NEUROPHYSIOLOGICAL, BEHAVIOURAL AND GENETIC MARKERS OF BEHAVIOURAL PROBLEMS IN EARLY CHILDHOOD

by

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ABSTRACT

The work presented in the present thesis investigated the neural, behavioural and genetic markers that may be associated with the manifestation of behavioural problems during the early years of life. Across four different empirical studies, and by incorporating, behavioural, neurophysiological and genetic investigations, it was demonstrated that: (1) there are neurophysiological signatures that may be associated with the manifestation of behavioural problems early in life; (2) common genetic variations that determine serotonin variability are strongly associated with affectivity-related patterns of frontal brain activation; and that (3) normal genetic variations that modulate serotonin availability and neuroplasticity are each associated with affectivity-related patterns of visual scanning behaviours in response to faces and aversive scenes. Taken together, the results illustrate the existence of robust neural, genetic and behavioural markers that may be associated with the manifestation of behavioural problems in early childhood and prompt further investigation of the area by generating novel hypotheses. Together, the empirical findings of the thesis provide a first stage contribution to the complex mechanisms that may yield risk and resilience for behavioural problems during the early years of life by generating a more comprehensive insight on the field of affectivity.

Η κραυγή που γροικάς δεν είναι δική σου. Δε μιλάς εσύ. Μιλούν αρίφνητοι πρόγονοι με το στόμα σου. Δεν πεθυμάς εσύ. Πεθυμούν αρίφνητες γενιές απόγονοι με την καρδιά σου.

Νίκος Καζαντζάκης, Ασκητική

The cry is not yours. It is not you talking, but innumerable ancestors talking with your mouth. It is not you who desire, but innumerable generations of descendants longing with your heart.

Nikos Kazantzakis, Spiritual Exercises

(Trans. Kimon Friar)

Στους Γονείς μου,

Ιωάννη & Αλεξάνδρα Χρήστου

To my Parents,

Ioannis & Aleksandra Christou

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LIST OF ACRONYMS AND ABBREVIATIONS

(Alphabetically)

ACC	Anterior Cingulate Cortex
ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analyses Of Variance
ASD	Autism Spectrum Disorders
BAS	Behavioural Activation System
BAS-II	British Ability Scales-II
BDNF	Brain Derived Neurotrophic Factor
BIS	Behavioural Inhibition System
CBCL	Child Behavioural Checklist
CBT	Cognitive-Behavioural Therapy
CD	Conduct Disorder
COMT	Catechol-O-methyltransferease
DLPFC	Dorsolateral Prefrontal Cortex
DNA	Deoxyribonucleic Acid
DSM	Diagnostic and Statistical Manual
EEG	Electroencephalogram
ERP	Event Related Potential
FFT	Fast Fourier Transform
fMRI	Functional Magnetic Resonance Imaging
fNIRS	functional Near-Infrared Spectroscopy
G×E	Gene × Environment
GCA	General Conceptual Ability
GWAS	Genome Wide Association Studies
Hz	Hertz

mPFC	Medial Prefrontal Cortex
OFC	Orbitofrontal Cortex
PET	Positron Emission Tomography
PFC	Prefrontal Cortex
PSD	Power Spectral Density
RoI	Region of Interest
SCQ	Social Communication Questionnaire
SNP	Single Nucleotide Polymorphism
vmPFC	ventro-medial Prefrontal Cortex
vPFC	ventral Prefrontal Cortex
WHO	World Health Organization
WS	Williams Syndrome

CHAPTER 1

Early childhood and vulnerability for behavioural problems: an introduction

1.1. Preface

The chapter provides an overview of the thesis' empirical studies, providing a rationale for the ensuing work. Moreover, a review of concepts, models, and research relevant to the empirical work described in this thesis is illustrated. Existing empirical evidence that investigates the complex gene by environment interactions on the development of early behavioural problems, along with the relevant models, are then reviewed. This chapter also comprises an introduction on the role of multivariable investigations and their importance on the field of early behavioural problems. Finally, the key areas for further investigation are highlighted.

1.2. Thesis Overview

The present thesis starts with a broad introduction of the main definitions in the field of risk and resilience, the genetic and environmental influences of behaviour, and the existing models that account for gene by environment interactions, which may predict behavioural outcomes. Continuing with this, Chapter 2 begins with an overview of the available research, models and methods used to investigate the development of emotion regulation in typical and atypical development, including the role of frontal EEG as a neurophysiological index of early affectivity. To continue, the first empirical study utilizes frontal EEG in order to derive measures of frontal activation that are believed to be reliable indexes of early behavioural problems. Taking into account previous evidence that has highlighted the existence of strong association between negativity patterns of frontal Electroencephalogram (EEG) asymmetry, and the manifestation of behavioural problems in young children, the study sought to further delineate the nature of frontal EEG patterns of early affectivity, to replicate the most reliable current knowledge, and also through the utilization of a novel experimental design to provide new insights in the field.

In Chapter 3 the same EEG investigation and experiment is utilized using a larger sample and age range of children. Through the incorporation of genetic investigations, neurobiological mechanisms are unveiled, which may account as an influence of frontal brain-based patterns that are associated with negative affectivity, or patterns that relate to positive affectivity. The main aim of this study is to reveal the putative role of common genetic variations that determine serotonin availability in influencing the positivity versus negativity patterns of frontal EEG activation during early childhood. To date, there have been no direct investigations on the role of serotonin availability in modulating the long-standing index of early affectivity, frontal EEG asymmetries, in early childhood.

Chapter 4 starts with a critical overview of various neurophysiological and behavioural methods, and the relevant theoretical concepts that have been used to measure emotional reactivity in child, adolescent, and adult literature. Furthermore, aiming to further delineate the genetic influences of early reactivity in a group of young children, eye-tracking technology is employed, where eye gazes towards angry, happy, and neutral facial expressions are recorded by employing a novel paradigm. Through the investigation of the neuropsychological, behavioural and genetic correlates (i.e., neuroplasticity, serotonin genetic variations) of visual scanning of emotional facial expressions and facial features, new insights will be provided on the individual role of candidate genes on behavioural traits that relate to early reactivity.

In the last empirical study of the thesis (Chapter 5), taking into account the important role of early atypicalities on the processing of affective stimuli, the genetic underpinnings of preferential looking behavioursare investigated in response to aversive versus positive scenes, in a the same group of young children tested in Chapter 4. The main aim of the study is to investigate the role of a common genetic variation that determines serotonin availability in modulating looking behaviours in response to different types of threatening stimuli in early childhood.

Chapter 6 summarizes the main findings of the present thesis and brings together the contributions of the substantive chapters to the neural, behavioural and genetic mechanisms that are present during early childhood and may account as markers associated with putative vulnerability constructs of better and worse psychological outcomes later in life.

Collectively, the outcomes of the investigations can provide novel insights into the complex constructs of early affectivity, and may further contribute into explaining the interindividual differences associated with psychological difficulties and problems. To this end, the overreaching aim of the present thesis is to critically approach the current understanding of the nature of early affectivity, and the ensuing work will act as a springboard for the development of novel hypotheses, as well as theoretical and early therapeutic models in the near future, targeting the most vulnerable individuals.

1.3. Investigating early behavioural problems

1.3.1. Definition of Risk and Resilience

The term 'risk' describes a range of variables that may increase an individual's likelihood of psychological problems, or increase their vulnerability to negative outcomes in their lifespan (Goyos, 1997). Some individuals may be better at managing environmental stressors, probably due to the availability of a repertoire of disposition resources and copying styles. In line with this concept, resilience has been previously defined as an individual's capacity to effectively recover, adapt, and remain unaffected when exposed in adverse environmental conditions (Masten, Best, & Garmezy, 1990). Moreover, the umbrella term 'resilience' has also been used to describe the environmental and genetic influences that may act as protective factors against psychopathology (e.g., Werner & Smith, 1992). Risk variables may include variables that reside within the individual (e.g., temperament, neurophysiology) and variables that come from an individual's external environment (e.g., poverty, nurturing environment). Interestingly, early work mainly focused in investigating children who were believed to be at increased environmental or genetic risk for the development of neuropsychiatric problems. However, these studies provided evidence to suggest great variability among children at the same level of risk, which suggested the existence of resilience markers (e.g. environment, genes) against psychological maladjustment, which resulted in the development of the risk-resilience model. Nowadays, research in risk and resilience represents a broader systems transformation in child psychology and developmental science (e.g., Lerner, Easterbrooks, & Mistry, 2012; see also Masten, 2014) and psychopathology (Cicchetti, 2013a).

The risk and resilience model has emerged simultaneously with the establishment of the developmental psychopathology field, since both aim to explore the development of human behaviour (Cicchetti, 2006; Masten, 2007). The term developmental psychopathology describes the study of the development of psychological disorders through the life course (Cicchetti, 1989) and has a particular focus on the investigation of the complex interplay between socio-emotional and biological underpinnings in both typical and atypical development across the life span (Cicchetti, 1993; Cicchetti & Toth, 1998; Rutter & Sroufe, 2000; Sameroff, 2000). A more recent definition described the field of developmental psychopathology as the extensive study of the human behavioural health and adaptation, in the context where the individual lives, by adopting a constant developmental perspective (Masten, 2006).

1.3.2. Environmental Influences for Behavioural Problems

The investigation of the early environmental influences that may contribute to the manifestation of behavioural problems in young children is a core area of research on the scientific field of developmental psychology. The most prominent environmental factors that have been considered as potential influences of psychological maladaptation include the broad family environment (e.g., mother-child interactions; Du Rocher, Schudlich & Cummings, 2007; Elgar, Mills, McGrath, Waschbusch & Brownridge, 2007), and the contextual factors where the child grows up (e.g., early adversity, poverty; see Lukkes *et al.,* 2009). These factors have been widely documented as having a critical influence on the establishment of temperamental styles to produce certain patterns of adaptive or maladaptive behaviour early in life (Rubin, Hymel, Mills & Rose-Krasnor, 1991).

1.3.3. Genetic influences for behavioural problems

Advancements in the field of molecular genetics have confirmed the existence of gene-mediated influences of psychopathology, and human behaviour in general (e.g., Caspi *et al.,* 2002, 2003; Cicchetti & Blender, 2004; Kaufman *et al.,* 2004). Aiming to uncover the heritability of psychiatric disorders and behavioural problems, during the last four decades, traditional research has focused on investigating the interplay between heritability and experience in shaping social and emotional functioning (e.g. for a review see Kendler & Baker, 2007). More specifically, linkage analysis studies have been testing the associations between genetic polymorphic markers and the presence of psychiatric disorders within families, with the strongest correlations to be believed to be associated more with the disease. However, linkage studies have shown to be unsuccessful in identifying strong associations between underlying genetic effects for most complex diseases (for a review see Merinkangas & Risch, 2003). Moreover, a candidate gene approach has also emerged, aiming to investigate the role of common genetic variations that involved in the neural circuits of emotion regulation and affectivity which may interact with environmental stressors to predict behavioural reactivity, and vulnerability versus resilience for affective disorders (Canli *et al.,* 2006; Caspi & Moffitt, 2006; Canli & Lesch, 2007).

In addition to linkage and candidate gene studies, Genome Wide Association Studies (GWAS) have been widely used as a more powerful research approach. GWAS is an examination of a range of common genetic variants in large populations of individual to see if any variant is associated with a trait. Interestingly, GWAS studies have provided numerous replication findings that were not evident in candidate gene studies, providing support for the existence of strong genetic associations with psychiatric disorders (for a review see Collins & Sullivan, 2013).

It is widely known that Deoxyribonucleic acid, or DNA, is contained in all known living organisms, with the four DNA nucleobases [the purine bases adenine (A) and guanine (G), and the pyrimidines thymine (T) and cytosine (C)] being responsible for the encoding of genetic information. When a single nucleotide in the genome differs between members of a biological species, or paired chromosomes in an individual, then a DNA sequence, or Single Nucleotide Polymorphism (SNP) is occurring. The majority of the functional studies available, however, have examined the function of SNPs in coding regions (for a review see Ng & Henikoff, 2003), due to their importance of such regions in influencing phenotype by altering the encoded proteins that associated with each gene region. However, although there are studies with SNPs in the non-coding regions of genes suggesting their involvement in the transcriptome (for a review see Ng & Henikoff, 2003), their exact function is not yet known. In addition to SNPs, Variable Number Tandem Repeats (VNTR) have also widely investigated for their role in predicting behavioural outcomes. VNTRs are widely used as markers in linkage analysis since they have polymorphic nature, and each of those consists of multiple copies of short repeated DNA sequences that vary from individual to individual (e.g., Brookes, 2013).

From a psychological perspective, research evidence suggests that variation in candidate genes are responsible for variations on human phenotype, by influencing vulnerability of certain disorders, and therefore may assist in facilitating early diagnosis, prevention and ideally treatment of various psychopathologies (Saxena, 2007). However, the importance and size of the effects of individual genetic variation in moderating or mediating human behaviour is still debatable. More specifically, a factor may account as a mediator variable when accounting for the relationship between the predictor and the outcome, which may be a behavioural or neurobiological variable. Conversely, a variable can be accounted as moderator when it is represented as an interaction between a major predictor and another variable with specific properties (e.g., a subpopulation). This is particularly important for candidate gene studies, where there is a likelihood that a single gene may explain a range of behavioural phenotypes, which limits the possibility for the existence of neurobiological explanations of specific traits (for a critical discussion see Munafo, 2006).

In addition to the individual genetic contributions in behavioural diversity, during recent years, the investigation of the cross-over interactions between, genes, brain, and behaviour has also emerged. Interestingly, Cicchetti (1990) as part of his early observations, stressed that the field of developmental psychopathology needs to adopt a multidisciplinary approach in order to unveil the complex interplay underlying adaptation and maladaptation, as well as to influence prevention and early intervention for psychopathology. In the following section, I provide an overview of the various models, methods and problems that exist when conducting multivariate investigations of human behaviour and the importance of such investigations in delineating the nature of psychological affectivity early in life.

1.4. Investigating Gene × Environment interactions

From a historical perspective, the genetic and environmental influences of human behaviour have been studied in isolation from one another, due to methodological differences adopted from the two fields (for a critical review see Dick, 2011). Originally the concept of genetic predisposition was evident in the field of medical sciences, where a specific genetic profile was associated with a disease phenotype at the genetic level. However, studies with monozygotic twins have underlined the role of environmental influences (e.g., microbes) on the cause of diseases, such as autoimmune or inflammatory diseases (Bach, 2005). In line with this, in recent years the limited progress in the genetics of common diseases has been acknowledged (Buchanan, Weiss, & Fullerton, 2006) which is overly expected to help in identifying the genetic background of diseases and develop early prognosis. In the field of psychology and psychiatry, due to the increasing evidence suggesting the involvement of environmental influences in the genetic vulnerability for behavioural and psychiatric problems empirical studies have started to integrate different variables coming from the human organism (e.g., genetic mechanisms) and from the individual's external environment (e.g., poverty, parental behaviour) under the same investigation, aiming to explain variation in human behaviour (Gottlieb, 1992). More specifically, Gottlied (1992) defined the complex interplay between genotype (i.e., normal genetic variations among individuals of the same species), phenotype (i.e., variations on behavioural traits) and environment as not predetermined, and therefore may bi-directionally influence the human behaviour over the course of development with cross over interactions between the human genome and the environment (Gottlieb, 1992).

In the field of developmental psychopathology, aiming to investigate both affected populations, as well individuals at increased vulnerability for various psychopathologies, early work in the field did not adopt a specific theoretical consideration to account for all the observed individual differences (Rutter & Sroufe, 2000), instead, knowledge came from different disciplines was integrated on the same framework by employing multiple levels of analysis (Cicchetti & Blender, 2004; Cicchetti & Dawson, 2002). To this end, the Gene × Environment (G×E) interaction model was generated, with the main argument being that individuals are not passively affected by the influences of their environment, but they generate themselves their experiences in a constantly changing world (Cummings, DeArth-Pendley, Du Rocher, Schudlich, & Smith, 2000). G×E interactions occur when an individual's positive or negative affective response to an environmental stressor depends on the individual's resilient or vulnerable genetic make-up (Caspi & Moffitt, 2006; for a review see Duncan & Keller, 2011). Depending on the environmental adversity, individuals vary in their degree of genetic predisposition, and may react differently. For instance, previous research reported that maltreated children whose genetic make-up predisposes them to negative affectivity and aggression (i.e., variations in the MAO-A gene), were more likely to exhibit antisocial behaviour and develop conduct disorder (see Caspi *et al.,* 2002).

Moreover, compared to G×E interactions, G×E correlations (rGE) reflect genetic influences when individuals are exposed to specific environmental conditions. rGEs have originally been conceptualized by behavioural geneticists who observed that genetic influences impacting on specific environments may make these environments heritable themselves (Kendler & Eaves, 1986; for a review see Jaffe & Price, 2007). For example, a genetic predisposition for stress reactivity may be mediated by life events or personality traits that may strengthen the environmental influence (Moffitt *et al.,* 2006). Therefore, interactions, or correlations (for a critical review see Dick, 2011; Moffitt, Caspi & Rutter, 2006), between genes and environmental influences may impact upon an individual's daily functioning and well-being.

Nowadays, it is generally accepted that neuropsychiatric disorders are the result of the interplay between genetic and environmental factors. In addition, the gradual unveiling of the dynamic interplay between early life experiences and brain functional development the recent years (Black, Jones, Nelson & Greenough, 1998; Greenough, Black & Wallace, 1987) has influenced the development of new theoretical concepts to interpret diversity in neurophysiology and affectivity. Therefore, the alongside investigation of genes, brain and behaviour early in life may provide a conclusive answer regarding the risk and resilience for the development of affective disorders.

1.4.1. From Genotype to Endophenotype

During the last three decades a distinct line of research has started to investigate the neurophysiological underpinnings of behavioural manifestation aiming to bridge the gap between the behavioural manifestation of a psychopathology and the genotypic variations that mediates this manifestation (for a review see Heatherton, 2011). The previously defined term of 'endophenotype' describes a range of internal processes of the human organism that include physiological, biochemical and psychological components of reactivity (Gottesman & Shields, 1973; Gottesman & Gould, 2003). In the field of psychiatric genetics, the term endophenotype is defined by the presence of specific criteria, such as to be heritable, to be associated with a psychiatric disorder, or to be present when the disorder is not present (i.e.

state independent), and to be reported in higher prevalence in healthy family members of affected individuals compared to the general healthy population (Gottesman & Gould, 2003; for a review see Flint & Munafo, 2007).

Aiming to derive a direct insight on the neurobiological basis of affective disorders, technological advancements in recent years in the field assisted in the increasing evidence to report insights of the human endophenotype, especially the human brain (e.g., Amso & Casey, 2006; Ciaranello *et al.,* 1995). The recruitment of such cutting-edge techniques to measure the neurobiology of human affectivity has provided valuable scientific knowledge on the structure and function of the human brain, such as knowing about brain connectivity and distinguishing white and grey matter, as well as measure changes in brain cells, such as function of neurotransmitter receptors (e.g., Thomas, 2003). Among the most widely used methods is the Positron Emission Tomography (PET), functional Magnetic Resonance Imaging (fMRI), Electroencephalography (EEG), as well as functional Near-infrared Spectroscopy (fNIRS), which led to a significant increase in knowledgeabout how the human brain interprets experience.

In addition to the studies examining brain development and reactivity, psychology and psychiatry research has also started to employ technologies that allow the measurement of eye movements to derive an endophenotypic index of psychological reactivity across various populations (for a review see Flint & Munafo, 2007). Most notably, cognitive models of depression have previously highlighted that biased processing of emotional information may contribute significantly on the manifestation of the depressive symptomatology (Teasdale & Barnard, 1993; Williams, Watts, MacLeod & Mathews, 1997). Thus, atypical attenuation to an environmental stressor may relate to an individual's inability to control the arousal resulted by the stressor, and therefore lead to difficulties with emotion regulation (Joormann & Gotlib, 2007). To this end, the employment of eye-tracking technologies to measure eye movements has gradually become a valuable method in psychology and psychiatry to understand the mechanisms of visual processing (for a review see Weierich, Treat, & Hollingworth, 2008). Taken together, the gradual unveiling of the existence of complex mechanisms that may influence human development and behaviour had as a result the generation of multiple theoretical accounts for the explanation of the complex interplay between genes, brain and behaviour.

1.4.1.1. Models of G×E Interactions

Vulnerability is a term that describes the increased likelihood for being affected from negative environmental influences that may place an individual in higher risk for developing a trait. However, this only highlights the negative side of an individual's plasticity in response to the environmental influence. For instance, a "vulnerable" child may benefit disproportionately from positive environmental influences that may suggest the need to generate more neutral concepts to describe such as susceptibility or plasticity (see also Pluess, 2015). The diathesis-stress model supports the existence of a dual-mode interaction between pre-existing neurobiological vulnerability and negative life experiences that may produce negative behavioural outcomes (Alloy, Hartlage & Abramson, 1988). More specifically, the diathesis-stress model describes the biological variables as fixed risk factors that under specific negative environmental interactions can reliably predict per se negative outcomes (see Figure 1.1). This model has been widely tested and supported in both clinical and healthy populations (e.g., Shell *et al.,* 2014). Conversely, the differential susceptibility framework proposes a more conclusive framework by introducing the concepts of sensitivity (Boyce & Ellis, 2005; Belsky & Pluess, 2009) and susceptibility factors (Belsky, Bakermans-Kranenburg & van Izendoorn, 2007) to describe complex interactions among different variables. More specifically, the differential susceptibility model has proposed the independence of the behavioural outcome from the biology-mediated susceptibility factors, allowing for cross-over interactions between biological and environmental factors.

The development of the differential susceptibility model may represent the gradual understanding on the field of psychology and psychiatry, that there are no single causal risk factors that may lead to psychological maladjustment, as well as that not all the individuals that carrying a potential risk factor will ultimately manifest the negative outcome in their later life (e.g., Cicchetti & Rizley, 1981; Kraemer, Stice, Kazdin, Offord & Kupfer, 2001; Luthar, Cicchetti & Becker, 2000). This novel insight in the field moves forward from the traditional conceptualization of the risk factors as static through the development (Kraemer *et al.,* 1997), and suggest a dynamic interplay among different susceptibility mechanisms through the course of development (e.g., Cicchetti, 1999; Cicchetti & Lynch, 1993).

As shown in Figure 1.2, a putative genetic vulnerability component (e.g. low serotonin concentrations) may lead to positive outcomes when followed by positive experience (e.g. caring nurturing environment). Moreover, the bright side of the susceptibility has been previously described on the Vantage Sensitivity framework (Pluess & Belsky, 2013). More specifically, it has been previously suggested that individuals may also vary in their responses to exclusively positive environments, which may be due toa range of endogenous variables, including their genetic make-up (for a recent discussion see Pluess, 2015). Furthermore, the neurosensitivity hypothesis suggests that there are different factors involved in environmental sensitivity that may include genetic, psychological and physiological factors (Belsky & Pluess, 2009). Taken together, the existence of multiple neurobiological mechanisms that may infer vulnerability versus protection for psychological problems may involve the contribution of mechanisms that reside within the individual, acting as internal mechanisms of environmental sensitivity (e.g., brain function and structures), also referred to as endo-environmental influences (e.g. Schmidt, Fox, Perez-Edgar & Hamer, 2009). To this end, the observed physiological and behavioural reactivity outputs may result from the interaction between direct and indirect effects of genetic and environmental influences (for a review see Pluess, 2015).

Figure 1.1. Graphical display of the Diathesis-stress model showing a unidirectional prediction of the interplay between neurobiological factors, experience and behavioural outcomes. As illustrated below, a negative biological predisposition can only predict negative outcomes.

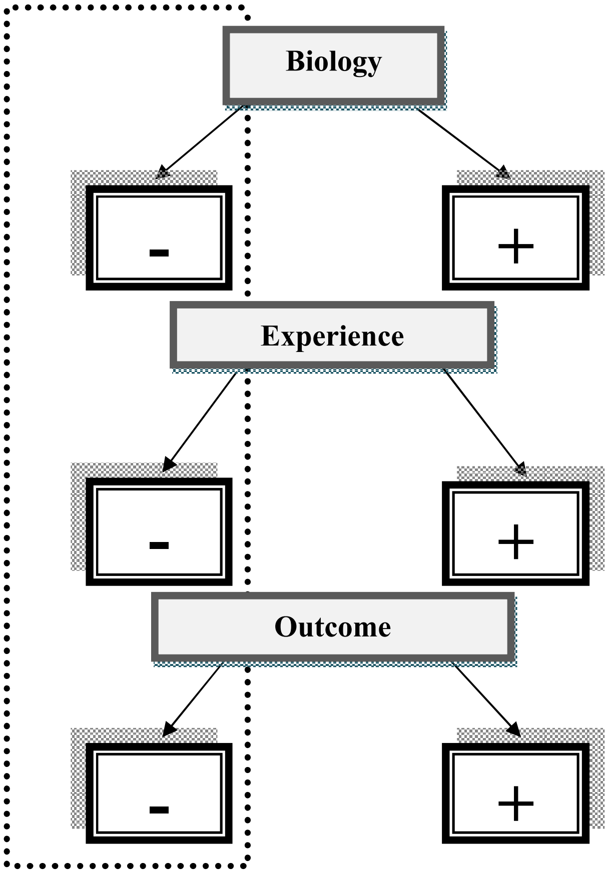
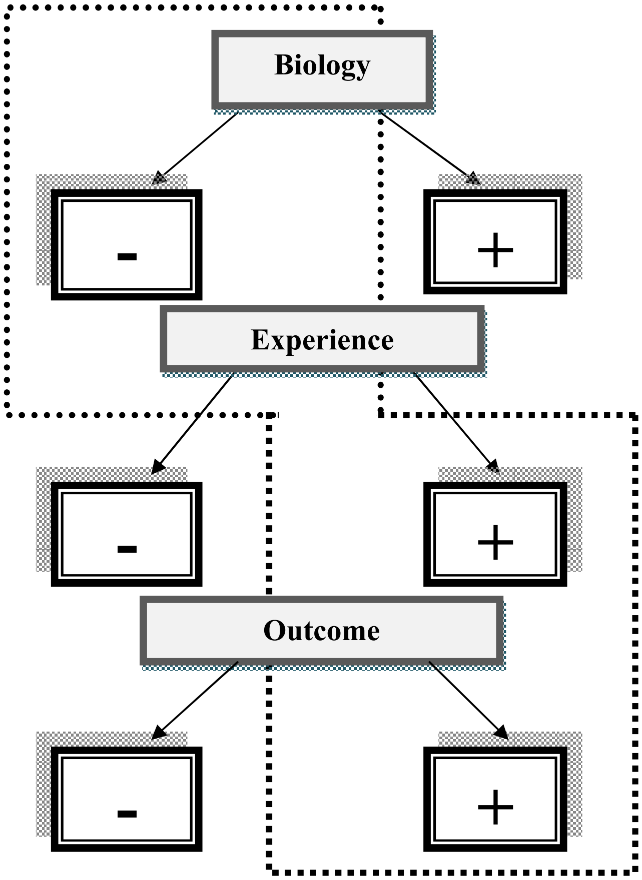


Figure 1.2. Graphical display of the Differential Susceptibility model showing a bidirectional prediction of the interplay between neurobiological factors, experience and behavioural outcome. As illustrated below, a negative biological predisposition can predict positive outcomes under favourable environmental conditions.



1.4.2. Measuring G×E in Early Childhood

There is increasing evidence to suggest that normal genetic variations and environmental influences may interact to predict not only vulnerability for psychopathological problems but also protection against them. In line with this claim, it has been previously suggested that genes are continuously interacting with the environment to determine positive or negative behavioural outcomes (Fox *et al.,* 2005; Segalowitz & Schmidt, 2008). In line with this notion, research evidence coming from studies on brain functioning and studies on the genetic underpinnings of human behaviour have been synthesised in recent years, to unveil the genetic influences of brain development and functioning in both children and adults. The development of this approach, widely known as neuroimaging genetics approach, investigates how genetic information is linked to individual variation in brain functioning, especially in neural networks and regions that are critical for psychological maladjustment (Meyer-Lindenberg, 2010; Hariri, Drabant & Weinberger, 2006).

In neuroimaging genetics studies, the utilization of neuroimaging techniques, such as fMRI and EEG, provide a neurofunctional phenotype, which is compared to genotype to derive the neurobiological basis of human behaviour. Therefore, through the employment of these methods the genetic influences of human brain functioning may be more accurately and reliably observed (Meyer-Lindenberg & Tost, 2012). There is now a decade of neuroimaging genetics research in both clinical studies with adults (e.g., Tan *et al.,* 2007), healthy adults (e.g., Papousek *et al.,* 2013), and children (Wiggins *et al.,* 2012), suggesting that the increasing incorporation of new sources of biological information coming from both brain structures and the human genome opens up the possibility for the establishment of this field as a prominent translational enterprise to influence the development of cutting-edge therapeutic approaches (Meyer-Lindenberg & Tost, 2012). Interestingly, the investigation of the interplay between the environment, the genome and brain functioning in children, may help to go a step further in understanding the aetiopathogenesis of specific developmental disorders (Moffitt, 2005).

1.4.2.1. Principles and problems in G×E research

A main challenge in research, which is linking genetic profile, brain functioning and behavioural outcomes, is the fact that human brain is a complex system, which is dynamically changing, especially in the developing brain. This complex process of ontogenetic development is a barrier on the overwhelming plans to use the genome aiming to uncover the specific function of genetic mechanisms (for a review see Casey, Soliman, Bath & Glatt, 2010; Karminoff-Smith, 2009). Moreover, neuroimaging genetics studies have low replicability rate which may be due to the fact that genetic risk for a specific disorder may be distributed across multiple genetic variants which may make observations with single genetic variants difficult to replicate (for a review see Casey, Soliman, Bath & Glatt, 2010; de Zubicaray *et al.,* 2008; Hariri *et al.,* 2002).

Another issue concerning candidate gene studies is the ancestry effects on the frequency of the polymorphic alleles; also known as stratification effects. For instance, studies have reported that the frequency of the E4 allele of the Apolipoprotein E (APOE) ranges from 5% in populations in Taiwan and Sardinia but has a high frequency of 40% in Pygmies (e.g., Corbo & Scacchi, 1999). Therefore, an allele with low frequency in a population is difficult to interpret, but also to replicate in other population, which makes the importance of controlling participants' ancestry imperative (for a review see Casey, Soliman, Bath & Glatt, 2010). Current and future investigations are vital to control the factor of ancestry variations that will allow higher replication validity of the studies in the field (for a recent protocol review see Culverhouse *et al.,* 2013).

In addition to unveiling the normal genetic variations that may infer susceptibility for better and for worse outcomes, it is important to underline the issues of missing heritability (Maher,

2008) in the fight of tackling affective disorders. Advancements in GWAS have provided influential evidence on some of the genetic mechanisms that drive neuropsychiatric disorders (e.g., O'donavan *et al.,* 2008). However, for other disorders, the particular genetic contributions remain largely unknown (Moskvina *et al.,* 2009), which is referred as missing heritability. To this end, G×Estudies are believed to be an important contributor towards unveiling missing heritability. More specifically, it is believed that the discovering of the environmental contributions that may affect only a subgroup of individuals with specific genetic profile, may allow the unveiling of the particular components of a complex mixture of effects (Thomas, 2010), such as the effects of air pollution (Hunter, 2005). Moreover, research has suggested that the clear understanding of the replication problems in GWA studies, may help in identifying real homogeneity among individuals, and therefore increase understanding of disease complexity (Greene, Penrod, Williams & Moore, 2009; Ioannidis, 2007). This later contribution of G×E, when applied using risk prediction models, will have an important implication in the field of public health, as well in advancements in personalised medicine (Thomas, 2010).

CHAPTER 2

The relationship of frontal EEG asymmetries and behavioural problems in early childhood

2.1. Preface

The chapter provides an overview of the current knowledge on the development of early behavioural problems in typical and atypical development, which includes the contribution and variability of several markers for affective disorders, as observed in early childhood. Empirical research as well as methodological considerations delineating the measuring of behavioural problems in early childhood is summarized, providing a rationale to the ensuing empirical work. In the prospective empirical study the neurophysiological constructs associated with early behavioural problems are investigated, by recording the rates of behavioural problems from parent reports, as well as by indexes of early affectivity as recorded from a novel EEG paradigm. The study aims to replicate previous knowledge on the development of early behavioural problems, as well generate new insights in the field.

2.2. Background and Rationale

2.2.1. Introduction

Over the last three decades there has been great interest in the investigation of why some people appear to be predisposed in different forms of psychopathologies where others are not. Self-regulation, which broadly refers to an individual's ability to modulate an affective or behavioural response (Blair & Diamond, 2008; Kopp, 1982), is believed to have a critical contribution to the development of early behavioural problems and, ultimately, to the development of psychopathological problems. To this end, the aspects of human behaviour that relate to the development of behavioural problems have been previously investigated, in both infancy and childhood, as vulnerability factors for developmental psychopathology. Although there is a plethora of research on the field, where several theoretical models and methodologies have been developed, to date, there is not a single conclusive account available to explain the individual differences that may shape the manifestation of affective problems. A study in this area can provide important information on the particular brain and behaviour associations, and aid a better understanding of the early behavioural constructs of affective disorders.

2.2.2. Development, personality, and behavioural problems

2.2.2.1. Classification of behavioural problems

With regards to the early manifestation of problematic behaviours, there are typically two broad domains of childhood maladjustment that are investigated in the field of developmental science; internalizing and externalizing problems. Internalizing problems are broadly defined as inner-directed problems that include distressful and over controlled behaviours, such as sorrow, fear, guilt and worry (Achenbach & McConaughy, 1992) typically conceptualized in child, adolescent and adult literature as core components of psychopathology (e.g., Fonseca & Perrin, 2001). More specifically, internalizing problems include behaviours such as withdrawal, anxiety and depression problems, and inhibition that affecting an individual's internal psychological environment (Campbell *et al.,* 2000). Conversely, externalizing problems are described as a range of overt problems that mainly include aggressive, impulsive, and hyperactive behaviours (Hinshaw, 2002) and relate to a child's negative response to the external environment (Campbell, Shaw, & Gilliom, 2000; Eisenberg *et al.,* 2001). Externalizing problems have been previously documented as major risk factors for later juvenile delinquency, criminal behaviour and violence (Betz, 1995; Farrington, 1989; Moffitt, 1993). It is now widely accepted that externalizing problems in toddlerhood and early childhood can be utilized as robust predictors of psychological maladjustment in the later school-age years (Campbell, Shaw & Gilliom, 2000). However, the dichotomic classification between these two clusters of behavioural problems is not exclusive. For example, a child's internalizing behaviour may have a negative impact on other individuals in the environment (e.g., siblings). In a similar vein, a child who exhibits externalizing behaviour may also have internalizing problems, with substantial comorbidity to have been reported previously for the two clusters of problems (Hinshaw, 2002).

The early establishment of cognitive-affective balance between approach and withdraw-related behaviours may have a critical contribution to an individual's affective management in response to challenging situations. For instance, cognitive emotion regulation strategies may aid an individual in handling emotional arousal and effectively keeping control of environmental stressors (Thompson, 1991). This highlights the importance to delineate the complex behavioural processes that may exacerbate early onset behavioural problems.

2.2.2.2. Measuring behavioural problems in early childhood

In order to understand the particular constructs of the development of early behavioural problems, previous and current research has been employing standardised parent-rated questionnaires to measure early affectivity and behavioural traits (for a review see McClelland & Cameron, 2012). In addition, EEG has been widely used as a complementary method to provide a direct index of endophenotypic variation of early manifestation of behavioural problems in children. In the following section, I examine the key issues surrounding the neurophysiological basis of early manifestation of behavioural problems, as well as the various methods of measuring the endophenotype of early affectivity mechanisms via these neural pathways.

2.2.3. Developmental cognitive neuroscience of behavioural problems

Currently, it is widely accepted that the frontal lobes of the brain are critically involved in humans' ability to regulate their emotions. Lateral hemispheric activation, or lateralization, is describing the differing activation of the right and left hemisphere, depending on the cognitive processes that an individual is undergoing. Research evidence has showed lateralization over the left prefrontal cortex (PFC) to mediate approach-related behaviours and positive affectivity, while right frontal hemispheric lateralization to mediate withdraw-related behaviours and negative affectivity (Davidson, 1993; Fox, 1991). In a similar vein, research into the neural origins of asymmetric electrocortical activity suggests that measures of frontal asymmetry (difference between relative right and left frontal activation; alpha power) reflect activity mainly over the dorsolateral prefrontal cortex, or DLPFC[2] (Pizzagalli, Sherwood, Henriques & Davidson, 2005).

