003). In the present study, we examined the relation between observed positive emotionality and parental reports of child behavior and executive functioning using a longitudinal design. Since parents were blinded to the results of temperamental observations, the combination of both methods provided a valid assessment of child's temperament and behavior. In addition, we controlled the analyses for factors that could possibly interfere with obtaining valid parental ratings on child behavior.

The present study had several strengths including a large sample size from the general population and considering the confounding effect of a wide range of pre and postnatal factors. However, the study sample was population-based and, thus, relatively healthy, and few children scored above the threshold for internalizing problems. Any generalization of negative findings regarding the prediction of psychopathology based on children's temperament should be done cautiously.

In the present study, we showed a relation between low positive emotionality at age three and withdrawn problems at age six years. In addition, our findings provided partial support for a role of executive functioning, particularly shifting problems, in this relation. Low positive emotionality early in life may lead to inflexibility and rigidity, which in turn may affect the degree at which children engage with their (social) environment, resulting in withdrawn behavior. This finding is consistent with the vulnerability hypothesis behind the association between temperament and psychopathology.

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CHAPTER 6

GENERAL DISCUSSION

The present series of investigations focuses on emotion recognition and temperament in the early preschool years, as both are important aspects of young children's emotional functioning and have consistently been implicated in psychopathology. The first part of the thesis described the relations between early risk factors and preschoolers' emotion recognition and temperament, while the second part of the thesis discussed how emotion recognition and temperament are linked to common internalizing and externalizing problems in preschoolers. The current chapter highlights the main findings as presented in the previous chapters, and provides an interpretation of these findings in a broader context. Following a discussion of relevant methodological issues, this chapter concludes with implications for clinical practice and future research.

MAIN FINDINGS

In Chapter 2, we examined how different task demands affect young children's ability to recognize emotional facial expressions. To this end, we employed age–appropriate computerized FER paradigms, reliant on children's verbal and nonverbal (i.e., visuospatial) abilities in a large sample of typically developing 3-year-olds. Children showed differential performance on the verbal and nonverbal FER tasks, especially with regard to the recognition of fearful faces. More specifically, fearful faces were amongst the most accurately recognized facial expressions when matched nonverbally and the least accurately recognized expressions when labeled verbally. These findings suggest that 3-year-olds' FER accuracy, in particular that of recognizing fear, strongly depends on the extent to which the task relies on verbal or visuospatial abilities, even after controlling for basic perceptual matching or labeling ability. In addition, young children committed systematic errors in FER such that happy expressions were often confused with fearful expressions, while negative emotions were often confused with each other. In line with previous studies, the non-random errors in preschoolers' FER suggest that emotion-categories are initially recognized broadly, grouping together emotions of the same valence (Widen & Russell, 2003). Our findings may guide neuroimaging work on the underlying neurobiology of FER. Most studies on the neurobiology of emotion perception highlight the role of the amygdala in processing emotionally salient information, particularly fear (Guyer et al., 2008; Hare, Tottenham, Davidson, Glover, & Casey, 2005; Thomas et al., 2001). Adult and adolescent neuroimaging studies have shown that different neural pathways underlie FER depending on the paradigms used for assessment. Studies using task paradigms similar to our nonverbal FER task have consistently reported increased bilateral amygdala activation, whereas labeling facial expressions has been associated with attenuated amygdala activation and increased prefrontal activation. Although the amygdala has strong connections to these prefrontal areas, it is also directly connected to brain areas which are implicated in swift autonomic and motor responses to threatening information (Morris et al., 1998). This may be particularly relevant in case of processing fear cues, which signal potential danger in the environment. The finding that children were particularly proficient at processing fear cues

nonverbally, may enable them to swiftly react to perceived danger in the environment, even if conscious appraisal of fear cues develops only later (LeDoux, 1995).

In Chapter 3, we addressed the influence of maternal depressive symptoms on young children's developing FER and temperament. We reported that maternal depressive symptoms predicted less accurate emotion-labeling in children and this association was not mediated by observed maternal sensitivity (Chapter 3.1). Nevertheless, maternal sensitivity was associated with improved emotion-matching performance in children, independent of maternal depressive symptoms. In Chapter 3.2, we observed that more chronic and severe depressive symptoms of the mother were related to lower positive emotionality (particularly in a social interaction context) and fearlessness (in situations eliciting a startle response) in the offspring, independent of maternal harsh discipline. Several potential mechanisms have been identified through which maternal depression may influence child outcome. Firstly, maternal depression may partially be genetically determined (Rutter, 1997). According to most twin studies, heritability of depression is low in childhood and increases to moderate in adolescence maintaining fairly stable levels up to adulthood (Eley & Stevenson, 1999). Furthermore, relative to healthy controls, infants of depressed mothers already exhibit depressive features such as inferior orienting behavior, lower activity level, lethargy, and weariness at the neonatal stages (Abrams, Field, Scafidi, & Prodromidis, 1995). A second important mechanism through which risk is transmitted from depressed mother to child is through their interactions. Depressed women spend less time looking at their infants, touching them and talking to them, and they exhibit less positive and more flat or negative emotions toward their children than non-depressed women (Campbell, Cohn, & Meyers, 1995; Cohn, Campbell, Matias, & Hopkins, 1990; Murray, Kempton, Woolgar, & Hooper, 1993). There is converging evidence that postpartum depression has adverse effects on mothers' interactional competence and sensitivity, which in turn impact child development (Beck, 1995; Lovejoy, Graczyk, O'Hare, & Neuman, 2000). The third possible causal route is via environmental adversity. Maternal depression often occurs in the context of social and personal adversity such as overcrowding, poverty, young maternal age, and low levels of social and emotional support, particularly in the partnership (Murray & Cooper, 1997). The exact underlying risk mechanism through which maternal depression leads to adverse child outcome may also depend on the specific outcome under investigation. Our findings with regard to preschoolers' FER and temperament are more suggestive of the genetic transmission model, because the observed effect of maternal depressive symptoms in both Chapters 3.1 and 3.2 was independent of general contextual risk factors and parenting measures (e.g., observed maternal sensitivity and harsh discipline). However, it is highly likely that our measures of contextual risk and parenting did not fully capture the whole range of variance explained by these factors, thus, residual confounding was still present in the data. Furthermore, in neither of the chapters did we officially examine the mediating role of contextual risk factors in the association between maternal depressive symptoms and child outcome. Although there was no meaningful drop in the effect estimates of maternal depressive symptoms when we incorporated contextual risk measures in the statistical

models, we cannot officially exclude the possibility that certain contextual risk measures did, to some extent, mediate the association between maternal depressive symptoms and children's FER or temperament. Taken together, the present thesis is not able to specify the exact underlying mechanisms involved in the relationship between maternal depressive symptoms and children's FER or temperament, but it did provide important insights into the relatedness of an important risk factor (i.e., maternal depressive symptoms) to core measures of emotional functioning in the preschool period.