Emerging literature demonstrates that the DLPFC region is involved in a range of cognitive activities, such as working memory, decision-making and planning abilities (e.g., Bardey, Krueger & Grafman, 2009; Fan, McCandliss, Fossella, Flombaum & Posner, 2005; Murray & Ranganath, 2007). In addition, fMRI studies have shown that DLPFC is linked with child and adolescent behavioural problems, with aggressive behaviours being associated with a reduced activation over the DLPFC region, which suggests the existence of a brain-mediated mechanism of impaired regulation of anger-related emotional impulses (for a review see van Goozen *et al.,* 2007). Research has shown that alpha activation acts as an inhibition contributor, therefore lower frontal asymmetry rates represent relatively less compared to right frontal cortical activation (Towerns & Allen, 2009). Studies investigating the role of frontal lobe asymmetries suggest that frontal EEG may reflect a reliable index of affectivity. More specifically, a synchronous activity of frontal EEG oscillations is seen as an indication of the underlying activation over subcortical neural structures (Shagass, 1972). This assumption is further supported by studies employing fMRI and Positron Emission Tomography (PET) that have shown a decrease in cortical blood flow when alpha power was increasing with increasing alpha power (Cook *et al.,* 1998; Goldman *et al.,* 2000).

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[2] DLPFC region is located in the middle frontal gyrus of the human brain (i.e., Brodmann's areas 9 and 46; Miller & Cummings, 2007).

Recent studies with children have confirmed the utilization of EEG as an index of affectivity. More specifically, in a study with children aged 6–13 who had mothers reporting a history of depression was found that children of depressed mothers with relatively less left frontal asymmetry (more right asymmetry) during the processing of emotional films but not in rest when compared to children with non-depressed mothers (Lopez-Duran, Nusslock, George & Kovacs, 2012). These findings agree with a line of research suggesting that individual differences in frontal EEG asymmetry are more pronounced when individuals are processing tasks with emotional component, as opposed to during rest (for a recent review see Allen & Reznik, 2015).

Furthermore, although there are now more than three decades of research investigating the association between individual differences in frontal EEG asymmetry and behavioural affectivity (for recent reviews see Gander & Buchheim, 2015; Harmon-Jones, Gable & Peterson, 2010) there is discrepancy in the literature regarding whether frontal EEG represents a disorder-specific endophenotype or not. Moreover, studies with children have shown associations between internalizing problems and greater relative right asymmetry, as well as externalizing problems and greater left asymmetry (e.g., Gatzke-Kopp, Jetha, & Segalowitz, 2014; Smith & Bell, 2010). Other studies have reported the opposite (Baving, Laucht, & Schmidt, 2002; Santesso, Reker, Schmidt, & Segalowitz, 2006) or did not find significant frontal EEG and behaviour associations (Fox, Schmidt, Calkins, Rubin, & Coplan, 1996; Theall-Honey & Schmidt, 2006). Interestingly, a recent meta-analysis found that psychosocial risk factors, but not early behavioural problems, were associated with the presence of greater right frontal asymmetry in studies with infants and children (Peltola *et al.,* 2014). However, although this study did not report publication bias, the effects reported were relatively underpowered. The documented inconsistencies in the literature may be due to methodological or sample-related issues, such as a small sample, heterogenity (Coan & Allen, 2004) or comorbidity (e.g., Heller & Nitschke, 1998) of the studied clinical groups, gender effects affecting EEG asymmetry, or may be a result of the behavioural measures used across different studies (for a review see Thibodeau, Jorgensen & Kim, 2006). To this end, to date, there is not an available developmental model of frontal EEG asymmetry to account for early reactivity and psychopathology. Future studies in this area of inquiry will be needed to delineate the nature of frontal EEG asymmetry and it will need to be determined whether it can be utilised as a reliable index or biomarker for affectivity.

2.2.3.1. Characteristics of frontal EEG asymmetry

The most widely investigated and reliable correlate of frontal activity is found in the frontal asymmetry difference score, which reflects the difference between homologous measures of EEG alpha power measured over left and right frontal electrode sites (i.e., Allen & Kline, 2004; Coan & Allen, 2004; Davidson, 2004). Frontal EEG asymmetry in adults is represented with changes in electrocortical activity over the prefrontal cortex, with a frequency of 8 to 13 Hertz (Hz), and is also known as alpha power. Alpha power has been documented to be present during attentive and awake states, but is significantly suppressed when the individual performs a cognitive task (Schaul, 1998). In general, studies agree on the exact Hz band boundaries of alpha power, where in infants and children the boundaries of corresponding bands appear to be lower, compared to adults. There is now more than three decades of research in frontal EEG asymmetries, in a variety of populations, with the majority of the available evidence strongly supporting the existence of a reliable neural signature, which reflects positive and negative affectivity (Davidson *et al.,* 1990; for a review see Harmon-Jones, Gable & Peterson, 2010). At large, more right frontal asymmetry has been associated with withdraw-related behaviours and negative affectivity, whereas more left asymmetry has been associated with approach-related behaviours and positive affectivity (Davidson, 2004).

An important characteristic of alpha power is that it is inversely proportional to cortical activation (Coan & Allen, 2003; Davidson, Jackson & Kalin, 2000; Schaul, 1998). For example, a decrease in alpha power in the EEG recorded from the left side of the scalp, relative to power in the right side, represents increased activation in the left frontal region or 'left frontal asymmetry'. Conversely, a decrease in alpha power over right frontal region reflects an increased activation in the right frontal region, or 'right frontal asymmetry'. As will be reviewed later, currently, frontal EEG activation and asymmetry remains a useful measure to explain interindividual differences in emotional reactivity (e.g., Allen & Kline, 2004; Goodman *et al.,* 2013; Jackson *et al.,* 2003), especially concerning the early identification of vulnerability patterns that may place an individual in higher risk for negative affectivity.

2.2.3.2. Theories of frontal EEG activation

During the last three decades, there have been three main contextual and theoretical concepts that have been developed in relation to frontal EEG asymmetry and behavioural problems (for a review see Harmon-Jones, Gable & Peterson, 2010). First, a proportion of research has emerged; focusing on the role of the affective valence of the environmental cues in influencing positive and negative affectivity patterns of frontal EEG activation (e.g., Kop *et* *al.,* 2011; Schmidt, Fox, Schulkin & Gold, 1999; Theall-Honey & Schmidt, 2006). More specifically, based in this line of research, individuals exhibiting a relatively greater right frontal asymmetry have increased affectivity in response to negative environmental cues, but also limited affective response to positive stimuli, whereas individuals with left asymmetry are biased towards positive stimuli (e.g., Tomarken, Davidson, Wheeler, & Doss, 1992). This evidence supports the notion that EEG asymmetry may be indicative of the moderation of individual differences in response to identical emotional stimuli (see also Coan & Allen, 2004). This line of research agrees with neuroimaging studies that suggest an involvement of prefrontal systems with reactivity in response to emotional information (Ochsner *et al.,* 2009), with recent evidence suggesting a specific sensitivity of these neural structures in emotional information, which requires social interpretation (Sakaki, Niki, & Mather, 2012).

In addition, the concepts of motivational direction (i.e., approach versus withdrawal), as well as the behavioural activation (i.e., activation versus inhibition), have also emerged. More specifically, frontal EEG asymmetries have been originally interpreted within the approach-withdrawal framework (e.g., Davidson, 1993; Tomarken & Keener, 1998). This line of research has originally emerged from studies with infants and children, initially introduced by the work of Fox and Davidson, who investigated the involvement of approach and withdrawal tendencies and behaviours in shaping infants' emotional development and regulation (Fox & Davidson, 1984). More specifically, in this line of research, left frontal EEG asymmetry is associated with approach-related behaviours and positive affectivity (Pizzagalli, Sherwood, Henriques, & Davidson, 2005), whereas right asymmetry with withdrawal tendencies and negative affectivity (Sutton & Davidson, 1997). Interestingly, research studies that accounted for the differentiation of valence and approach-withdrawal motivation, show that only approach and withdraw tendencies are evident, as lateralization over the frontal and prefrontal brain areas (Berkman & Lieberman, 2010; Carver & Harmon-Jones, 2009).

Regarding the activation versus inhibition concept of frontal EEG asymmetries, behavioural activation system (BAS) has been proposed to be responsive to reward-related stimuli of the environment, by eliciting responses of positive emotionality (Gray & McNaughton, 1996) and leading to active approach-related behaviours. On the other hand, the behavioural inhibition system (BIS) has been proposed to respond to punishment-related stimuli as well as novel and fearful stimuli, producing negative responses (Gray & McNaughton, 1996; for a review see also Briesemeister, Tamm, Heine, & Jacobs, 2013). However, is important to highlight that the BIS/BAS system is theoretically parsimonious, for which, to date, there is very limited empirical evaluation of its significance. Therefore, further research is required to delineate the significance of these theoretical concepts in justifying the validity of frontal EEG activity as an indicator of emotion dysregulation.

2.2.3.3. Alpha EEG asymmetries and psychopathology

In studies with clinical populations, a typical finding is that relatively greater right hemisphere activation is found in individuals diagnosed with depressive and anxiety disorders, compared to controls (Baving, Laucht & Schmidt, 2003; Blackhart, Minnix & Kline, 2006; Thibodeau, Jorgensen & Kim, 2006). Interestingly, numerous studies have highlighted greater right frontal activation as a putative marker of an individual's affective dysregulation, which may infer vulnerability to developing depression (Coan & Allen, 2003; Dawson *et al.,* 1995; Nusslok *et al.,* 2011; Tomarken, Dichter, Garber & Simien, 2004). On the other hand, although more left asymmetry has been associated with reduced symptoms of depression (Deslandes *et al.,* 2008), research has suggested a link between left asymmetry and the presence of externalizing problems in healthy adults (Stewart, Levin- Silton, Sass, Heller & Miller, 2008). However, findings for externalizing symptoms have been less consistent (Baving *et al.,* 2003; Santesso *et al.,* 2006).

Aiming to provide a greater specification on how frontal EEG asymmetry moderates outcomes of psychopathology, two main theoretical hypotheses for the role of EEG asymmetry have been developed: (i) EEG asymmetry as a state marker; and (ii) EEG asymmetry as a trait marker. More specifically, based on the first account, also known as the capability model, individual differences in frontal EEG activation may be more proactively emerging due to interaction between the emotional demands of a specific situation with the individual's capacity to respond emotionally in the same context (Coan, Allen, & McKnight, 2006). These effects may be expressed as a function of right or left frontal EEG asymmetry and may be utilized as an index of an individual's vulnerability for the development of affective psychopathology (Coan & Allen, 2004; Feng *et al.,* 2012). For instance, a study has shown that frontal EEG asymmetry during a session with fear induction was a better predictor of greater right asymmetry (negativity) compared to baseline (Coan, Allen, & McKnight, 2006). Therefore, the context where affectivity is measured may be an important link between affective responsivity and frontal EEG activation.

On the other hand, a significant amount of research has utilized frontal EEG as a trait marker or frontal EEG, to describe the automatic behavioural frontal activation due to emotional arousal, which is independent to the nature of the state-dependent emotional arousal (e.g., for a review see Coan & Allen, 2004). More specifically, based on this line of research, greater right frontal EEG activity has been associated with a disposition for withdraw-related behaviours, where relatively greater left asymmetry with disposition for approach-related behaviours (e.g., Coan & Allen, 2004; Harmon-Jones & Allen, 1997; Hugdahl & Davidson, 2003; Schmidt & Fox, 1998).

However, the disposition and capability model are not mutually exclusive from one another and it has been suggested that frontal EEG may reflect both a context-specific emotional demand, but also the regulatory abilities of the individual in response to the emotional demand (Coan *et al.,* 2006). To avoid this conflict it has been previously suggested that the most reliable practice for the field would be to consider both the state and trait indices of frontal EEG asymmetry (Jackson *et al.,* 2003; Theall-Honey & Schmidt, 2006).

2.2.3.4. Development, Frontal EEG, and early behavioural problems

In typically developing children, the ability to regulate emotions partially relates to the inhibition abilities (e.g., Jahromi & Stifter, 2008; Murray & Kochanska, 2002) that are largely managed by the frontal lobe (Stuss & Knight, 2002). Interestingly, early accounts suggested associations between patterns of frontal EEG asymmetry and children's levels of internalizing and externalizing behaviours that are present as early as toddlerhood (Calkins & Dedmon, 2000; Feng *et al.,* 2008; Fox 1991; 1994; Smith & Bell, 2010). More specifically, early observations based on the approach-withdrawal model, have suggested greater right frontal asymmetry to be associated with internalizing problems, and greater left frontal asymmetry to be associated with a lack of ability to control approach behaviours, which might lead to externalizing behaviours (Davidson, 1993; Fox, 1991). Furthermore, children who have been reported to have high levels of frontal EEG stability across their development, starting from infancy, have been found to manifest both higher internalizing and externalizing problems in childhood (for a review see Smith & Bell, 2010). In research with adults has been found moderate long-term stability in frontal EEG asymmetry (Vuga *et al.,* 2006; Hagemann *et al.,* 2002; Tomarken *et al.,* 1992). However, recent meta-analysis studies on the predictive validity of stable EEG asymmetry across different time points of development was estimated to be low to moderate during childhood (Vuga *et al.,* 2008), as well as in adults (Vuga *et al.,* 2006). There are several factors that may influence the stability of frontal EEG asymmetry, including gender differences, which may impact upon neural structures, handedness, as well as the history of traumatic life events or parental depression (e.g., Negri-Cesi *et al.,* 2004). Moreover, methodological factors may have an impact on the stability results, where studies with children between 0-3 years of age have studied small groups, and therefore potential gender effects might be understudied due to statistical power confounds (Jones *et al.,* 1997; Fox *et al.,* 1992). However, other studies suggest that the stability of frontal EEG asymmetry among youngsters may be influenced by neural developmental pathways during early critical periods (e.g., Kanemura *et al.,* 2003). Therefore, this evidence may provide support for the hypothesis that EEG can be utilised as trait susceptibility marker early in life, which may be independent of a disorder-specific phenotype (see also Vuga *et al.,* 2008). To this end, the use of frontal EEG in developmental science may account as a reliable index for unveiling associations with behaviour, but such associations may fluctuate during development, where critical neurophysiological changes can occur. Further longitudinal studies in the field are required to delineate this area.

In addition to the approach-withdrawal model of frontal EEG, other studies have adopted an alternative approach to determine the predictive validity of greater right or left asymmetry, mainly because of the inconsistencies in the literature on defining the constructs of internalizing/externalizing behaviours. Most notably, as a recent account suggests, aggressive behaviour in children may not always be associated with approach-related behaviours, especially when the expression of anger is impossible or socially inappropriate, where individuals may inhibit, instead to express their anger (Kelley, Hortensius & Harmon-Jones, 2013). To this end, right asymmetry has also been conceptualized as a predictor of negative affectivity that may include both inner-directed behaviours such as anxiety but also externalizing behaviours, such as anger. In line with this claim, Baving and colleagues (2003) found that children with higher reported externalizing behaviours exhibited significantly greater right frontal EEG activity at rest, compared to children with less externalizing problems.

In line with this later negative versus positive affectivity concept of frontal EEG asymmetries, a study has underlined the putative moderating role of additional temperamental characteristics in modulating frontal asymmetries. It was shown that shy children are more likely to exhibit internalizing problems when displaying a right frontal brain asymmetry, whereas in highly sociable children, the same pattern of frontal activation externalizing problems were found (Santesso *et al.,* 2006). In a similar vein, Fox *et al.* (1996) reported that children who have been reported to have higher rates of externalizing problems exhibited relatively greater right frontal asymmetry, compared to children who exhibited relatively greater left frontal asymmetry. This was interpreted in a cognitive capability basis, where it was proposed that the availability of a broad range of cognitive capabilities that are modulated by the frontal lobe (i.e., language skills, analytic-based strategies, decision making techniques) could potentially determine the frontal asymmetries. Taken together, it is believed that children high in approach behaviours may be more likely to develop problems with aggression because of a possible inability to control the negative emotions associated with their approach behaviours, and more specifically their aggressive-related behaviours (Smith & Bell, 2010).

2.3. The current study

The current study investigates the role of state versus trait characteristics in frontal EEG asymmetry and its associations with early manifestation of behavioural problems by placing participants into two state contexts: social video watching and non-social video watching.

More specifically, in addition to the utilization of frontal EEG as an index of affectivity in typically developing children, and children with affective traits, frontal EEG has also been utilized in studies with children with Autism Spectrum Disorders (ASD), which is a neurodevelopmental disorder characterised by profound social deficits. Extensive, robust evidence has shown that social stimuli are of critical value and importance for humans from birth through the lifespan (for a review see Grossmann & Johnson, 2007; Ronald, Happe, & Plomin, 2005). Influenced by the social motivation theory of autism, it has been previously suggested that early impairments in social attention might lead to deficits in social learning, and that the resulting imbalance in attending to social (e.g. people speaking) versus non-social stimuli (e.g. dynamic toys) may further disrupt social cognitive development (e.g., Dawson *et al.,* 1995; Schultz, 2005). Interestingly, several studies have reported atypicalities in visual processing of both social and non-social stimuli in infants in high-risk for ASD (Elsabbagh & Johnson, 2007; McCleery, Allman, Carver & Dobkins, 2007; McCleery, Akshoomoff, Dobkins & Carver, 2009; Dawson *et al.,* 1995). However, is now widely accepted that activation of frontal lobe is not ASD-specific (Burnette *et al.,* 2011) with increasing scientific consensus to report that EEG asymmetry is independent of clinical status and can serve as a trait marker of susceptibility for affectivity (Gotlib, 1998). Further investigation of the effects of social versus non-social information processing in patterns of frontal EEG activation is required, in order to delineate the nature and manifestation of these early traits.

2.3.1. Aim 1: To examine the effect of processing social versus non-social information on frontal EEG activation in early childhood.

The first aim of the present study is to explore the association between affective patterns of frontal EEG activation in response to the social and non-social conditions. More specifically, it is expected that the previously documented associations between withdraw-related patterns of frontal EEG activation will be dependent on whether children watch social versus non-social videos (state utilization of frontal EEG). In the case that the condition will not have an effect on frontal EEG activation would mean that the social versus non-social state cannot have an effect to positive/approach versus negative/withdraw-related patterns of reactivity and therefore frontal EEG will be utilized as a trait marker.

2.3.2. Aim 2: To examine frontal EEG measures of behavioural problems in early childhood

The second aim of the study is to investigate associations between the presence of elevated rates of externalizing and internalizing problems and state-dependent frontal EEG activation. It is expected that the present study will provide further insight into the differential activation of frontal lobe in response to social versus non-social stimuli and early affective problems in young children. In the case of the null effect of viewing social versus non-social videos on the patterns of frontal EEG activation, it would mean that alternative trait-specific pathways may drive elevated rates of behavioural problems early in life, which may be independent of viewing videos with social versus non-social component. Moreover, if contrary to the field's expectations there are not evident associations between rates of behavioural problems and state or trait patterns of frontal EEG, it may suggest that frontal EEG may not be a reliable index of affectivity early in life.

2.3.4. Hypotheses

There are two main hypotheses that are tested as part of the second aim of the study. Based on the previous EEG evidence in adults, which suggests an association between greater right frontal hemisphere activation in individuals with depressive and anxiety disorders compared to controls (Baving *et al.,* 2003; Blackhart *et al.,* 2006; Thibodeau *et al.,* 2006), it is hypothesised that negativity-related patterns of greater right asymmetry during social processing will relate to elevated anxiety/depressive rates in young children. More specifically, it is hypothesised that children with elevated rates of anxiety/depressive problems will exhibit negativity-related patterns of frontal EEG activation as a way of inhibiting the arousal that the social demands of the stimuli may provoke.

Moreover, it is hypothesised that higher rates of aggression problems will be also related to higher right EEG asymmetry during the processing of social information. Taking into account previous evidence, which highlights an association between social competences, aggressive behaviour, and frontal asymmetries (Santesso *et al.,* 2006), the present study seeks to investigate the social influence of these associations by employing a novel EEG paradigm. More specifically, it is hypothesised that children with elevated aggressive behaviour will present a negative pattern in response to social stimuli; probably due to an inability to control the negative emotions associated with their approach behaviours in particular anger (Smith & Bell, 2010).

2.4. Methods and Materials

2.4.1. Participants

A total of 52 children aged between 3 ½ and 5 years contributed to this study (Mean age in months = 54.78, SD = 8.18; males n = 23). Participants were recruited through a local community research participation advertisement/outreach program as part of an on-going procedure at the Infant and Child Laboratory, at the University of Birmingham. The sample size was calculated on the basis of the study's hypotheses. Power analysis suggested that the sample size required to achieve a power of $1-\beta = 0.90$ for the one-sided chi-square test at significance level $\alpha = 0.050$ requires at least 36 participants. The parents or guardians/carers of all participants reported that the child had no history of a neurological or psychiatric disorder and that they had normal or corrected to normal vision. Exclusion criterion included if participants scored below a certain range (IQ < 75) on the British Ability Scales (BAS-II; Elliot, Smith & McCulloch, 1996), a standardised assessment of intelligence/developmental age. All participants had English as their first language. Outside of these limitations described above, participants were not excluded from participation based on their ethnicity or gender. In general, these characteristics were represented in the participant population in the same manner as they are represented in the greater Birmingham / West Midlands region, or the UK more generally. Families who expressed interest in the study were screened in an initial telephone contact, where the child's age, diagnostic history, and English language criteria were confirmed. If these criteria were met, a laboratory intake appointment was arranged. Families who met study acceptance criteria were provided with compensation of £10 towards their travel expenses.

2.4.2. Data collection procedures

Written informed consent was gained from parents prior to the initial assessments. Ethical consent was gained from the University of Birmingham Ethical Committee. Children were told that they were going to watch a range of interesting videos on a computer screen and that if they remain still they will get a gift at the end. The vast majority of the assessments took place in one laboratory visit.

2.4.2.1. Behavioural Measures

As part of the study, measures of rates of autism symptomatology and cognitive development were undertaken. For the assessment of rates of autism symptomatology, the Social Communication Questionnaire-Lifetime Edition was completed by parents (SCQ; Rutter, Bailey & Lord, 2003). SCQ is a valid and well standardised screening assessment, completed by a parent or other primary caregiver, and is based on the Autism Screening Questionnaire (Berument, Rutter, Lord, Pickles & Bailey, 1999). In the SCQ's Lifetime Form there are 40 yes/ no questions that aim to measure aspects of the child's developmental history. A total score is provided after the administration that is interpreted based on the measure's cut off criteria[1]. The cut-off score indicates that a child may have an Autism Spectrum Disorder (ASD) and suggests that further clinical assessments may need to be conducted. SCQ has reported to have good psychometric properties with the reliability coefficient for the total scale to be reported *a* = .90, suggesting excellent internal consistency (Berument *et al.,* 1999).

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[1] The SCQ provides a total score between 0-39; the first question that relates to current language abilities is not calculated for the extraction of the total score. The cut-off score for ASD is ≥15, thus scores of 15 and above were considered as clinically significant through the thesis's studies.

In the original standardisation, all items to total score correlations were reported to be in the range of *r* =.26 - .73, where 23 items out of 39 to be over *r* = .50 (Berument *et al.,* 1999).

For the assessment of children's cognitive ability the BAS-II, Early Years was employed (Elliot *et al.,* 1996). BAS-II is an age-standardized cognitive ability test, normed in UK children between 2:6 and 7:11 years of age. BAS-II has three main subscales that are used to assess the most significant aspects of development, following particular scoring procedures: verbal reasoning, non-verbal reasoning and spatial abilities[3]. The assessment of the BAS-II scales is estimated to take 40 minutes, on average, to complete. This was in line with the study requirements to minimise burden of the participants and with the overall time the experimenter had available to allocate for cognitive assessment during the 2-hour laboratory visit.

2.4.2.2. Measure of behavioural problems

For the assessment of children's rates of behavioural problems, the Child Behavioural Checklist was filled by parents (CBCL; Achenbach & Rescorla, 2001). CBCL includes two different versions: the early years version (for children between 1½ - 5 years of age) and the school age version (children and adolescents aged 6–18 years). The CBCL 1½ -5 is an empirically based checklist that is filled in by parents, and includes 99 items that describe areas of behavioural, emotional, and social problems that characterize preschool children. Items are on a 3-point scale ranging from not true to very true/often true, including open-ended items for the description of additional problems. The scale has two main sub-scales,

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[3] Mean age standardised t-score values for BAS subscales have a mean of 100 and a standard deviation of 15.The scores of each ability cluster are combined to give an overall General Conceptual Ability (GCA) which is equivalent to IQ score.

which are structurally independent from each other (Achenbach & Rescorla, 2001), that map externalizing and internalizing problems (see Appendix 2.1 for items included in each subscale). Higher total scores in each sub-scale or in each behavioural problems category suggest the existence of more problematic behaviours. Using the scales raw scores age-adjusted t-scores (M = 50, SD = 10) can be extracted providing a similar measure for all scales. However, as the authors highlight, the use of raw scores as opposed to t-scores is encouraged for statistical analyses due to effects of data truncation. CBCL has reported to have good psychometric properties and has a robust procedure for classifying behavioural problems in each sub domain[4]. Parents were provided with introductory information as well as detailed instructions on how to complete the scale forms, whilst their child completed other assessments.

2.4.2.3. Electrophysiological Recordings

After taking consent from parents, parent and child were escorted in a sound-attenuated, dimly lit room and the stimuli were presented on a 17-inch computer monitor. The child was asked to sit in a comfortable chair facing the monitor, located approximately 70 cm away. Subjects were instructed to refrain from movement and were monitored for eye and head movements via a video camera. When possible, the parent was seated in an adjoining room, out of the child's line of vision. Also children were monitored for alertness and attention to the videos during EEG collection to provide a record of potential movement artefact.

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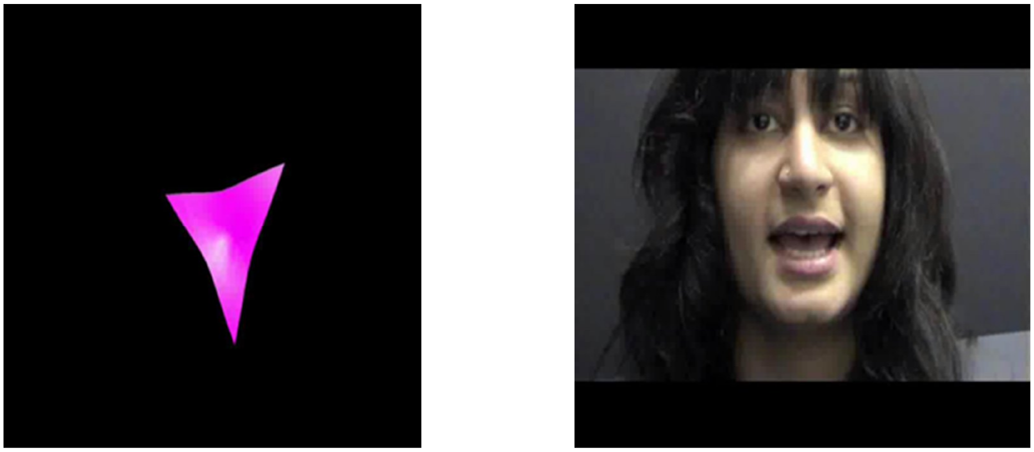
[4] CBCL 1½-5 has reported to have good internal consistency ranging from $a = 0.89$-0.95, test- retest reliabilities range from $r = 0.87$ to 0.95, and inter-rater reliabilities of $k = 0.48$-0.67 (Achenbach & Rescorla, 2001). There is one set of norms provided for the CBCL 1 ½ -5. Symptomatology that is the "Clinically significant" is defined by t-scores ≥ 64, where the "Borderline" classification ranges from 60 to 63 (Achenbach & Rescorla, 2001).

EEG data were collected while children watched videos of social stimuli (adults speaking nursery rhymes) and non-social stimuli (dynamic computer-generated objects moving with contingent sounds). Similar stimuli have previously been used in infant and child EEG studies on temperament and emotion regulation (Hane & Fox, 2006; Marshall, Bar-Haim & Fox 2002). The videos were in Windows Media Video format and were recorded using a digital camera with a resolution of 720 × 576 colour pixels and with a frame rate of 25 frames /s, and therefore subtended a visual angle of 22.6° horizontal by 13.5° vertical. The following parameters were used for all of the video recordings: data rate of 768 kbps, total bit rate of 89 kbps, frame rate of 25 frames/sec, audio bit rate of 128 kbps, stereo audio samples rate of 44 kHz. Videos were presented with an average volume of 68 dB recorded at the child's head, using 2.1 Hz audio speakers.

Figure 2.1. Example stimuli frames extracted from the video clips for the social (right frame) and the non-social (left frame) experimental conditions.



All video clips lasted a total duration of 30 seconds. Each condition lasted 6 minutes in total, with 20 different videos presented in each condition (see Figure 2.1 for examples). Social and Non-social conditions were counterbalanced between participants, and each video was played once during the experiment, giving a total of 12 minutes data collection (6 minutes social videos, 6 minutes non-social videos).

2.5. Analysis

2.5.1. Analyses of Behavioural data

For the measures of cognitive abilities (BAS-II), mean standardized IQ-scores were assessed. All children in this study had CBCL t-scores of less than 60 (below subclinical threshold). Raw scores from the two CBCL clusters of behavioural problems (i.e. internalizing and externalizing problems) were used for statistical analysis following the authors' guidelines (Achenbach & Rescorla, 2001, p. 89). Autism symptomatology (SCQ) mean sum scores were calculated on the basis of raw scores. All children had an SCQ mean sum score of 12 or less.

2.5.2. EEG Recordings and Analyses

EEG was recorded continuously using a 128-channel Hydrocel Geodesic Sensor Net (HCGSN; Electrical Geodesics, Inc., Eugene, Oregon), referenced to a single vertex electrode, Cz (sample rate = 500 Hz), using Net Station 4.3 data acquisition software. The stimuli were presented using E-Prime 2.0 software (Psychology Software Tools Inc., Sharpsburg, PA, USA).

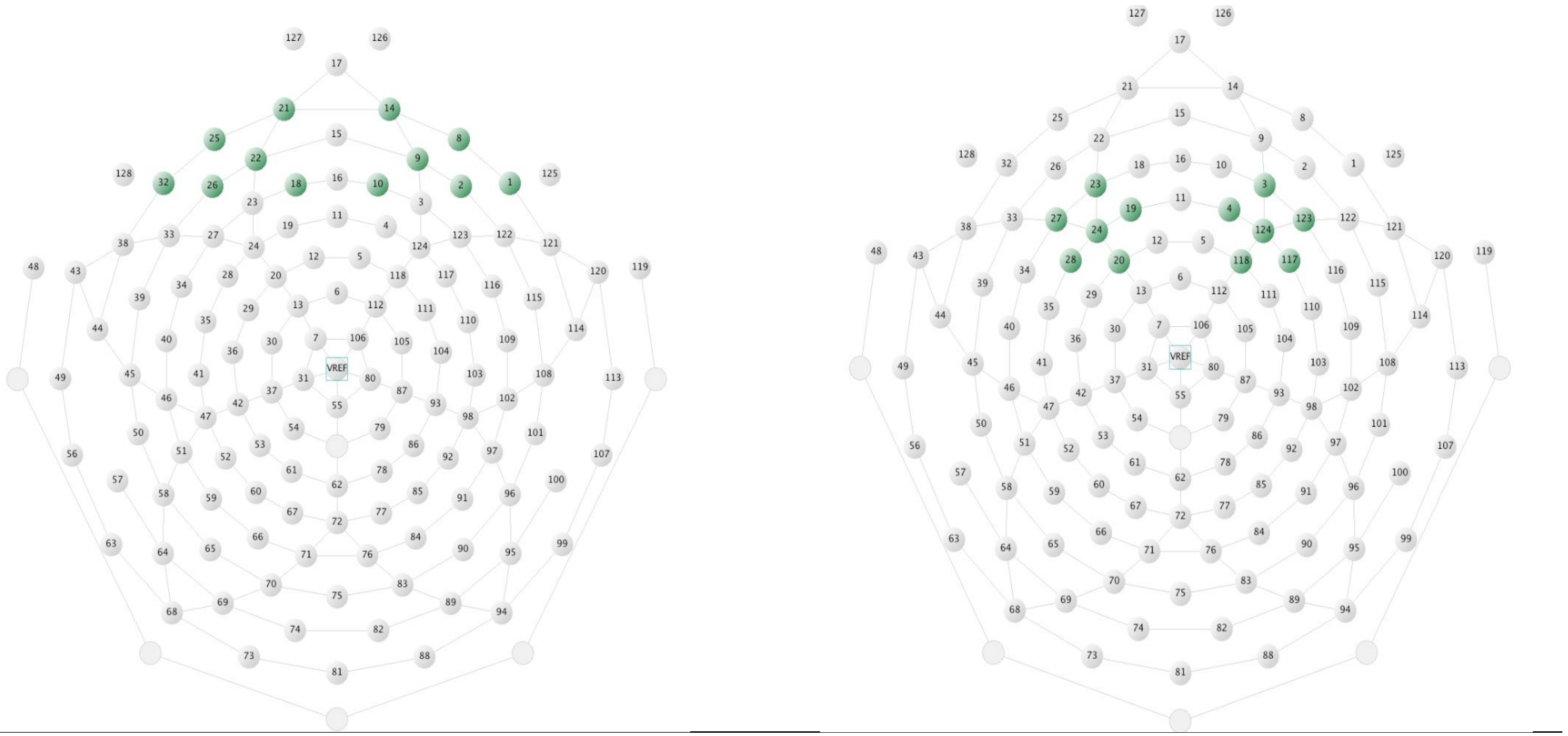
EEG recordings were processed offline using Net station 4.5.1 software. The data were filtered offline with a high pass filter at a cut off frequency of = 0.1 Hz, and with a 50Hz Notch filter, prior to processing. Each data file was processed with a clinical segmentation tool that segregated the EEG according to condition. Consistent with previous frontal EEG asymmetry research, after the overall inspection of the recording, where bad electrodes were identified, portions of data containing artefacts, including eye blinks, and participant movement were manually identified and removed for each condition separately (e.g., Forbes *et al.,* 2008; Smit, Posthuma, Boomsma & de Geus, 2007). During this procedure, up to 12 bad electrodes were identified per participant, and the data from bad channels were replaced using a spherical spline interpolation algorithm (Srinivasan *et al.,* 1996). Similarly to previously published research using the same EEG system that was utilized in the current experiment, the algorithms used are effective at replacing data in up to 10% of EEG electrode channels (e.g., Oberman, Hubbard, McCleery *et al.,* 2005; Oberman, McCleery *et al.,* 2007), which would be 12 electrodes out of the 128 utilized in the current experiment. Therefore, segments with more than 12 bad channels were eliminated from further analysis. In the case of a considerable proportion of bad channels was located in the areas of interest (more than 3), the segment was also eliminated from further analysis. However, no participant had more than three bad electrodes in the areas of interest, and therefore no participant partial or entire EEG data was excluded based on this criterion, or due to poor EEG data. The remaining artefact-free only data were combined for each condition and participant and average referenced. To assess EEG Power Spectral Density asymmetry, the EEG data were exported in RAW format for use in a purpose-built MATLAB-based program for data analysis. The MATLAB Version 7.1.0 program split the EEG data into one second epochs. Fast Fourier Transforms (FFTs) were then calculated for each epoch using a 500 ms window with 60% overlap. Power Spectral Density (PSD) in the alpha band was logged and averaged across epochs for each electrode group, in preparation for statistical analyses. Based on inspection of the power distribution at the mid-frontal sites and previous developmental findings (Marshall, Bar-Haim, & Fox, 2002), the alpha band was defined as 7-11 Hz for the 3 ½- to 5-year-old participants of the study. The research assistants who analysed the EEG data were systematically trained and blind to any participant details.

Clusters of left/right hemisphere electrodes (six on each hemisphere) corresponded to positions F3 (electrode number 24) and F4 (electrode number 124) on the EGI index of coordinates, as well as additional clusters of left/right hemisphere electrodes (6 left and 6 right) over the positions F1 (electrode number 22) and F2 (electrode number 9; equivalent to the international 10-10 EEG coordinate system; see Luu, & Ferree. 2000; see Figure 2.2). In accordance with long-standing practice, frontal asymmetry was computed as the power in the right hemisphere minus the power in the left hemisphere, which indexes the relative activation of left (over right) mid-frontal sites (Davidson et al. 2000; Coan & Allen, 2003). Thus, a negative asymmetry index score represents right EEG frontal alpha asymmetry (increased activity in the right frontal region), while a positive index score represents the left EEG frontal alpha asymmetry (increased activity in the left frontal region).

2.5.3. Statistical Analyses

Descriptive statistics were conducted in order to describe the sample’s demographic information, such as gender, age and distribution of cognitive abilities. Raw data from the behavioural and cognitive scales were examined for normality using skewness tests and Kolmogorov–Smirnov test. CBCL subscales did not meet criteria for normal distributions (Kolmogorov-Smirnov test $p < 0.05$). Therefore, to further examine possible correlations between age, gender, IQ and scores on the behavioural measures, Spearman’s Rho non-parametric correlation coefficients tests were also performed. Further correlation analyses were conducted to investigate possible correlations between raw EEG data and asymmetry ratios for each condition and hemisphere in the two regions of interest (separately but also averaged across the regions), with participants' age and gender (Spearman's Rho correlations

Figure 2.2. Electrode layout of left/right hemisphere electrodes over areas F3-F4 (right) and F1-F2 (left). Data collected using Geodesic Sensor Net Hydrocell 128-channel the paediatric medium, large and adult small sizes, based on standard sizing procedures for head circumference (Electrical Geodesics: Eugene, OR).



Kolmogorov-Smirnov test $p < 0.05$), as well as with participants overall IQ score (Pearson correlation; Kolmogorov-Smirnov test $p > 0.05$). In order to examine differences in frontal EEG asymmetry in responses to social and non-social videos, a three way analyses of variance (ANOVAs) with Condition (Social, Non-social), Hemisphere (Left, Right) and region (F3-F4; F1-F2) as within factors and gender as between factor were performed. For the ANOVAs, the PSD values were studied.

Moreover, in line with the study's first hypothesis, correlation analyses between PSD values/asymmetry ratios and rates of anxiety/depressive problems were also conducted. Furthermore, backward elimination regression analysis was utilized to assess the specificity of the anxiety/depressive rates to predict alpha raw PSD power and EEG asymmetry scores above and beyond participants' age, gender and IQ. The above analyses were repeated separately to test the study's second hypothesis regarding the PSD/asymmetry values and aggression problems. Compared to other regression methods, such as multiple regression, where a clear prediction of the effect of each variable is available, in the current study there was not a clear prediction from the literature on the potential effect and its size of each of the examined demographic characteristics. Similar analytical practice has been employed in previous studies with children with roughly the same sample size (e.g., Butler, Rizzi & Handwerger, 1996) Therefore, taken the exploratory nature of this later analysis the backward elimination regression analyses was deemed as the most suitable, to measure the predictive validity of the rates of behavioural problems above and beyond other demographic characteristics in predicting frontal EEG asymmetry patterns, as the least significant variables are eliminated from the model in an iterative process. The statistical software package SPSS 20.0 was used for all the analyses.

2.6. Results

2.6.1. Demographic Characteristics

Participants included 52 children (males n = 29) between 3 ½ and 5 years of age. Tables 2.1 and 2.2 demonstrate participants' main demographic characteristics, such as gender, age and age equivalent cognitive abilities. Pearson's correlation analyses showed no significant correlations between age or gender and participants' cognitive development rates or early behavioural problems. Further Spearman's Rho correlation showed a significant correlation between attention problems and higher autism symptoms ($r = .370$, $p = .007$), as well as a strong correlation between internalizing and externalizing problems ($r = .350$, $p = 0.11$). Co-occurrence between internalizing and externalizing clusters has originally reported on the

Table 2.1. Participants' Demographic characteristics.

N		**52**
Gender	% Male *(n)*	55.8 *(29)*
	% Female *(n)*	44.2 *(23)*
Handedness	% Right	78.8
	% Left	21.2
SCQ Total Score	Mean *(SD)*	4.53 *(3.25)*
	Range	0-12
BAS-II[4] Total Score	% Below Av.	3.8
	% Average	65.4
	% Above Av.	25.0
	% High	5.8

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[4] Based on the BAS-II standardisation the following GCA-based classifications of ability (IQ equivalent) are applied though the Thesis: Low: 70-79; Below Average: 80-89; Average: 90-109; Above Average: 110-119; High: 120-129; Very High: 130 and above.

CBCL scales standardisation (Achenbach & Recorla, 2001) as well as in a range of other developmental studies (e.g., Card & Little, 2006; Marsee & Frick, 2007; Dietz, Jennings, Kelley & Marshal, 2009). According to Achenbach and Rescorla (2001), even though these behaviours may co-occur, some children primarily exhibit internalizing while others primarily exhibit externalizing problems. Finally, correlation analyses revealed significant negative correlations between age and right frontal EEG activation during social (r = -.429, p = .001) and non-social processing (r = -.437, p = .001), but also on left frontal activation during social processing (r = -.425, p = .002).

Table 2.2. Participants' IQ and developmental ages.

Chronological Age*	Mean *(SD)* Range	56.7 *(8.1)* 44-71
Overall Ability**	Mean *(SD)* Range	105.6 *(8.7)* 84-123
Verbal Ability	Mean *(SD)* Range	101.2 *(11.4)* 69-121
Non-verbal Ability	Mean *(SD)* Range	108.8 *(10.8)* 90-123
Developmental Age[5]	Mean *(SD)* Range	58.1*(9.8)* 42.5-82.5
Developmental Verbal Ability	Mean *(SD)* Range	55.7 *(11.3)* 36.5-87.5
Developmental Non Verbal Ability	Mean *(SD)* Range	61.1 *(13.0)* 35-91

* Age data presented in months

**Overall ability is calculated from the overall BAS-II total score and Verbal and Non-verbal ability form the BAS-II clusters of abilities. Values represent GCA.

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[5] Through the thesis developmental ages are assessed through the BAS-II standardised tables of age-equivalent scores. Moreover the developmental verbal and non-verbal ability is assessed using each sub-scale's specified standardised tables of age-equivalent scores.

2.6.2. Behavioural problems and EEG alpha activation/asymmetries

A three-way analyses of variance (ANOVA) with Condition (Social, Non-social), Hemisphere (Left, Right) and Region (F3-F4; F1-F2) as within factors, and Gender as between factor revealed a significant main effect of Region [(F(1,50) = 26.54 , η_p^2 = .347, p < .001)] (see Table 2.3 and Table 2.4), as well as a two-way Hemisphere by Condition interaction effect [F (1, 50) = 4.15 , η_p^2 = .077, p =.047)], where relatively higher activation was evident for the left hemisphere during the social processing, which was associated with positivity during social processing (see Table 2.3 and 2.4; see also Appendix 2.2 for histograms of individual PSD data).

In addition, a significant two-way Gender by Region interaction was evident [F(1, 50) = 4.39, η_p^2 = .081, p = .041)], where females exhibited more activation over the F3-F4 areas, represented with lower alpha power (M = 1.20, SD = 0.03) compared to males (M = 1.38, SD = 0.02). To further evaluate the two-way Gender by Region effect the activation across the two hemispheres and conditions was averaged for each region. However, a t-test for the F3-F4 areas (PSD) data normally distributed; Kolmogorov-Smirnov test p > 0.05) did not confirm a significant difference between males and females [t(50) = -9.13, p = .366)]. Similarly, when conducting a Mann-Whitney U test for the data from the F1-F2 areas (PSD data not normally distributed; Kolmogorov-Smirnov test p < 0.05), no effect of gender in region activation was evident (U = .319.00, p = .789)]. It is possible that gender effects may be involved in neurophysiological signatures of frontal EEG indexes of affectivity early in life. This hypothesis requires further investigation.

Table 2.3. Alpha power as recorded in each region, per hemisphere and condition. Lower alpha power over the right hemisphere is observed for the F1-F2 areas during the non-social condition.

	Social		**Non-social**	
	Right	**Left**	**Right**	**Left**
Alpha Power[*]	Mean *(SD)*		Mean *(SD)*	
F3-F4	1.35 *(0.80)*	1.30 *(0.70)*	1.29 *(0.72)*	1.26 *(0.65)*
F1-F2	1.02 *(0.86)*	1.00 *(0.78)*	0.91 *(0.70)*	0.94 *(0.70)*
Total	1.10 *(0.64)*	1.10 *(0.67)*	1.15 *(0.71)*	1.18 *(0.79)*

[*] Alpha power = ln [7–11 Hz] power spectral density ($\mu V^2/Hz$)

Regarding the first hypothesis of the study aiming to unveil whether frontal EEG activation will be dependent upon the viewing of social versus non-social videos (state utilization of EEG), the null effect of the Condition suggests that frontal EEG is not specific to the viewing of this type of videos and therefore may be utilised as a trait measure of affectivity. Furthermore, to determine if parent reports of children's rates of behavioural problems were related to EEG alpha activation, and taking into account the null main effect of the Condition on the original ANOVA analysis, PSD values were averaged across the two conditions for left and right hemisphere, and across the two regions of interest (normally distributed; Kolmogorov-Smirnov $p > 0.05$). The resulted average left and right PSD activation was also computed for the F3-F4 and F1-F2 areas separately, and both met normal distribution criteria (Kolmogorov-Smirnov $p > 0.05$), therefore Spearman's Rho correlation analyses were conducted, in the first instance, to investigate possible correlations between frontal EEG activation and CBCL scores. To investigate the study's two hypotheses, this was done separately for the anxiety/depressive and aggressive behaviour.

Table 2.4. Results of the repeated measures ANOVA with condition and hemisphere and region as within factors, and gender as between factor. Significant differences ($p < 0.05$) are highlighted in bold.

Effect	**F**	**df**	***P* value**
Hemisphere	.426	1.000	.517
Condition	2.19	1.000	.144
Region	26.5	1.000	**.001**
Region * Gender	4.39	1.000	**.041**
Hemisphere * Condition	4.15	1.000	**.047**

As shown in Table 2.5, a negative correlation between rates of anxiety/depressive problems and higher right (r = -.344, p = .012) but also left (r = -.294, p = .034) PSD activation across the two regions was evident. However, correlation analyses for each region separately revealed that higher rates of anxiety/depressive behaviour correlated with bilateral activation on the F1-F2 areas, whereas rates of anxiety/depressive problems was evident only for the right F3-F4 region (see Table 2.5). Complementary analyses using the rates from the broader internalizing CBCL subscale as predictor of EEG activation did not provide any significant correlation with any of the above frontal EEG variables. Additional Spearman's Rho correlation analyses did not show significant correlations between higher rates of anxiety/depressive problems and asymmetry ratios (see Table 2.6).

To further investigate the specificity of children's early anxiety/depressive behaviour in predicting frontal activation and asymmetry, backward elimination regression analyses were conducted in which parent report of behavioural problems, age, gender and IQ were entered as predictors of EEG asymmetry and activation. This analysis was conducted separately for the average raw PSD activation and average asymmetry ratios for both regions, but also for each region separately. As illustrated in Table 2.7, higher rates of anxiety/depressive problems uniquely predicted greater right frontal PSD activation over the F1-F2 areas at the last model of the regression analysis [F(1,51) = 5.50, adjusted R^2 = .081, *p* = .023] and the model was accounting for approximately 8% of the variance in the sample. In addition, higher rates of anxiety/depressive problems significantly predicted greater right frontal activation over the F3-F4 areas on the last model of backward elimination regression [*F*(2,51) = 8.06, adjusted R^2 = .217, *p* = .044]. However, within the same model age was also significantly predictive of right frontal activation (*b* = -.404, *p* = .002), which suggests a strong association between maturation and trajectories of frontal activation. In addition, although further regression analyses showed that higher rates of anxiety/depressive problems were predictive of asymmetry when averaged across regions [*F*(1,51) = 4.68 , adjusted R^2 = .067, *p* = .036], separate analysis for each region showed that only activation over the F3-F4 areas was predicted from elevations on anxiety/depressive rates [*F*(1,51) = 5.42 , adjusted R^2 = .066, *p* = .037] above and beyond age, gender and IQ. Overall, this model was significant accounting for 7% of the variance in the population (see Table 2.7).

Regarding the second hypothesis of the study, correlation analyses did not reveal any significant effect with the PSD values. However, when complementary Spearman's Rho correlation analyses were conducted with the asymmetry ratios collapsed across the two regions (Table 2.6; see also Appendix 2.3 for scatter plots), it was shown that higher rates of aggressive behaviour was correlated with relatively higher right asymmetry (*r* = -.293, *p* = 0.35). Moreover, separate analysis for each region's asymmetry ratios suggested that only the F3-F4 areas significantly correlated with the presence of higher aggressive behaviour (*r* = -.322, *p* =.020). Interestingly, when the rates coming from the externalizing subscale were

Table 2.5. Results from Pearson's correlation coefficients between anxiety/depressive rates and PSD activation. Significant differences ($p < 0.05$) are highlighted in bold.

Effect	Pearson's Correlations	*P* value
Attention problems × SCQ	.370	**.007**
Internalizing × Externalizing	.350	**.011**
Average Right PSD × A/D*	-.344**	**.012**
Average Left PSD × A/D	-.294	**.034**
Right PSD (F3-F4) × A/D	-.291	**.037**
Left PSD (F3-F4) × A/D	-.254	.070
Right PSD (F1-F2) × A/D	-.315	**.023**
Left PSD (F1-F2) × A/D	-.330	**.017**

*Anxiety-Depressive Problems

**Negative correlations suggest the existence of significant correlations between elevated behavioural problems and lower alpha in the regions of interest (higher frontal activation) and vise versa.

entered as predictor, the same pattern of correlation was evident for the F3-F4 asymmetry ratios (r = -.297, p = .033) but not for the F1-F2 areas asymmetry ratios (r = -.122, p = .388). No further correlations between asymmetry and other measures were evident.

Table 2.6. Results from Spearman's Rho correlation coefficients between anxiety/depression and aggression rates and asymmetry ratios. Significant differences ($p < 0.05$) are highlighted in bold.

Effect	**Spearman's Rho**	***P* value**
Average Asymmetry × A/D*	-.230**	.101
Average Asymmetry × Aggressive problems	-.293	**.035**
Asymmetry (F3-F4) × A/D	-.125	.376
Asymmetry (F1-F2) × A/D	-.259	.063
Asymmetry (F3-F4) × Aggressive problems	-.322	**.020**
Asymmetry (F1-F2) × Aggressive problems	-.108	.447

*Anxiety-Depressive Problems

**Negative correlations suggest the existence of significant correlations between elevated behavioural problems and more negative values of asymmetry in the region of interest (right frontal activation) and vise versa.

Furthermore, backward elimination regression revealed that higher aggression rates predicted higher right asymmetry over the F3-F4 areas [$F(1,51) = 8.1$, adjusted $R^2 = .122$, $p = .006$] above and beyond age, gender and IQ (Table 2.7 and Figure 2.3). Overall, this model was significant accounting for 12% of the variance in anxiety/depression rates.

Table 2.7. Summary of results of backward elimination regression analysis with average asymmetry ratios as the dependent variable and Pout < 0.10 as the removal criterion. Significant effects of the predictors ($p < 0.05$) are highlighted in bold.

	Anxiety/Depression			**Aggressive Problems**		
	Beta[a]	***P* value**	**Adjusted R^2**[6]	**Beta**	***P* value**	**Adjusted R^2**
Average Asymmetry	-.292	**.036**	.067	-.206	.143	.000
Asymmetry (F3-F4)	-.290	**.037**	.066	-.374	**.006**	.122
Asymmetry (F1-F2)	-.215	.132	.027	-.030	.842	-.043
Average Right PSD (F3-F4)	-.117	**.044**	.217[b]	-115	.388	.158
Average Left PSD (F3-F4)	-.189	.132	.225	.000	.999	.169
Average Right PSD (F1-F2)	-.315	**.023**	.081	-.065	.656	-.032
Average Left PSD (F1-F2)	-.249	.075	.043	-.044	.766	-.025

[a] Beta standardised coefficients

[b] Values presented at final regression model where anxiety depressive rates accounted as a predictor of PSD. However, age revealed to be a better predictor ($b = -.404$, $p = .002$) of PSD here.

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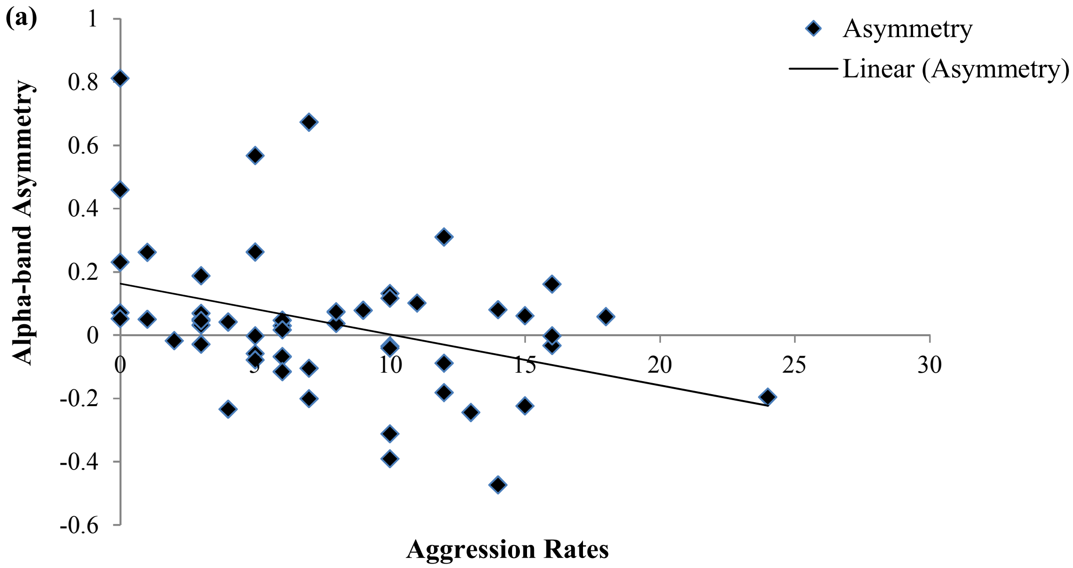
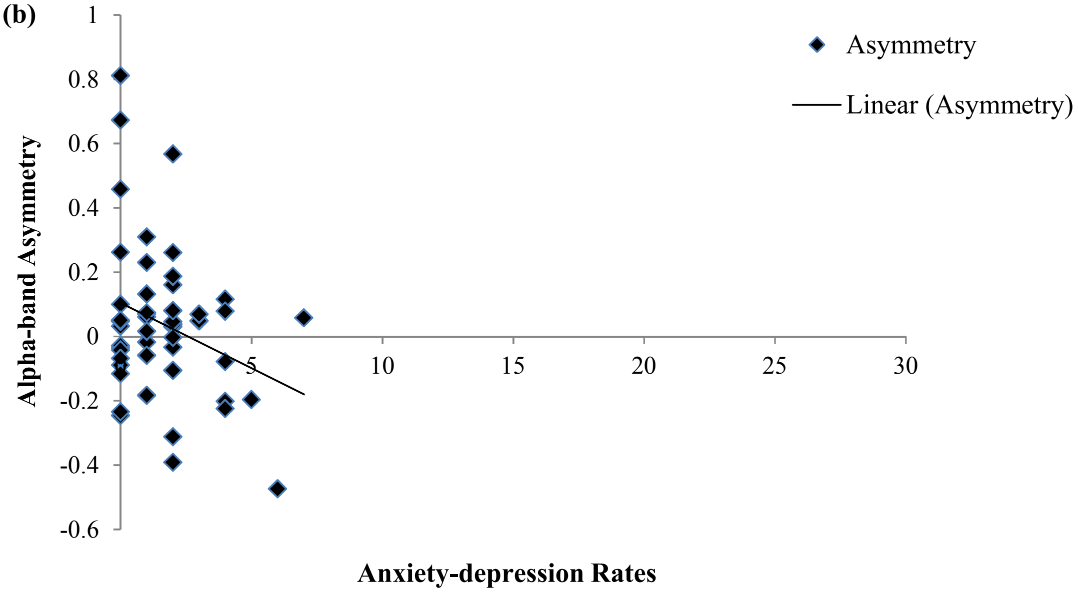
[6] The adjusted R^2 informs about the percentage of variation explained by only those independent variables that truly affect the dependent variable and penalizes for adding independent variable(s) that do not belong in the model.

Figure 2.3. Scatter plot illustrating the association between alpha F3-F4 asymmetry and (a) aggression as well as (b) anxiety-depression problems. Negative values on Alpha-band asymmetry represent greater right frontal asymmetry.





2.7. Discussion

The primary aim of the present study was to examine the associations between frontal EEG asymmetry and the manifestation of early rates of behavioural problems in a group of typically developing young children. The difference of relatively greater frontal brain activation in response to social and non-social videos across two frontal regions in early childhood relative to the presence of early behavioural problems was explored. The two main hypotheses of the study were partially supported. More specifically, associations between negativity-related patterns of frontal EEG activation and rates of early behavioural problems were evident, which was not associated with the viewing of social versus non-social videos. To this end, the study provided evidence for a trait utilization of frontal EEG.

In line with the first hypothesis of the study, an association between trait frontal activation and rates of anxiety/depressive problems was evident. Regarding the asymmetry ratios, the study showed that only greater right asymmetry over the F1-F2 areas was uniquely predicted by elevated rates of anxiety/depression problems. Moreover, although A/D rates found to be significant predictors of frontal activation over the F3-F4 areas, age has been shown to be a better predictor of activation in the specific region. Therefore, the study may suggest that maturational processes may also modulate the activation over the frontal region and be involved in each individual's rates of behavioural problems. This area of inquiry requires further investigation.

Moreover, consistent with previous evidence with older children and the second hypothesis of the study, the findings support the existence of an association between relatively greater right frontal activation at rest and the presence of increased rates of aggressive behaviour in early childhood (Baving *et al.,* 2003; Santesso *et al.,* 2006). More specifically, the study showed that elevated rates of aggression problems were uniquely predictive of greater right asymmetry over the F3-F4 areas, above and beyond age, gender and IQ. This pattern of results may suggest that children high in approach behaviours may be more likely to develop problems with impulsivity and aggression because of a possible inability to control the negative emotions associated with their approach behaviours, specifically anger (Smith & Bell, 2010).

The present study, suggest that early in life some neurophysiological patterns may exist that may link to affective traits and contribute towards understanding early affectivity. More specifically, the study suggests the existence of some degree of brain-behaviour associations early in life, which is independent of viewing social versus non-social videos, suggesting a trait utilization of EEG. Given that the present investigation studied a healthy young group of children, previous evidence that suggested a role of viewing social versus non-social videos in brain activation of atypical populations, such as children with autism, may relate to a disorder-specific response to social and non-social information. This hypothesis requires further investigation in studies that recruit both typically and atypically developing populations.

Limitations

The associations between rates of behavioural problems and frontal EEG activation that reported in the present study explain a small variance of the sample. However, the documented small representation of the findings in the present study is equivalent to other studies examining individual differences in brain-behaviour mechanisms in typically developing children (e.g., Harmon-Jones & Allen, 1997). Although the pattern of findings suggest the existence of an association between greater right asymmetry and negative affectivity, expressed as elevated rates of internalizing/externalizing behaviours is consistent with part of the literature (Baving *et al.,* 2003; Santesso *et al.,* 2006), the particular role of frontal EEG asymmetries in predicting early affective problems requires further investigation.

Moreover, as discussed in the introduction section, there is inconsistency in the field on the frontal EEG-behavioural affectivity associations, where contrary to the present study's findings, there is a considerable line of research to suggest that a link between left asymmetry and the presence of externalizing problems in healthy adults (Stewart, Levin- Silton, Sass, Heller & Miller, 2008), although these findings are less consistent compared to the evidence on the internalizing behaviours (Baving *et al.,* 2003; Santesso *et al.,* 2006). To this end, based on the study's results, although the utilisation of frontal EEG have been widely used as a valuable method to index affectivity in various population, there is not yet clear the extent of the validity of this technique as a biomarker of behavioural affectivity. As Saby and Marshall (2012) highlight, there is currently very limited knowledge on the ontogenic nature of frontal EEG asymmetry and the individual differences observed. Moreover, to date, there is no available developmental account for frontal asymmetry to explain the individual differences observed and their importance in early affectivity and later manifestation of behavioural problems. This area of inquiry requires further investigation.

Moreover, a number of methodological issues may also account for the findings of the present study. In contrast to previous studies that utilized EEG as an index of temperament and used a similar, but more ecological valid social stimuli (Hane & Fox, 2006; Marshall *et al.,* 2002), the present study did not provide significant effect of the type of videos viewed on the modulation of frontal EEG activation. This pattern of findings is in favour of the literature suggesting a reliable trait, instead of state, utilization of frontal EEG activation. However, it is worth highlighting the evidence supporting that an EEG procedure itself can be experienced as an affective situation for some individuals that may influence brain asymmetries accordingly (Blackhart *et al.,* 2006). To this end, children's patterns of brain activation that relate with negative and positive affectivity may be influenced by minimum environmental stimulation, compared to adults.

Furthermore, it is known that resting EEG effects and associations are strongest with eyes closed and a proportion of EEG studies employ this kind of baseline resting state condition, which helps drawing better comparisons and conclusion across conditions. However, the fact that children as young as 4 years old experience difficulties sitting with their eyes closed during the EEG assessment, the employment of a baseline condition would potentially result in increased risk for data loss, as well as in a final sample consisting by a group of children with specific abilities. Moreover, alternative reasons to explain the null effect of the social non-social videos in frontal brain activation may relate to the information included in the videos that may elicit more eye movement artefacts or activate more memories that may interfere with the passive viewing of videos. Moreover, it is possible the information included on the videos to elicit specific memories in children that may impact upon frontal EEG activation. These cognitive processes have not been controlled here for their role in mediating frontal EEG activation; therefore the role of other cognitive processes in frontal EEG activation during the processing of social versus non-social information requires further research.

Interestingly, in a recent meta-analysis Peltola et al. (2014) was not confirmed the previously documented association between greater left asymmetry and externalizing problems and right with externalizing. Interestingly, the study suggested that outcome measure employed in the majority of these studies, including the Child Behavior Checklist (CBCL) may also contribute on the absence of strong effects using subdomains of internalizing symptoms, i.e. emotional reactivity, anxiety/depression, somatic complaints, and withdrawal that are of different nature of approach and withdraw motivation. Therefore, future studies need to be explicit and specific on which aspects of internalizing symptoms are investigating in relation to frontal EEG asymmetry (Peltola *et al.,* 2014). In addition, other recent accounts have suggested that the observed inconsistency among these later studies may be due to the variation in the analytical procedures of EEG data (Keil *et al.,* 2014).

In conclusion, taken that the present findings explain limited variations in the studied sample, it is vital that there is an investigation of the genetic underpinnings that may account for individual differences in affective brain activation during these critical periods of development. These genetic mechanisms may interact with temperament and pre-existing endophenotypic markers of frontal lobe activation and may result in mechanistic relationships of plasticity for behavioural problems.

CHAPTER 3

Variation in 5-HTTLPR Short/Long genotype modulates frontal EEG asymmetries in young children

3.1. Preface

In the previous chapter, research investigating the neurophysiological signatures of the development of behavioural problems was reviewed. A number of areas for future research on the manifestation of early behavioural problems were identified. This included a pressing need for the further delineation of the neurobiological underpinnings of the development of affective problems in early childhood. The present chapter extends the already presented empirical research, by reporting data from a broader age range of children, aiming to assess gene-mediated mechanisms of early affective behaviours. By keeping the previously used EEG methodology constant and through the employment of genetic investigations, which will allow comparison between different genotype groups, it is anticipated that novel insights on the neurobiological basis of early affectivity will be generated.

3.2. Introduction

3.2.1. Background and Rationale

There has been increasing interest in recent years in the examination of G×E interactions in the context of developmental susceptibility for psychiatric outcomes in humans. Based on the Differential Susceptibility hypothesis, individuals are differentially affected by experiences or qualities of the environment that they are exposed to over the course of development, due to pre-existing heightened biological sensitivity factors (e.g., Belsky *et al.,* 2007; Belsky, 1997). The differential susceptibility hypothesis extends the description of individual and biological variables as fixed risk factors (e.g. Diathesis/Stress model), byadopting the concepts of sensitivity (Boyce & Ellis, 2005; Belsky & Pluess, 2009) and susceptibility factors (Belsky *et al.,* 2007) to describe complex developmental interactions among them. More specifically, the evolution-inspired theory ofdifferential susceptibility has proposed the independence of the behavioural outcome from the biology-mediated susceptibility factors, allowing for cross-over interactions between biological and environmental factors. Based on the evolutionary roots of the differential susceptibility model, and in light of uncertain future developmental environments during rearing, natural selection has made the human organism maintain genes that can be adaptable in both conditional but also alternative developmental strategies (see Ellis *et al.,* 2011). The differential susceptibility model has been extensively useful to date in evaluating the differing susceptibility constructs and their interactions, which may lead to vulnerability or resilience for affective problems and disorders, and has been confirmed from various studies as a reliable concept for studying individual differences (e.g., Roth & Fonagy, 2005).

On the other hand, the diathesis-stress model suggests that there is a two-level interaction between heightened biological sensitivity and environmental influences that may be responsible for negative outcomes in an individual's life (Alloy, Hartlage & Abramson, 1988). More specifically, the diathesis-stress model highlights that the early influences of adverse experiences, such as parenting style, in an individual's environment may interact with the vulnerable make-up of an individual (i.e. diatheses) that may place him or her at increased risk for maladaptive behavioural outcomes. Based on this model, the amount of a diathesis or vulnerability in an individual is disproportional to the stress required to trigger certain maladaptive behaviours. For instance, the more an individual has a genetic make-up that predisposes him/her to affective disorders, the less environmental influence is required from the environment for the affective behaviour to be evident.

Studies supporting the diathesis-stress model have identified several potential diatheses that an individual may have, such as temperament as well as genetic polymorphisms, such as the Short allele of the serotonin transporter gene (e.g., Roisman, Newman, Fraley, Haltigan, Groh, & Haydon, 2012). In contrary, the differential susceptibility model points out that many of these putative vulnerability factors not only link to maladaptation when interacting with adverse environmental conditions, but also may infer increased probability for positive adaptation in the face of positive environmental experience (Belsky & Pluess, 2009). Therefore, the latest model suggests that these factors may be better conceptualised as plasticity factors, instead of per-se vulnerability factors (Belsky, 1997).

During the past two decades, the majority of the studies in the field of differential susceptibility have mainly utilized longitudinal observations to measure behavioural and genetic interactions that may predict affective outcomes (for a review see van IJzendoorn, Belsky, & Bakermans-Kranenburg, 2012). However, in a recent re-conceptualization of the differential susceptibility model Belsky and Hartman (2014) paid extra focus on the importance of exo-environmental influences (beyond the individual's choice) in shaping behavioural outcomes. More specifically, the authors suggested that because environmental experiences is a matter ofindividual preference, rather than external assignment, the previously documented gene-environment interactions in observational data, may in fact be gene-environment correlations (Belsky & Hartman, 2014; see also Section 1.2). In addition, recent developments in the field have begun to redefine 'environment' as not only a range of factors originating from the external environment (Caspi*et al.,* 2002, 2003; Fox *et al.,* 2005; Rutter, Moffitt & Caspi, 2006), but also factors arising from the individual's endogenous environment (i.e., brain functioning), which are considered to play an equally important influence in human behaviour (Schmidt, Fox, Perez-Edgar & Hamer, 2009). Together, the field of differential susceptibility has gradually started to expand from the observational methods to the investigation of the complex neurobiological constructs that may relate to affectivity aiming to derive a more direct picture for the mechanistic relationships and interactions between genes, brain and behaviour.

3.2.1.1.5-HTTLPR genotype as an early susceptibility marker

With regard to genetically-mediated risk markers for psychological problems, a great deal of attention has been drawn to the hypothesis that brain mechanisms involved in the manifestation of various psychopathologies may be mediated by complex interactions associated with otherwise normal variations in genes that code for neurotransmitter systems, neurotrophic factors, or neural plasticity (e.g., Duman, Heninger& Nestle, 1997, Manji, Drevets & Charney, 2001, Nestler *et al.,* 2002). One of the most commonly studied of these is the 5-HTTLPR polymorphism, which is a degenerate repeat polymorphism in the promoter region of the serotonin transporter gene (5-HTT). This region is characterized by pairs of Short (S) and Long (L) alleles (i.e., Short/Short, Short/Long, Long/Long; Lesch *et al.,* 1996). Although the Long and Short polymorphisms produce the same protein, the Short allele has been associated with an approximately three times lower basal activity than the Long allele (Hariri *et al.,* 2002; Lesch *et al.,* 1996).

Early accounts based on the diathesis-stress model (see also Section 1.3.1.1) observed that the presence of one (Caspi *et al.,* 2003) or two (Pluess, Belsky, Way & Taylor, 2010) copies of the Short 5-HTTLPR allele werea significant moderator of depressogenic effects that resulted from the exposure to stressful events. Recent evidence has suggested that youth with at least one Long allele manifest behavioural resilience against affective disorders, whereas youth homozygous for the 5-HTTLPR Short allele appear to be more susceptible to psychological problems (Bogdan, Agrawal, Gaffrey, Tillman & Luby, 2014; Hankin *et al.,* 2011). Interestingly, compared to the evidence that support the diathesis-stress model that highlights serotonin Short allele as vulnerability allele (Burmeister *et al.,* 2008; Rutter, 2006), other studies that are supporting the differential susceptibility hypothesis holdthat the serotonin-transporter gene does not only increase vulnerability to contextual risk, but under positive environmental influences is associated with disproportionate positive response, that may suggest that the Short allele can be seen as plasticity gene (Belsky *et al.,* 2009; Belsky & Pluess, 2009).

However, compared to the considerable inconsistency in studies with adults suggesting an association between the promoter region of the serotonin transporter gene and depression vulnerability (Caspi *et al.,* 2010; Munafo *et al.,* 2009; Risch *et al.,* 2009; Uher & McGuffin, 2010; for a meta-analysis see also Karg, Burmeister, Shedden & Sen, 2011; but see also Risch *et al.,* 2009), evidence arising from studies with young populations are much more consistent (for reviews see Brown & Harris, 2008; Karg *et al.,* 2011; Uher & McGuffin, 2008, 2010). Serotonin affects neural circuits that reach maturation during the late adolescent years (Kobiella *et al.,* 2011; Lenroot & Giedd, 2006). Therefore, the more consistent findings among studies with young populations may be explained by the vulnerability of the neural regions that undergo maturational procedures early in life (Sibille & Lewis, 2006).

In addition to the evidence suggesting behavioural associations with variations on the 5-HTTLPR genotype in children, adolescents and adults, a recent meta-analysis also supported the hypothesis that individuals carrying the less efficient Short allele of the 5-HTTLPR, compared to individuals homozygous for the Long allele, exhibit an atypical neurophysiological pattern of higher amygdala reactivity when exposed to negative or arousing environmental conditions (Munafo *et al.,* 2009; Murphy *et al.,* 2013; Walsh *et al.,*2012). This line of evidence is consistent with that coming from studies with adults reporting that individuals homozygous for the Short 5-HTTLPR allele exhibit reduced gray matter volume in both the amygdala and the perigenual cingulate cortex (Pezawas *et al.,* 2005). Taken together, these findings suggest that the presence of one or two copies of the Short allele may be associated with increased vulnerability for psychopathology, following exposure to a negative life event, perhaps as a result of an atypical amygdala-cingulate system.

Interestingly, there is research to suggest that the serotonin transporter genotype may have a critical impact early in life which reflects an effect on the maturation trajectories of neural networks that link to the risk for the manifestation of depressive psychopathology (Parsey *et* *al.,* 2006). In line with this evidence, research suggests that carriers of the Short allele, who have been exposed to childhood maltreatment, have manifested increased stress sensitivity in later life (Stein *et al.,* 2007). It is now widely documented that early adversities may have a more profound impact upon an individual's brain development, personality and emotional sensitivity that stressful life events alone might have (e.g., Stevens *et al.,* 2009). This evidence highlights the possibility that carriers of the Short allele may be at greater risk for developing psychiatric disorders when exposed to early adverse life experience.

Interestingly, there is considerable proportion of studies examining the effects of the 5-HTTLPR polymorphism that provided support for the differential susceptibility hypothesis. More specifically, there is evidence to suggest that 5-HTTLPR Short allele carriers have the worst outcomes when exposed to adverse environmental conditions, but the best outcomes in supporting environments (Belsky *et al.,* 2009; Belsky & Pluess, 2009). In a similar vein, studies have shown that 5-HTTLPR Short allele and positive parenting has interacted to predict positive affectivity in middle childhood years and early adolescence, suggesting that children with such plastic genetic make-up were more likely to respond positively to positive parenting compared to the carriers of two copies of the Long 5-HTTLPR allele (Hankin *et al.,* 2011). Interestingly, the vantage sensitivity that is linked to the 5-HTTLPR Short allele is also evident with studies with adults (e.g. Pluess, Belsky, Way & Taylor, 2010), with evidence to suggest a link between the experience of positive life events and lower rates of neuroticism.

Taken together, there is now a plethora of evidence to suggest that the serotonergic system plays a critical role in brain development, synaptic plasticity and neurogenesis, with evidence to suggest an important influence of 5-HTTLPR polymorphism upon adult (Pezawas *et al.,* 2005; Hariri & Holmes, 2006) as well as child and adolescent brain structure (Wiggins *et al.,* 2012). Therefore it is likely that an individual's vulnerability versus resilience for affective disorders, such as depression to depend on the combination of childhood and adult life experiences (see also Grabe *et al.,* 2012).

3.2.1.2. Frontal EEG asymmetries as an early vulnerability marker

Simultaneous to research on normal variation in 5-HTTLPR as a genetic risk marker for later psychopathology, frontal EEG asymmetry has similarly been evaluated as a putative marker for endogenous risk versus resilience for affective disorders (e.g., Schmidt *et al.,* 2009; for a recent discussion see Schmidt & Moscovic, 2013). Lateralized differences in electro-cortical activity recorded over the dorsolateral prefrontal cortex, with a frequency of 8 to 13 Hz, or 'alpha band activity', haveshown to be heightened during attentive and awake states, but suppressed when an individual performs a cognitive task (Schaul, 1998; see also Section 2.2.1). There is now more than two decades of research using the frontal EEG activation and asymmetry measure as an index of affectivity in a variety of populations (for recent reviews see Gander & Buchheim, 2015; Harmon-Jones, Gable & Peterson, 2010; see also Section 2.2.1), with results that strongly support the hypothesis that this neural measure reflects cognitive and behavioural tendencies towards approach versus withdrawal (Davidson *et al.,* 1990). Specifically, right frontal asymmetry has been associated with withdraw-related behaviours and negative affectivity, whereas approach-related behaviours and positive affectivity have been associated with more left frontal asymmetry (Davidson, 2000; see also Section 2.2.3).