In Chapter 4, we examined potential genetic influences on several key indices of emotional functioning in the preschool period (i.e., FER, attachment, temperament, and emotional problems). We reported a role for the 5-HTTLPR polymorphism in young children's FER abilities (Chapter 4.1) and developing emotional problems (Chapter 4.3). Results indicated that the 5-HTTLPR polymorphism selectively impacted the recognition of fearful faces, which was already apparent in very young typically developing children (Chapter 4.1). This finding may signal an early genetic vulnerability, which in the presence of adverse environmental exposures may lead to more global deficits in recognizing facial expressions as well as to the emergence of emotional problems (Chapter 4.2). These findings are consistent with the vulnerability model of psychopathology (Burmeister, McInnis, & Zollner, 2008), which holds that vulnerability genes influence disease risk either more generally (i.e., through 'main effects') or under specific environmental conditions (i.e., through 'gene-environment interactions'). The latter process is also known as the diathesis-stress model (Rutter, 2006). Alternative to the diathesis-stress model is the differential-susceptibility perspective, which posits that putative vulnerability genes may rather function as plasticity genes, rendering certain individuals more responsive than others to both positive and negative environmental experiences (Belsky et al., 2009). Since it was not tested in the present thesis whether the differential-susceptibility model holds for the 5-HTTLPR polymorphism in relation to preschoolers' FER, I can only speculate about it. In Chapter 3.1, we reported a beneficial effect for a positive environmental exposure (i.e., observed maternal sensitivity during mother-child interactions) on young children's FER. It is not unimaginable that this association is moderated by children's 5-HTTLPR status. Future studies investigating the effect of the 5-HTTLPR polymorphism on children's developing FER abilities could benefit from including both negative and positive environments in their design to fully capture the range of environmental influences on children's FER. In complex behavioral phenotypes like FER, attachment, and temperament, environmental factors probably operate in combination with polygenic effects. Thus, there are still ample opportunities to identify new genes in relation to these complex traits, and as suggested in Chapter 4.3 GWA and pathway analysis approaches may offer promising avenues in this quest. Importantly, these gene-discovery approaches should additionally also include careful assessment of the environment, since the most important effects might be hidden in gene-environment interactions.

In Chapter 5, distinct FER profiles were outlined in relation to specific syndromes of internalizing and externalizing behavior in the preschool period (Chapter 5.1). This

study was the first attempt to explore longitudinal associations of early internalizing and externalizing problems with FER in a large sample of preschoolers. Results suggested a qualitatively different relationship between internalizing and externalizing behavior problems and children's FER, such that internalizing problems contributed to emotion-specific differences in FER, while externalizing problems were associated with more general FER deficits. Knowledge of the specific FER profiles associated with internalizing and externalizing behavior problems can prove useful for the refinement of emotion-centered preventive interventions. A better understanding of the associations between internalizing and externalizing problems and facial expression recognition early on is important for identifying subtypes of children who might be particularly at risk for developing psychiatric disorders. In addition, these findings extend our conclusions in Chapter 2.1 by showing the importance of employing FER paradigms that rely on verbal and visuospatial abilities when examining FER differences in relation to behavior problems. More specifically, children's performance on the emotion-labeling and emotion-matching tasks differed as a result of their behavior problems. Emotion-specific differences related to internalizing problems consistently emerged for emotion-labeling ability, whereas the emotion-matching task better captured global FER deficits related to externalizing problems than the emotion-labeling task. As discussed in Chapter 2.1, the emotion-labeling task relies more on attention processes than the emotion-matching task due to its elements of cognitive appraisal and labeling. Since emotion-specific attention biases are consistently implicated in internalizing problems (e.g., anxiety and depression), emotion-labeling paradigms may be more sensitive to detect any resulting emotion-specific differences in recognition accuracy related to internalizing problems than emotion-matching paradigms.

In Chapter 5.2, we reported that difficulties in attention shifting, a specific domain of executive functioning, was causally involved in the mechanism through which low positive emotionality lead to the development of internalizing problems (i.e., socially withdrawn behavior) in a nonclinical sample of preschool children. It is important to briefly address here the similarities between executive functioning and the temperamental trait effortful control, as these include partially overlapping processes. Furthermore, effortful control has previously been implicated in prosocial behavior (Eisenberg et al., 2003), the regulation of behavioral approach and withdrawal tendencies, and as a resiliency factor against developing psychopathology in the presence of temperamental vulnerabilities (e.g., high negative emotionality or low positive emotionality; Caspi & Shiner, 2008; Nigg, 2006; Rothbart & Ahadi, 1994). Given the above, one might argue that our findings reflect the interplay between low positive emotionality and low effortful control in the development of social withdrawal. Thus, it is important to briefly discuss the similarities and differences between the two constructs. Both concepts describe aspects of self-regulation but from different perspectives. Effortful control is a temperament-based approach, while executive functioning is a neuropsychological approach. Effortful control refers to the ability to inhibit a dominant response in favor of a less salient response. Its factors involve inhibitory control, attention focusing, low intensity pleasure, and perceptual sensitivity (Raver, 2004;