Despite the clear similarities and overlap in the areas of 5-HTTLPR and EEG research, there is currently very limited research on the putative relationship between the 5-HTTLPR genotypes and frontal EEG activation and asymmetry.

3.2.2. Neuroimaging genetics and psychopathology

Recent advancements on the field of psychology, psychiatry and neuroscience have started to adopt the main principles of neuroimaging genetics research (see Section 1.2.2) aiming to unveil the mechanism that may influence vulnerability for affective disorders. Most notably, in a recent fMRI study, Wiggins et al. (2012) investigated and reported the moderating effects of 5-HTTLPR genotype on children's and adolescents' connectivity of the right superior medial frontal cortex during rest. Moreover, although the existence of increasing evidence to suggest the distinct involvement of both frontal EEG activation and 5-HTTLPR variations in modulating affectivity, to date there is very limited evidence on the role of serotonin availability in frontal activation in both adults and children. Only the results of one recent EEG study with healthy adults suggested that S/S homozygotesexhibit a pattern of more withdrawal/right frontal asymmetry in response to negative emotion cues, compared with carriers of the Long allele (Papousek *et al.,* 2013). However, unlike the vast majority of EEG asymmetry research, which has focused on the right asymmetry as a trait (versus state) marker, this study reported that effects were only evident when participants were exposed to a video containing traumatic content, and not during the observation of a neutral visual display (Papousek *et al.,* 2013). Similarly, an additional study reported an impact of 5-HTTLPR Short allele in frontal activation over the areas F1/F2 when interacted with the presence of Major Depressive Disorder (Bismark *et al.,* 2010). Similarly, inthe same study, subjects homozygous for the serotonin HTR1A susceptibility allele had significantly greater relative right frontal activity at sites F7/F8, F5/F6, and F1/F2, when compared to subjects with at least one resilience-related allele. Moreover, fMRI studies have previously shown that variation in the serotonin transporter has been previously associated with inter-individual differences in vPFC and amygdala activation (Hariri *et al.,* 2002; Heinz *et al.,* 2007).

In addition to the serotonergic effects of early affectivity, and subsequently the effects on functional brain development and function, further polymorphisms on the dopaminergic system have also been associated with early affectivity, as well with the presence of affective disorders in the later life. Most notably, Catechol-O-m ethyltransferease (COMT) is an enzyme that is involved withdopamine degradation (Lachman *et al.,* 1996) and its genetic variations, where the best-studied Val^{158}Met has reported to modulate dopamine signalling in the frontal lobes, with an intermediate effect in executive cognitive functions (Bruder*et al,.* 2005). Specifically, the Met variant appears to be three to four times less active than the Val variant, resulting in less efficient breaking down of dopamine in the prefrontal cortex (e.g., Lachman *et al.,* 1996; Shehzad, DeYoung, Kang, Grigorenk & Gray, 2012; Tunbridge, Harrison & Weinberger, 2006). Consistent with this assumption, different studies have reported strong associations between the Val^{158}Met and specific neuropsychiatric disorders, such as schizophrenia (Caspi *et al.,* 2005) and autism (James *et al.,* 2006) or in placing an individual at higher risk for psychopathology when faced with life stressors (Evans *et al.,* 2009). In regards to the neurophysiological involvement of the Val^{158}Met polymorphism, evidence suggest the expression of the polymorphism in the amygdala (Herrmann *et al.,* 2009), an area of the brain important for socio-emotional functioning.

While recent findings provide evidence for the existence of putative pathways in genetic and brain processes that may relate to differential vulnerability for affective and other disorders (Bismark *et al.,* 2010; Papousek *et al.,* 2013); the particular mechanisms via which these genetic and environmental factors function and interact remain largely unknown. Given the developmental nature of existing models of risk/resilience for childhood, adolescent, and adult onset psychopathology (for a discussion see Belsky & Pluess, 2009), studying relationships between different susceptibility factors in children is critical to furthering our understanding of the developmental pathways to the manifestation of affective disorders. However, direct studies involving children as participants are notably absent from the extant literature. Moreover, although twin and family studies have reported a high heritability estimates of up to 90 % of the neurophysiological pattern of EEG activation (Anokhin, Heath & Myers, 2006; Gao, Tuvblad, Raine, Lozano & Baker, 2009; Smit, Posthuma, Boomsma & de Geus, 2007), to date there is very limited research in delineating the genetic underpinnings of frontal lobe activation.

3.2. The current study

Previous studies have documented 5-HTTLPR as a genetic risk variant that contributes to variability in outcomes of psychopathology. In order to investigate whether normal genetic variations on the 5-HTTLPR polymorphism and frontal EEG asymmetry are associated with one another, the present study seeks to investigate frontal EEG hemispheric asymmetries in relation to 5-HTTLPR genotyping in 4- to 6-year old children.

3.3.1. Aim 1: To examine EEG measures of behavioural problems in early childhood

Taking into account that the EEG methodology employed here is the same with the one in Chapter 2, and taking into consideration the considerable overlap between the samples of the present and the study post-posed (i.e., 68.5 %)[7], here there is expected a difference in children's responses on the two experimental conditions to be evident. To this end, the age range of the sample is further extended here to include 6 year olds; aiming to provide new putative associations between developmental trajectories of early behavioural problems and frontal EEG activity in response to social and non-social videos. Compared withthe sample investigated in Chapter 2, where the vast majority of the children consisted of children in their early fourth year of life, the present study examines the putative maturational effects, as resulted from an individual's environmental adjustments (school transition; change on the societal inhibition expectances from older children) as a factor that may contribute significantly on this area of inquiry.

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[7] The present study employed only a subset from the sample used in the previous study. This was done for two main reasons : (a) the study in Chapter 2 was conducted 6 months apart from the current study and (b) for the study in Chapter 2 was employed a more diverse sample of children from various ethnic backgrounds and therefore more representative of the wider community. Taken the ancestry constrains associated with the conduct of genetic studies, the unavailability of some of the families to return to the laboratory when invited to participate, and the need to extend the sample size the samples between the two studies overlaps by 68.5 %.

3.3.2. Aim 2: To examine 5-HTTLPR effects on frontal EEG asymmetries during early childhood

Individual variations in both 5-HTTLPR genotype and frontal EEG hemispheric asymmetry have been highlighted in previous research as separate susceptibility markers for better and worse outcomes later in life. A second aim of the present study is to investigate for the first time the inter-individual variability as determined by the 5-HTTLPR genotype on the activation of frontal lobe in children between 4 and 6 years of age. The first aim of the present study is to explore the association between affective patterns of frontal EEG activation in response to the social and non-social conditions. More specifically, 5-HTTLPR by frontal EEG associations will be dependent on viewing videos of social versus non-social components (state utilization of frontal EEG).

3.3.3. Hypotheses

There are two main hypotheses that are tested in the present study. The study employs a larger sample of children with a wider age range of children, compared with the sample investigated in the previous study (Chapter 2). To this end, the study sought to further investigate the potential involvement of the processing of social versus non-social information in frontal EEG activation and the presence of early behavioural problems. Although, the results of the previous study show no effect of condition on the reported brain-behaviour associations, the present study aims to examine the putative maturational effects as a factor that may contribute to this area of enquiry. Previous evidence has highlighted the existence of an association between relatively greater right frontal activation at rest and the presence of increased rates of aggressive behaviour in early childhood (Baving *et al.,* 2003; Santesso *et al.,* 2006). More specifically, it is hypothesised that children with elevated aggressive behaviour will present a state-specific negative pattern of frontal brain activation during the processing of social information, probably because of an inability to control the negative emotions associated with their aggressive behaviour (Smith & Bell, 2010). Moreover, a range of studies have reported atypicalities in visual processing of both social and non-social stimuli in infants in high-risk for ASD (Elsabbagh & Johnson, 2007; McCleery, Allman, Carver & Dobkins, 2007; McCleery, Akshoomoff, Dobkins & Carver, 2009; Dawson *et al.,* 1995). However, it is now increasingly accepted that individual differences in EEG asymmetry is independent of clinical status, and can serve as a trait marker for behavioural problems (Gotlib, 1998). More specifically, it is expected that the negativity-related patterns of greater right frontal EEG activation will be dependent on whether children watch social versus non-social videos (state utilization of frontal EEG). In the case that the video watching will not have an effect on frontal EEG activation, it would mean that the social versus non-social state cannot have an effect to positive versus withdraw-related patterns of frontal brain activation, and therefore frontal EEG will be utilized as a trait marker.

Secondly, it is hypothesised that there is a selective relationship between the 5-HTTLPR and state-dependent (social versus non-social videos) frontal EEG asymmetry, with the presence of two copies of the short allele to be associated with a negative pattern of relatively greater right frontal asymmetry during social but not non-social processing. Extensive, robust evidence has shown that social stimuli are of critical value and importance for humans, from birth through the full life span (for a review, see Grossmann & Johnson, 2007; Ronald, Happe & Plomin, 2005). Moreover, there is evidence that reported the moderating effects of 5-HTTLPR genotype on children's and adolescents' connectivity of the right superior medial frontal cortex during rest (Wiggins *et al.,* 2012) as well as a pattern of more withdrawal/right frontal asymmetry in response to negative emotion cues in healthy adults carrying two copies of the Short allele (Papousek *et al.,* 2013). Thus, if frontal EEG asymmetry associations with genotype vary according to social versus non-social video context, then this will suggest that the relationships observed are state dependent. Alternatively, if frontal EEG asymmetry associations with genotype are robust to the social versus non-social video context, then this will suggest that the relationships observed are trait dependent.

3.4. Methods and Materials

The known as candidate gene approach has emerged aiming to investigate the role of common genetic variations that involved in the neural circuits of emotion regulation and affectivity, which may interact with environmental stressors to predict behavioural reactivity, and vulnerability versus resilience for affective disorders (Canli *et al.,* 2006; Caspi & Moffitt, 2006; Canli & Lesch, 2007). However, it is worth noting that replication–related problems do exist in candidate gene studies (e.g. Gillespie, Whitfield, Williams, Heath, & Martin, 2005; Surtees *et al.,* 2006) that may contribute toslowing down the delineation of the biological underpinnings of human affectivity.

In the current study, was conducted a focused, hypothesis-driven examination of the relationship of variation in genotype on a genetic polymorphism (5-HTTLPR) and a particular, pre-determined marker of neural functioning (frontal EEG asymmetry). This research design and method represents a recently established approach to understanding genetic mediation of brain mechanisms that may shape human behaviour (e.g. Deary, Penke & Johnson, 2010).

3.4.1. Participants

The study's sample size (N= 70) was calculated on the basis of the study's hypotheses. Power analysis suggested that the sample size required to achieve a power of $1-\beta = 0.90$ for the ANOVA test at significance level $\alpha = 0.050$ requires at least 33 participants. In this regard, the current study utilizes a larger sample relative to most previous neuroimaging genetic studies with children and adolescents that employed fMRI (Stollstorff *et al.,* 2010; Wiggins *et al.,* 2012) or EEG/ERP (Althaus *et al.,* 2009; Beroletti, Zanoni, Giorda & Battaglia, 2012).

A total of 70 children aged between 4 and 6 years contributed to this study (Mean age in months = 60.8, SD = 11.6; males n = 38). Participants were recruited through a local community research participation advertisement/outreach program,as part of the on-going procedure at the Infant and Child Laboratory at University of Birmingham. The parents or guardians of all participants reported that the child had no history of a neurological or psychiatric disorder, and that they had normal or corrected to normal vision. Exclusion criterion included if participants scored below a certain range (IQ < 75) based on the BAS-II (Elliot *et al.,* 1996), a standardised assessment of intelligence/developmental age. Finally, because genes vary by ancestry (e.g., Freedman *et al.,* 2004), all children in the sample were from Caucasian/White British ancestry. In addition, all participants had English as their first language. Informed consent was obtained from the parents/guardians of all participants prior to participation in the study in accordance with an ethical protocol approved by the University ethical committee.

In the current study, EEG alpha was recorder power over left and right prefrontal cortex (F1-F2, F3-F4) while children watched videos with social stimuli (adults speaking nursery rhymes) and non-social stimuli (dynamic computer-generated objects moving with contingent sounds).

3.4.2. Data collection procedures

See Section 2.4.2.

3.4.2.1.Behavioural Measures

See Section 2.4.2.1.

3.4.2.2. Measure of behavioural problems

For the assessment of children's rates of behavioural problems the CBCL scales (Achenbach & Rescorla, 2001) were completed by parents. CBCL includes two different versions: the early years version (for children between 1½ - 5 years of age) and the school age version (children and adolescents aged 6–18 years). Both the early years and school age versions were used inthe present study. Details on the CBCL 1½ -5checklist are provided in Section 2.4.2.2 and Appendix 2.1. The CBCL 6-18 years version has 113 items, and although it describes the same aspects of behaviour as in the early years version, the content of some items varies, aiming to capture developmental changes and behaviours that are unique to each age. It has been reported that the CBCL 6-18 version has good psychometric properties[8] and has a robust procedure for classifying behavioural problems in each subdomain. Both versions' items are on a three-point scale, ranging from not true to very true/often true, including open-ended items for describing additional problems. The scale has two main sub-scales that are structurally independent from each other (Achenbach & Rescorla, 2001), whichmap externalizing and internalizing problems (see Table 2.1). Despite these differences, previous studies with children have reported the findings of the two broad sub-scales of the behaviours

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[8] CBCL 6-18 scale has been reported to have high test-retest reliabilities (r = 0.73-0.94), good internal consistency reliabilities (a=0.63-0.97), as well as good inter-rater reliabilities (k = 0.57 - 0.88) based on the original standardisation data (Achenbach & Rescorla, 2001).

coming from the two different versions (e.g., Stanger, Ryan, Hongyun & Budney, 2011). Inthe present study, parents completed the age-appropriate version of the scale.

3.4.2.3. Electrophysiological Recordings

See Section 2.4.2.3.

3.4.2.4. DNA Preparation

Genomic DNA was extracted from saliva samples using the Oragene OG-500 self-collection kit (Oragene, DNA Genotek Inc., Canada), according to the manufacturer's recommendations. DNA concentrations ranged from 65-962 ng/ul and the 260/280 ratio was between 1.8 and 2 for all samples. Genotyping results were successfully obtained for all 70 subjects.

3.5. Analysis

3.5.1. Analysis of Behavioural data

The CBCL 1½ -5 and 6-18 versions provides raw values that can be converted to age-adjusted t-scores if needed. All children in this study had t-scores of less than 60. Raw scores from the two clusters of behavioural problems (i.e., internalizing and externalizing problems) were used for statistical analysis following the authors' guidelines (Achenbach & Rescorla, 2001, p. 89). Higher total scores in each CBCL subscale suggest the existence of more problematic behaviours. Autism symptomatology (SCQ) mean sum scores were calculated on the basis of raw scores. For the measures of cognitive abilities (BAS-II), mean standardized IQ-scores were assessed.

3.5.2. EEG Recordings and Analyses

All the EEG recording and analyses procedures that conducted in the present study were the same as for the study presented in Chapter 2 (see Section 2.5.2). Moreover, in the current study aiming to further investigate if the 5-HTTLPR genotype effects were specific to the frontal region, additional clusters of electrodes over the P3 (electrode number 52) and P4 (electrode number 92) parietal areas were selected for analyses.

3.5.3. Analysis of Genetic Material

<u>3.5.3.1. 5-HTTLPR Genotyping</u>

Direct bidirectional sequencing was used to genotype the 5-HTTLPR polymorphism. The region containing the 43bp insertion polymorphism was amplified using primers described (Hu*et al.,* 2006) producing a 528bp amplification product from the L allele and a 485bp product from the S allele. Polymerase Chain Reaction (PCR) was performed using Megamix PCR solution (supplied by Microzone UK Ltd) in a total volume of 25ul, containing 25pmol of each primer and 3ul of betaine. An initial denaturation step at 95ºC for 5 minutes was followed by 30 cycles of PCR (95ºC 1 minute, 58ºC 1 minute, 72ºC 1 minute) and then a final extension at 72ºC for 10 minutes. PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (according to manufacturer's instructions). 10ul sequencing reactions were generated containing 0.25ul BigDye Terminator (v3.1, Applied Biosystems), 1.9ul molecular grade water, 3pmol of forward or reverse primer and 1ul purified HTTLPR PCR amplicon (diluted 1 in 2). Cycle conditions for sequencing included an initial denaturation step at 95ºC for 5 minutes followed by 30 cycles of (95ºC 30 seconds, 50ºC 10 seconds, 60ºC 4 minutes) and reaction products were purified using CleanSEQ® beads (Agencourt) in a 1:1 ratio as described by the manufacturer. Products were re-suspended in 70ul molecular grade water and analysed on a 3730 Genetic Analyser (Applied Biosystems).

Allele frequencies across participants for the 5-HTTLPR was n = 59 (42.1 %) for the Short allele and n = 81 (57.9%) for the Long allele. Different genotype classifications were used inthe present study with three [S/S (N = 13), L/S (N = 33), L/L (N = 24)], as well as with two groups of participants: one with homozygous for the Long allele (L/L; N = 24) and one with heterozygotes and homozygous for the low uptake Short allele (S/S, S/L; N = 46). 5-HTTLPR genotype frequencies were in Hardy-Weinberg Equilibrium [$x^2(1) = .077$, $p = .780$], as calculated with a reliable online tool that can be found here:http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%20calculator.xls . In follow up analyses, taking into account the variation in group classification in 5-

HTTLPR studies (e.g., the S/L group being classified differently in different studies; see for example Hariri *et al.,* 2002; Lee & Ham, 2008) and aiming to provide a greater theoretical precision to the moderating effects of 5-HTTLPR gene variation (Walsh *et al.,* 2012), S/L participants were excluded, and compared the two groups of homozygotes [i.e., S/S (N = 13), L/L (N = 24)].

3.5.3.2. COMT Val^{158}Met Genotyping

Direct bidirectional sequencing was used to genotype the single nucleotide polymorphism within the COMT gene (rs4680). PCR primers were designed to flank the polymorphism producing a 250bp amplification product. Sequences of the primers are as follows: forward GGGCCTACTGTGGCTACTCA and reverse GGGTTTTCAGTGAACGTGGT. PCR was performed using Megamix PCR solution (supplied by Microzone UK Ltd) in a total volume of 25ul containing 25pmol of each primer. An initial denaturation step at 95ºC for 5 minutes was followed by 30 cycles of PCR (95ºC 1 minute, 58ºC 1 minute, 72ºC 1 minute) and then a final extension at 72ºC for 10 minutes. PCR products were purified and sequenced as described above for 5-HTTLPR genotyping.

Allele frequencies for the COMT Val^{158}Met was n = 71 (50.7 %) for the Val allele and n = 69 (49.3 %) for Met allele. Following a similar strategy with the 5-HTTLPR genotype grouping, a classification with three genotypes was employed [M/M (N = 14), V/M (N = 41),V/V (N = 15)], as well as by including the homozygous participants for the more active Val allele (V/V; N = 15) in one group in the sample compared to a group of heterozygotes and homozygotes of the low uptake allele (M/M, M/V; N = 55). COMT Val^{158}Met genotype frequencies where in Hardy-Weinberg equilibrium [x^2 (1) = 2.06, *p* = .150] as measured from the same tool used for the 5-HTTLPR genotype. Consequently, in aiming to provide a greater theoretical precision to the moderating effects of the COMT Val^{158}Met gene variation in follow up analyses, heterozygous participants were excluded to compare the two homozygous groups [i.e., Val/Val (N = 15); Met/Met (N = 14)].

3.5.3. Statistical Analyses

Descriptive statistics were conducted in order to describe the sample's demographic characteristics such as, gender, age, and distribution of cognitive abilities. Raw data from the behavioural and cognitive scales were examined for normality using Kolmogorov–Smirnov tests. CBCL subscales did not meet criteria for normal distributions (Kolmogorov–Smirnov, $p < 0.05$). Therefore, to further examine possible correlations between age, developmental age, gender, IQ and behavioural scores, Spearman's Rho non-parametric correlations coefficients tests also performed. Further correlation analyses conducted to investigate possible correlation between Raw EEG recording and asymmetry ratios, with participants' demographic characteristics. In addition, Spearman's Rho correlations were conducted between the two clusters of internalizing and externalizing problems and the PSD and ratio values from the EEG data for each condition and hemisphere separately.

Pearson correlation analyses were conducted to determine if a correlation among demographic characteristics or cognitive performance and genotype group was evident. Further one-way ANOVAs were conducted to investigate possible correlations between 5-HTTLPR or COMT Val^{158}Met genotypes and demographic, cognitive development rates and affective problems in the sample. Moreover, correlation analyses between asymmetry group (left versus right asymmetry) and demographic characteristics were also conducted.

Furthermore, to examine if the artefact-free EEG data was systematically differ among the 5-HTTLPR and COMT Val^{158}Met genotype groups, separate one-way ANOVAs were conducted. Furthermore, to assess if excessive frontal or parietal artefact (i.e., number of bad electrodes in the areas of interest) systematically differ among the three 5-HTTLPR and COMT Val^{158}Met genotypes, additional one-way ANOVAs were conducted.

In order to examine differences in frontal EEG activation in multiple frontal sites in response to social and non-social stimuli, a three-way ANOVA with Condition (Social, Non-social), Hemisphere (Left, Right) and region (F3-F4; F1-F2) as within factors and gender (female, male) and 5-HTTLPR genotype (S/S versus L/S versus L/L) as between-groups factors was conducted. Repetition of the same analyses with different genotype classification (i.e., L/L versus S/-) was also conducted. For the initial analysis, the PSD were studied. As a control analyses, to investigate whether the 5-HTTLPR genotype effects are specific to the frontal region we also conducted a two-way ANOVA with Condition (Social, Non-social) and Hemisphere (Left, Right) using PSD data from parietal regions (i.e., P3-P4).Then the initial omnibus ANOVA was followed up with analysis of EEG asymmetry scores, by conducting post-hoc tests for each SNP using an average asymmetry ratio for each participant. When the data did not satisfied Kolmogorov-Smirnov tests for normality, Mann-Whitney U or Kruskal-Wallis tests were performed, instead of t-tests. In order to evaluate the specificity of the 5-HTTLPR effect, this same analysis process was repeated with equivalent COMT Val^{158}Met polymorphism classifications (i.e., M/M versus M/V versus V/V; M/- versus V/V) as a control analysis. The statistical software package SPSS 20.0 was used for all the analyses.

3.6. Results

3.6.1. Demographic Characteristics

Participants included 70 children (males n = 38) between 4 and 6 years of age. Tables 3.1 and 3.2 demonstrate the participants' main demographic characteristics, such as, gender, age, and cognitive abilities. One-way ANOVAs showed no significant correlations between 5-HTTLPR or COMT Val^{158}Met Genotypes and demographic and cognitive characteristics (see Appendix 3.1). Similarly, One-way ANOVAs showed no significant correlations between 5-HTTLPR or COMT Val^{158}Met Genotypes and rates of affective problems. Moreover, Asymmetry groups did not differ in demographic characteristics.

Table 3.1. Sample size and demographic characteristics of sample.

N		**70**
Gender	% Male *(N)*	55.8 *(29)*
	% Female *(N)*	44.2 *(23)*
Handedness	% Right *(N)*	82.9 *(58)*
	% Left *(N)*	17.1 *(12)*
SCQ total score	Mean *(SD)*	4.25 *(3.13)*
	Range	0-12
BAS-II	% Below Av.	2.9
Total Score	% Average	67.1
	% Above Av.	22.9
	% High	7.1

Furthermore, Spearman's Rho correlation showed asignificant positive correlation between rates of internalizing and externalizing problems (*r* = .350, *p* = 0.11). Co-occurrence between internalizing and externalizing clusters has originally been reported on the CBCL scales original standardisation (Achenbach & Recorla, 2001) as well as in a range of other studies (e.g., Card & Little, 2006; Marsee & Frick, 2007; Dietz, Jennings, Kelley & Marshal, 2009). No further correlations between demographic characteristics and behavioural scores were evident.

Table 3.2. Participants General and Age equivalent cognitive ability.

Chronological Age*	Mean *(SD)*	60.8 *(11.6)*
	Range	48-82
Overall Ability**	Mean *(SD)*	105.8 *(8.6)*
	Range	84-127
Verbal Ability	Mean *(SD)*	101.6*(13.3)*
	Range	58-127
Non-verbal Ability	Mean *(SD)*	109.5 *(12.9)*
	Range	86-144
Developmental Age*	Mean *(SD)*	64.2 *(12.9)*
	Range	42-89
Developmental Verbal Ability	Mean *(SD)*	62*1.7(14.9)*
	Range	38-96
Developmental Non Verbal Ability	Mean *(SD)*	66.9*(15.0)*
	Range	42-96

*Age data presented in months

**Overall ability is calculated from the overall BAS-II total score and Verbal and Non-verbal ability form the BAS-II clusters of abilities. Values represent GCA.

3.6.2. Behavioural problems and EEG alpha activation/asymmetries

Spearman's Rho analyses did not reveal any significant correlation between children's internalizing and externalizing scores and frontal EEG activation when using the raw PSD scores or the asymmetry ratios. This finding is contrary to the findings reported in the previous study in Chapter 2 which employed the same behavioural and experimental/neurophysiological measures. However, as noted earlier (see also Footnote 7) the sample in the present study had a 68.5 % overlap with the sample in the study presented in Chapter 2. The first investigation was conducted with younger children mainly from diverse ethnic background (Chapter 2), whereas only older children frontal Caucasian ancestry were included in the present study. Moreover, compared to the previous study were detailed scales of symptomatology was possible to be extracted and calculated (early years CBCL was employed) the current analyses were based in the behavioural rates that were calculated from the two main sub-clusters only (i.e., internalizing and externalizing) due to the fact that both the two versions of early and school-age years CBCL measure was employed. Taken together with the fact that the sample consisted of young unaffected children, this may have contributed to the absence of the previously reported brain by behaviour associations.

3.6.3. 5-HTTLPR Genotype Group Differences in Frontal Alpha Asymmetry

A one-way ANOVA showed that the time of artefact-free EEG data did not differ systematically among the three 5-HTTLPR genotype groups (see also Appendix 3.1; Table 3.4) for the social [$F(2) = 1.65$, $p = .199$] as well as for the non-social condition [$F(2) = 0.96$, $p = .385$]. In a similar vein, time of artefact-free EEG data did not differ systematically among the three COMT Val^{158}Met genotype groups for the social [$F(2) = .298$, $p = .744$] as well as for the non-social condition [$F(2) = .426$, $p = .655$]. Moreover, an additional one-way ANOVA showed that the number of electrodes of interest that were marked as bad channels did not systematically differ among the three 5-HTTLPR genotypes for the frontal [$F(2) = 1.74$, $p = .182$] and the parietal [$F(2) = 0.63$, $p = .850$] selected areas of interest.

Similarly, no systematic difference was observed for the COMT Val^{158}Met genotype groups the frontal [$F(2) = .019$, $p = .981$] and the parietal [$F(2) = 1.39$, $p = .254$] selected areas of interest.

The ANOVA revealed a main effect of Region [$F(1,64) = 35.50$, $\eta_p^2 = 0.35$, $p < .001$] and a two-way Hemisphere by Condition interaction[$F(1,64) = 5.08$, $\eta_p^2 = .007$, $p < .028$]. Regarding the between-groups effects the ANOVA revealed an additional Hemisphere and 5-HTTLPR genotype interaction [$F(2, 64) = 5.69$, $\eta_p^2 = .151$, $p = .005$], indicating different frontal activation between the three 5-HTTLPR genotype groups (see Table 3.4; Figure 3.1). No further effects or interactions were observed. The same Hemisphere by 5-HTTLPR interaction was confirmed when repeating the ANOVA after grouping carriers of at least one Short allele in one group (i.e., S/- versus L/L) [$F(1, 66) = 8.95$, $\eta_p^2 = .120$, $p = .004$].No further main or interaction effects were evident (see also Table 3.3).

Table 3.3. Average frontal PSD activation per hemisphere, condition and frontal region.

	Social		**Non-social**	
Alpha Power*	**Left** Mean *(SD)*	**Right** Mean *(SD)*	**Left** Mean *(SD)*	**Right** Mean *(SD)*
F3-F4	1.15 *(0.72)*	1.19 *(0.78)*	1.15 *(0.66)*	1.17 *(0.72)*
F1-F2	0.91 *(0.73)*	0.92 *(0.81)*	0.90 *(0.64)*	0.88 *(0.64)*
Total	1.03 *(0.70)*	1.05 *(0.77)*	1.03 *(0.63)*	1.03 *(0.66)*

* Alpha power = ln [7–11 Hz] power power spectral density (µV2/Hz)

A Kolmogorov-Smirnov test revealed that the PSD data used in this analysis, as well as the asymmetry ratio data were not normally distributed (Kolmogorov–Smirnov, $p < 0.05$), this analysis was followed up with complementary non-parametric tests using the left/right asymmetry ratios. A Kruskal-Wallis test was performed to further investigate the mean frontal asymmetry score ratio differences by the Genotype (S/S versus L/L versus S/L). This analysis revealed a significant effect of 5-HTTLPR genotype group on the frontal EEG asymmetry ratios [$x^2(2) = 8.65$, $p = .013$], providing confirmatory support for the statistical interaction between Hemisphere and 5-HTTLPR that observed on the initial ANOVA (see Figure 3.1; Table 3.4). Specifically, the genotype group homozygous for the Short allele (i.e., S/S) manifested a frontal alpha activity more to the right, whereas a group with 5-HTTLPR L/L exhibited more left activation (see Table 3.4). Similarly, a Mann-Whitney U test with the alternative genotype classification (S/- versus L/L) also showed significant differences among 5-HTTLPR genotypes in predicting frontal asymmetry ($U = 199.00$, $p = .010$).

In order to evaluate the specificity of a link between frontal EEG activation patterns and 5-HTTLPR genotypes, the same analysis was repeated for the COMT Val^{158}Met polymorphism. No significant interaction effect was observed of this genotype with frontal hemisphere activation [$F(2, 64) = 0.85$, $\eta_p^2 = .026$, $p = .429$] when comparing the three COMT genotypes (i.e., M/M versus M/V versus V/V), or for the V/V versus M/- classification [$F(1, 66) = 1.06$, $\eta_p^2 = .016$, $p = .305$] (see Table 3.4; Figure 3.1).

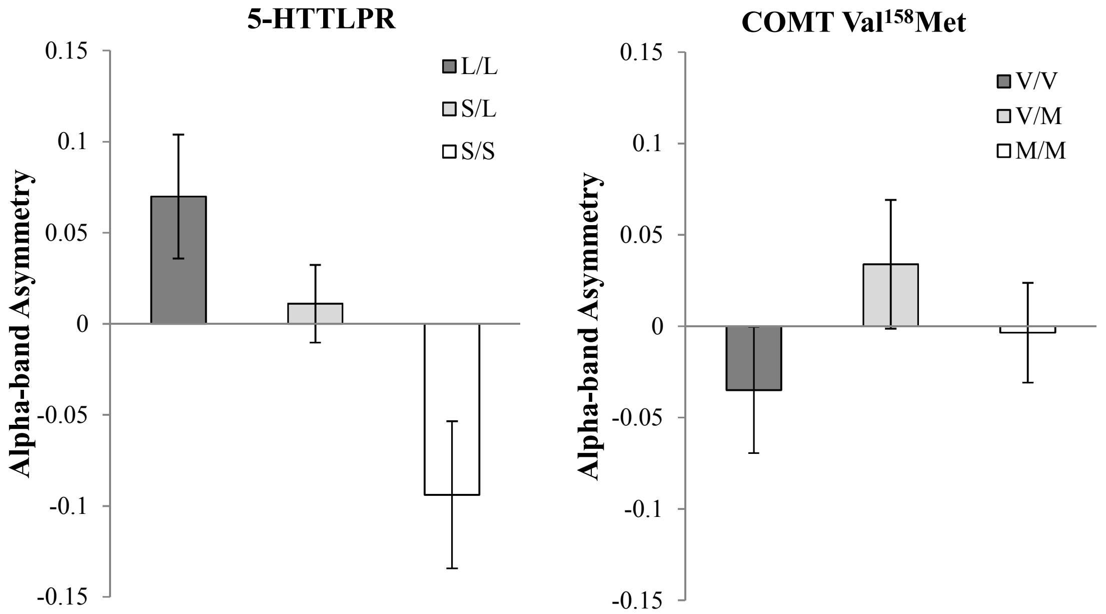
Furthermore, in order to further investigate the specificity of the effects of 5-HTTLPR genotype to the frontal region, an additional two-way ANOVA with Condition (Social, Non-social) and Hemisphere (Left, Right) using alpha band PSD data recorded over left and right parietal regions was conducted (i.e., P3-P4 region). The results of this ANOVA did not reveal any 5-HTTLPR by parietal hemisphere activation [$F(1, 66) = .284$, $\eta_p^2=.004$, $p = .596$] or other main or interaction effects.

Table 3.4. Means and standard deviations (in brackets) of the logged alpha power spectral density in the frontal region among 5-HTTLPR and COMT Val^{158}Met genotype. The mean PSDs were consistently lower for the Short-allele carriers compared to participants homozygous for the Long carriers, especially over the right hemisphere, suggesting a more withdrawn pattern of brain activation in participants with at least one Short allele.

SNP	**Social**		**Non-Social**	
	Left	**Right**	**Left**	**Right**
5-HTTLPR				
S/S	1.05 *(0.57)*	0.97 *(0.48)*	0.92 *(0.48)*	1.03 *(0.62)*
S/L	0.99 *(0.63)*	1.00 *(0.67)*	0.97 *(0.59)*	0.98 *(0.64)*
L/L	1.08 *(0.87)*	1.17 *(1.02)*	1.10 *(0.69)*	1.15 *(0.77)*
COMT Val^{158}Met				
M/M	0.88 *(0.83)*	0.90 *(0.90)*	0.97 *(0.69)*	1.00 *(0.65)*
M/V	1.13 *(0.66)*	1.18 *(0.76)*	1.08 *(0.60)*	1.10 *(0.66)*
V/V	0.88 *(0.70)*	0.85 *(0.66)*	0.90 *(0.69)*	0.87 *(0.66)*

Taking into account the variations in the classifying methods for the 5-HTTLPR genotypes [e.g., in some studies the Long allele is re-categorized as an S because when it is present alongside the G-allele of rs25531 SNP, it behaves like the Short allele (Hu *et al.,* 2006; Wendland *et al.,* 2006; Zalsman *et al.,* 2006), and aiming to achieve a better understanding of the moderating effects of 5-HTTLPR gene variation (Walsh *et al.,* 2012), the heterozygotesparticipants were excluded (i.e., L/S), and the initial analysis was repeated by comparing the two groups of homozygotes, for the 5-HTTLPR [i.e., S/S (N = 13), L/L (N = 24)].

Figure 3.1. Mean asymmetry index for 5-HTTLPR (left) and COMT (right) allelic variants. The data were collapsed across social/non-social condition. The frontal asymmetry was computed as the alpha power in the right hemisphere minus that in the left hemisphere. The error bars denote one standard error of the mean.



Furthermore, the same analyses was also conducted using the homozygous groups of the COMT Val^{158}Met genotypes [e.g., Val/Val (N = 15) and Met/Met (N = 14)]. Kolmogorov-Smirnov tests for these subgroups' data revealed that the PSD and asymmetry ratio data metcriteria for anormal distribution (Kolmogorov–Smirnov, $p > 0.05$). Consistently with the results from the initial classification, the results of this follow-up ANOVA confirmed a two-way interaction between 5-HTTLPR Genotype and Hemisphere [$F(1, 33) = 7.99$, $\eta_p^2 = .195$, $p = .008$], which was absent for the COMT Val^{158}Met genotype [$F(1, 25) = 1.16$, $\eta_p^2 = .070$, $p = .324$]. Finally, consistent with the initial results, a t-test revealed a significant lower average frontal EEG asymmetry scores in the S/S compared to the L/L 5-HTTLPR group [$t(35) = 2.97$, $p = .005$)].

3.7. Discussion

The present study was designed to examine the relationship between 5-HTTLPR genotypes and frontal EEG activity in young children. Consistent with the study's hypothesis, the results indicated that normal variation in the 5-HTTLPR genotype is associated with frontal lobe hemispheric activations in young children, such that children homozygous for the Short genotype exhibited right (negativity-related) frontal EEG hemispheric asymmetries, whereas those who carried the Long allele exhibited more left (positivity-related) frontal EEG asymmetries. The current results provide evidence for the existence of a neurobehavioural mechanism in individuals carrying the Short allele of the 5-HTTLPR polymorphism, which may act as a context-specific risk factor for later psychological maladjustment and negative affectivity for young children. However, taken the inconsistency that is evident among different frontal EEG studies (e.g., Coan & Allen, 2004), the evidence suggesting a differential susceptibility for both positive and negative effects in carriers of the Short allele (Belsky & Pluess, 2009), and the absence of context-specific investigation in the present study the present findings can be only accounted as a first-stage contribution on the putative genetic effects in early brain functioning and affectivity.

An additional aim of the study was to investigate putative associations between early affective internalizing/externalizing problems and frontal EEG activation in response to social and non-social videos. Somewhat inconsistent with previous research, no significant correlation between measures of early affectivity and trait or state-specific frontal EEG activation in response to social videos was evident. A possible explanation for this null effect might be the fact that the variation in the children of the study's sample were in the normal range on internalizing and externalizing problems, with an exclusion criterion of the study to be the elevated behavioural problems above the subclinical threshold of the measure. Compared to previous studies with youth, where significant behaviour by frontal EEG associations were evident in children with a particular set of symptoms or traits, such as anxiety or shyness (e.g., Santesso *et al.,* 2006), the normal range of internalizing/externalizing problems of the study's sample may explain the results of the study. However, it is worth mentioning at this point that research has shown that EEG frontal asymmetry is more directly associated with approach/withdrawal tendencies than with measures of internalizing/externalizing problems (for a review see Coan & Allen, 2003). Taken the results presented in the study of Chapter 2, where behavioural-brain associations were evident in younger children, but accounted for a small variation of the sample, future research is needed to delineate the link between early behavioural patterns of approach/withdraw and their putative link with internalizing and externalizing during the early years of life. As in the study of Chapter 2, given that the present investigation studied a healthy young group of children, previous evidence that suggested a role of viewing social versus non-social videos in brain activation of atypical populations, such as children with autism, may relate to a disorder-specific response to social and non-social information. This hypothesis requires further investigation.

Furthermore, partially inconsistent with the hypothesis of the study there was no significant effect of viewing social versus non-social videos on frontal EEG activation relative to 5-HTTLPR genotype. However, the study provided evidence for the trait utilization of frontal EEG and its associations with variations in the 5-HTTLPR genotype. To this end, the previously documented atypicalities on the processing of social and non-social information in young children in higher risk for ASD (Elsabbagh & Johnson, 2007; McCleery, *et al.,* 2007; McCleery, *et al.,* 2009; Dawson *et al.,* 1995), may relate to the manifestation of the social difficulties that primarily associated with this specific disorder. The sample of the present study consisted of healthy young children whodo not present a particular set of disorders that may affect the way they processing social versus non-social information. Conversely, the findings of the study that suggest a trait utilization of EEG as a function of a genotype involved in serotonin availability agrees with previous research highlighting that individual differences in EEG asymmetry is independent of clinical status and can serve as a trait marker for behavioural problems (Gotlib, 1998). Finally, other methodological aspects of the study, such as the nature of the stimuli used may have also contributed on the absence of the investigated effects (see also Section 2.7).

The results of the study open up the possibility that one of the key mechanisms via which carriers of two copies of the Short 5-HTTLPR genotype generates susceptibility for later negative and positive affectivity is through serotonin-based mediation of right versus left frontal cortex activity. This pattern of results is consistent with previous developmental evidence to show that carries of one (Caspi *et al.,* 2003) or two (Pluess *et al.,* 2010) copies of the Short 5-HTTLPR allele had increased depressogenic effects, compared to Long allele homozygotes, that resulted from the exposure to stressful events. In particular, evidence suggests that carriers of at least one Short allele of the 5-HTTLPR, compared to individuals homozygous for the Long allele, exhibit a neurophysiological pattern of higher amygdala reactivity when exposed to negative or arousing environmental conditions (Munafo *et al.,* 2009; Murphy *et al.,* 2013; Walsh *et al.,* 2012).Therefore, through these mechanisms and under disadvantageous context this genotype may predispose the individual toward the reductions in approach-related motivations and increased negative affectivity that are characteristics of individuals with more right frontal EEG asymmetries (Coan & Allen, 2003; see also Dason *et al.,* 1995; Nusslock *et al.,* 2011; Tomarken *et al.,* 2004).

Furthermore, based on the differential susceptibility hypothesis, carriers of at least one copy of the low serotonin uptake-related Short allele would be expected not only to exhibit a more negative response in face of adversity but also a positive response under favourite circumstances (e.g., Belsky & Pluess, 2009). The present findings that suggest that the existence of an associative mechanism between the short 5-HTTLPR allele and right frontal asymmetry may suggest that the susceptible individuals carrying this dyad of susceptibility markers would be expected under adverse conditions, such as traumatic life events to be more vulnerable for the manifestation of maladaptive behaviours, but under positive environmental influences would be predicted to exhibit the better adaptation compared to individuals carrying the long uptake allele and exhibit relatively greater left asymmetry. This hypothesis requires further longitudinal investigation that will start early in life and will include the investigation of the environmental context that is necessary to be included on this equation.

Furthermore, the current findings suggest that carriers of two copies of the Long allele that is associated with high uptake of serotonin were manifesting a relatively more left frontal asymmetrypattern, which is associated with positive emotionality and approach-related behaviours. This pattern of findings is consistent with previous research that suggests that the presence of at least one 5-HTTLPR Long allele may serve as a protective factor against negative affectivity for affective psychopathologies (Bogdan *et al.,* 2014; Hankin *et al.,* 2011; Pluess *et al.,* 2010). However, there is evidence to suggest an association between relatively greater left frontal EEG asymmetry and increased rates of aggressive behaviours in children (e.g., Gatzke-Kopp, Jetha, & Segalowitz, 2014; Smith & Bell, 2010). To this end, the current aspect of findings need to be interpreted with extra caution and can only account as a first stage contribution on the neurobiological underpinnings of positive affectivity and protection. To test whether such speculation for a protection-related association between the presence of two copies of the Long 5-HTTLPR allele and greater left asymmetry,it is critical for further studies to be conducted that will test the way in which adverse versus positive contexts may effect individuals with such different neurobiological profiles.

The direction of the current results where a stable pattern of negativity of frontal EEG activation was evident for participants homozygous for the Short allele, is similar to that of previous -state dependent- EEG evidence in adults reporting an association between homozygocity in the Short allele and higher right frontal EEG activity in response to observing aversive film scenes (Papousek *et al.,* 2013). In addition, the present finding is consistent with previous evidence from functional magnetic resonance imaging (fMRI) studies with older children reporting that the connectivity of the right superior medial frontal cortex during rest is particularly sensitive to 5-HTTLPR variations (Wiggins *et al.,* 2012). The relationship between the serotonin transporter-linked polymorphic region (5-HTTLPR) and frontal activation in early childhood observed in the current study may help to bridge theexisting gap between the previously reported structural and functional MRI evidence on the effects of the 5-HTTLPR genotype and neural structures with lateralization of activity in the frontal lobe. This is the first study with young children to report 5-HTTLPR genotype effects in frontal EEG. Compared withrecent evidence in adults reporting small (Papousek *et al.,* 2013) or absent effects (Bismark *et al.,* 2010) of 5-HTTLPR in frontal EEG during rest, the present findings may be attributable to the fact that the current sample consists of unaffected young children, who are in an early developmental and neurobiological maturation stage of emotion regulation where, compared to adults, approach-withdraw related patterns of frontal brain activation may be influenced by minor environmental influences. Future cross-sectional and longitudinal research, in which the same experimental paradigms and procedures are used with groups of individuals at different ages, will shed critical light on the role of 5-HTTLPR genotypes in influencing state and trait indices of frontal EEG asymmetry, across development.

Limitations

Consistent across many neuroimaging genetic studies, one primary limitation of the present study is the small sample size. However, taken into account the time constrains for the completion of the study, as well as the restrictions associated with children's age and ancestry, it is challenging to recruit a relatively large sample especially within one geographic location. Despite these constraints, the study was able to recruit a sample of 70 children. Furthermore, taken the very limited previous empirical studies on the field, the purpose of the current study was to examine the role of normal 5-HTTLPR genotype variations in functional brain activation in young children. Therefore, it was beyond the remit of the present study to investigate the same patterns of neurofunctional development in a group of atypically developing children. However, it is acknowledged that the results of the present empirical study can act as a springboard for future research in atypical development. In a similar vein, further research using a larger sample is required to further delineate the genetic factors that may drive the early precursors for these behaviours and the complex interactions between genes, brain, development, and behaviour.

In sum, the results of the current study suggest that two putative markers that relate to plasticity for behavioural outcomes, 5-HTTLPR genotype and frontal EEG hemispheric asymmetry, are related to one another during childhood. The current findings further open the possibility for a pathway from 5-HTTLPR-mediated differences in the availability of serotonin to risk versus protection against later psychological problems through frontal brain activity patterns that establish negativity and positivity-related cognitive-behavioural tendencies in childhood.

CHAPTER 4

Genetic influences on the visual scanning of faces in young children

4.1. Preface

The previous chapter investigated the putative associations between frontal brain activation and a genotype that relates to serotonin uptake, providing evidence for mechanistic associations between these two independent markers that may link with plasticity for behavioural outcomes early in life. These research outputs may account as a first-stage contribution towards understanding the development of affectivity early in life. Aiming to further delineate the neurobiological underpinnings of early reactivity and its putative associations with the manifestation of problematic behaviours, the current chapter provides an overview of the current knowledge on the human visual scanning processes in response to emotional face stimuli and their relationship to human affectivity and problematic behaviours. To this end, in this prospective study, eye-tracking technology is employed, along with genetic investigations in candidate genes to investigate the neurobiological underpinnings of visual processing of faces in a group of typically developing young children.

4.2. Development of Facial Emotion Recognition

Effective processing of vast amounts of the incoming information in our daily lives requires a range of cognitive skills, such as filtering of stimuli, timely disengagement from negative cues, as well alertness for best and most meaningful incoming cues to emotion (Beevers, Clasen, Stice & Schnyer, 2010). It has been previously suggested that cognitive processes involved in the modulation of visual scanning processes in response to emotions may have a particularly important impact on the development of early affectivity (Pine, Helfinstein, Bar-Haim, Nelson & Fox, 2009). More specifically, there is evidence to suggest that difficulties to disengage from negative stimuli may also relate with emotion regulation problems (e.g., MacLeod, Rutherford, Campbell, Ebsworthy, & Holker, 2002). Interestingly, current models of emotion regulation incorporate a range of cognitive regulatory strategies that may assist effective emotion regulation, such as distraction of attention, selection of a specific environmental situation, as well as rumination (for a review see Gross *et al.,* 2011; Gyurak, *et al.,* 2011). As Gross (1998) highlights, an effective way for an individual to regulate his/her emotions is by shifting eye gaze to certain emotional stimuli in the environment. For instance, in face of a negative trigger some individuals may look away from the affective stimuli as a way to inhibit the arousal that the stimuli provokes to them, which may also help them to preserve positive emotionality (e.g., Isaacowitz, 2005; Xing & Isaacowitz, 2006).

In line with this concept, it has been suggested that biased processing of emotional stimuli is part of the intermediate phenotype for affective disorders (Hasler, Drevets & Charney, 2004), such as depression and anxiety (Gross & Munoz, 1995). More specifically, there is evidence to suggest that atypicalities in the processing of facial emotions may relate with the increased manifestation of a range of social and affective symptoms (for a review see Bourke, Douglas

& Porter, 2010) but also cognitive deficits (Gotlib & Joormann, 2010) that are present in depressive symptomatology. Moreover, associated changes on the patters of processing facial emotions have been previously documented with effective prediction of treatment responsivity in major depressive disorder (Venn *et al.,* 2005). Interestingly, a study shows that healthy female adults with a family history of depression as well as affected female participants, exhibit more problems inhibiting negative stimuli (i.e., latency of naming the colour of emotionally charged words) in an emotional Stroop paradigm (van Oostroom *et al.,* 2013), which may suggest that biases in affective processing may account as a trait characteristic that contributes to the onset of depressive disorders.

There are several theoretical models that have been developed to measure reactivity in response to environmental stressors. It is believed that emotional stimuli in the environment may trigger immediate behavioural responses that can be recorded experimentally. Among the most widely used index to assess emotional affectivity in response to face in both child and adult literature, is eye movements. To this end, the employment of eye-tracking technologies has been shown to provide a reliable neuropsychological measure of affectivity in response to environmental stressors. In the following section, different models and corresponding studies are reviewed that have primarily employed eye-tracking techniques to measure affectivity in various populations.

4.2.1. Measuring visual scanning behaviour

Over the last three decades, various theoretical concepts and experimental paradigms have been proposed and utilized in an effort to examine and compare visual scanning behaviour in anxious and healthy populations (Cisler & Koster, 2010). A typical finding in these studies is that adults diagnosed with anxiety disorders exhibit a biased visual scanning tendency (often referred to as attentional bias) to orient toward threat-related stimuli (Bar-Haim *et al.,* 2007; Armstrong, Olatunji, Sarawgi & Simmons, 2010; Buckner Maner & Schmidt, 2010; Koster, Verschuere, Crombez & Van Damme, 2005; Van Damme & Crombez, 2009). However, evidence from developmental studies has been relatively inconsistent (Mogg & Bradley, 1998; Le Doux, 2000; for a review see Puliafico & Kendall, 2006; also see Shechner *et al.,* 2013). One possible explanation for this may be the fact that the effective control of visual processing is affected by known, but poorly understood, maturational effects in fronto-cortical circuits that undergo notable development during the late adolescent years (Hare, Tottenham, Davidson, Glover & Casey, 2005; Pessoa, 2010).

Studies in recent years have employed eye-tracking methods as a reliable reference index of visual emotional preference to assess vigilance versus avoidance behaviour to emotional stimuli in various populations. The main aim in this area of inquiry is the investigation of the increase versus decrease in the orienting of visual processing, also known as vigilance-avoidance (Mogg & Bradley, 1998), through the measurement of fixation time among different types of emotional stimuli (Armstrong, Olatunji, Sarawgi & Simmons, 2010; Weierich, Treat, & Hollingworth, 2008; for an overview see Duchowski, 2007). In eye-tracking studies, among the most commonly used observations is the mean fixation duration, or dwell time, that has been used as a reliable index of individual differences in visual processing control (Colombo, Mitchell, Coldren & Freeseman, 1991). Compared with other behavioural motor-dependent tasks, that measure reaction time to detect probes (i.e., in the dot-probe task) or tasks that relate to colour-naming threat words (i.e., Stroop task), eye-tracking has lower processing constraints (for a review see Puliafico & Kendall, 2006; also see Shechner *et al.,* 2013) allowing the recording of fixations towards and away a stimuli in ms (for a review see Bradley, Mogg, & Millar, 2000). As a result, maturational factors that may affect reaction time are likely to be reduced or controlled. Therefore, the employment of eye-tracking techniques to derive time-course of orienting biases and visual scanning pathways in youths may be a valuable method for the identification of early precursors that may relate to the manifestation of affective problems.

At this stage, it is worth underlining that eye-tracking studies have examined additional patterns of eye movements over time, such as the mean proportion of fixations, number of fixations and average fixation duration(e.g., Gamble & Rapee, 2010; Garner *et al.,* 2006), that can provide valuable information on the visual patterns of processing emotional information. In a similar vein, given the evidence that suggests that critical visual scanning information is included in segments of a second or longer (3-4 fixations in each second; Rayner, 1998), studies would be important to incorporate the analyses of multiple indexes on their visual scanning investigations [9].

4.2.1.1. Theoretical concepts of visual scanning

Based on the negative selectivity hypothesis, it is suggested that anxious individuals exhibit a preferential orientation towards threatening stimuli (for a review see Ruiz-Caballero & Bermudez, 1997; Bradley *et al.,* 2000). More specifically, behavioural (e.g., Vasey, Daleiden, Williams & Brown, 1995; Vasey, El-Hag & Daleiden, 1996; Watts & Weems, 2006) and eye-tracking studies (Reid, Salmon & Lovibond, 2006) of anxious youth have provided support

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[9] For the needs of the present study, and in line with the procedures of other eye-tracking studies with young populations (e.g., de Wit *et al.,* 2008; Farzin, Rivera & Hessi, 2009), and especially to keep consistency with the study that employed similar paradigm on the laboratory where the present study was conducted (Crawford *et al.,* 2015), only overall time spent looking at emotional faces as well as on the additional regions of interest (RoIs) from the total dwell time have been calculated and reported.

for the negative selectivity hypothesis, with a visual preference towards threatening stimuli to be evident for the affected populations, whereas other behavioural observations have reported a pattern of avoidance of threat in anxious youth (e.g., Mogg *et al.,* 1997; Monk *et al.,* 2006; Stirling, Eley & Clark, 2006).

More recent accounts have suggested dual-stage processing of emotional stimuli, where anxious individuals are quicker in shifting their visual scanning orientation towards negatively valenced stimuli during early stages of processing (e.g. 0-500 ms) compared to controls, but in the later stages of processing (e.g. 1000-1500 ms) exhibit an avoidance pattern (Koster, De Raedt, Goeleven, Franck & Crombez, 2005). This model, known as the vigilance-avoidance model, is believed to represent the manifestation of automatic visual orienting to threat-related information, which is later followed by strategic avoidance of the affective stimuli, in an effort to suppress the negative arousal resulted from exposure to the negative stimuli (Mogg, Philippot & Bradley, 2004; for a recent review see Armstrong & Olatunji, 2012). A range of eye-tracking studies, have confirmed a vigilance-avoidance pattern of visual processing in adults with anxiety (Bar-Haim *et al.,* 2007; Calvo & Avero, 2005) or anxiety traits (Hermans, Vansteenwegen & Eelen,1999; Rohner, 2002), where other behavioural detection studies have failed to confirm such an association in anxious individuals (e.g., Bradley *et al.,* 2000; Bradley, Falla & Hamilton*.,*1998).

Furthermore, eye-tracking studies in youth have been largely inconsistent to date. More specifically, in an eye-tracking study with younger children with social phobia between 5-12 years of age, reported that vigilance versus avoidance pattern of looking angry faces was dependent on the degree of anxiety rates (Waters, Mogg, Bradley, & Pine, 2011). Moreover, other studies have reported avoidance of threat in anxious children, independently of their behavioural rates (Monk *et al.,* 2006; Stirling *et al.,* 2006). Moreover, In-Albon *et al.* (2010) have reported that children with separation anxiety disorder, compared to controls, looked more at threatening/separation scenes after 1000ms of presentation, but looked away after 3000ms of presentation. It has previously been suggested that methodological variations among different studies, such as samples with comorbid conditions, maturational procedures, and duration of stimuli presentation are potential explanations for the inconsistencies in the field (In-Albon *et al.,* 2010, Puliafico & Kendall, 2006; Waters *et al.,* 2008).

Interestingly, due to the importance of specific emotions on the facilitation of urgent responses in human behaviour, a line of research has been developed to explain the human behaviour in response to facial expressions of anger.

4.2.2. The anger-superiority hypothesis

Due to the importance of the human face as an explicit signal to aggression and, subsequently, in detection of immediate social threat, the use of facial expressions of anger has become established in the field as a reliable index of early fear-related social affectivity. The corresponding 'anger superiority hypothesis' has thus emerged to highlight a documented pattern of preferential processing of angry faces versus facial expressions of other emotions (Hansen &Hansen, 1988; Holmes *et al.,* 2009; Öhman, Juth & Lundqvist, 2010). Interestingly, the majority of the studies that have examined visual scanning of angry versus other emotional faces (i.e., sad) have agreed on the superiority in quicker speed of detection of angry faces compared to happy and neutral facial expressions (Fox & Damjanovic, 2006; Gilboa-Schechtman, Foa & Amir, 1999; Horstmann & Bauland, 2006; Lipp, Price & Tellegen, 2009; Öhman *et al.,* 2010; Pinkham, Griffin, Baron, Sasson & Gur, 2010; Susa, Pitica, Benga & Miclea, 2012). However, a smaller proportion of studies with adults have not confirmed systematic differences in the detection of angry compared to happy faces (e.g., Williams *et al.,* 2005), or have reported inverse effects, suggesting the superiority of detection of happy faces (Calvo & Nummenmaa, 2008; Juth, Lundqvist, Karlsson & Öhman, 2005; Öhman *et al.,* 2010). Furthermore, it has been shown previously that individual differences in attention-related biases towards angry faces may contribute significantly to the maintenance of affective disorders.

4.2.2.1. Processing of facial expressions of aggression in affected populations

Among eye-tracking observations there is evidence to show that adults with social anxiety exhibit a pattern of vigilance-avoidance when scanning emotional faces, independently of the valence, compared to matched controls (Garner, Mogg & Bradley, 2006). In studies with anxious youth, behavioural studies exploring the time course of processing have shown that affected young individuals exhibited an increased looking preference towards angry facial expression during early stages of the stimulus presentation compared to non-anxious youth (for a review see Shechner *et al.,* 2013). In addition, an eye-tracking study with young children and adolescents reported a vigilance-avoidance pattern of visual scanning of negative emotions independently of their anxiety symptomatology when processed emotional/neutral face pairs for 3s (Gamble & Rapee, 2009). Moreover, increased symptoms of social phobia have been found to be associated with misidentification of facial expressions of anger in young children (Battagglia *et al.,* 2004). In sum, this evidence highlights that early in life neuropsychological patterns of affectivity may exist, that can be indexed my accessing the visual scanning patterns of individuals in response to environmental stressors.

In addition to the visual scanning patterns of looking emotional faces, there is another line of research that has focused on the individual differences of looking facial features that are critical for the establishment of effective social interaction. More specifically, this literature investigates atypicalities on the visual scanning of features, especially the eyes and mouth region that is believed to represent a reliable index of social-related affectivity and withdraw-related tendencies.

4.2.2.2. Atypical Gaze towards Eyes and Mouth

Attending to the eyes region of faces has been highlighted as a critical component of successful facial identification (Gold, Tadin, Cook & Blake, 2008), as well as for the detection and classification of another individual's facial emotions and intentions (Baron-Cohen, Wheelwright & Jolliffe, 1997). Healthy individuals have been observed to first fixate on the eyes, and to subsequently spend relatively more time looking at the eye region compared with the mouth region of the face (for a review see Itier & Batty, 2009). Interestingly, avoidance of looking the eye region of angry faces has been reported by eye-tracking studies with adults with social anxiety (Horley, Williams, Gonsalvez & Gordon, 2004; for a review see Crozier & Alden, 2005). However, other studies, specifically examining socially anxious women, have reported the inverse results (Wieser, Pauli, Alpers & Muhlberger, 2009).

Interestingly, studies employing simultaneous fMRI and eye-tracking methods, reported that amygdala hyper-responsiveness was associated with gaze orientation toward the eye region when processing fearful faces (Gamer, Zurowskia & Büchela, 2010; Gamer & Buchel, 2009). Moreover, a line of eye-tracking research with children with autism has found that compared to typical controls, children diagnosed with autism spent more time looking at the mouth region than the eyes during the scanning of negative only (e.g., de Wit, Falck-Ytter & von Hofsten, 2008). Emerging research has also highlighted atypical looking to the eye region of faces in individuals with Fragile X syndrome, a genetically defined neurodevelopmental disorder associated with social and communication impairments, and social anxiety (Crawford, Moss, Anderson, Oliver & McCleery, 2015; see also Farzin e*t al.,* 2009; Dalton *et al,* 2008; Holsen *et al.,* 2008). Together, this evidence suggest a link between negative affectivity and looking the eyes versus mouth region of human faces, although, todate, has not yet been established whether these behavioural manifestations may represent disorder-specific phenotypes or not.

Beyond the behavioural personality characteristics that may relate to emotion regulation abilities, there is a line of research that has been investigating the neurobiological underpinning of emotion regulation and affectivity. Based on this line of research, normal variations in candidate genes that relate to effective emotion regulation may explain the individual differences observed in reactivity in response to environmental stressors, especially those with social significance. In the following section, the function of two genetic polymorphisms in relation emotion reactivity in response to facial emotions is discussed.

4.2.3. Genetics of Emotion Face Processing

4.2.3.1. Brain-derived Neurotropic Factor and Emotional Processing

BDNF is a secreted protein present in the human brain that has been reported to mediate affective responses to emotional stimuli. BDNF is part of the neurotrophin growth factor family and has been observed to be involved in the regulation of survival and differentiation of neurons, as well as synaptic plasticity (Lu, 2003). In both developing and mature brains, BDNF is expressed at high levels in the Prefrontal Cortex and the hippocampus (Lu & Gottschalk, 2000; Pezawas *et al.,* 2004), and acts as an important factor for the development and plasticity of the central nervous system (Chao, 2003; Huang & Reichardt, 2001; for a review see also Murray & Holmes, 2011). More specifically, BDNF has a range of diverse actions with evidence to illustrate its influence on axonal and dendritic remodeling (e.g., Yacoubian & Lo, 2000), synaptic efficacy (e.g., Kafitz *et al.,* 1999) and synaptogenesis (Alsina *et al.,* 2001). Moreover, BDNF has been shown to have vital involvement in learning and memory (e.g., Broad *et al.,* 2002).

Most notably, the BDNF Single Nucleotide Polymorphism Val^{66}Met results in a change from Guanine (G) to Adenine (A) at nucleotide position 196 in the protein coding sequence of the gene, as well as subsequent change in amino acid from valine to methionine at position 66 (rs6265). This leads to decreased availability of BDNF in the brain due to decreased secretion of the variant form of BDNF (Egan *et al.,* 2003). In addition, there are various reports that suggest that Val^{66}Met is involved in shaping the developmental trajectories of particular brain structures, including the hippocampus, amygdala, and anterior cingulate cortex, which have each been implicated in emotional regulation and affective processing (e.g., Joffe *et al.,* 2009; Lang *et al.,* 2007; Van Wingen *et al.,* 2010). In consistent, structural and functional magnetic resonance imaging (MRI) studies, have shown that BDNF Val^{66}Met Met allele was linked to smaller hippocampal volumes when compared to subjects homozygous for the Val allele (e.g., Pezawas *et al.,* 2004), as well as with differences to hippocampal activation (e.g., Egan *et al.,* 2003). Similarly, in a study with adults Val/Val individuals were observed to preferentially seek positive emotions (e.g. happy faces) and have stronger regional fMRI activation over the orbitofrontal cortex, amygdala, and hippocampus regions in response to aversive stimuli (Gasic *et al.,* 2009).

Moreover, there has been increasing scientific consensus in recent years to support the involvement of BDNF Val^{66}Met variants in modulating behaviour, including stress reactivity and depressive symptomatology. For example, the presence of the low activity Met allele of the Val^{66}Met SNP has been examined for associations with affective psychopathology (Egan *et al.,* 2003; Gatt *et al.,* 2009; Xie *et al.,* 2012), whereas individual homozygous for the Val allele exhibit behaviours that have been associated with protection-related mechanisms against forms of psychopathology (e.g., Zhang *et al.,* 2014). Similarly, a recent study employing a spatial cueing task observed that Met allele carriers had greater difficulty in turning attention away when viewing positive cueing words, as recorded by the speed of key pressing, compared with Val allele homozygotes (Gong *et al.,* 2013). This finding was interpreted as a practice to disengage from negative stimuli in Met allele carriers, as a way to reduce the arousal that the negative stimuli induced to the individual.

Studies of children have produced results that suggest that the BDNF Met allele may act as a vulnerability factor for affective disorders (e.g., Beevers, Wells & McGeary, 2009), especially in combination with early life stressors (Gatt *et al.,* 2009). In a recent study a gene–environment interaction was observed, whereby children who teamed up with aggressive peers in childhood showed significantly increased vulnerability for becoming aggressive during adolescence if they were carriers of the Met allele, compared with Val homozygotes (Kretschmer, Vitaro & Barker, 2014). Similarly, BDNF Met allele carriers with a history of childhood stressful life events have been found to reduce grey matter volume (Gerritsen *et al.,* 2011; Scharinger, Rabl, Sitte & Pezawas,2010) and to show greater neural responses during emotion-processing tasks, when compared with Val homozygotes (Montag *et al.,* 2008; Schofield *et al.,* 2009; Lau *et al.,* 2010).

Despite these results, however, there are some controversies in the literature regarding BDNF Val^{66}Met still remain (for a recent review see Groves, 2007). Most notably, there is evidence to suggest the existence of differential susceptibility in carriers of the low neuroplasticity Met allele, where institutionalised children carrying at least one copy of the low uptake BDNF Met allele and two copies of the low serotonin uptake 5-HTTLPR Short allele exhibited most indiscriminate behaviour when placed in the usual caring environment but the least indiscriminative in enhanced caring environment (e.g., Drury *et al.,* 2012). To this end, depending on the environmental influence, some individuals may be affected disproportionately to both positive and negative life experiences, which may result greater responsivity to adversity but also to positive environmental conditions (e.g., Ellis, *et al.,* 2011; see also Section 1.4.1.1).

4.2.3.2. Serotonin Transporter and emotional processing

In addition to the role of neuroplasticity in affective response to emotional faces, associations between common genetic variation in the serotonin transporter gene (5-HTT) and individual differences in visual scanning of emotional faces also exist (e.g. Battaglia *et al.,* 2005; Lau *et al.,* 2009). 5-HTT has been documented to be involved in emotion regulation abilities, by influencing the availability and signalling of serotonin over the pre- and post-synaptic receptors that are mainly located in neurons in affective corticolimbic circuitry (for a review see Hariri & Holmes, 2006; see also Section 3.2.1.1). This polymorphism is represented by two variants: a short (S) allele; and a long (L) allele, with the Short allele associated with significant decreases in serotonin reuptake (Lesch *et al.,* 1996). In combination with the exposure to life-threatening situations, individuals carrying at least one copy of the Short allele have been reported to be at increased vulnerability for negative cognitive, behavioural, and neurophysiological outcomes (Caspi *et al.,* 2003; Disner *et al.,* 2013; Mercer *et al.,* 2012; Xie *et al.,* 2009).

A recent meta-analysis has also shown a strong association between the Short allele and increased amygdala reactivity in response to angry or fearful facial expressions, suggesting a reliable influence of the polymorphic region on corticolimbic circuitry and subsequently in human emotion regulation behaviour (Munafo, Brown, & Hariri, 2008; Carver, Johnson, & Joormann, 2009). Interestingly, a recent eye-tracking study was shown that the low serotonin uptake 5-HTTLPR genotype exhibited greater accuracy of classifying emotional faces (Boll & Gamer, 2014). From a developmental perspective, children as young as 9 years of age carrying the Short 5-HTTLPR allele have been found to exhibit greater neural activation in response to fearful and angry faces than children homozygous for the Long allele, in various brain regions previously linked to attentional control in adults (Thomason *et al.,* 2010). In line with this neurophysiological evidence, a range of behavioural studies in both children and adults have measured behavioural reaction times, and reported that the presence of two copies of the high activity Long 5-HTTLPR allele is associated with positive affectivity (shorter reaction times) toward happy facial stimuli compared with neutral facial stimuli, whereas carriers of the Short allele with elevated reactivity (for a review see Homberg & Lesch, 2010). Although, these studies are consistent with the notion that the 5-HTTLPR Short allele is associated with high reactivity in response to negative stimuli, there is currently limited information regarding the particular characteristics and nature of the critical visual behaviours associated with the processing of these types of stimuli in young populations.

However, there are studies that highlight that the serotonin-transporter 5-HTTLPR polymorphism does not only associated with increased vulnerability to contextual risk but, under positive circumstances, may relate to disproportionate positive response, that may provide a plasticity-related function to the Short allele (Belsky *et al.,* 2009; Belsky & Pluess, 2009; Homberg & Lesch, 2011; see also Section 3.2.1.1). In line with this concept, there is evidence to support that carriers of the Short 5-HTTLPR allele have difficulty disengaging from both negative and positive emotional stimuli (Beevers, Wells, Ellis & McGeary, 2009; Beevers, Ellis, Wells & McGeary, 2010; Perez-edgar *et al.,* 2010) which has been previously conceptualised as a behavioural hypervigilance pattern in this genotype group, in response to environmental stimuli (for a review see also Homberg & Lesch, 2011). Moreover, in experimental studies, it has been also reported that the Short 5-HTTLPR allele presented strong biases towards positive and negative emotional stimuli (Fox *et al.,* 2011; Beevers *et al.,* 2009, 2010). To this end, increased sensitivity to negative stimuli to this genotype group may be linked to increased vulnerability for affective problems, whereas sensitivity for positive stimuli may potentially ameliorate risk (Belsky *et al.* 2005; see also Section 3.2.1).

4.3. The current study

The current study utilized eye-tracking technology in order to examine the potential role of common genetic variation in candidate genes for influencing the processing of faces in children aged 4 and 7 years. To this end, face stimuli expressing different emotions (NimStim; Tottenham *et al.,* 2009) were presented, including aggressive, happy, and neutral facial expressions. Eye gaze indices of automatic visual orientation in response to different emotional expressions, were examined alongside normal variations in genes associated with neural/environmental plasticity (i.e. BDNF Val^{158}Met) and those involved in social-emotional regulation (i.e. 5-HTTLPR). In addition, parent report measures of the children's rates of early behavioural problems were employed in an effort to examine how early rates of affective problems (i.e., elevated rates of internalizing/externalizing problems; empathic abilities) may contribute to these affect-related behaviour patterns. Through these methods, the current study aimed to provide novel insights into how genetically-mediated neural plasticity and socio-emotional genetic mechanisms might modulate emotional reactivity in response to different emotional cues.

4.3.1. Aim 1: To examine the behavioural associations of socio-emotional abilities with the processing of emotional faces

The present study aims to investigate the associations between the development of early affective traits and the experience of positive or negative affectivity in young children. The study is employing eye-tracking technologies, along with ecologically valid and reliable parent report measures to assess children' s early rates of affective problems. Throughout this investigation, is also investigated the time course of visual scanning of emotional faces by analysing data from eye gazes towards and away happy and angry faces at different time points.

4.3.2. Aim 2: To investigate genetic influences on fixation patterns in response to emotional faces

There has been increasing scientific consensus in recent years that suggests an association between affective responses to emotional faces and the neurobiological underpinning of variations in these behaviours, as a reliable endophenotype for current or later maladaptive behaviour. However, to date, there has been very limited evidence on the genetic influences on these behaviours in children. The present study aims to further delineate the role of normal genetic variations on the neuroplasticity-related BDNF Val^{66}Met SNP in modulating the fixation duration in response to emotional faces during early childhood. Furthermore, the role of the serotonin uptake 5-HTTLPR genotype on the visual scanning of emotional faces is also investigated.

4.3.3. Aim 3: To investigate genetic influences on fixation patterns in response to facial features

A supplementary aim of the study involves the investigation of the putative link between variations on the BDNF Val^{66}Met and 5-HTTLPR genotype and eye gazes towards the eye and mouth regions of neutral faces and their corresponding associations with the early manifestation of social deficits and negative affectivity in general. Eye gaze in eye and mouth regions were calculated from relative fixation duration of looking the neutral faces. In addition, due to the fact that seventy out of eighty experimental trials were baseline/habituation neutral face pairs, the use of these trials for these analyses were chosen as the most representative of the under measure eye gaze behaviour.

4.3.4. Hypotheses

Three main hypotheses were tested as part of this study. Taking into account previous evidence to suggest vigilance-avoidance patterns of visual scanning of emotional information in young children with social phobia (In-Albon *et al.,* 2010) it was hypothesised that behavioural measures of elevated rates of early behavioural problems, and more specifically anxiety traits, would be significantly correlated with vigilance-avoidance patterns of visual scanning of angry, but not happy facial expressions.

Moreover, considering the evidence suggesting a moderating role of the BDNF Val^{66}Met for emotional reactivity (Montag *et al.,* 2008; Schofield *et al.,* 2009; Lau *et al.,* 2010), an additional hypothesis of the study was related to this genotype's effect in modulating visual scanning of emotional faces. More specifically, it was hypothesised that carriers of the low neuroplasticity Met BDNF allele, when compared to children carrying two copies of the high activity Val allele, would exhibit vigilance-avoidance patterns in the time spent looking toward the facial expressions of anger. Furthermore, taking into account evidence suggesting modulation of reactivity in response to facial emotions by 5-HTTLPR genotype (Thomason *et al.,* 2010), it was hypothesized that carriers of at least one low serotonin uptake 5-HTTLPR Short allele would similarly display vigilance–avoidance pattern in response to angry facial expression.

For the third hypothesis of the study was taken into account recent evidence that suggested the existence of avoidance-related patterns of attention towards the eyes in atypically developing populations (Crawford, *et al.,* 2015; see also Farzin e*t al.,* 2009; Dalton *et al,* 2008; Holsen *et al.,* 2008), and the evidence that suggest the existence of high reactivity in carriers of the Short 5-HTTLPR (Thomason *et al.,* 2010) and Met- BDNF allele (e.g. Montag *et al.,* 2008; Schofield *et al.,* 2009; Lau *et al.,* 2010). Therefore, the third hypothesis was that carriers of at least one 5-HTTLPR Short allele would spent significantly less time looking at the eyes versus the mouth region of neutral face pairs, compared with the high serotonin uptake Long/Long genotype. Finally, was tested the hypothesis that carriers of at least one low plasticity-related BDNF Met allele would similarly spent less time looking at the eyes versus the mouth of neutral face pairs, compared with individuals homozygous for the Val allele.

As reviewed earlier, the stimuli durations have been previously considered as a potential factor accounting for these patterns of visual scanning (In-Albon *et al.,* 2010, Puliafico & Kendall, 2006; Waters *et al.,* 2008). To this end, and taken the absence of previous evidence from child studies directly examining these areas of inquiry, the direction of the hypotheses of the study was determined based on the previous studies that employed similar experimental design and conducted investigations with young children at the same age (e.g., Crawford, *et al.,* 2015; Farzin e*t al.,* 2009).