Rothbart & Ahadi, 1994). Executive function refers to a collection of cognitive processes (e.g., attention shifting, working memory, inhibitory control) that are utilized in planning, problem solving, and goal-directed activity (Miyake, Friedman, Emerson, Witzki, & Howerter, 2000). The primary overlap between the two constructs is the inhibition of prepotent responses in favor of less salient responses. Executive function, however, focuses on volitional control of cognitive self-regulatory processes mainly under affectively neutral circumstances, whereas effortful control also includes the appetitive and aversive nature of the conditions under which control is required (i.e., the nonconscious component of emotional reactivity and regulation; Blair & Razza, 2007). Nevertheless, recent work has suggested that some of the cognitive processes related to executive function may be influenced by the conscious or nonconscious representation of the motivational or affective significance of the stimuli (i.e., by emotional arousal; Bechara, 2004; Gray, Braver, & Raichle, 2002). Despite this overlap, developmental studies of preschool children suggested only moderate correlations between executive function and effortful control, which points to the importance of differences between the two concepts (Carlson & Moses, 2001; Davis, Bruce, & Gunnar, 2002). Based on results presented in Chapter 5.2, we cannot conclude on the role of effortful control in the association between low positive emotionality and internalizing problems. Studying this was outside the scope of the present thesis. Future studies, however, should address this question in more detail.

In summary, the present work showed that early risk factors influence preschoolers' emotional functioning which, in turn, is associated with common internalizing and externalizing behavior problems in young children. The most important early risk factors examined in the present thesis were maternal depressive symptoms (measured prospectively from before birth until the early preschool years of the child) and children's 5-HTTLPR genotype. More specifically, maternal depressive symptoms were linked to low positive emotionality (in a social interaction context), low fearfulness (in a startle response provoking situation), and decreases in performance for labeling emotional expressions. In addition, the short allele of the 5-HTTLPR polymorphism was associated with decreased nonverbal fear recognition ability in young children as well as it moderated the effect of environmental adversity on children's accuracy to nonverbally recognize emotional expressions and to develop emotional problems. Emotional functioning in the preschool period was also associated with common emotional and behavior problems. Distinct FER patterns were characteristic of early and concurrent internalizing and externalizing problems in preschool children, and low positive emotionality was associated with the development of socially withdrawn behavior in young children, partly mediated by difficulties in the attention shifting domain of executive functioning. Taken together, these findings enhance our understanding of emotional functioning in the preschool period, and emphasize its pervasive significance in the psychological well-being of young children.

ASSESSING EMOTION RECOGNITION: TASK PARADIGMS AND EXPERIMENTAL STIMULI

There are a number of important methodological issues which need to be addressed in relation to the assessment of emotion recognition. One such topic is the use of diverse task paradigms in this area. For instance, research focusing on infants' abilities to discriminate among discrete emotion signals usually employs experimental task paradigms (i.e., habituation and preferential looking procedures). Although these procedures may represent the best manner to assess FER in young babies, employing such techniques can be problematic for a number of reasons. Firstly, such studies generally present stimuli including complex features, which makes it challenging to isolate the specific aspects of the stimulus to which infants are attending and responding (Saarni, Campos, Camras, & Witherington, 2006). Secondly, the paradigms used in preverbal infants are quite different from those applied in older children, which makes comparison across development problematic. In addition, it raises uncertainty as to whether the same construct of emotion recognition is being measured over development (Herba & Phillips, 2004; McClure, 2000). As children acquire language, researchers can use a wider range of techniques to evaluate children's ability to recognize emotions. By age 3 years, approximately 93% of children regularly use the words happy, sad, angry, and scared in their conversations. This enables researchers, for instance, to ask children to apply these words in tasks assessing their FER (Ridgeway, Waters, & Kuczaj, 1985). Cross-sectional studies measuring emotion recognition during the preschool period, childhood, and adolescence have most frequently used (a) situation discrimination, (b) matching discrimination, (c) forced choice labeling, and (d) free labeling paradigms. These paradigms differ markedly according to the cognitive skills they require as well as their dependency on the development of emotion vocabulary and visual discriminatory skills (Markham & Adams, 1992). For instance, situation discrimination requires matching facial expressions to emotion-inducing situations, which relies very little on specific emotion vocabulary but rather taps the individual's understanding of the affective meaning of expressions. Free labeling of emotional expressions requires an adequate vocabulary and sufficient recall memory for emotion words, while the recognition of emotion labels (e.g., happy, sad) does not necessitate recall skills rather comprehension of the verbal labels provided by the experimenter (Camras & Allison, 1985). The emotion-matching task employed in the present thesis required the child to match a target expression with one of two emotional expressions. This nonverbal matching discrimination task bypasses the child's emotion vocabulary. In order to successfully solve this task, the child does not need to access a prototype of each emotion from memory, but to simply match particular facial features from the target face to the comparison faces. Although this may seem an easy task, it requires feature to feature matching, which can be quite difficult when faces vary in age, race, sex, or other characteristics (Markham & Adams, 1992). The emotion-labeling task used in the present thesis required the child to select from several emotional expressions the one that corresponded with the emotion label the child heard.

In order to successfully solve this task, the child needs to be able to comprehend the verbal labels, but does not need to recall a particular emotion label from his own vocabulary. Furthermore, the task puts only minimal load on children's working memory, as all choices are presented simultaneously (Markham & Adams, 1992). Importantly, both the emotion-matching and emotion-labeling tasks were presented using a touch-sensitive monitor to make sure that the young participants could complete all tasks on the computer; a button-box may have been too difficult for some of the 3-year-old children.

Another methodological issue concerns the large variety of task stimuli used in developmental studies of emotion recognition, as this can also complicate comparison between study results. Emotional information can be obtained via a number of different channels; it may include auditory cues (e.g., acoustic information, semantic information, prosody), visuospatial cues (e.g., facial expressions, bodily postures, gestures), and in most cases a combination of all of the above. Because it is virtually impossible to ensure that signal intensity is equated across the different modalities, attempts to make direct comparisons, for instance, between facial and vocal expressions may be misleading (Saarni et al., 2006). The vast majority of studies have employed facial expressions, as these are powerful nonverbal cues of emotion (Herba & Phillips, 2004). Some studies have utilized line drawings of emotional expressions. These drawings have a lower ecological validity compared to the more realistic and well validated photographs of facial expressions (Ekman & Friesen, 1967; Tottenham et al., 2009) and may measure a more general ability to discriminate forms (Goss & Ballif, 1991). A common feature of line drawings and photographs is that they both display facial expressions as static (Gross & Ballif, 1991; Herba & Phillips, 2004). During real-life social interactions emotions occur in rapid sequential patterns and involve subtle changes in facial expressions. Therefore, studies applying static facial expressions need to be cautious when generalizing their results to naturally occurring interactive situations, as the amount of available emotional information is very different in the two situations (Gross & Ballif, 1991). Dynamic and static displays of emotion may be processed differently and served by distinct underlying neural structures (Haxby, Hoffmann, & Gobbini, 2000; 2002). Future research is necessary to gain satisfactory evidence for this hypothesis.