4.4. Methods and Materials

4.4.1. Participants

Forty-nine children from Caucasian ancestry participated in the study (24 males; 25 females; Mean age in months = 70.8, SD = 11.5, age range 4-7 years of age). Power analysis suggested that the sample size required to achieve a power of $1-\beta = 0.90$ for the ANOVA test at significance level $\alpha = 0.050$ requires at least 33 participants. All the children participated in the present study have been invited from the pool of 70 children that have participated in the study presented in the study presented in Chapter 3. This was done due to the study's hypotheses, but also due to the prior availability of the genotype information for this sample. Parents or guardians of all participants reported that the child had no history of a neurological or psychiatric disorder and that they had normal or corrected to normal vision. Exclusion criterion included if participants scored below a certain range (IQ < 75) on the British Ability Scales II, Early years (BAS-II; Elliot, Smith, & McCulloch, 1996), a standardised assessment of intelligence/developmental age and abilities. No participants met this exclusion criterion (see Table 4.2). All participants had English as their first language. Informed written consent was obtained from the parents/guardians of all participants before participating in the study. In addition, children aged 7 provided written assent to participate in the study. Families who expressed interest in the study were scheduled to attend a laboratory intake appointment. Families were provided with compensation of £10 towards their travel expenses. Ethical consent was gained from the University of Birmingham Ethical Committee.

4.4.2. Data collection procedures

Children were told that they are going to see a range of interesting photos on a computer screen, while a special camera recorded their eye movements. The eye-tracking and parent report assessments took place during one laboratory visit.

4.4.2.1. Behavioural Measures

For the assessment of children's cognitive ability the BAS-II, Early Years was employed (see also Section 2.4.2.1). Taken that the children who consisted the sample have undergone a BAS-II assessment as part of the study presented on Chapter 3 which took place in a period between 6-8 months before the occurrence of the present study, the data from the initial BAS-II assessment and the corresponding age-equivalent abilities are reported here. Although there is no straight forward practice in the literature about a safe minimum between assessments, that may vary based on individual circumstance and each child's abilities, the decision not to repeat the assessment within this time window in the present study, is in line with the BAS-II manual's recommendations, suggesting a gap of more than 6 months gap between repeat assessments (Elliot *et al.,* 1996). This is also congruent with recent longitudinal evidence suggesting high stability of IQ measured at the age of 4 within shorter interval during early childhood (i.e., up to the age of 7; Schneider, Niklas & Schmiedeler, 2014)

4.4.2.2. Measures of behavioural problems

For the assessment of children's rates of behavioural problems the CBCL scales were used (Achenbach & Rescorla, 2001). Both the Early Years (for children between 1 ½ - 5 years of age) and School Age (children and adolescents aged 6–18 years) versions were used here (see Sections 2.4.2.2 and 3.4.2.2). For the assessment of autism symptomatology rates the Social Communication Questionnaire-Lifetime Edition was completed by parents (SCQ; Rutter *et* *al.,* 2003; see Section 2.4.2.1 for details on the measure).

4.4.2.3. Eye-tracking assessment

Stimuli

A total of 80 trials of coloured happy-neutral, angry-neutral and neutral-neutral face pairs constructed the experiment. All the face stimuli were selected from the MacBrain Face Stimulus Set[1] (Tottenham *et al.,* 2009) and were matched in terms of gender, race and age. Available validity data for the MacBrain Face Stimulus Set in both children and adults have been reported high inter-rater agreement for the emotion that is displayed in these facial expressions (Tottenham *et al.,* 2009). Pairs of faces were presented simultaneously side-by-side, with emotional faces presented equally on the right and the left side of the screen. In order to determine whether increased or reduced fixation duration towards the emotional faces (critical trials) resulted from heightened orientation, difficulty in disengaging from emotional stimuli, or both, the experiment was constructed using baseline neutral-neutral face pair trials (baseline; N = 70), and critical trials of emotional-neutral face trails (i.e. 5 Angry-Neutral Pairs; 5 Happy-Neutral Pairs; N = 10).

The experiment started with seven baseline trials (pairs of neutral-neutral faces), where at least four baseline trials were presented between the critical trials (pairs of emotional-neutral faces). Baseline and critical trials were pseudorandomly allocated across the experiment in line with previous behavioural studies (e.g., Arndt & Fujiwara, 2012; Crawford *et al.,* in press; Mogg *et al.,* 2004; Salemink, van den Hout & Kindt, 2007). The eye-tracking experiment was programmed using Experiment Builder software for EyeLink (SR research, Ontario, Canada). The facial stimuli consisted of 38 colour photographs of male and female faces[10] (1024 × 768 pixels) depicting one of three expressions (neutral, happy, and angry). Although some of the neutral face pairs were repeated across the experiment, the neutral face stimuli used during the critical trials were not used elsewhere during the experiment. Therefore, face familiarity did not affect face preferences during critical trials

Each trial began with a fixation point (in the shape of an animated dolphin), measuring 2.81 × 2.08 degrees of visual angle in the middle of the screen which was displayed for 1000 ms (except in the case of mini calibration; see Procedure section). This was followed by a pair of faces presented side by side in a white background for 2500 ms. The inter trial interval was 1000 ms (see Figure 1). The gap between the two faces was 7.2 degrees of visual angle. Each stimuli pair was presented with a visual angle of 14.3 × 18.6 degrees.

Procedure

Participants' eye movements were recorded using an Eyelink 1000 Tower Mount eye-tracking system and the stimuli were presented on a 19-inch CRT with a resolution of 1024 × 768 pixels. The eye-tracker sampled eye position at 500 Hz (i.e. every 2 ms). Average spatial accuracy is between 0.25° and 0.5° of visual angle. Participants were seated in a dimly lit room, 60 cm away from the display screen and they had their head positioned against a head rest and their chin placed on a chinrest to minimize the possibility of movements. Viewing was binocular, but only data from the right eye were collected.

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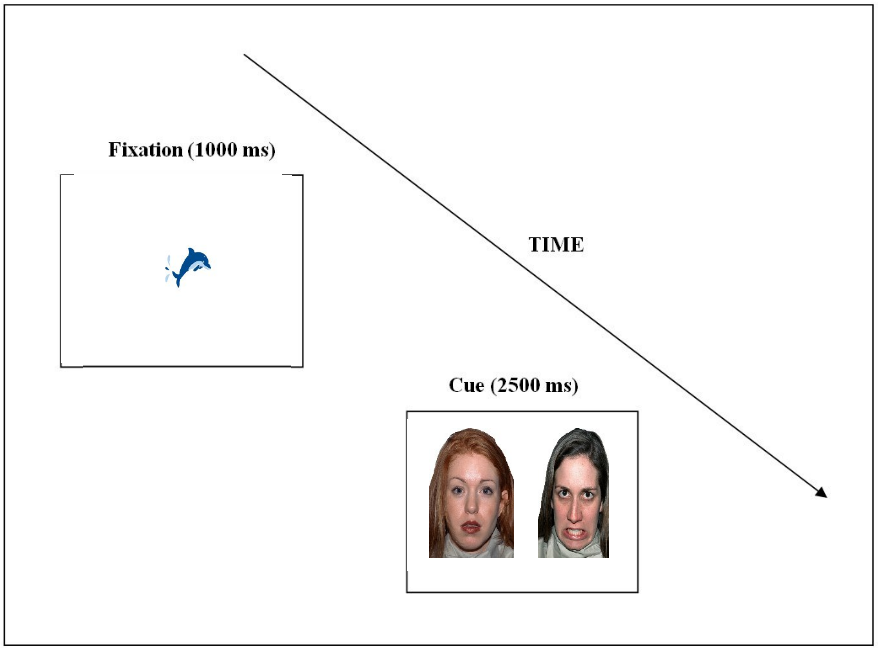
[10] The MacBrain Face stimuli that used here are as follow: *Angry Faces:* 01F, 05F, 06F, 20M, 22M; *Happy Faces:* 08F, 11F, 12F, 24M, 26M; *Neutral Faces:* 02F, 03F, 07F, 09F, 10F,13F, 14F, 15F, 16F, 17F, 18F, 19F, 21M, 23M, 25M, 28M, 29M, 30M, 31M, 32M, 33M, 34M, 35M, 36M, 37M, 38M.

Figure 4.1. An example of the face stimuli pairs used in the eye-tracking experiment and an illustration of a trial structure.



During calibration the EyeLink recorded the eye position at 5 target locations, providing the required reference data for computing gaze positions to ensure a point of fixation error rate of less than 0.5 degrees. A mini calibration was repeated every 5 trials in order to ensure that eye movement data were adjusted for small-scale movement of the head. In the case of unsatisfactory eye-tracking, a 5-point calibration was repeated.

4.5. Analysis

4.5.1. Analysis of Behavioural Data

The procedures for analysing the CBCL and BAS-II scores were the same as described on Sections 2.5.1 and 3.5.1.

4.5.2. Reduction of Eye-tracking data

Fixations were calculated using the EyeLink online detection analysis algorithm when eye movement met the following four criteria: a) velocity threshold of 30 °/sec, b) a motion threshold of .1°, c), a 8000 $°/sec^2$ acceleration threshold, d) and the pupil was not missing consecutively for three or more times from a sample [11]. Trials were classified as 'invalid' if a child did not look at all at the faces during the trial. In addition, if more than 40% invalid trials were evident the participant's data were excluded from further analyses. No participant met this exclusion criterion; therefore, all 49 participants provided valid eye-tracking data.

For analyses, each 2500 ms trial was divided into five 500 ms intervals. The relative mean proportions of viewing time for the angry and happy faces were then calculated for each 500 ms time interval of watching during the critical trials. This was done by subtracting the overall dwell time of the neutral stimuli (for each critical trial) from the overall dwell time

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[11] The EyeLink 1000 parser is available from SR Research and is designed for on-line, accurate identification of saccades and blinks. The parser computes velocity and acceleration of the eye data and these are compared to the prespecified thresholds that ensure accuracy of the observations. More specifically, the velocity threshold is the velocity that an eye-movement needs to exceed in order for a saccade to be accurately detected, that is especially useful for the detection of small saccades. Acceleration data is noisier than velocity data, and thresholds of and $8000°/sec^2$ for cognitive research is recommended. Finally, the saccadic motion threshold is used to delay the onset of a saccade until the eye has moved significantly, with a threshold of 0.1° to 0.2° to be suggested to be sufficient for shortening saccades (SR Research).

looking at the emotional (happy or angry) face. In addition, this was done separately for each subject and for each happy and angry critical trial. Average dwell time of looking for each emotion type (i.e., angry, happy) was later calculated for each subject. Two additional regions of interest (RoIs) for the eyes and mouth region were identified. For this analysis, the neutral only/baseline pairs were used, where the coordinates of gaze for each eye as well as the mouth region were identified and extracted using the EyeLink Data viewer software. The overall amount of time spent (in ms) looking at the eye and mouth regions was divided by the amount of time spent (in ms) looking at the whole neutral face. This was done separately for each neutral baseline trial (for the overall 2500 ms), and then averaged across the baseline trials for each participant.

For both of these analyses, after the subtraction positive values represented a visual preference for the emotionally expressive face (versus neutral) or facial feature and negative values represented visual patterns that relate to avoidance behaviour for the emotionally expressive face (versus neutral) or facial feature.

4.5.3. Analysis of Genetic Material

4.5.3.1. BDNF Genotyping

Direct bidirectional sequencing was used to genotype the single nucleotide polymorphism within the BDNF gene (rs6265). PCR primers were designed to flank the polymorphism producing a 249bp amplification product. Sequences of the primers are as follows: forward AAACATCCGAGGACAAGGTG and reverse AGAAGAGGAGGCTCCAAAGG. PCR was performed using Megamix PCR solution (supplied by Microzone UK Ltd) in a total volume of 25ul containing 25pmol of each primer. An initial denaturation step at 95ºC for 5 minutes was followed by 30 cycles of PCR (95ºC 1 minute, 58ºC 1 minute, 72ºC 1 minute) and then a final extension at 72ºC for 10 minutes. PCR products were purified and sequenced as described for the 5-HTTLPR genotyping (see section 3.5.3.1).

Allele frequencies for the BDNF Val^{66}Met was n = 24 (25.5 %) for Mel alleles and n = 74 (75.5 %) for Met alleles respectively. To this end, three genotype groups resulted; one with Met allele homozygotes (i.e. M/M; N = 3), heterozygotes V/M (N = 18), as well as homozygotes for the Val allele (i.e. V/V; N = 28). BDNF Val^{66}Met genotype frequencies where in Hardy-Weinberg equilibrium [x^2 (1) = .002, *p* = .962] as calculated with a reliable online tool that can be found here: http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%20calculator.xls. However, taken the small sample of participants homozygous for the low activity Met allele (N = 3) and the previous evidence associating the presence of at least one Met activity with behavioural outcomes (e.g., Wichers *et al.,* 2008) here carriers of at least one Met allele [i.e. Heterozygotes (Met/Val), and Homozygotes for the Met allele (Met/Met), were grouped in one 'Met allele carriers' group (i.e. M/-). Additional classifications with three genotype groups where also employed (i.e. V/V versus V/M versus M/M).

4.5.3.2. 5-HTTLPR Genotyping

The procedures for the 5-HTTLPR genotype preparation and DNA extraction are identical as presented on section 3.5.3.1). Allele frequencies across participants for 5-HTTLPR was n = 42 (42.8 %) for Short allele and n = 56 (57.2%) for Long Allele. To this end three genotype groups where resulted, one with Short allele homozygotes (i.e. S/S; N = 10), heterozygotes L/S (N = 22), as well as homozygotes for the Long allele (i.e. L/L; N = 17). 5-HTTLPR genotype frequencies where in Hardy-Weinberg equilibrium [x^2 (1) = .340, *p* = .559] as calculated with a reliable online tool that can be found here: http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%20calculator.xls. Similar to the BDNF Val^{66}Met genotype, carriers of at least one Short allele [i.e. Heterozygotes (S/L), and Homozygotes for the Short allele (S/S), were grouped in one 'Short allele carriers' group (i.e. S/-; N = 32) and compared with the remaining homozygous participants for the high serotonin uptake Long allele (L/L; N = 17). Additional classifications with three genotype groups where also employed (i.e. L/L versus L/S versus S/S).

4.5.4. Statistical Analysis

Preliminary Analyses

Descriptive statistics were conducted in order to describe the sample's demographic characteristics such as, gender, age, and distribution of cognitive abilities. Raw data from the behavioural and cognitive scales were examined for normality using Kolmogorov–Smirnov tests. The CBCL subscales did not met criteria for normal distributions (Kolmogorov–Smirnov, *p* < .005). Therefore, to further examine possible correlations between age, gender, IQ, and scores on the behavioural measures, Spearman's Rho non-parametric correlations coefficients tests were performed. Moreover, Pearson correlation analyses were conducted to determine if a correlation among demographic characteristics or cognitive performance and genotype group was evident, and Spearman correlation analyses were conducted to investigate possible correlations between BDNF Val^{66}Met and 5-HTTLPR Genotypes and demographic, cognitive, and rates of affective problems in the sample.

Behavioural Ratings and Eye Gaze Patterns

The primary analysis examined whether children's behavioural scores were correlated with fixation duration towards particular emotional faces at each time point, and fixation duration towards facial features. For the cognitive abilities (BAS-II) measures, mean standardized IQ-scores were calculated. Furthermore, correlation analyses were conducted to investigate possible correlations between dwell time looking at the emotional faces and participants' demographic characteristics for each emotion and face feature separately. Furthermore, in the case of significant correlations between scanning pathways and behavioural rates, backward elimination regression analysis was utilized to assess the specificity of the behavioural rates to predict visual scanning pathways in beyond participants' age, gender and IQ.

Genetics and visual scanning of faces

To assess the looking preference towards and away from the emotional faces, the overall dwell time spent fixating on the emotional face minus the overall dwell time spent fixating on the accompanying neutral face was computed for 5 time intervals (dependent variables): 0-500 ms (T_1), 501-1000 ms (T_2), 1001-1500 ms (T_3), 1501-2000 ms (T_4), and 2001-2500 ms (T_5). A 2 (Emotion: positive vs. negative) × 5 (Time: 0-500 ms vs. 501-1000 ms vs. 1001-1500 ms vs. 1501-2000 ms vs. 2001-2500 ms) mixed ANOVA with Gender (female, male) and Genotype (BDNF V/V versus M/-) as between-groups variables was conducted. The same analysis was repeated separately with different BDNF genotype classification (i.e. M/M versus M/V versus V/V), as well as with the three 5-HTTLPR genotype (S/S versus S/L versus L/L) as between-groups factor as a control comparison. All within subjects effects that violated the assumption of sphericity (Mauchly's test of sphericity $p > 0.05$) were adjusted using the Greenhouse-Geisser correction. To further evaluate the time course of attention, independent samples t-tests were conducted to determine whether there was a looking preference towards or away from the emotional images of a specific genotype group at any of the 500 ms time intervals. This was done for each SNP (BDNF Val^{66}Met and 5-HTTLPR) and each facial expression (happy and angry), separately, after the initial ANOVA. When the data did not satisfy Kolmogorov-Smirnov tests for normality, Mann-Whitney U tests were performed instead of t-tests.

Furthermore, to investigate looking preference towards the eye and mouth regions, a separate two-way mixed ANOVA with the repeated factor RoI (i.e., eyes, mouth) and genotype group (i.e. S/- versus L/L) and gender as independent factor was conducted to examine gaze behaviour for each face region for the baseline trials only (neutral-neutral face pairs). Moreover, the same analysis was repeated with the three 5-HTTLPR genotype (S/S versus S/L versus L/L). Finally, as a control analysis the same analysis was repeated with two (i.e., M/- versus V/V) but also three BDNF genotype classification (i.e. M/M versus M/V versus V/V). After the omnibus ANOVA, and because eye gaze data were non normally distributed, a Mann-Whitney U test was conducted, to investigate the 5-HTTLPR genotype effects on the overall viewing time for the eyes and mouth region respectively. The statistical software package SPSS 20.0 was used for all the analyses.

4.6. Results

4.6.1. Demographic Characteristics

Tables 4.1 and 4.2 present the participants' main demographic characteristics, including gender, age, and cognitive ability. Correlation analyses did not reveal any significant correlation between demographic characteristics and behavioural measures, or correlations between demographics, rates of early behavioural problems and genotype. Moreover, t-tests showed that the two 5-HTTLPR genotype groups did not differ in terms of Age [$t(47) = -.037$, $p = .971$], Gender [$t(47) = .994$, $p = .325$], IQ [$t(47) = -1.17$, $p = .245$], developmental age [$t(47) = -.245$, $p = .808$], or other behavioural measures. Similarly, the two BDNF Val^{66}Met genotype groups did not differ in terms of Age [$t(47) = .000$, $p = 1.00$], Gender [$t(47) = .162$, $p = .872$], IQ [$t(47) = -.427$, $p = .671$], or developmental age [$t(47) = -.223$, $p = .824$].

Table 4.1. Sample size and demographic characteristics of sample.

N		**49**
Gender	% Male *(N)*	48.9 *(24)*
	% Female *(N)*	51.1 *(25)*
Handedness	% Right*(N)*	77.3*(39)*
	% Left*(N)*	22.7*(10)*
SCQ	Mean*(SD)*	3.63*(2.77)*
Total Score	Range	0-12
BAS-II	%Below Av.	3.8
Total Score	% Average	65.4
	%Above Av.	25.0
	% High	5.8

Moreover, task engagement was calculated by subtracting the relative looking time away from the areas of the stimuli from the time looking the face stimuli. This analysis shows that participants spent consistently more than 60% of the time looking the face stimuli [M(SD) = 0.63 (0.24)] and a Mann-Whitney U test show that these rates did not differ between BDNF M/- and V/V genotypes (z = -1.37, *p* = .702) as well as when comparing three BDNF genotypes [Kruskal-Wallis test; $\chi^2(2) = 1.941$ *p* = .379]. In a similar vein no difference on the task engagement rate where evident between the two (z = -0.63, *p* = .950) or three 5-HTTLPR genotypes [$\chi^2(2) = 1.140$, *p* = .565].

Table 4.2. Participants' general and age-equivalent cognitive abilities.

Chronological Age*	Mean*(SD)* Range	70.8 *(11.5)* 55-91
Overall Ability**	Mean*(SD)* Range	106.8*(8.7)* 86-125
Verbal Ability	Mean*(SD)* Range	103.5*(13.9)* 58-127
Non-verbal Ability	Mean*(SD)* Range	110.8*(13.8)* 86-144
Developmental Age*	Mean*(SD)* Range	63.9*(13.1)* 42-88
Developmental Verbal Ability (Months)	Mean*(SD)* Range	64.9*(15.5)* 35-96
Developmental Non Verbal Ability (Months)	Mean*(SD)* Range	66.6*(15.5)* 35-96

*Age data presented in months
**Overall ability is calculated from the overall BAS-II total score and Verbal and Non-verbal ability form the BAS-II clusters of abilities. Values represent GCA.

Correlation analyses revealed a positive correlation between externalizing problems and rates of autism symptomatology (*r* = .339, *p* = .017) was revealed. No further relationships of participants' demographic characteristics in cognitive development or early affective problems were observed. Furthermore, Spearman's Rho correlation showed a significant positive correlation between rates of internalizing and externalizing problems (r = .524, p< .001). Moreover, Pearson correlation analyses revealed a positive correlation between rates of externalizing problems and children's age (r = -.362, p = .011).

4.6.2. Behavioural effects in Fixation Duration

Since the eye movement data varied in terms of normality across different time points of processing (i.e. Happy T_2, T_3, T_4 and Angry T_2, T_5 were $p > 0.05$; Happy T_1, T_5 and Angry T_1, T_3, T_4 were $p < 0.05$ in Kolmogorov-Smirnov test of normality), both parametric and non-parametric correlation analyses were conducted with CBCL rates and looking dwell time spent for each time point and each type of emotion separately. A negative correlation between age and time spent fixating angry faces at T_3 (r = -.408, p = .004), T_4 (r = -.338, p = .017) and T_5 (r = -.526, p < .004) was documented. No further significant correlation were evident. No further relationships of participants' demographic characteristics in cognitive development or rates of early affective problems were observed. Finally, parametric correlation analyses with behavioural rates and fixation duration towards the eye (normally distributed) and non-parametric for the mouth region (not normally distributed) did not revealed any significant correlation.

4.6.3. Genotype effects in Fixation Duration for Emotional Expressions

A 2 (Emotion: positive vs. negative) by 5 (Time: 0-500 ms versus 501-1000 ms versus 1001-1500 ms versus 1501-2000 ms versus 2001-2500 ms) mixed analysis of variance (ANOVA) with Gender (female, male) and Genotype (BDNF M/- versus V/V) as between-groups factors revealed a main effect of Emotion, [F (1, 45) = 7.10, $\eta_p^2 = .136$, $p = .011$], a main effect of Time [F(4, 180) = 46.89, $\eta_p^2 = .758$, $p < .001$], and a two-way Emotion by Time interaction [F(4, 180) = 13.07, $\eta_p^2 = .535$, $p < .001$]. In terms of genotype effects, a two-way Time by BDNF Genotype [F(4, 180) = 4.01, $\eta_p^2 = .082$, $p = .004$], as well as a three-way Emotion by Time by BDNF genotype interaction [F(4, 45) = 3.52, $\eta_p^2 = .073$, $p = .009$], were evident (see also Appendix 4.1. for scatter plots of dwell data). No further interaction effects were observed. Repetition of the same analysis when comparing three BDNF genotype groups (i.e., V/V versus M/V versus M/M) also revealed a significant Time by BDNF Genotype [F(4, 172) = 2.18, $\eta_p^2 = .092$, $p = .031$], as well as a three-way Emotion by Time by BDNF genotype interaction [F(8, 172) = 2.55, $\eta_p^2 = .106$, $p = .012$; see Appendix 4.2.]. The omnibus ANOVA was repeated with the 5-HTTLPR genotype (i.e. L/L versus S/-) as a between factor. Contrary to the BDNF genotype effects, this analysis did not revealed any significant Time by 5-HTTLPR Genotype [F(4, 180) = 2.55, $\eta_p^2 = .017$, $p = .537$], or a three-way Emotion by Time by 5-HTTLPR genotype interaction [F(4, 180) =.152, $\eta_p^2 = .003$, $p = .962$; see Table 4.3], or any other interaction effect. Similarly, when comparing three 5-HTTLPR genotype groups (L/L versus S/L versus S/S) no significant Time by 5-HTTLPR Genotype [F(8, 172) = .787, $\eta_p^2 = .035$, $p = .615$], or a three-way Emotion by Time by 5-HTTLPR genotype interaction [F(8, 172) =.471, $\eta_p^2 = .021$, $p = .876$] was evident (see Appendix 4.2). No further effects were detected from this analysis.

To further delineate the observed Time by BDNF genotype effect, the dwell time at each of the five time points was averaged across the two emotions. A Kolmogorov-Smirnov test of normality showed that the averaged data at each time point were normally distributed ($p > .005$) therefore t-tests were conducted at each time point of visual scanning averaged across the two emotions. This analysis revealed a significant difference between the two genotype groups (i.e. M/- versus V/V) on the time spent looking at emotional stimuli during T_4 [$t(47) = -.205$, $p < 0.05$)]. Moreover, to delineate the three-way Emotion by Time by BDNF interaction, follow up analyses were conducted to determine whether there was a preference towards or away each emotion at each of the time intervals. Due to the fact a Kolmogorov-Smirnov test revealed that the relative viewing time between stimuli in specific time points were normally distributed (e.g. Happy T_2, T_3, T_4 and Angry T_2, T_5 where $p > 0.05$, where Happy T_1, T_5 and Angry T_1, T_3, T_4 where $p < 0.05$ in Kolmogorov-Smirnov test of normality), this analysis was followed up with complementary parametric and non-parametric analyses at each Time Point separately.

For the time points with not-normally distributed data, a Mann Whitney-U test revealed a significant difference between the two BDNF genotypes in the dwell time towards the facial expressions of Anger at T_4 ($U = 157.00$, $p = .010$; see Figure 4.2 and Table 4.3). Similarly, a Kruskal-Wallis test with the three BDNF genotype groups (M/M versus M/V versus V/V) also revealed a significant difference between genotype groups on the dwell time spent fixating the angry face during 1501-2000 ms [$x^2(2) = 8.50$, $p = .028$)]. Moreover, for the normally distributed time points a t-test for T_5 was shown that the carriers of the low neuroplasticity Met allele spent significantly less time looking at the angry faces [$t(47) = -2.10$, $p = .041$], which was absent for the happy faces. In contrast, carriers of two copies of the

Val allele exhibited an increase in time looking to the angry faces. Conversely, a one-way ANOVA with three BDNF genotype groups did not revealed any significant difference on the time spent fixating angry faces during 2001-2500 ms [$F(2) = 2.20$, $p = .122$], suggesting that the presence of one low neuroplasticity Met allele moderate visual scanning of angry faces when contrasted to the high uptake Val/Val group (see Appendix 4.2).

Table 4.3. Relative dwell time in ms and standard deviations (in brackets) viewing angry and happy faces in different genotype groups, showing an aggression-specific vigilance-avoidance patterns of attention allocation in the Met/- genotype group.

Time Interval	**BDNF**		**5-HTTLPR**	
	M/- (N=21)	**V/V (N=28)**	**S/- (N=32)**	**L/L (N=17)**
Facial expressions of Anger				
T1	-8	-7	-3	-16
	(49)	*(49)*	*(45)*	*(55)*
T2	220	134	175	164
	(138)	*(168)*	*(156)*	*(173)*
T3	292	438	399	332
	(331)	*(233)*	*(252)*	*(343)*
T4	258	465	410	314
	(312)	*(237)*	*(259)*	*(336)*
T5	46	191	147	94
	(242)	*(236)*	*(236)*	*(270)*
Facial expressions of Happiness				
T1	45	18	14	60
	(181)	*(63)*	*(51)*	*(204)*
T2	162	174	154	196
	(291)	*(175)*	*(193)*	*(290)*
T3	181	199	172	228
	(338)	*(218)*	*(204)*	*(376)*
T4	107	135	116	136
	(306)	*(251)*	*(243)*	*(331)*
T5	44	23	31	33
	(231)	*(181)*	*(136)*	*(294)*

Figure 4.2. BDNF genotype differences in fixation duration to facial expressions of Anger (left) and Happiness (right) relative to the neutral face. Carriers of at least one Met allele, are initially fixating more the angry faces, but later spent significantly less time looking the angry faces. Subsequently V/V participants look less at angry faces early but later, looked more at the affective faces. The error bars denote one standard error of the mean.

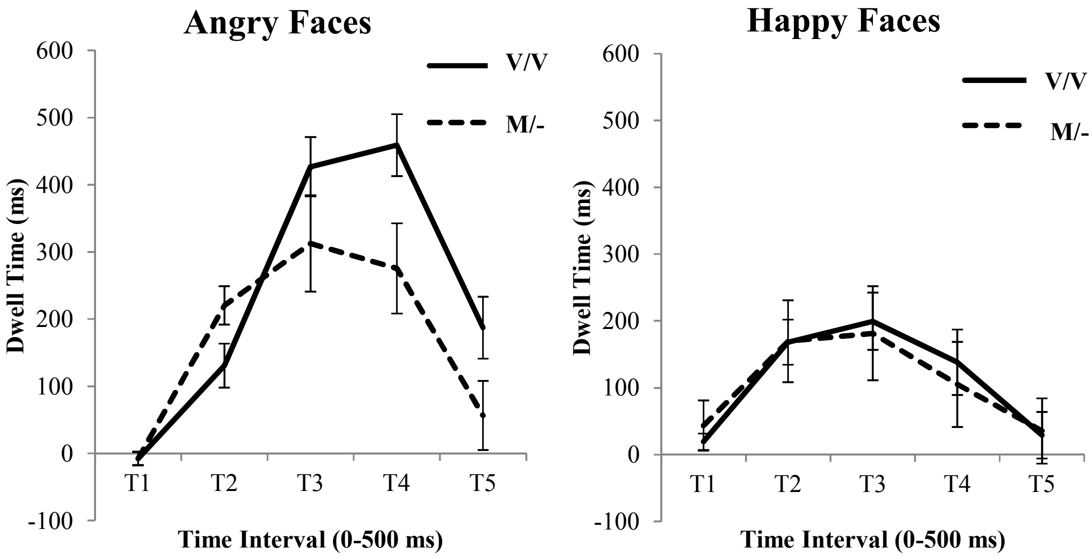
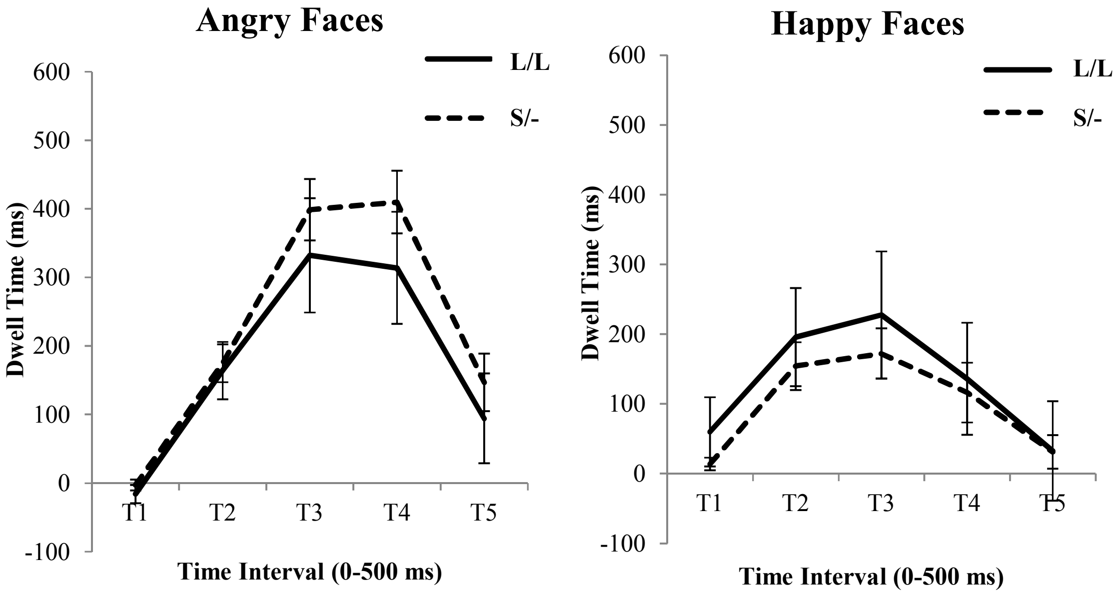


Figure 4.3. 5-HTTLPR genotype differences in fixation duration to facial expressions of Anger (left) and Happiness (right) relative to the neutral face. Genotype groups are not differing at any Time point across the two types of emotional faces. The error bars denote one standard error of the mean.



4.6.4. Genotype effects on atypical gaze patterns

A two-way mixed ANOVA with the repeated factor RoI (eyes, mouth) and genotype group (S/- versus L/L) and gender as independent factors, examined gaze behaviour for each face region on the baseline trials, which showed a significant main effect of RoI for the face areas of interest [$F(1,45) = 126.11$, $\eta_p^2 = .737$, $p < .001$], whereby children spent more time looking the eye region of the neutral faces (see Table 4.4). Moreover, a significant interaction between RoI and 5-HTTLPR genotype group was evident [$F(1,45) = 7.25$, $\eta_p^2 = .139$, $p = .010$]. Repetition of the initial analysis with three 5-HTTLPR genotype groups also revealed a significant two-way genotype by region interaction [$F(1,43) = 3.57$, $\eta_p^2 = .143$, $p = .037$]. Repetition of the same analyses with the BDNF Val^{66}Met genotype group (V/V, M/-) and gender as independent factors, did not revealed a significant interaction between RoI when comparing two [$F(1,45) = 0.74$, $\eta_p^2 = .016$, $p = .393$] or three [$F(1,43) = .396$, $\eta_p^2 = .018$, $p = .396$] BDNF genotype groups (see Appendix 4.3).

To further examine the 5-HTTLPR genotype effects observed in the ANOVA, and given that the data for the eyes region met normal distribution criteria (Kolmogorov-Smirnov test $p > 0.05$), complementary parametric tests were conducted. Therefore, an independent samples t-test was performed to further investigate the association between viewing time for the eye region and the Genotype (L/L, S/-). This analysis revealed a significant effect of Genotype group on viewing time for the eye region [$t(47) = 27.15$, $p = .008$], providing evidence that Short allele carriers spent relatively less time viewing the eye region compared to participants homozygous for the Long allele. This evidence also provides support for the statistical interaction observed in the initial ANOVA (see Figure 4.4; Table 4.4).

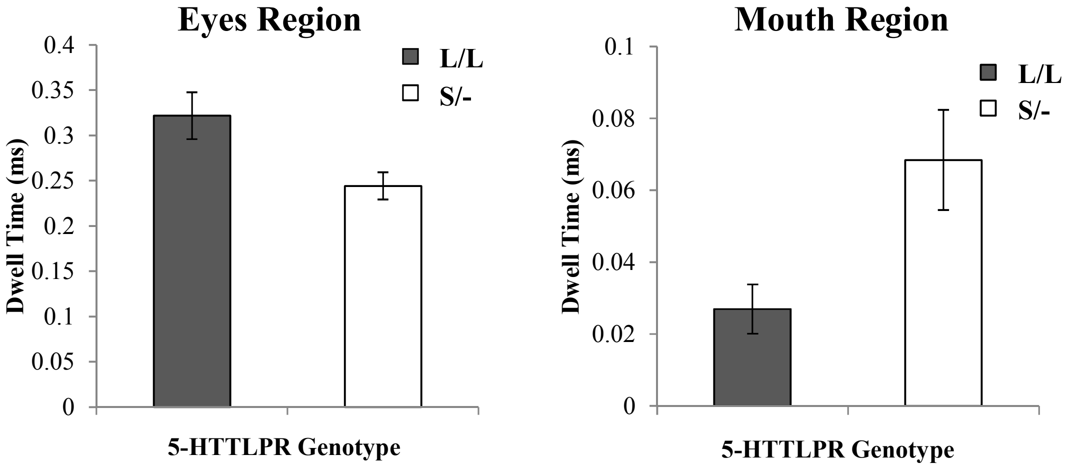
Table 4.4. Means and standard deviations (in brackets) of the BDNF Val^{66}Met and 5-HTTLPR genotype differences in visual scanning patterns towards eyes and mouth region on neutral faces (in ms), relative to the time spent looking the whole face. The S/- genotype group is spending significantly less time looking the eyes region, whereas spend more time fixating the mouth region of neutral faces.