In the present thesis, we used prototypical photographs of facial expressions, which may be of less ecological validity than alternative emotional stimuli such as emotional vocalization or dynamic display of emotional gestures. On the other hand, earlier research has demonstrated that basic emotions can most reliably be recognized from facial expressions, and that neural circuits involved in emotional processing are consistently engaged in response to pictures depicting prototypical facial expressions (Adolphs, 2002; Hariri et al., 2000, 2003). Additionally, our results can be more readily interpreted within the context of previous work on the development of FER measured behaviorally and neurally (Guyer et al., 2007).

In summary, the relative difficulty of FER varies when the same stimulus is used but with different procedures (Harrigan, 1984), and when similar procedures are used but with different stimulus material and subjects (Camras & Allison, 1985). In order to gain a good understanding of emotion processing one should include tasks tapping into both verbal and

visuospatial emotion processing, as these are suggested to follow distinct developmental pathways, while preferably accounting for children's basic verbal and visuospatial abilities (Gross & Ballif, 1991; Vicari, Reilly, Pasqualetti, Vizzotto, & Caltagirone, 2000). This approach was carefully followed in the present thesis by applying a nonverbal emotion-matching and a verbal emotion-labeling task to assess young children's FER. Importantly, control tasks assessing general matching and labeling abilities were also included in order to account for the basic cognitive abilities necessary for the successful completion of our FER tasks.

ASSESSING TEMPERAMENT: SOURCE OF INFORMATION

The validity of any empirically based inference is always influenced by the nature of the observations (Kagan & Fox, 2006). There are several ways to collect information on child temperament with most approaches falling into two broad categories. *Informant reports* (i.e., questionnaires or interviews) of child temperament are most commonly acquired from parents but they can also be collected from teachers, peers, and in older children via self-reports. Alternatively, child temperament can also be directly observed by independent, trained raters. These *behavioral observations* may take place either in a laboratory, where parameters are controlled and standardized tasks are used to elicit the behaviors of interest, or in naturalistic settings (e.g., at home or in the school), where children are observed in their everyday environment participating in regular activities.

Each method has its relative advantages and limitations. For instance, parent reports tap the extensive knowledge base of parents, who have seen their child in many different situations on multiple occasions (Rothbart & Bates, 2006). Thus, the final score reflects responses across a broad range of situations and times, and includes infrequently occurring behavior which may be critical in defining a particular temperamental trait (Rapee, 2002; Rothbart & Bates, 2006). In addition, parent report measures have been developed to map onto modern theoretical models of temperament which emphasize continuity between child and adult personality (e.g., Rothbart, Ahadi, Hersey, & Fisher, 2001). However, parent reports also have several limitations. For instance, they have low convergent validity with laboratory measures, naturalistic observations, or teacher reports (Kagan, 1998), and poorer predictive validity for later adjustment than self- and teacher reports (Mesman & Koot, 2001). Importantly, they may also be subject to dysphoria-related biases (Youngstrom, Izard, & Ackerman, 1999). Parent report measures are confounded by at least two processes which may exhibit differential stability across development: child temperament and parental interpretations of child behavior. The latter is influenced by stable characteristics of the parent (Bates, 1994), as well as parental perceptions and motivations such as the parents' desire to maintain a consistent view of their children (Kagan, 1998). Consequently, parent report measures may result in inflated stability estimates of child temperamental characteristics (Durbin, Hayden, Klein, & Olino, 2007).

Naturalistic observations have a high degree of ecological validity. They are generally more objective than informant reports, since behavior is coded using objective criteria, thereby

avoiding the problem of parental interpretation of child behavior. Nevertheless, unlike laboratory observations, they cannot control for the influence of context on the child's behavior (Durbin et al., 2007). Other limitations of naturalistic observations include their relative expense and low day-to-day reliability, the latter of which makes it difficult to collect an adequate set of relevant behaviors (Rothbart & Bates, 2006). In addition, naturalistic observations are highly influenced by situational factors in ways which are difficult to record and take account of when the coder's attention is focused on the child (Rothbart & Goldsmith, 1985).

Laboratory observations have several unique advantages compared to informant reports and naturalistic observations. For example, lab tasks use standardized stimuli designed to elicit behaviors that are relevant for the traits of interest. Thereby, they also make it possible to observe behaviors that are expressed at a lower base rate, such as fear. In addition, they allow for a sharper differentiation of individual differences from the contexts in which they are observed (Durbin et al., 2007). Laboratory tasks also allow for fine-grained analysis of specific behaviors and for linking these behaviors with brain functioning (Caspi & Shiner, 2008). Behaviors during lab tasks are reliably coded using objective criteria. However, this objectivity comes at a cost of considerable investment of time and expense, and assessment of generally a single time point only (Rapee, 2002). Repeated testing necessary to measure a complex trait may be unfeasible or involve carryover effects. The ecological validity of laboratory measures is lower than that of naturalistic observations and informant reports, and it can be challenging to design measures that are equally valid for tapping temperament across developmental periods (Durbin et al., 2007).