RoI	**BDNF**		**5-HTTLPR**	
	M/- (N=21)	**V/V (N=28)**	**S/- (N=32)**	**L/L (N=17)**
Eyes Region	0.25 *(0.09)*	0.28 *(0.09)*	0.24 *(0.08)*	0.32 *(0.10)*
Mouth Region	0.06 *(0.08)*	0.04 *(0.04)*	0.06 *(0.07)*	0.02 *(0.02)*

Due to the fact a Kolmogorov-Smirnov test revealed that the data for the mouth region data were not normally distributed ($p > 0.05$), this analysis was followed up with complementary non-parametric tests using a Mann-Whitney U test in order to further investigate the way in which 5-HTTLPR genotype groups (i.e. L/L versus S/-) differ in the time spent looking the mouth region.

This analysis revealed a significant effect of Genotype group on the viewing time for the mouth region ($U = 139.0$, $p = .005$), indicating that Short allele carriers spent relatively more time viewing the mouth region compared to participants homozygous for the Long allele. Moreover, a significant effect of genotype on looking the eye region was evident, where Short allele carriers spent significant less dwell time fixating the eye region ($U = 168.0$, $p = 0.29$), when compared to carriers of two copies

Figure 4.4. Differences of overall time spent looking at the eyes (left) and mouth region (right) among 5-HTTLPR genotype groups, relative to the time spent looking the whole face. Carriers of at least one Short allele, are fixating less the eyes region, but spending more time looking the mouth region of neutral faces. The error bars denote one standard error of the mean.



of the Long allele. This evidence provides support for the statistical interaction (see Figure 4.4; Table 4.4) observed in the initial ANOVA. In consistent a Kruskal-Wallis test with three 5-HTTLPR genotype groups showed that 5-HTTLPR genotype modulated the dwell time spent fixating the mouth region [$x^2(2) = 8.50$, $p = 0.14$] but not significant differences on viewing the eye region of neutral faces ($x^2(2) = 4.88$, $p = .087$; see Appendix 4.3). This later observation suggests that the presence of at least one copy of the Short allele modulates visual scanning of facial features when compared with carriers of two copies of the high uptake 5-HTTLPR Long allele.

4.7. Discussion

The present study examined associations of normal variations in genetic SNPs involved in both neural plasticity (BDNF Val^{66}Met) and serotonin availability (5-HTTLPR) with visual scanning of faces in typically developing young children. The present study show that normal variations on the BDNF Val^{66}Met and 5-HTTLPR genotype early in life may account for individual differences in the visual scanning of faces. Specifically, it was shown that children carrying the low activity BDNF Met allele, exhibited a visual scanning pattern that may relate to vigilance-avoidance of viewing angry but not happy faces. Conversely, carriers of two copies of the high plasticity Val allele spent less time looking the angry faces in early stages of processing but spent significantly more time in the later stages. Moreover, in a separate analysis was shown that carriers of the low activity 5-HTTLPR Short allele, compared to the participants homozygous for the high serotonin activity Long allele, spent significantly less time looking at the eye region relative to the whole face, when at the same time they spent more time looking at the mouth region.

There is increasing evidence to highlight the involvement of BDNF Val^{66}Met on the structural formation of brain structures, that play an important role on the affective processing (e.g., Joffe *et al.,* 2009; Lang *et al.,* 2007; Van Wingen *et al.,* 2010). Consistent with studies of children to suggest heightened neurophysiological sensitivity of Met allele carriers in response to negative environmental stressors (Scharinger *et al.,* 2010; Gerritsen *et al.,* 2011; Montag *et al.,* 2008; Schofield *et al.,* 2009; Lau *et al.,* 2010), and in line with the study's hypothesis, it was shown that Met allele carriers spent more time fixating angry versus neutral facial expressions during early stages of processing (501-1000 ms), which decreased during later stages of processing (1501-2000 ms; 2001-2500 ms).On the other hand, participants homozygous for the high activity Val allele did not show similar patterns of avoidance. Instead, they spent significantly more time looking at the angry faces after 1501 ms, relative to the neutral facial expression. Although, this pattern of findings is consistent with the direction of the study's hypothesis, showing that the two genotype groups spent equal time looking the Happy-Neutral face pairs, for the Angry-Neutral face pairs is possible that the time-specific significant effect may be mainly driven from a differentiation of the high plasticity Val/Val group when processing angry faces, who exhibited an increased interest into viewing the angry facial expressions. More specifically, it might be argued that participants in the Met/- genotype group have viewed the angry facial expressions in a similar fashion to their viewing of happy relative to neutral faces. Taken that the data here represent dwell time of looking the emotional face, relative to the neutral face, this may also suggest that participants in the low plasticity Met/- group directed their gaze towards the neutral face rather than simply avoiding negative stimuli. However, it is possible that Met carriers may have viewed neutral faces as negative or threatening, which would be consistent with biases observed among people with high trait and state anxiety (Yoon & Zinbarg, 2007, 2008; see also Beevers *et al.,* 2011).

Conversely, the pattern of findings may suggest that the high plasticity Val/Val genotype group was shown to exhibit an increased interest towards exploring the negative facial expressions, without switching their eye gaze away to explore the neutral stimuli in the trial. Although there is evidence to suggest the involvement of the BDNF Val^{66}Met polymorphism in modulating responses to environmental stressors (Scharinger *et al.,* 2010; Gerritsen *et al.,* 2011; Montag *et al.,* 2008; Schofield *et al.,* 2009; Lau *et al.,* 2010), it is not yet clear from the present findings, how the increased time spent looking the angry faces relative to the neutral in the high plasticity Val/Val allele relates with the modulation of neural pathways that involved in emotion reactivity. Moreover, taking into account previous evidence to show reduced fear conditioning (i.e. startle response potentiation) in Met/Met homozygotes, which was interpreted in the basis of alterations in fear acquisition in this genotype group (Hajcak *et al.,* 2009), the evident reduced viewing of the fearful stimuli in the present study may relate to deficits in eliciting defensive response to appropriate environmental stressors in the Met/- group. This area of inquiry requires further investigation.

To this end, although the study provide evidence for the moderating effects of the BDNF genotype on the visual scanning pathways early in life, due to the sample size limitations of the study, the present findings need to be considered with great cautiousness. It is not yet clear from the current investigation, or other available evidence in the literature, whether the spending of more versus less time exploring the affective stimuli may suggest per se risk or resilience for affective problems. Early in life young children may exhibit a particular interest in specific set of stimuli, but since this area is severely understudied can not be concluded of what each of these behaviours account for. As previously reviewed, there is a discrepancy in the current literature on the specific visual scanning pathways of affected young children in response to emotional faces (e.g., In-Albon *et al.* 2010). To this end, the present study provides an important, but first-stage dimension, in existing work that support the hypothesis that variations in the BDNF Val^{66}Met genotype may relate to early manifestation of atypical physiological responses to environmental stressors. Further research will be needed to show how these differences may relate with context-specific susceptibility for behavioural outcomes.

The current study provides a novel contribution to the neurobiological underpinnings of affectivity that may be due to critical influences of the BDNF Val^{66}Met on the connectivity between the amygdala and the PFC (Carlson *et al.,* 2013). Conversely, the analyses did not reveal a similar effect of 5-HTTLPR genotype on the processing of emotional faces. While this later finding may be considered inconsistent with some previous neurophysiological and behavioural studies of children and adults that have suggested 5-HTTLPR effects related to responses to emotional faces (Homberg & Lesch, 2010; Thomason *et al.,* 2010), it is possible that developmental effects of the sample, or differences in the material used, may have contributed to these inconsistencies.

In addition to the effects of the BDNF Val^{66}Met genotype in predicting preferential looking, a separate analysis suggested a role of the serotonin transporter 5-HTTLPR polymorphism in modulating gaze direction towards the eye and the mouth regions of faces posed in neutral expressions in the current study. Consistent with a plethora of studies suggesting the existence of neurobiological sensitivity for negative affectivity, such as stress reactivity, in carriers of the Short 5-HTTLPR allele (e.g. Caspi *et al.,* 2003; Disner *et al.,* 2013; Mercer *et al.,* 2012; Thomason *et al.,* 2010) the pattern of results of the present study show that early in life, the presence of the Short 5-HTTLPR may be related to individual differences in face scanning behaviour that has previously been associated with pervasive anxiety and/or shyness (e.g., Horley *et al.,* 2004). More specifically, the study showed that the carriers of the low activity Short allele spent significantly less time looking at the eye region relative to the rest of the face, compared to the participants homozygous for the high serotonin activity Long allele. These individuals also spent more time looking at the mouth region.

One possible explanation for the observed pattern of looking behaviour is that Short allele carriers diverted their eye gaze away from the eye region of neutral faces, and swift their attention away into looking the mouth region of the face perhaps as a compensatory mechanism to down-regulate heightened reactivity when processing the eyes region. Conversely, Long allele homozygotes may be less reactive to socially demanding stimuli and therefore less urged to switch their eye gaze towards the mouth region of the face (see also Beevers *et al.,* 2011).The possibility that 5-HTTLPR Short allele carriers, known to experience higher vulnerability for poor reactivity to distressing negative emotional cues, may help to link with the literature that suggests that reduced looking to the eye region is evident in individuals with social anxiety (Crawford *et al.,* 2015; Farzin e*t al.,* 2009). However, although the sample size and size of effects is similar to the ones previously reported, the present pattern of genetic findings needs to be interpreted cautiously. To this end, the previously documented plasticity function of the 5-HTTLPR Short allele that is associated with disproportionate response to negative and positive environmental influences, may be partially reflected in this early eye gaze pattern towards the eyes region early in life, which in conjunction with other factors, such as negative life events, may increase the risk versus resilience for later affective problems. This hypothesis requires further investigation, which will potentially incorporate the longitudinal measurement of behavioural outcomes.

Moreover, contrary to the study's hypothesis, the present study results did not uncovered associations between parent reports of early affective problems and overall fixation duration towards emotional faces or facial features. One potential explanation for this may be related to the study's sample age, which consisted of young and unaffected young children compared to observations with older children (see Battaglia *et al.,* 2004) or adolescents (Gamble & Rapee, 2009). As already reviewed in the present study, there is a discrepancy between child and adult studies (see also In-Albon *et al.,* 2012), which may be due to methodological or maturational effects of the studied samples. Although there is evidence to suggest that during the later stages of early childhood, performance in face recognition increases (e.g., Tremblay, Kirouac & Dore, 2001), to date there is very limited knowledge on the developmental trajectories of such behaviours and their relations with the early manifestation of affective problems (for a review see Thomas, De Bellis, Graham & LaBar, 2007). This area of inquiry requires further investigation. Another possibility that may explain the absence of effects of early behavioural problems in modulating visual scanning of facial emotions and features may be the material and the experimental design used in the present study. For instance, previous studies have used various negative emotional faces (Gamble & Rapee, 2009), as opposed to angry-only negative emotional faces, or longer periods of angry-neutral face pair presentations (Gamble & Rapee, 2010). As Bons and colleagues (2013) indicate both these variables may be critical in shaping the pattern of findings in studies looking into individual differences and may contribute to the discrepancy among studies in typical and atypical development. Future investigations that will account for the consistency on the material used among studies will shed light in this area of inquiry. Moreover a longitudinal investigation of the developmental trajectories of early reactivity in both typical and atypical development will further delineate the particular neurobiological constructs of early affectivity early in life.

Limitations

A limitation of the present study is the relatively small sample size. However, through a fine grained and hypothesis-driven analysis, reliable data were generated. Moreover, the study adopted a recently established approach to understand the neurobiological basis of behavioural problems via the investigation of the normal variation of candidate genes (Wiggins *et al.,* 2012). In this regard, the current study utilizes a larger sample relative to most previous developmental neuroimaging genetic studies that employed fMRI (Stollstorff*et al.,* 2010; Wiggins *et al.,* 2012) or equal to the ones employing EEG/ERP methods (Bertoletti *et al.,* 2012). In addition, the evidence provided here is the first known to show the existence of serotonin-mediated mechanistic influences of gaze allocation during the processing facial features of neutral faces. Although previous evidence has highlighted an atypical pattern towards the eyes region in affected young populations, the present evidence generates a novel question on whether these atypicalities represent general plasticity for behavioural outcomes, as opposed to a disorder-specific phenotype.

In conclusion, although the employment of eye-tracking technologies provides a direct neuropsychological index of reactivity in response to emotional stimuli, the ecological validity of the measure needs to be further justified. Future studies in this area of inquiry would be necessary to incorporate complementary behavioural measures, such as structured observation, so the potential behavioural outcomes of the observed mechanisms that may relate to susceptibility for better and for worse could be directly and reliably measured. In addition to the evidence presented here to highlight the role of the serotonin 5-HTTLPR genotype in modulating the eye gaze patterns towards facial features; variations on the same polymorphism have also been associated with negative affectivity in response to aversive information in adults. Therefore, further research on the role of serotonin uptake on the processing of aversive information in young children would be critical to be conducted, in order to unveil the particular neurobiological constructs of early reactivity in response to social versus non-social aversive threat.

In summary, the current study's results suggest that normal variation in genetic single-nucleotide polymorphisms contributes to the manifestation of individual differences on early patterns of visual scanning towards faces and face features early in life. Overall, the outcomes of the study are consistent with existing adult, adolescent, and child psychopathology research literatures suggesting a contribution of both BDNF Val^{66}Met and 5-HTTLPR to variations in emotional reactivity that may relate with early onset behavioural problems. The current findings further offer particular insights into cognitive/behavioural mechanisms of gene-mediated early plasticity and affectivity, that in conjunction with other environmental factors, may influence the development of psychological problems in the later adolescent and adult life.

CHAPTER 5

Serotonin 5-HTTLPR genotype modulates visual scanning of aversive stimuli in young children

5.1. Preface

In the previous chapter, the effects of plasticity and serotonin availability-related genes in modulating children's visual scanning towards emotional faces and facial features were shown. A number of areas for future research on the context of the neurobiological basis for behavioural problems were identified. This included a need for further investigation of the modulating role of variations in candidate genes in the visual scanning pathways of threat-induced information. Therefore, the present chapter aims to assess the putative associations between behavioural as well as genetic markers and visual scanning pathways in response to aversive stimuli, on the same young population of children as on the study presented in Chapter 4. Through the employment of a novel eye-tracking paradigm designed to measure visual scanning of social and non-social aversive and positive stimuli and the alongside investigation of the impact of normal genetic variations on the visual scanning behaviour, novel insights into the early patterns of emotional reactivity will be generated.

5.2. Processing of affective stimuli and psychopathology

The ability to detect threat in the environment is the ability that humans acquire early in life, which has been linked to influences of human evolution (Seligman, 1971). More specifically, the accurate and timely effective detection and evaluation of threat are critical for survival purposes, where an individual needs to employ an immediate response strategy when exposed on a dangerous and life-threatening situation. However, atypical patterns of the visual scanning of aversive information have been widely investigated as a putative prominent component on the manifestation and establishment of affective problems (for a review see Boyer & Bergstrom, 2011). More specifically, patterns of preferential looking, towards or away, threat-related information are believed to significantly contribute to the manifestation but also on the maintenance of anxiety disorders in the adult life (e.g., Beck, 2008; Eysenck, 1992; Mathews, May, Mogg & Eysenck, 1990).

Aiming to delineate the manifestation of affective behaviours, during the last two decades, empirical studies with both healthy and clinical populations have highlighted the role of visual scanning pathways as a component of behavioural sensitivity that is present in individuals affected from a range of affective disorders (Gotlib *et al.,* 2004; Joormann & Gotlib, 2007). Most notably, an eye-tracking investigation using negative stimuli presented side-by-side with neutral stimuli for three seconds, showed that a young adult population with dysphoria exhibited a prolonged period of viewing the affective stimuli compared to controls (Caseras, Garner, Bradley & Mogg, 2007). Similarly, other eye-tracking investigations with longer periods of processing (i.e., 30 seconds) of different types of emotional versus neutral stimuli showed that young adults diagnosed with depression exhibited prolonged eye-gaze duration in response to dysphoric images compared to matched controls (Eizenman *et al.,* 2003;

Kellough, Beevers, Ellis & Wells, 2008). It has been suggested these gaze biases towards negative stimuli and avoidance of the positive stimuli that were evident in individuals with depression (Kellough, Beevers, Ellis & Wells, 2008), are driven by effortful control deficits that relate with reactivity and emotion regulation, both being core part of the depressive symptomatology (Hartlage, Alloy, Vazquez & Dykman, 1993).

However, to date, atypicalities on the visual scanning behaviour of negative stimuli have not yet been associated with the particular phenotype of a specific disorder. Conversely, research suggests that atypical visual scanning pathways of aversive scenes may reflect a wider behavioural trait of overactivity that may also be evident in individuals at increased risk for affective symptomatology (for a review see Ellis, Boyce, Belsky, Bakermans-Kranenburg, & van IJzendoorn, 2011). Most notably, in a behavioural response task was showed that individuals with spider phobia exhibited a vigilance-avoidance pattern of behaviour, where they automatically approached –quicker responses– in spider-related stimuli, but later avoided the affective stimuli compared to the non-anxious control group (Rinck & Becker, 2006). Moreover, other studies have supported the negative selectivity hypothesis (see also Section 4.2.2.1), based on which individuals diagnosed with anxiety have shown an overall preferential orientation towards threatening stimuli (for a review see Ruiz-Caballero & Bermudez, 1997; Bradley *et al.,* 2000).

However, in contrast to the plethora of studies with adults, the very limited evidence coming from studies with children does not allow conclusions about the developmental significance for each of these accounts. The current cognitive models of anxiety suggest the existence of a threat-specific processing system that may be responsible for the prioritization of threat processing compared to other emotions, and subsequently aid on the manifestation of affective traits (Beck & Emery, 1985). To date, it is still unclear what the particular constructs that determine the individual variation on these behaviours are and their putative role in the manifestation of affective problems. Delineating the nature of the manifestation of early affectivity may help in detecting those at increased risk for affective disorders early in life and design tailored therapeutic interventions.

5.2.1. Measuring visual processing of affective stimuli

Studies employing eye-tracking have provided support for the vigilance-avoidance model in anxious adults (Garner *et al.,* 2006; Mogg *et al.,* 2000; Pflugshaupt *et al.,* 2005; Rinck & Becker, 2006; see also Section 4.2.2), although a proportion of studies have provided partial support to the model, reporting avoidance but not vigilance in anxious populations (e.g., Hermans *et al.,* 1999; Rohner, 2002). Similar to the literature on emotional face processing, evidence on the field of processing aversive information in both healthy and clinical populations has exhibited an heterogeneity, perhaps due to the various methodologies, experiment structure and theoretical concepts that have been proposed and adopted over time (for a review see Bar Haim *et al.,* 2007; also see Section 4.2.2 for a review on this area). However, more recent eye-tracking studies have produced more consistent outcomes, and it has been suggested this may be due to the employment of common experimental properties (i.e., stimulus duration; stimuli material; for a review see Kellough *et al.,* 2008). Most notably, in these eye movement studies, longer durations of stimuli have been used (e.g. 1000 ms), as opposed to brief presentation that was usually employed in earlier studies (for a review see Kellough *et al.,* 2008). Numerous studies have highlighted the importance of long stimuli duration (i.e. > 1000 ms) on the sustained processing of threat-related stimuli documented in individuals diagnosed with affective disorders, such as depression (Calvo & Avero, 2005; Rohner, 2002; Mogg & Bradley, 2005; Siegle, Granholm, Ingram & Matt, 2001).

Visual scanning behaviours are typically conceptualised in the context of negative and positive affectivity, with the negativity bias to represent increased fixation duration towards aversive information and negative affectivity, whereas the positivity bias to represent the stronger response to positive or neutral stimuli and positive affectivity (Ito & Cacioppo, 2005; Larsen, Norris, McGraw, Hawkley & Cacioppo, 2009; Norris, Larsen, Crawford & Cacioppo, 2011). It has been previously suggested that if positive affectivity that reflects adaptive responses is not effectively established early in life, it may lead to maladaptive responses and emotion dysregulation (Norris *et al.,* 2011). To this end, it is critical to investigate the early mechanisms that may contribute on the establishment of visual scanning pathways of threat-inducing environmental stimuli that may contribute on the manifestation of maladaptive behaviours in an individual's later life (Donnelly, Hadwin, Menneer & Richards, 2010).

5.2.1. Affective processing in typical and atypical development

There are several studies in both typically and atypically developing populations that have investigated visual scanning behaviour of aversive stimuli. In this area of inquiry, atypical processing patterns of negative information, such as threat, have been associated with emotional, temperamental (Pérez-Edgar *et al.,* 2010), and genetic markers (Pergamin-Hight *et al.,* 2012). Although previous research has underscored that behavioural patterns of threat avoidance are established well before adulthood (Muris *et al.,* 2003), to date, the vast majority of the available evidence in child and adolescent literature is largely inconsistent (for a discussion see Vasey & MacLeod, 2001). This may be due to variations in sample's characteristics across studies (i.e., age, abilities) that may further complicate the delineation of the developmental parameters of early reactivity in response to threat. For example, a recent study, in measuring latency of touch in response to emotional targets found that healthy children were quicker in detecting aversive stimuli, such as snakes, compared to frogs or other distractors (LoBue & DeLoache, 2008). However, there is inconsistency among studies with children diagnosed with anxiety, with a proportion of research examining probe detection of emotional-neutral pairs of words to show a preferential response of children with anxiety towards threatening stimuli (e.g., Vasey, Daleiden, Williams & Brown, 1995; Vasey, El-Hag & Daleiden, 1996), whereas others primarily report common patterns of threat avoidance in both anxious children and healthy controls (e.g., Kindt, Bierman & Brosschot, 1997).

In addition to the above difficulties, there is very limited evidence on the time course of visual scanning of aversive stimuli early in life. The investigation of the processing of aversive stimuli in the child literature has been overshadowed in the literature, perhaps due to the reluctance of the research community to provoke threat in young children through the exposure to threatening scenes. Most notably, a recent eye movement study showed that compared to healthy controls, children with separation anxiety exhibited vigilance-avoidance patterns of scanning separation pictures (In-Albon *et al.,* 2010). Additional ERP investigations of the brain correlates of threat processing have also used aversive scenes as experimental stimuli, that showed differential activation of the emotion-related late positive potential between positive and negative emotions in groups of young healthy children (Solomon, De Ciccob & Dennisa, 2011; Dennis & Hajcak, 2009). Moreover, a recent ERP investigation has also reported developmental effects between pre-school and older school age children, with faster neural processing of negative stimuli to be evident in older children (Leventon, Stevens & Bauer, 2014). Taken together, understanding the developmental trajectories of threat processing may be particularly important to delineate the individual differences in early affectivity. Interestingly, in recent years a separate distinction has emerged in the relevant literature, which suggest the existence of differential processing pathways for the processing of threat with social versus non-social component.

5.2.1.1. Processing of social versus non-social threat

An additional distinction has also emerged in the field, highlighting the particular constructs of fearful information i.e., fearful information that conveys social component (e.g. human actions or faces), and stimuli that includes non-social fear (e.g. aggressive animals such as bears, snakes, spiders).The neural basis of this assumption is based on evidence that highlights that subcortical neural pathways such as amygdala function to be a key component for social processing (e.g., Adolphs, 2009; Vuilleumier & Pourtois, 2007). Interestingly, an fMRI study with healthy adults has shown significantly reduced amygdala activation during the processing of affective socially relevant stimuli, such as faces, as opposed to non-social affective stimuli (Kirsch *et al.,* 2005). This differential amygdala activation in response to social relevance agrees with other primate lesion (Prather *et al.,* 2001) and human studies (Meyer-Lindenberg *et al.,* 2005), suggesting the existence of distinct neural systems for the processing of social versus non-social fear.

In addition, children diagnosed with Williams Syndrome (WS), a genetic syndrome which is characterised by hypersociability (e.g., Klein-Tasman & Mervis, 2003; Meyer-Lindenberg, Mervis & Berman, 2006), have shown to exhibit increased amygdala reactivity when processing non-social fearful scenes when compared with IQ-matched controls. In contrast, in a recent study that measured reaction times based on motor responses young children with elevated shyness exhibited increased sensitivity (higher reaction times) towards social threats (e.g. faces) but did not differ from the low shyness group in the processing of non-social threats (LoBue & Perez-Edgar, 2014), which was interpreted as a pattern of increased sensitivity in the group with elevated shyness. Further research in needed to delineate the nature of differentiated neural and behavioural responses of social versus non-social threat and their role to early manifestation of affective problems. However, the absence of real-time recording of preference towards or away threat in these behavioural studies, does not allow the drawing of clear conclusions about temperamental variability (see also Section 4.2.2). To this end, the distinction between social and non-social threat through robust neuropsychological measurements, such as eye-tracking, may be of critical importance in the in the extant literature of emotional processing and reactivity.

In addition to the behavioural associations with visual scanning behaviours in response to threat, there is increasing evidence in the adult literature for the existence of gene-mediated pathways of preferential looking towards emotional contexts. Visual scanning behaviour and their intermediate endophenotypes have been proposed to be temporally stable with a profound biological component (Ito & Cacioppo, 2005; Norris *et al.,* 2011). Further investigation into the physiological and neurobiological correlates of the individual differences that may drive heightened sensitivity to negative cues from the information will shed light on the underlying role of these early precursors in predicting later emotion dysregulation.

5.2.3. Genetic influences in affective processing

5.2.3.1 Serotonin Transporter and affective processing

There are now two decades of research on the individual differences of negativity and positivity biases in response to emotional stimuli (e.g., for a review see Hamann & Canli, 2004). Most notably, serotonin and its variations have been highlighted as an important parameter involved in psychological maladjustment, with alterations on the serotonin transmission, and subsequently serotonin availability, to be documented to modulate affective responses (Carver, Johnson & Joormann, 2009; Gonda *et al.,* 2009). Among the most commonly studied genetic polymorphism that may influence human reactive behaviour is the promoter region of the serotonin transporter gene (5-HT), known as 5-HTTLPR. The 5-HTTLPR polymorphism is characterized by pairs of short (S) and long (L) alleles (i.e., short/short, long/short, long/long; Lesch *et al.,* 1996), with the Short allele to be associated with an approximately three times lower basal activity when compared to the long allele (Hariri *et al.,* 2002; Lesch *et al.,* 1996; see also Section 3.2.1).

Evidence, coming from neuroimaging studies in healthy populations, has shown that carriers of the Short allele exhibit heightened neurophysiological reactivity when processing aversive stimuli on brain structures that relate with the processing of fear (Bertolino *et al.,* 2005; Hariri *et al.,* 2002; Hariri *et al.,* 2005; Munafo *et al.,* 2008; for a recent study see Jonassen and Landrø, 2014). Consistently with this notion, a range of studies with children has emerged the recent years to show that carriers of the low activity Short 5-HTTLPR allele exhibited increased neurophysiological sensitivity in response to negative environmental stressors (Bogdan *et al.,* 2014; Caspi *et al.,* 2003; Hankin *et al.,* 2011; Pluess *et al.,* 2010).

In addition to the neuroimaging studies, recent studies have employed eye-tracking technologies to record the putative role of serotonin availability in modulating visual scanning patterns of threatening versus positive information. Most notably, in an eye-tracking investigation Beevers et al. (2010) reported that Short 5-HTTLPR allele homozygotes selectively fixated more to positive emotion scenes when simultaneously processed four different emotional stimuli in 30s trials, suggesting a pattern of selective avoidance of negative stimuli in an effort to regulate heightened reactivity to negative stimuli. However, this finding is not consistent with evidence from behavioural studies that measure reaction times. Most notably, following the presentation of pairs of aversive/neutral pairs of stimuli participants homozygous for the high serotonin uptake 5-HTTLPR Long allele have been shown to exhibit positive affectivity (i.e., higher reaction times) in response to positive and neutral stimuli, and a selective avoidance/negative affectivity when processing negative stimuli (Fox, Ridgewell & Ashwin, 2009; Perez-Edgar *et al.,* 2010). In line with this, in a study that employed a dot-probe task of pairs of spiders and neutral controls, it was shown that 5-HTTLPR Short allele carriers exhibited selective preferential looking at non-social fearful stimuli when presented for 2000 ms (Osinsky *et al.,* 2008). Although well designed behavioural measurements may inform about the nature of positive and negative affectivity in response to aversive stimuli, the employment of eye-tracking methodology may also provide a reliable neuropsychological index of the direction and the time-course of the reactivity. This will also increase the current understanding of the impact of neurobiology on behaviours associated with early affectivity.

Despite the increasing evidence coming from eye-tracking studies in adults, currently there is no available eye-tracking study on the potential moderating effects of 5-HTTLPR genotype on visual scanning of affective stimuli in children. There are only a few available behavioural studies that have used negative facial emotions as an index of early affectivity and revealed intermediate effects of 5-HTTLPR genotype in the visual scanning behaviour. Most notably, Gibb and colleagues (2009) reported that children who carried at least one Short 5-HTTLPR allele when their mothers reported increased depressive symptoms, exhibited avoidance behaviour in response to sad faces when presented side-by-side with neutral faces (Gibb, Benas, Grassia, & McGeary, 2009). Consistent with this, a recent meta-analysis on the moderating effects of 5-HTTLPR genotype on the avoidance of negative stimuli reported more significant effects from evidence coming from adult studies compared with data coming from studies with children and adolescents (Pergamin-Height *et al.,* 2011). Although this may be due to the limited number of studies with young populations in the field, it also raises some critical questions on whether maturational effects may also be involved in affective response. Compared to adults, where a priority in processing negative stimuli is evident across studies, children may exhibit different patterns of processing of threat-induced stimuli, perhaps due to immatureness in the inhibitory control system that has been previously associated with the processing of threat (Morren, Kindt, van den Hout & van Kasteren, 2003). Therefore, it is possible that young children may look away when exposed to a threatening stimulus or situation, instead of further exploring the arousing stimuli, as a way to inhibit their overall arousal response (Susa, Pitica & Benga, 2008).

In addition, there is a proportion of research highlighting the involvement of a polymorphism that relates to neuroplasticity, BDNF Val^{66}Met, with individual differences to aversive processing.

5.2.3.2. BDNF and affective processing

There is a line of research highlighting the role of the BDNF gene on the modulation of affectivity in response to environmental stressors. BDNF is a secreted protein that is involved on the release of survival and growth promoting factors (see also Section 4.2.5.1), and normal allelic markers within the gene have been associated with the development of depression and anxiety symptomatology in adolescents (Aguilera *et al.,* 2009, Kaufman *et al.,* 2006 and Wichers *et al.,* 2008). Moreover, BDNF has been shown to have a critical involvement on the regulation of neural development, connectivity, as well as neural plasticity (Martinowich, Manji & Lu, 2008). Most notably, a functional variation of the BDNF gene, the single nucleotide polymorphism BDNF Val^{66}Met (Bath & Lee, 2006), has been widely investigated in relation to affective disorders and the associated behavioural traits. In humans the polymorphism produces a valine-to-methionine substitution at codon 66 (Chen *et al.,* 2006), with the Met allele to be evident to be associate with increased vulnerability for affective disorders (e.g., Sarchiapone *et al.,* 2008; for a review see also Section 4.2.5.1).

Interestingly, emerging evidence has shown effects of the BDNF Val^{66}Met polymorphism on amygdala and hippocampal activation during the response to emotional tasks in adults (Montag *et al.,* 2008; Schofield *et al.,* 2008) and in adolescents (Lau *et al.,* 2010). There is emerging evidence to show increased rumination (Hilt *et al.,* 2007; Beevers *et al.,* 2009) and deficits in fear conditioning (Hajcak *et al.,* 2009) in adult carriers of the BDNF Met allele. However, comparing data coming from adult and child or adolescent studies may be problematic as complex gene-by-brain mechanisms may be confounded by developmental trajectories that are currently poorly understood (Webster *et al.,* 2002). Future research would be critical to be conducted in young children and adolescents to spread extra light on the maturational contributions of variations in the BDNF gene and their potential role on the development of early affective.

5.3. The current study

The current study utilizes eye-tracking technology aiming to explore the potential role of the common genetic variation in the serotonin transporter-linked 5-HTTLPR and neuroplasticity related BDNF Val^{66}Met polymorphism in modulating fixation durations during the processing of affective stimuli in children aged 4 to 7. In addition, aiming to unveil association between rates of early affective problems and processing of aversive scenes, parent-report measures of children's social-emotional development were also employed. In this study, positive, negative, and neutral stimuli were selected from the International Affective Picture System (Lang, Bradley & Cuthbert, 2008).

5.3.1. Aim 1: To investigate the role of early behavioural problems on fixation patterns in response to affective stimuli

The study aims to investigate the putative associations between rates of early behavioural problems, especially internalizing problems, with visual processing of negative information, by calculating the relative viewing dwell time of looking the negative versus positive stimuli. Taking into account previous evidence that reported vigilance-avoidance patterns of processing threatening pictures in children with separation anxiety (In-Albon *et al.,* 2010), the present study aims to investigate whether the same patterns of processing may be also associated with elevated rates of behavioural problems, especially internalizing problems, that may aid as a precursor of affectivity early in life. This is the first known study to test these associations in young children, by employing reliable eye-tracking technologies as well as ecologically valid parent measures to assess children' s early affectivity.

5.3.2. Aim 2: To investigate genetic influences on fixation patterns in response to affective stimuli

A second aim of the study is to unveil the putative effects of 5-HTTLPR and BDNF Val^{66}Met genotypes on the time course of the time spent looking positive versus aversive stimuli. Taking into account recent studies suggesting a direct role of the 5-HTTLPR (Beevers *et al.,* 2010; Gibb *et al.,* 2009) and BDNF Val^{66}Met genotype (e.g. Hajcak *et al.,* 2009) in modulating the processing of affective stimuli, the study sought to unveil the modulating role of these two candidate polymorphisms on the affective responses early in life. By distinguishing social and non-social negative and positive scenes and calculating the relative dwell time spent fixating each type of stimuli, the gene-mediated constructs that relate to early reactivity were sought to be unveiled.

5.3.3. Hypotheses

There are two main hypotheses that are tested as part of this study. Firstly, taking into account the eye-tracking evidence in adults with dysphoria (Caseras *et al.,* 2007) and depression (Kellough *et al.,* 2008) showing prolonged visual scanning of negative stimuli, as well as the previously reported vigilance-avoidance patterns of processing threatening pictures in children with separation anxiety (In-Albon *et al.,* 2010), it is hypothesised the presence of elevated rates of internalizing problems in children, will be significantly correlated with vigilance-avoidance patterns of scanning negative stimuli. More specifically, it is expected that children that reported to have elevated anxiety and depressive problems will initially fixate more to the negative stimuli but later will spend less dwell time fixating the same stimuli, providing support for the vigilance-avoidance hypothesis.

With regards to the second aim of the study, taking into account emerging evidence highlighting the role of variations in the 5-HTTLPR polymorphism (Beevers *et al.,* 2010; Gibb *et al.,* 2009) in modulating the processing of affective stimuli, it is hypothesised that carriers of the low serotonin 5-HTTLPR Short allele will exhibit a vigilance-avoidance pattern of looking negatively valenced stimuli, compared to the high serotonin uptake Long allele group, providing support for the vigilance-avoidance model. More specifically, it is expected that carriers of the low serotonin uptake Short allele, compared to Long allele homozygotes, will initially fixate more on the negative stimuli but on the later stages of processing will spend significantly less dwell time fixating the negative stimuli when compared to Long allele homozygotes. In a similar vein, taken that carriers of the low neuroplasticity Met BDNF Val^{66}Met allele (e.g. Hajcak *et al.,* 2009) have been shown to exhibit increased reactivity in response to environmental stressor is hypothesised that compared to the high neuroplasticity Val/Val group, Met allele carriers will exhibit vigilance avoidance patterns of processing the aversive stimuli. The study aims to initiate important steps in integrating specific neurocognitive and genetic factors that may account as risk markers of reactivity early in life.

5.4. Methods and Materials

5.4.1. Participants

The final sample consisted of 49 children from Caucasian/White British ancestry (24 males; 25 females; Mean age = 70.8 months, SD = 11.5). Power analysis suggested that the sample size required to achieve a power of 1-β = 0.90 for the ANOVA test at significance level α = 0.050 requires at least 33 participants. See Section 4.4.1 for detailed information on participant demographics.

5.4.2. Data collection procedures

See Section 4.4.2.

5.4.2.1. Behavioural Measures

See Section4.4.2.1.

5.4.2.2. Measures of behavioural problems

See Section3.4.2.2.

5.4.2.3. Eye-tracking assessment

Stimuli

Developmentally appropriate coloured pictures that have been previously used in studies with children of the same age (Dennis & Hajcak, 2009; Solomon *et al.,* 2011; Leventon *et al.,* 2014) were selected from the International Affective Picture System (IAPS; Lang *et al.,* 2008). Many studies in adults have examined preferential looking gaze patterns on emotional pictures from the IAPS picture set, which is documented to be a well standardised set of emotional stimuli. However, in studies with young children, where it was impossible to obtain subjective valence and arousal ratings of the IAPS pictures because of the children's difficulty in understanding the self-assessment mannequin rating techniques (Lang *et al.,* 2008), a subset of developmentally appropriate IAPS pictures has been used (Dennis & Hajcak, 2009; Solomon *et al.,* 2011; Leventon *et al.,* 2014).

It has been previously reported that children respond in a similar manner as adults to complex developmentally appropriate subset of images/emotional stimuli from the IAPS (Lang *et al*., 2008). To this end, in the present study is was deemed appropriate to use a subset of IAPS pictures that have been previously used on these studies with children and adolescents, that included pleasant scenes[12] (e.g. smiling faces, sport scenes, pleasant animals and scenes and family moments), unpleasant scenes[13] (e.g. faces with negative expressions, attack pictures or disasters), and also neutral scenes[14] (e.g. neutral faces, household objects or nature). Additional neutral stimuli were selected to match the requirements of the experimental design.