To date, none of the above methods has become so established that it can be seen as the gold standard of temperament measurement. Ideally, always more than one method should be used in order to exploit the different advantages these methods can offer and to counteract the weaknesses inherent in each (Caspi & Shiner, 2008). It is also desirable to include more than one informant when possible, and assess more than one trait to provide evidence of divergent validity for the traits of interest. It is of note that if investigators exclusively rely on one type of information, be it informant report or behavioral observation, the validity of their inferences is restricted to that particular class of information and is not a proxy for alternative source of data (Kagan & Fox, 2006). However, the available resources are often limited, and one cannot afford to use multiple techniques to assess child temperament. In such cases, it is important to consider certain aspects of the study design when selecting the optimal measurement. One such aspect is the specific traits under investigation; certain measurements may be more effectively tapping a particular temperamental trait than others. For example, due to children's rare exposure to fearful situations in everyday life, it may be advisable, as done in the present thesis, to observe temperamental fearfulness in the laboratory using tasks which are designed to elicit fearful reactions from the child. Other factors to consider when deciding in favor of a method may involve sample size, available financial resources, and time frame. For instance, large-scale, prospective cohort studies with a focus on longitudinal assessment of temperament would likely benefit from using questionnaires. Questionnaires are relatively inexpensive and simple to administer, due to

which they provide convenient and efficient means to repeatedly gather information on a broad range of temperamental traits in large populations over time. Another significant issue to consider when deciding on the type of temperament assessment in a particular study is the problem of shared method variance. Shared method variance arises when the predictor and outcome share a common method or agent of assessment (e.g., both are based on parent reports). In Chapter 3.2, we examined the potential effect of maternal depressive symptoms on young children's temperament. Since maternal depressive symptoms were self-reported, using laboratory observations to assess child temperament reduced any potential biases which might have arisen due to shared method variance.

FALSE POSITIVE FINDINGS IN GENOME WIDE ASSOCIATION STUDIES

The study presented in Chapter 4.3 represented the first attempt in an effort to use state of the art gene analysis tools and go beyond the usual genetic suspects to expand our knowledge on the underlying genetics of individual differences in child attachment and temperament. The application of this genetic approach in medical research has exploded in recent years and we expect that it will gain a similar popularity in the field of child development. Although in Chapter 4.3 we reported only one nearly genome wide significant association in the case of disorganized attachment, it is important to address here the common methodological concern of GWA studies, namely the high likelihood of false positive findings they generate. In a GWA setting, a false positive finding occurs when there is no genuine association between the polymorphisms and the trait of interest, nevertheless the GWA study does find spurious relations which achieve formal statistical significance (Ioannidis, 2007).

False positive findings may occur due to a number of reasons. Firstly, they may arise due to chance variability. GWA studies are typical examples of massive discovery-oriented testing, where hypothesis-free investigation of several hundred thousand genetic variants is now feasible (Ioannidis, 2007). Even the definition of what constitutes a GWA is subject to change, given the continuous increase in the number of polymorphisms that can be covered by available genotyping platforms (Ioannidis, 2007). In hypothesis-generating research with massive testing, where tested relationships can exceed true ones 1000-fold, false positive findings are extremely common, despite efforts made to adjust for the enormous number of multiple comparisons (Ransohoff, 2004; Todd, Goldstein, Ge, Christie, & Palmer, 2011; Wakefield, 2012). Secondly, false positive findings can arise as a result of biases in study design, data analysis, or reporting of findings (Ioannidis, 2005). Such examples may include differential or non-independent errors in phenotyping and genotyping, confounding, population stratification due to ethnic admixture, and selective or distorted reporting of research findings. A third possibility of spurious positive results in a GWA setting is when an association that was proposed by a single GWA study attracts so much attention that intense replication attempts are made using very large studies (Ioannidis, 2007). As research efforts become more globalized, large sample size is practically the rule, especially in the

case of GWA studies, where most newly discovered variants have subtle effects and explain only a small percentage of the risk variance in complex traits (Pereira, Patsopoulos, Salanti, & Ioannidis, 2009). A nominally statistically significant result of a single GWA study with borderline statistical significance in a very large-scale replication does not increase the credibility of the postulated association (Ioannidis, 2007).

Fortunately, there are ways to reduce the likelihood of false positive findings. Firstly, low-bias meta-analyses may be helpful, as their findings tend to approximate the underlying "real truth". Secondly, research efforts should be made concerning questions, for which the pre-study probability of a true finding is already high (e.g., large degree of heritability shown in previous studies; Ioannidis, 2005). Thirdly, the totality of evidence should be more important than the statistically significant findings of any single study (Todd et al., 2011). Instead of chasing statistical significance, investigators should consider what the chances are that they are testing a true relationship. In summary, whenever ethically acceptable, large studies with minimal bias should be performed on research findings that are considered relatively established to see how often they are confirmed (Ioannidis, 2005). In addition, further replications and collaborative meta-analyses (joint analyses) are critical to reduce the probability of false findings.

The study reported in Chapter 4.3 provided only preliminary insights into the use of state of the art gene analysis tools in child attachment and temperament research. Nevertheless, we hope that our approach invites researchers in the field of child development to pool their samples in an attempt to replicate and extend our preliminary findings.

CLINICAL IMPLICATIONS

Children's temperamental dispositions as well as their ability to accurately recognize emotional expressions should be considered desirable targets of prevention and intervention efforts. The reason for this is that both constructs are implicated in childhood behavior problems and influenced by classic risk factors of psychopathology, even before psychiatric symptoms emerge. To date, a few preventive intervention techniques have exploited these associations (e.g., Greenberg et al., 1995; Izard, 2002); my recommendations concern the further refinement of these techniques. For instance, the preschool period (between 2 and 5 years) is identified as a sensitive period for developing a solid foundation for accurate perception and labeling of emotions of oneself and others (Izard, Fine, Mostow, Trentacosta, & Campbell, 2002). An impoverished emotion vocabulary and the inability to perceive and label emotion cues in this period may be used as indicators for emotion-centered preventive interventions (Izard et al., 2002). The goal of emotion-centered prevention techniques is to enhance children's ability to accurately detect and label emotion signals. Techniques include practice using emotion recognition and labeling tasks, and interactive reading of emotion stories to encourage labeling and articulation of emotions (Greenberg et al., 1995; Izard, 2002). These preventive interventions are mainly carried out in school settings by teachers, especially among student populations who are at risk for

behavior problems and psychopathology (Izard et al., 2002). Further refinement of such programs may include differential training of children displaying specific bias patterns of FER (e.g., general deficits or emotion-specific biases). In addition, I also recommend that attention-modification techniques (e.g., attention-bias training), which have successfully been applied in the treatment of pediatric anxiety disorders, be applied as a preventive intervention, targeting children who are at risk for developmental psychopathology. In the future, these children may be identified based on their FER profiles or temperamental characteristics, and offered more personalized attention-bias trainings. For instance, children who show increased attention toward negative emotions can systematically practice to focus their attention on positive emotional stimuli, or children who show an attentional shift toward threat-related cues can practice to deliberately allocate their attention to other, less threatening cues in the environment (Fox & Pine, 2012). Similarly, children with problems identifying distress cues could perhaps be specifically sensitized to them. Another advantage of attention-bias modification therapies is that they are firmly grounded in cognitive neuroscience theory (Pine, Helfinstein, Bar-Haim, Nelson, & Fox, 2009). Temperament-based preventive interventions generally operate in two different and complementary ways: by modifying basic tendencies or by helping children and their parents to better cope with those tendencies (Caspi & Shiner, 2008). Example for the former may include training sessions for children with difficult temperament aimed at practicing basic skills in emotion-regulation and self-control (Muris & Ollendick, 2005). Example for the latter may include a brief program for the parents in which they receive specific education and training about adequate parenting techniques tailored to their child's temperament (Rapee, Kennedy, Ingram, Edwards, & Sweeney, 2005). In addition to the more general school-based prevention programs, prevention and intervention efforts should also specifically target children of depressed parents. Techniques may include teaching children to seek out and anticipate rewards, and enhance or maintain positive emotions in order to prevent internalizing problems among this high-risk population (Silk, Shaw, Forbes, Lane, & Kovacs, 2006). Additionally, a brief screening device assessing depressive symptoms should be a standard component of prenatal and early postnatal maternal care and, if necessary, intervention should be accessible for women. Although the effectiveness of screening for depression in primary care is debated, screening women for depression in the prenatal and postnatal periods may be useful given the high prevalence of depressive disorders at both times, and because of evidence that depression can be effectively treated (Buist et al., 2002). Finally, targeted preventive interventions may be developed in the future for children with known genetic vulnerability for a specific psychopathology, especially if this is coupled with stressful living conditions and/or other risk factors. Nevertheless, it must be noted that the positive predictive value of individual susceptibility-conferring genotypes in relation to common, complex disorders is low (Holtzman & Marteau, 2000). Likewise, there is also very little scientific evidence supporting the positive predictive value of more comprehensive genomic profiles (i.e., information about the presence of multiple predisposing alleles) with regard to complex diseases (Becker et al., 2011; Janssens et al., 2008). The reason for

this is that most common disorders are caused by complex interactions among multiple genetic, environmental, and behavioral factors, each of which confers only minor increases in risk (Holtzman & Marteau, 2000). Sufficient understanding of this complex interplay might develop within the next decades, which will then revolutionize our current views on diagnosis, treatment, and prevention of common disorders (Bell, 1998). However, as long as genomic profiles are composed of low-risk susceptibility genes, the difference between high-risk and low-risk groups will be small enough for both groups to equally benefit from general interventions (Janssens et al., 2008).

FUTURE DIRECTIONS

Given that children's performance on FER tasks strongly depends on the task specifications, future research should continue to apply both verbal and visuospatial FER assessments, while accounting for basic verbal and visuospatial abilities (Székely et al., 2011; Vicari et al., 2000). Our current knowledge on the development of FER is almost exclusively based on cross-sectional studies. Drawing inferences about developmental processes from cross-sectional data is confounded by methodological problems. Repeated measures of FER in the context of longitudinal cohort studies such as the Generation R Study could provide important insights into the developmental trajectories of FER, which may also help resolve the issue of comparing underlying construct validity in infant and child/adolescent studies of FER (McClure, 2000). Additionally, longitudinal cohorts are also ideally suited for the prospective follow-up of children with aberrant FER patterns to investigate whether they will develop psychiatric disorders over time. Future studies of FER would also benefit from incorporating more subtle measures of emotion processing than recognition accuracy such as attention biases, error biases, or speed of processing (Van Beek & Dubas, 2008; Herba & Phillips, 2004). Such subtle measures of emotion processing may be particularly informative in studies of older children and adolescents, where recognition accuracy approaches ceiling levels. Furthermore, additional research is necessary to extend our knowledge on the effect of paternal depressive symptoms on children's FER/temperament, given that paternal depression is also associated with elevated rates of psychopathology in the offspring (Hammen, 2009). Currently, very little is known about the effect of paternal depression on children's emotional functioning or adjustment. However, there is some indication that maternal depression may be more strongly related than paternal depression to psychopathology in the offspring, particularly in younger children (Connell & Goodman, 2002; Tully, Iacono, McGue, 2008). Along similar lines, Durbin, Klein, Hayden, Buckley, and Moerk (2005) found that low positive emotionality in preschoolers was associated with mothers' but not with fathers' depression. To my knowledge, no studies to date have investigated the effect of paternal depression on children's FER ability. Finally, given the importance of gene-environment interactions in the manifestation of complex phenotypes, future studies applying a GWA approach to identify novel disease-susceptibility genes may benefit from incorporating environmental information in their study design. Such

an approach involves the study of gene–environment interaction in the context of GWA studies, termed as gene-environment-wide interaction studies. Longitudinal cohorts with precise measurements on both the phenotype and environmental exposure such as the Generation R Study will prove particularly useful to this end.

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CHAPTER 7

SUMMARY/SAMENVATTING

SUMMARY

Emotions are essential aspects of our adaptation to the physical and social environment. All emotions, even the negative ones, have significantly beneficial effects on our behavior, well-being, and adaptation. On the contrary, inappropriate and uncontrolled emotional responses are implicated in many forms of psychopathology and even in physical illness. In young children, temperament and the ability to accurately recognize emotions are key aspects of emotional functioning. The current thesis examined environmental and genetic predictors of children's temperament and facial expression recognition (FER) in the early preschool years, a period in which temperament begins to stabilize, and children develop a solid foundation for accurate perception and labeling of emotions. All studies in the present thesis were conducted within the context of the Generation R Study, a large-scale population-based prospective child cohort from fetal life onward in Rotterdam, the Netherlands. The aim of the Generation R Study is to identify early environmental and genetic causes of normal and abnormal growth, development, and health. The current thesis is based on a subsample of this cohort, consisting of approximately 1000 Dutch children and their parents, in whom more detailed measurements were available.

In **Chapter 2**, we examined how three-year-olds verbally label and nonverbally match facial expressions of basic emotions (*Chapter 2.1*). Children showed differential performance on the verbal and nonverbal FER tasks, especially with regard to the recognition of fearful faces; fearful faces were amongst the most accurately recognized facial expressions when matched nonverbally and the least accurately recognized expressions when labeled verbally. Thus, three-year-olds' ability to recognize emotional facial expressions, in particular fear, strongly depends on the extent to which the task relies on verbal or visuospatial abilities, even after controlling for basic labeling or perceptual matching ability. In addition, young children committed systematic errors in FER such that happy expressions were often confused with fearful expressions, while negative emotions were often confused with one another.

In **Chapter 3**, we addressed the influence of maternal depressive symptoms on young children's developing FER and temperament. We reported that maternal depressive symptoms predicted less accurate emotion-labeling in children (*Chapter 3.1*). This association was not mediated by measures of observed maternal sensitivity. Nevertheless, maternal sensitivity was associated with improved emotion-matching performance in children, independent of maternal depressive symptoms. In *Chapter 3.2*, we observed that more chronic and severe depressive symptoms of the mother were related to lower positive emotionality (particularly in a social interaction context) and fearlessness (in a situation eliciting a startle response) in the offspring, independent of maternal harsh parenting.

In **Chapter 4**, we examined potential genetic influences on several key indices of emotional functioning in the preschool period (i.e., FER, attachment, temperament, and emotional problems). We reported a role for the 5-HTTLPR polymorphism in young children's FER abilities (*Chapter 4.1*) and developing emotional problems (*Chapter 4.2*). Results indicated that the 5-HTTLPR polymorphism selectively impacted the recognition of fearful faces, which was already apparent in very young typically developing children

(Chapter 4.1). This finding may signal an early genetic vulnerability, which in the presence of adverse environmental exposures may lead to more global deficits in recognizing facial expressions as well as to the emergence of emotional problems (Chapter 4.2). In *Chapter 4.3*, we conducted genome wide association studies and gene pathway analyses in search for new genetic variants implicated in observed infant attachment and temperamental fearfulness in preschoolers. For attachment disorganization, we found one nearly genome wide significant association located in the CACNA2D3 gene, and evidence for the role of the dopamine receptor mediated signaling pathway. For temperamental fearfulness, a significant asparagine and aspartate biosynthesis pathway was found. Findings go beyond the usual genetic suspects in attachment and temperament and should be replicated in larger samples.

In **Chapter 5**, we outlined distinct FER profiles in relation to specific syndromes of internalizing and externalizing behavior in the preschool period (*Chapter 5.1*). Results suggested a qualitatively different relationship between internalizing and externalizing behavior problems and children's FER such that internalizing problems contributed to emotion-specific differences in FER, while externalizing problems were associated with more general FER deficits. Knowledge of the specific FER profiles associated with internalizing and externalizing behavior problems can prove useful for the refinement of emotion-centered preventive interventions. In *Chapter 5.2*, we reported that difficulties in attention shifting, a specific domain of executive functioning, was causally involved in the mechanism through which low positive emotionality lead to the development of internalizing problems (i.e., socially withdrawn behavior) in a nonclinical sample of preschool children. Findings provide important insight into the underlying mechanisms through which temperamental vulnerability may lead to emotional and behavior problems in early childhood.

Chapter 6 highlights the main findings and provides an interpretation of these findings in a broader context. In addition, the chapter discusses relevant methodological issues and concludes with implications for clinical practice and future research.

SAMENVATTING

Emoties zijn essentieel voor het kunnen aanpassen aan onze fysieke en sociale omgeving. Alle emoties, zelfs negatieve emoties, zijn belangrijk voor ons gedrag, welzijn en onze aanpassing aan de omgeving. Echter, ongepaste of ongecontroleerde emoties spelen een rol bij verschillende vormen van psychopathologie en zelfs bij organische ziektes. Temperament en emotieherkenning zijn belangrijke domeinen van het emotionele functioneren van jonge kinderen. In dit proefschrift onderzochten we verschillende genetische en omgevingsfactoren die van invloed kunnen zijn op het temperament en het emotieherkenningsvermogen van kinderen in de kleutertijd. Tijdens de kleutertijd begint het temperament geleidelijk te stabiliseren en ontwikkelen kinderen een stevige basis voor het herkennen en benoemen van emoties. Het onderzoek werd 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 met een Nederlandse nationaliteit, werden gedetailleerde metingen verricht waarop het huidige proefschrift is gebaseerd.

In **hoofdstuk 2** bestudeerden we hoe driejarige kinderen emotionele gezichtsuitdrukkingen kunnen herkennen (*hoofdstuk 2.1*). De prestaties van driejarige kinderen verschilden op de verbale en nonverbale emotieherkenningstaken, met name bij het herkennen van bange gezichten. Driejarige kinderen vonden het zeer gemakkelijk om bange gezichten met elkaar in verband te brengen (ten opzichte van andere emoties, zoals blijheid, boosheid en verdriet) maar ze vonden het veel moeilijker (ten opzichte van andere emoties) om bange gezichten te herkennen aan de hand van een verbale stimulus. Daarnaast maakten kinderen systematische fouten bij het herkennen van emoties; blije gezichten verwisselden kinderen het vaakst met bange gezichten terwijl negatieve emoties het vaakst met elkaar werden verward.

In **hoofdstuk 3** werd onderzocht of depressieve klachten van de moeder tijdens de zwangerschap en in de postnatale periode gerelateerd zijn aan het emotieherkenningsvermogen (*hoofdstuk 3.1*) en temperament van kleuters (*hoofdstuk 3.2*). Uit de resultaten bleek dat depressieve klachten, zoals vermoeidheid, interesseverlies en negatieve gedachten van de moeder geassocieerd waren met zowel het emotieherkenningsvermogen als het temperament van het kind. Kinderen van moeders die depressieve klachten hadden waren minder goed in het verbaal herkennen van emoties. Het effect van depressieve klachten van de moeder op de emotieherkenning van het kind was niet toe te schrijven aan de sensitiviteit van de moeder, die was gemeten op driejarige leeftijd met twee moeder-kind interactie situaties. In hoofdstuk 3.2 toonden we aan dat kinderen van moeders met depressieve klachten minder positieve emoties lieten zien tijdens het spelen met handpoppen en minder verlegen waren tijdens het spelen met een springende nepspin dan kinderen van moeders die geen depressieve klachten hadden. Deze relatie was niet toe te schrijven aan andere risicofactoren, zoals een lage sociaal-economische positie of een hardhandige opvoedingsstijl van de moeder.

In **hoofdstuk 4** hebben we ons gericht op genetische factoren die van invloed kunnen zijn op het emotionele functioneren in de kleutertijd. *Hoofdstuk 4.1* laat zien dat de

korte vorm van het serotonine transporter gen promotor polymorfisme (5-HTTLPR) bij zeer jonge kinderen het vermogen vermindert om bange gezichten te herkennen. Daarnaast zorgde de korte vorm van het 5-HTTLPR polymorfisme, in combinatie met een stressvolle omgeving, voor een verhoogd risico op emotionele klachten bij jonge kinderen (*hoofdstuk 4.2*). Dit betekent dat er een mogelijke genetische ontvankelijkheid bestaat bij kinderen met de korte vorm van het 5-HTTLPR polymorfisme, die al op een zeer jonge leeftijd in een negatieve, stressvolle omgeving tot verminderd emotieherkenningsvermogen en tot emotionele klachten kan leiden. In *hoofdstuk 4.3* hebben we genoom-brede associatie studies en genetisch netwerk analyses uitgevoerd om nieuwe genetische varianten te identificeren die gerelateerd zijn aan de kwaliteit van gehechtheid en een verlegen temperament bij jonge kinderen. We vonden een bijna genoom-brede significante associatie tussen gedesorganiseerde gehechtheid en het CACNA2D3 gen en bewijs voor de rol van de 'dopamine receptor mediated signaling pathway' in gedesorganiseerde gehechtheid. Daarnaast vonden we significante associaties tussen de asparagine en aspartaat biosynthese netwerken en verlegen temperament bij kinderen. Deze studie is vernieuwend omdat het laat zien hoe moderne genetische analytische technieken kunnen worden gebruikt in het onderzoek naar de psychologische en emotionele ontwikkeling van het kind.

In **hoofdstuk 5** beschreven we verschillende 'emotieherkenningsprofielen' in relatie met internaliserende en externaliserende gedragsproblemen bij jonge kinderen (hoofdstuk 5.1). In *hoofdstuk 5.1* toonden wij aan dat internaliserende gedragsproblemen bij jonge kinderen gepaard gingen met emotie-specifieke verschillen in emotieherkenning terwijl externaliserende gedragsproblemen waren geassocieerd met algemene beperkingen in de emotieherkenning. Deze bevindingen kunnen worden gebruikt bij het verfijnen van preventieve interventies met een focus op het herkennen van emoties. *Hoofdstuk 5.2* wees uit dat kinderen die op drie jaar minder geneigd waren om positive emoties te laten zien tijdens het spelen, op vijf jaar sociaal meer teruggetrokken waren. Deze associatie kon deels door een specifiek domein van het executieve functioneren op vier jaar worden verklaard, namelijk door een verminderd vermogen om de aandacht snel van richting te kunnen veranderen. Het idee is dat kinderen die minder positieve emoties laten zien, meestal ook minder geïnteresseerd zijn in hun omgeving wat op een latere leeftijd tot teruggetrokken gedrag kan leiden.

In **hoofdstuk 6** bespreek ik de resultaten van alle studies gepresenteerd in dit proefschrift in een bredere context. Bovendien bespreek ik enkele methodologische aspecten die van belang zijn bij dergelijke studies en doe ik aanbevelingen voor de klinische praktijk en toekomstig onderzoek.

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Eszter Székely was born on 14th of November, 1980 in Debrecen, Hungary. Having completed her high school education, she pursued a pre-Bologna System Hungarian university program (Bachelor's and Master's combined degree) in her native city in the field of Clinical Child Psychology. During her studies, Eszter was admitted to the university's 'Program for the Talented and Gifted,' and received a scholarship for outstanding student-athletes. After graduation, she was employed as a research assistant by the Health Promotion Group of the School of Public Health at the Medical and Health Science Center in Debrecen. She contemporaneously completed a post-graduate training in Family and Couple Counseling. In 2005, Eszter won the 'Utrecht Excellence Scholarship' to study at the Utrecht University Medical Center in the Netherlands, where she followed a research Master's program in 'Neuroscience and Cognition'. In 2007, she was admitted to the Ph.D. program of the Department of Child and Adolescent Psychiatry/Psychology at the Erasmus University Medical Center in Rotterdam. In parallel, she obtained a Master of Science degree in Epidemiology from the Netherlands Institute for Health Sciences. Her doctoral research was conducted as part of the Generation R Study, a population-based longitudinal cohort from the fetal stage onward. The research focused on identifying environmental and genetic predictors of children's emotional functioning in the preschool period, culminating in the present thesis. After receiving her doctoral degree, Eszter will be a Post-Doctoral Fellow at the Social and Behavioral Research Branch of the National Human Genome Research Institute of the National Institute of Health in Bethesda, Maryland, USA.

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Prevention Research	2007	0.7
Health Economics	2007	0.7
Conceptual Foundation of Epidemiologic Study Design	2008	0.7
Introduction to Decision-Making in Medicine	2008	0.7
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Classical Methods for Data Analysis	2007	5.7
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Genetics in Child Cohort Studies, Symposium, The Generation R Study Group	2010	0.3
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