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[12] The IAPS numbers for pleasant pictures are: 1460, 1610, 1710, 1920, 2070, 2091, 2224, 7325, 7330, 7400, 8031, and 8496.

[13] The IAPS numbers for threatening pictures are: 1050, 1120, 1201, 1300, 1321, 1930, 3280, 6190, 6300, 9490, 9582, and 9594.

[14] The IAPS numbers for neutral pictures are: 5130, 5210, 5220, 5201, 5250, 5390, 5551, 5611, 5631, 5711, 5740, 5750, 5800, 5820, 5870, 5890, 5900, 7002, 7000, 7004, 7009, 7010, 7025, 7030, 7031, 7035, 7041, 7050, 7100, 7140, 7150, 7175, 7190, 7224, 7233, 7235, 7236, 7496, 7512, 7502, 7545, 7560, 7580, 7595, 7632, 7705, 7900, 7950.

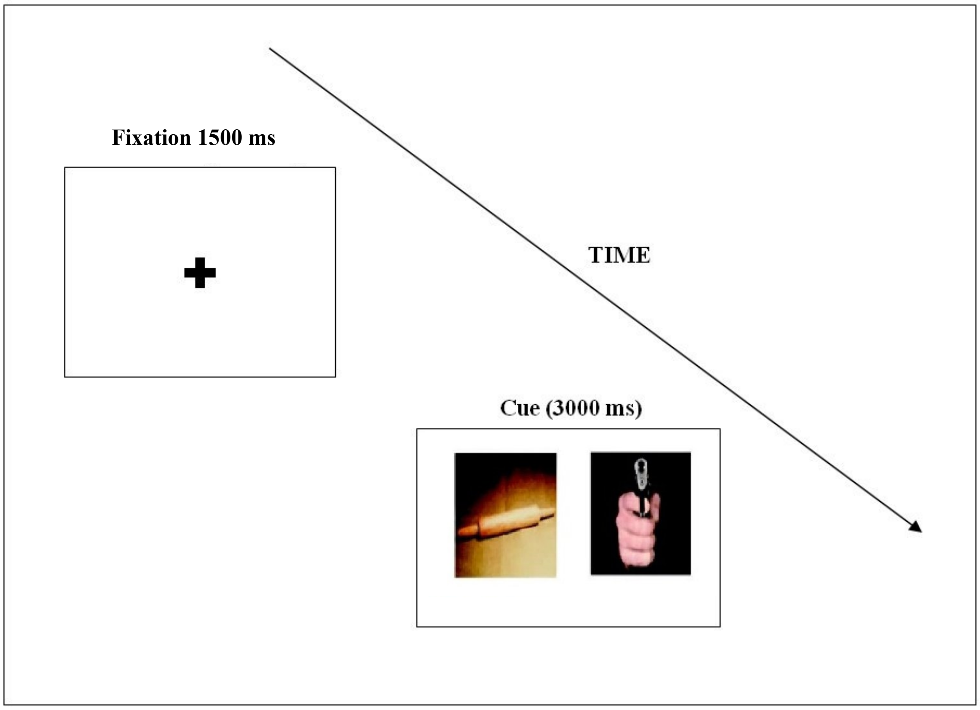
As in previous studies that used the same stimuli with young children, means and standard deviations for valence and arousal ratings for each picture were taken from the IAPS normative adult ratings (Lang *et al.,* 2008).

The IAPS normative ratings are rated on a 9-point scale, where higher ratings for valence represent increased pleasantness, and higher arousal ratings correspond to more arousing stimuli. Negative, positive and neutral pictures differed in terms of valence [Positive: Mean(SD) = 7.62(1.48); Neutral: Mean(SD) = 5.71(1.36); Negative Mean(SD) = 3.65(1.88)]. In a similar vein, the categories of the emotional pictures differed from neutral in terms of arousal [Positive Mean(SD) = 4.67(2.35); Neutral: Mean(SD) = 3.32(2.07); Negative: Mean(SD) = 6.14(2.01)]. A repeated measures ANOVA comparing the image categories (positive versus negative versus neutral) on valence and arousal ratings yielded a significant effect of image category on both valence ratings [$F(2, 22) = 100.28$, $\eta_p^2 = 0.97$, $p < .001$], as well as on arousal ratings, [$F(2, 22) = 39.61$, $\eta_p^2 =0.94$, $p < .001$]. As given by the means and standard deviations above, pictures with positive components had higher valence than neutral, where neutral had higher valence rates compared to negative stimuli. In contrast, negative images had higher arousal ratings than neutral, and in turn, neutral had higher arousal.

Forty-eight pairs of pictures of negative-neutral and positive-neutral pairs were selected for the present study. Stimuli pairs were matched based on colour, contrast, and complexity after having been reviewed by two independent investigators and were presented simultaneously side-by-side. As a general criterion, all the selected pictures can be seen by young children on a daytime television program or in the news. To investigate the potential role of social versus non-social components of the stimuli in affectivity, half of the pictures (i.e., n = 6) for negative and positive emotional presented scenes that involved people whereas the other half presented scenes that involved animals. In addition, the emotional-neutral pairs were matched in terms of arousal levels. The four different types of emotional pictures (i.e., negative social; negative non-social; positive social; positive non-social) were pseudorandomly distributed across the experiment, and each type presented equally over the left and right side of the screen. Moreover, to investigate the role of novelty in fixation durations, the first 24 pairs of the experiment consisted of novel affective stimuli (12 negative-neutral pairs, 12 positive-neutral), whereas in the second block the same emotional stimuli were each paired with a novel neutral picture. This was done to inform whether the effects of preferential looking of particular type of emotion would be able to hold across the two blocks, compared to the other types of stimuli, even when the stimuli have been previously seen.

The eye-tracking experiment was programmed using Experiment Builder software for EyeLink (SR Research, Ontario, Canada). Each trial began with a fixation cross, shown for 1500 ms, measuring 2.81 x 2.08 degrees of visual angle in the middle of the screen, which was displayed for 1000 ms (except in the case of mini calibration; see Procedure Section). This was followed by a pair of pictures presented side by side for 3000 ms (see Figure 5.1). Stimuli presented on a 19-inch CRT, in 1280 x 1024 dimensions with a gap of 4.3 cm between the two pictures. Each stimulus pair was presented with a visual angle of 14.3 x 18.6 degrees.

Figure 5.1. An example of the stimulus pairs used in the eye-tracking experiment, and an illustration of the trial structure.



Procedure

After taking consent from parents, children were escorted in a dimly lit room. All children initially participated on the face processing eye-tracking experiment that was presented in Chapter 4 (see also Section 4.4.2.3). After the completion of the first experiment, children were given a short break and were prepared to participate on the affective processing experiment. Both experiments took place during a single visit on the same experimental room using the same eye-tracking system. Children were told they were going to see different pictures on a computer screen, where their eye movements would be recorded with a special camera. During calibration, the EyeLink recorded the eye position a 9-target location calibration was conducted providing the required reference data for computing gaze positions to ensure a point of fixation error rate of less than 0.5 degrees. A mini calibration was repeated every four trials in order to ensure that eye movement data were adjusted for small-scale movement of the head. In the case of unsatisfactory eye-tracking, a 9-point calibration was repeated. The rest of the eye-tracking procedure was the same as on the emotional face processing experiment as described in section 4.4.2.3. Participants then completed the experimental trials. A mini calibration was repeated every five trials to ensure that eye movement data was adjusted for movement of the headset and/or body.

5.5. Analysis

5.5.1. Analysis of Behavioural Data

See Section 3.5.1.

5.5.2. Reduction of eye-tracking data

Data analysis aimed to measure the time-course of preferential looking towards and away positive and negative emotional information, relative to the neutral stimulus. The criteria for calculating valid fixations were the same as presented in the Section 4.5.2. For the investigation of the fixation durations towards and away positive and negative emotions, dwell time was calculated in ms after subtracting the overall dwell time of the neutral stimuli from the emotional stimuli. This was done separately for each subject and for each positivity and negativity-inducing trial. Average dwell time of looking for each emotion type (i.e., negative, positive) was later calculated for each subject. A separate calculation of social versus non-social trials, for the first and second block separately, was conducted after this analysis. All the above analytical procedures were conducted separately for the positive and the negative stimulus. After the subtraction, the positive values represented preference towards the emotion and negative values avoidance of the emotional stimuli. In the case of detection of more than 40% invalid trials participants were excluded from further analyses. However, no participant met this exclusion criterion; therefore, all 49 participants provided valid eye-tracking data.

Aiming to remain consistent with previous studies measuring proportion of fixations to emotional stimuli in adults (Rohner, 2002) and in children (Gamble & Rappe, 2009) where the 3-s stimulus exposure was divided into 1 s, in the present study proportion of fixations to the emotional picture was computed for each emotion type and each 1000 ms time interval. In line with this notion, recent meta-analytical reviews in the field of affective processing suggesting that the vigilance in the content of vigilance-avoidance hypothesis has been particularly captured after 500ms of presentation when multiple stimuli compete for attention (for a review see Weierich, Treat, & Hollingworth, 2008), which has been suggested to be due to initial fixation on a stimulus within the 0-1000 ms epoch (for a recent review see Armstrong & Olatunji, 2012). Together, taking into account the above evidence and due to the complexity of the affective stimuli and developmental age of the participant, the selection of three equal 1s time windows for the investigation of the vigilance-avoidance patterns of scanning affective stimuli was deemed as the most appropriate in the present study.

5.5.3. Analysis of Genetic Material

5.5.3.2. 5-HTTLPR Genotyping

See Section 3.5.3.1.

5.5.3.1. BDNF Genotyping

See Section 4.5.3.1.

5.5.4. Statistical Analysis

Preliminary Analyses

See Section 4.5.4.

Behavioural Ratings and Eye Gaze Patterns

The primary analysis examined whether children's behavioural scores (i.e., early behavioural problems; rates of autism symptomatology) were correlated with visual scanning patterns in response to particular emotional picture. Therefore, initial parametric and non-parametric correlation analyses were conducted with the overall viewing dwell time to the negatively and positively valenced pictures separately.

Genetics and visual scanning

Each 3-second trial was divided into three equal 1000 ms intervals. The relative viewing dwell time (in ms) of the emotional images was calculated for each 1000 ms interval and then averaged across trials for each emotion (i.e., positive versus negative), condition (i.e., social versus non-social) and block (i.e., block 1 versus block 2) separately. A 2 (Image Type: negative versus positive) by 3 (Time: 0-1000 ms versus 1001-2000 ms versus 2001-3000 ms) by 2 (Condition: social versus non-social) by 2 (Block: novel emotional versus familiar emotional) mixed analysis of variance (ANOVA) with 5-HTTLPR Genotype (L/L versus S/) and Gender as between-groups factors was conducted. The same analysis was repeated separately with different 5-HTTLPR genotype classification (i.e. S/S versus S/L versus L/L), as well as with two (i.e. V/V versus M/-) and three (M/M versus V/M versus V/V) BDNF Val^{66}Met genotype. All within subject, effects that violated the assumption of sphericity were adjusted using the Greenhouse-Geisser correction (adjusted degrees of freedom are noted as adj. df). To further evaluate the time course of attention allocation, independent samples t-tests were conducted to determine whether there was a looking preference towards or away from the emotional images of a specific genotype group at any of the 1000 ms time intervals. This was done for each SNP (i.e., 5-HTTLPR and BDNF Val^{66}Met) and each emotion, separately, after the initial ANOVA. When the data did not satisfy Kolmogorov-Smirnov tests for normality, Mann-Whitney U tests were performed instead of t-tests.

5.6. Results

5.6.1. Demographic Characteristics

See Section 4.6.1 for the sample's demographic characteristics.

Task engagement was calculated by subtracting the relative looking time away from the areas of the stimuli from the time looking the affective stimuli. This analysis show that participants spent consistently over 60% of the time looking the affective stimuli [M(SD) = 0.625 (0.31)] and a T-test test show that these rates did not differ between the 5-HTTLPR S/- and L/L genotypes [t(47) = .436, *p* = .665)] as well as when comparing three 5-HTTLPR genotypes (one-way ANOVA; *p* = .320). In a similar vein no difference on the task engagement rate where evident between the two [t(47) = .156, *p* = .887) or three BDNF genotypes (one-way ANOVA; *p* = .721; see also Appendix 5.3). Moreover, repetition of the same analysis for the engagement with negative and positive stimuli separately showed these rates did not differ between the 5-HTTLPR S/- and L/L genotypes [t(47) = .432, *p* = .661)] as well as when comparing three 5-HTTLPR genotypes (one-way ANOVA; *p* = .622). In a similar vein no difference on the task engagement rate where evident between the two [t(47) = .425, *p* = .772) or three BDNF genotypes (one-way ANOVA; *p* = .522).

5.6.2. Behavioural effects in fixation duration

Pearson correlation analyses revealed a negative correlation between looking time of the negatively valenced stimuli and age (*r* = -.559, *p* < .001), where younger children exhibited higher reactivity/attenuation towards negative stimuli. No further significant correlations between children's demographic characteristics and fixation duration for each emotion, block, condition, and time point were observed. Moreover, no significant correlation was evident with children's internalizing and externalizing problems and average fixation duration at any emotion, time point, condition or block.

5.6.3. Genotype effects in fixation duration towards affective stimuli

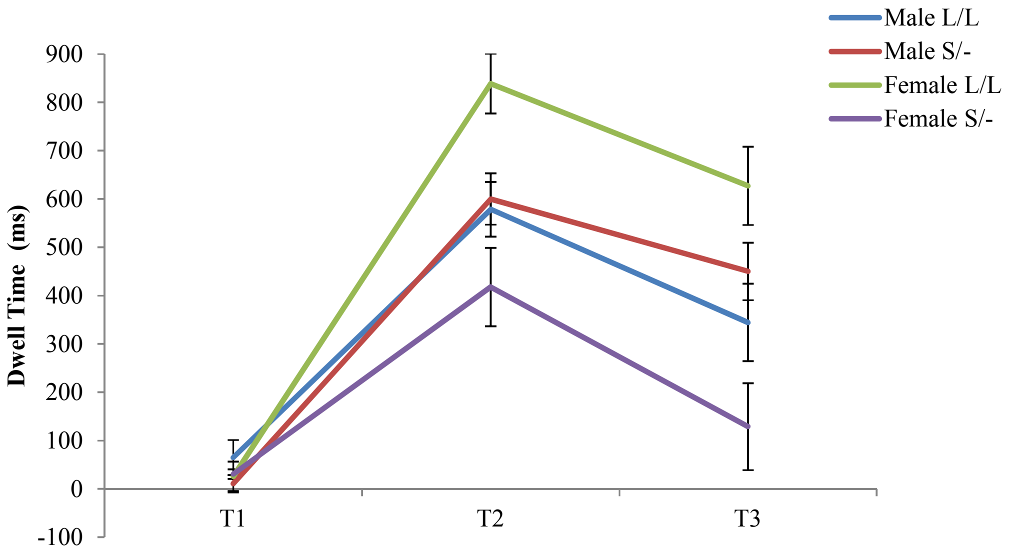
A 2 (Image Type: negative versus positive) by 3 (Time: 0-1000 ms versus 1001-2000 ms versus 2001-3000 ms) by 2 (Condition: social versus non-social) by 2 (Block: novel emotional versus familiar emotional) mixed ANOVA with Genotype (5-HTTLPR L/L versus S/-) and Gender as between factors revealed a significant main effect on Emotion [$F(1,45) = 6.27$, $\eta_p^2 = .122$, $p = .016$)], where participants exhibited a preferential looking pattern towards the positive stimuli compared to the negatively stimuli (see Table 5.1; see also Appendix 5.1 for raw data). A significant main effect of Time was also evident [$F(2,44) = 121.80$, $\eta_p^2 = .730$, $p <.001$] with visual scanning duration to be evident to peak at T_2 [1001-2000 ms] and declined on the later stage of processing (see Table 5.2). Moreover, a main effect of Block [$F(1,45) = 112.72$, $\eta_p^2 = .715$, $p <.001$] suggests that participants during Block 2 spent less time looking on the emotional/previously seen emotional stimuli (Block 1) and compensate the time by exploring the novel neutral stimuli (see Table 5.1). In addition, a significant two-way Time by Block interaction [$F(2,44) = 44.66$, $\eta_p^2 = .498$, $p < .001$] suggests that independently of the emotion valance children spent less time looking the emotional/previously seen stimuli on the second block, and spend more time exploring the novel neutral stimuli (see Table 5.2). Furthermore, a two-way Emotion by Time interaction effect was evident [$F(2,44) = 6.72$, $\eta_p^2 = .130$, $p = .002$] where participants spent more time looking at the positive stimuli across blocks and conditions relatively to the negative stimuli, difference which was more pronounced over T_2 (1001-2000 ms; see Table 5.2). Similarly, a two-way Emotion by Condition effect [$F(1,45) = 6.03$, $\eta_p^2 = .118$, $p = .018$] was observed with relatively lower visual scanning time to be evident for non-social negative stimuli (see Table 5.1). In addition, a three-way Emotion by Time by Condition interaction was observed [$F(2,44) = 5.69$, $\eta_p^2 = .112$, $p = .005$] with more time spent looking at the positive non-social stimuli (i.e., happy animals; sweets) than social and/or negative, which was more pronounced during 2001-3000 ms (T_3; see Table 5.2).

Table 5.1. Participants' mean time (in ms) and standard deviations (in brackets) spent per emotion, condition and block, averaged across time points.

	Social		**Non-Social**	
	Block 1	**Block 2**	**Block 1**	**Block 2**
Positive	495*(278)*	200*(298)*	588*(325)*	243*(299)*
Negative	448*(364)*	140*(363)*	344*(436)*	22*(354)*

With regards to the effects of serotonin transporter polymorphism, a two-way Time by 5-HTTLPR genotype was evident [$F(2,90) = 3.61$, $\eta_p^2 = .074$, $p = .031$] where Short allele carriers, compared to Long allele homozygotes, spent less time looking at the emotional stimuli independently of valence during T_2 (1001-2000 ms; see Figure 5.2). Moreover, a three-way Time by 5-HTTLPR by Gender interaction was evident [$F(2,90) = 10.79$, $\eta_p^2 = .193$, $p < .001$] where homozygous female participants for the high serotonin uptake Long allele spent significantly more time processing the emotional stimuli independently of the valence at T_2 (Mean = 838.53; SD = 163.10; see Figure 5.2) and female Short allele carriers spent less dwell time looking at the emotional stimuli during T_2(M = 417.61, SD = 345.77). No further effects of the 5-HTTLPR were evident for this analysis.

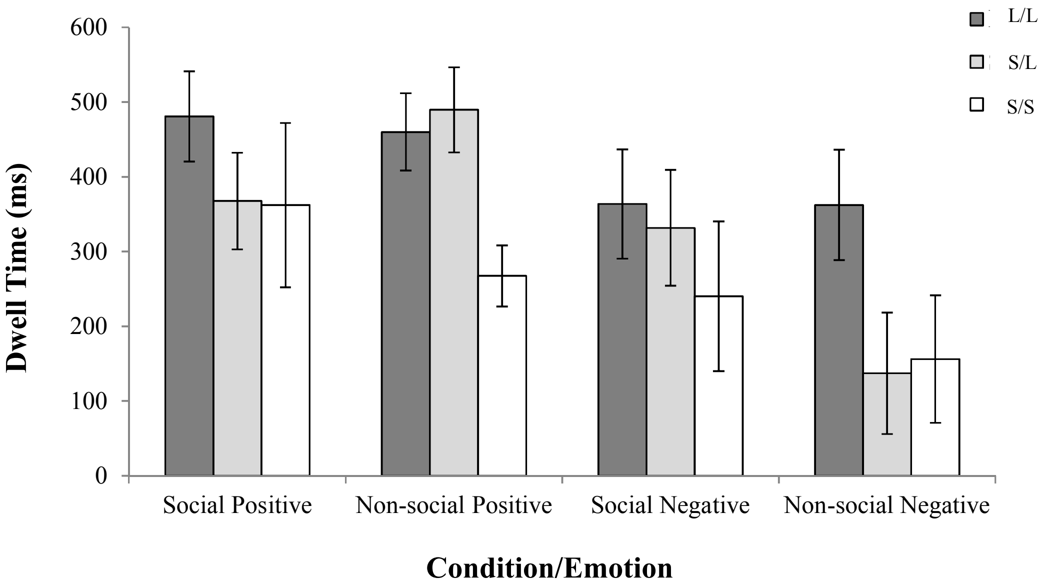
Figure 5.2. Genotype and gender effects on time course of visual scanning of emotional stimuli



To further investigate the documented two-way Time by 5-HTTLPR genotype (S/- versus L/L), the dwell time at each of the three time points was averaged across the two emotions. A Kolmogorov-Smirnov test of normality showed that the averaged data at each time point was normally distributed (p > .005). Therefore, a t-test was conducted at each time point, which showed Short allele carriers spent significantly less time looking at the emotional stimuli when compared to Long allele homozygotes during 1001-2000 ms [$t(47) = -2.28$, $p = .027$; see Table 5.3]. Interestingly, a two-way ANOVA at each of the three time points of processing emotional stimuli irrespectively of the valance, with both gender and 5-HTTLPR genotype as independent variables, show a significant Gender by 5-HTTLPR effect at T2 [F(1) = 7.94, $p = .007$, $\eta_p^2 = .150$] and T3 [F(1) = 11.15, $p = .002$, $\eta_p^2 = .199$]. This may suggest that gender when combined with genetic mechanisms may modulate behavioural reactivity in response to emotional information early in life.

This initial analysis was repeated with three 5-HTTLPR genotype groups (i.e. L/L versus L/S versus S/S) which revealed an additional three-way Emotion by Condition by 5-HTTLPR interaction [*F*(2,43) = 1.78, η_p^2 = .159, *p* = .024]. This analysis suggested that participants who were carriers of two copies of the Long allele, spent more time exploring the non-social threatening stimuli, where Short allele carriers (i.e. S/S, L/S) spent relatively less time looking at the negatively valenced non-social stimuli (see Figure 5.3. and Appendix 5.2).

Figure 5.3. 5-HTTLPR genotype effects on relative viewing time per emotion and condition. The presence of one Short allele was associated with avoidance pattern of non-social negative stimuli, whereas two copies of the genotype with two copies of the Short allele were associated with reduced looking at non-social positive stimuli.



The initial ANOVA was repeated with the BDNF Val^{66}Met genotype (i.e. V/V versus M/-), which did not revealed any Time X BDNF genotype effect [*F*(2,90) = 0.30, η_p^2 = .030, *p* = .506] or any additional effect (see Table 5.4). Similarly, when entering the three BDNF genotype groups (i.e. M/M versus V/M versus V/V) as between factor, no significant Time by

BDNF interaction [*F*(4,86) = 0.55, η_p^2 = .025, *p* = .693] or a three-way Emotion by Condition by BDNF Genotype [*F*(2,43) = 1.10, η_p^2 = .005, *p* = .896], or any other interaction was evident (see also Appendix 5.3).

To further delineate the 5-HTTLPR genotype effects on fixation duration towards social and non-social fearful stimuli, separate one-way ANOVAs were conducted after the relative dwell time was averaged for Negative-Non-Social, Positive Social and Positive Non-Social stimuli (normally distributed; Kolmogorov-Smirnov test $p > .05$) comparing the three 5-HTTLPR genotype groups. Since the data from the average fixation duration in response to Negative-Social stimuli were not normally distributed (Kolmogorov-Smirnov test $p < .05$), a Kruskal-Wallis test was conducted. The ANOVAs revealed a significant effect of 5-HTTLPR genotype on the time spent looking the negative non-social stimuli [*F*(2) = 4.04, *p* = .025] showing that Long allele homozygotes spent significantly more time looking at the non-social negative stimuli (see Figure 5.3 and Appendix 5.2). In contrast, the ANOVAs did not revealed any significant effect for the Non-social positive stimuli [*F*(2) = 2.66, *p* = .080] and social positive stimuli [*F*(2) = .844, *p* = .437]. In a similar vein the Kruskal-Wallis test did not revealed any significant effect of 5-HTTLPR genotype on the processing of negative stimuli with social component [$x^2(2)$ = 2.21, *p* = .330].

Table 5.2. Mean dwell time of participants (in ms) and standard deviations (in brackets) per Emotion, Block, Condition and Time Point. Participants are spending less time fixating the negative non-social stimuli across the two blocks compared to the social-related emotional stimuli.

	Block 1						**Block 2**					
	Social			**Non-Social**			**Social**			**Non-Social**		
	T1 Mean (SD)	**T2 Mean (SD)**	**T3 Mean (SD)**	**T1 Mean (SD)**	**T2 Mean (SD)**	**T3 Mean (SD)**	**T1 Mean (SD)**	**T2 Mean (SD)**	**T3 Mean (SD)**	**T1 Mean (SD)**	**T2 Mean (SD)**	**T3 Mean (SD)**
Negative	67	765	513	94	649	289	-25	346	97	2	92	-29
	(143)	*(503)*	*(617)*	*(166)*	*(613)*	*(730)*	*(110)*	*(512)*	*(603)*	*(105)*	*(488)*	*(606)*
Positive	57	867	561	48	956	759	-3	385	219	9	441	278
	(136)	*(411)*	*(443)*	*(173)*	*(423)*	*(557)*	*(100)*	*(442)*	*(504)*	*(93)*	*(419)*	*(490)*

Table 5.3. 5-HTTLPR genotype groups dwell time (in ms) and standard deviations (in brackets) per Emotion, Block, Condition, and Time Points. Carriers of at least one Short allele are spending less time fixating negative stimuli overall, across blocks, different which is more pronounced for the non-social threat stimuli.

		Block 1						**Block 2**					
		Social			**Non-Social**			**Social**			**Non-Social**		
		T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
5-HTTLPR													
L/L	Negative	80	905	719	129	945	617	-7	368	117	8	296	178
		(149)	*(448)*	*(586)*	*(207)*	*(602)*	*(626)*	*(140)*	*(500)*	*(514)*	*(74)*	*(401)*	*(543)*
	Positive	44	925	680	79	1065	877	23	501	274	35	479	225
		(163)	*(398)*	*(403)*	*(228)*	*(331)*	*(516)*	*(116)*	*(385)*	*(434)*	*(110)*	*(428)*	*(499)*
S/-	Negative	61	691	403	75	492	115	-34	335	87	-1	-16	-139
		(141)	*(521)*	*(614)*	*(140)*	*(567)*	*(730)*	*(92)*	*(526)*	*(653)*	*(118)*	*(501)*	*(616)*
	Positive	64	836	497	32	898	697	-16	324	189	-5	420	306
		(122)	*(420)*	*(456)*	*(137)*	*(459)*	*(575)*	*(89)*	*(464)*	*(542)*	*(82)*	*(419)*	*(491)*

Table 5.4. BDNF genotype groups dwell time (in ms) and standard deviations (in brackets) per Emotion, Block, Condition, and Time Points. No significant variations between the two genotypes observed.

		Block 1						Block 2					
		Social			**Non-Social**			**Social**			**Non-Social**		
		T1 Mean (SD)	**T2 Mean (SD)**	**T3 Mean (SD)**	**T1 Mean (SD)**	**T2 Mean (SD)**	**T3 Mean (SD)**	**T1 Mean (SD)**	**T2 Mean (SD)**	**T3 Mean (SD)**	**T1 Mean (SD)**	**T2 Mean (SD)**	**T3 Mean (SD)**
BDNF Val^{66}Met													
V/V	Negative	83	825	527	132	669	295	-40	398	149	5	181	86
		(134)	*(458)*	*(503)*	*(171)*	*(597)*	*(701)*	*(94)*	*(494)*	*(492)*	*(115)*	*(387)*	*(451)*
	Positive	111	966	546	85	938	762	-5	399	198	11	472	338
		(111)	*(433)*	*(481)*	*(160)*	*(427)*	*(617)*	*(101)*	*(466)*	*(525)*	*(95)*	*(330)*	*(438)*
M/-	Negative	49	692	495	47	625	281	-6	283	35	-1	-18	-170
		(154)	*(555)*	*(747)*	*(150)*	*(645)*	*(781)*	*(127)*	*(539)*	*(723)*	*(93)*	*(580)*	*(741)*
	Positive	-9	745	579	3	978	756	0	369	245	7	403	203
		(138)	*(355)*	*(401)*	*(182)*	*(426)*	*(487)*	*(100)*	*(422)*	*(488)*	*(94)*	*(513)*	*(549)*

5.7. Discussion

The present study was designed to examine the relationships between 5-HTTLPR and BDNF Val^{66}Met genotypes, rates of early behavioural problems and visual scanning of emotional stimuli in young children. The results indicated that children with at least one Short 5-HTTLPR allele exhibited increased reactivity in response to threat-related stimuli with non-social component, compared to participants with two copies of the Long 5-HTTLPR allele. Conversely, participants with two copies of the Long 5-HTTLPR allele were found to spend significantly more time fixating the non-social negative stimuli. Contrary, variation on the BDNF Val^{66}Met genotype did not accounted for individual differences on the visual scanning of affective stimuli.

While this pattern of findings agrees with previous evidence that reported avoidance but not vigilance towards negative stimuli in anxious populations (e.g., Hermans *et al.,* 1999; Rohner, 2002), the present pattern of findings may also suggest the existence of reward-seeking behaviour in carriers of the high serotonin uptake-related Long 5-HTTLPR allele. More specifically, the pattern of the eye movement behaviour suggest that carriers of two copies of the Long allele, compared to carriers of the Short allele consistently spend more time fixating the different types of negative but also positive stimuli. This pattern of findings reaches significant levels during the processing of non-social negative stimuli. Taken previous findings that reported positive association between the presence of the Long 5-HTTLPR allele and novelty seeking behaviours (e.g., Strobel, Lesch, Jatzke, Paetzold, & Brocke, 2003) future research would be critical to be conducted to differentiate between neuropsychological indexes of vigilance-avoidance and reward and novelty seeking, as well as positive approach.

With regards to the evident effect of 5-HTTLPR genotype on the visual scanning of negative non-social stimuli, although the pattern of findings was in principle consistent with the study's hypothesis, the vigilance-avoidance pattern was not evident. In contrast, the results suggest that participants carrying at least one low serotonin uptake-related Short allele, compared to Long allele carriers, exhibited a consistent selective avoidance pattern of looking the non-social negative stimuli across the 3,000 ms trial. This pattern of findings is partially consistent with eye movement data in adults that reported that carriers of the Short 5-HTTLPR allele spent significantly less time fixating negative stimuli and subsequently fixated more at positive emotion scenes, pattern of findings which interpreted as an effort to regulate heightened reactivity to negative stimuli (Beevers *et al.,* 2010). Interestingly, the present study shows that the reduced looking pattern was specific to aversive stimuli with non-social component. This finding agrees with a range of observations with adults (Kirsch *et al.,* 2005; Prather *et al.,* 2001; Meyer-Lindenberg *et al.,* 2005) and children (Susa *et al*., 2008) that reported a differential role of non-social fear in uniquely generating affective responses. Therefore, it is possible that children as young as 4 years old that carry the plasticity-related Short 5-HTTLPR allele may look away from the threatening situation, instead to further explore the aversive stimuli, as a way to inhibit their emotional arousal.

At this point is worth highlighting that the differential susceptibility hypothesis has been influential in the field in terms of adopting a more flexible framework to describe sensitivity for psychopathology (Boyce & Ellis, 2005). Based on this account, the Short 5-HTTLPR allele is not considered as per-se risk allele but as a plasticity allele that can interact with environmental factors to predict behavioural outcomes for better and for worse (see also Section 3.2.1). More specifically, recent meta-analyses have confirmed that the Short 5-HTTLPR allele was associated with risk for negative outcomes when exposed to adverse environment, but were also found to be associated with more positive influences when supported by positive environments (see van IJzendoorn *et al.,* 2012). Therefore, the documented neuropsychological index of reduced fixation duration in response to negative non-social stimuli in young carriers of the Short 5-HTTLPR allele in the present study, would be important to be measured along with environmental influences later in life to confirm whether these associations may account as differential early markers for negative but also positive affectivity. To this end, future research that will include the investigation of gene-mediated behavioural outcomes in both typical and atypical development will eliminate the inconsistencies in the extant literature (LoBue & Perez-Edgar, 2014; Munoz *et al.,* 2010) and will shed light on the particular constructs of early reactivity and behavioural affectivity.

However, in contrast to the study's hypothesis, the results did not show any significant effect of the BDNF Val^{66}Met genotype on the visual scanning of affective stimuli (Montag *et al.,* 2008; Schofield *et al.,* 2008; Lau *et al.,* 2010) As reviewed earlier although research has shown that both 5-HTTLPR and BDNF genotype are involved in similar aspects of affective responses, and taken the documented effects of BDNF on visual scanning of faces on Chapter 4, there is a possibility that early in life, differing genetic mechanisms may drive reactivity in response to faces versus aversive scenes. To this end, the documented effects of the two candidate polymorphisms on emotion reactivity in the present young and unaffected sample of children may be due to complex, but poorly understood pathways that undergoing maturation during these sensitive periods. It has been shown earlier that early manifestation of visual scanning behaviour in response to emotions may relate with individual differences in emotion regulation. However, taking into account previous evident that highlight 5-HTTLPR as plasticity variant (e.g., see Belsky *et al.,* 2009) that may be associated with both negative and positive outcomes depending on the context, it is difficult to interpret whether the direction of more versus less time spent looking emotional stimuli may related with a specific kind of affectivity. Further developmental research is required to delineate the putative aging effects on reactivity and the potential role of neurobiological mechanisms that modulate reactivity.

In addition to the effect of 5-HTTLPR genotype in modulating visual scanning pathways in response to non-social negative stimuli, an interaction between serotonin genotype and gender was evident on the time course of viewing emotional stimuli, irrespectively of the valence. More specifically, compared to females carrying the Short allele and males with either the genotype, female with two copies of the Long 5-HTTLPR allele, spent significantly more time looking the emotional stimuli during T_2 and T_3. The current observation for gender by genotype interaction may suggest the existence of a gender-specific biological contribution in the visual scanning of emotions early in life that may relate to increased susceptibility for behavioural problems. However, taken that there is no conclusive evidence on the gender effects of affectivity early in life, as well as the sample size limitation of the study, the present pattern of findings need to be interpreted cautiously. Although, research has previously highlighted the existence of gender differences on the manifestation of affectivity, especially in relation to depression, where increased susceptibility for affective disorders has been found in females compared to males (Nestler *et al.,* 2002), the particular gender underpinnings that may influence susceptibility for affective disorders are currently unknown (e.g., for a review see Bale, 2006). This hypothesis requires further investigation.

Moreover, the results of the present study did not provide significant correlations between parent reports of early affective problems, especially anxiety traits, and average fixation duration to emotional pictures. The previously documented association between early affective traits and atypicalities on visual scanning behaviours in youths are not confirmed here (Martin, Horder & Jones, 1992; Vasey *et al.,* 1995; Vasey *et al.,* 1996; LoBue & Perez-Edgar, 2014). As highlighted earlier, the sample of children that was employed in the present study consists of a young unaffected population. Therefore, the regulatory mechanism of visual scanning may differ between healthy children, as in the study's sample, and affected subgroups (e.g., LoBue & Perez-Edgar, 2014). However, the fact that children in each genotype group were matched on their early affectivity rates, allows for the outcomes coming from the eye movement data to be uniquely attributed on the variations of the serotonin transporter-linked 5-HTTLPR polymorphism. Future research that will include diverse groups of children (e.g., ability, genetic profile, nurturing environment) and in multiple developmental ages, will be important to be conducted in the future to inform about the putative developmental pathways and constructs of early affectivity.

In addition to the genotype and behavioural implications, some additional effects were documented that are informative for the field of affective processing. More specifically, the evident Emotion by Time interaction suggested a preferential pattern in response to stimuli with positive valence. Most notably, it was shown children spent more time looking at the positive (especially non-social) stimuli relatively to the negative stimuli, a difference which was more pronounced between 1000-2000 ms of processing. Recent studies with young populations have shown that increased preferential looking at threat only manifests in a subset of anxious children, while non-anxious children exhibit a preferential looking for positively valenced stimuli as opposed to negative (Eldar *et al.,* 2012). Compared to adults, where a priority in processing negative stimuli is evident across studies, the documented increased fixation duration in response to positive stimuli in the sample of young children on the present study may be explained by immatureness in the inhibitory control system that is associated with the processing of threat (Morren *et al.,* 2003).

Conversely, the study reported lower visual scanning allocation in response to negative stimuli with non-social components (e.g. animals) on the later stage of visual scanning. This is consistent with a plethora of neurophysiological studies that highlighted the differential role of non-social fear in uniquely generating affective neural responses (Kirsch *et al.,* 2005; Prather *et al.,* 2001; Meyer-Lindenberg *et al.,* 2005). The present study further supports the existence of a potential differential effortful control mechanism early in life in response to environmental stressors that may underpin the evolutionary influence of defence when an individual is exposed in a dangerous environmental situation. Interestingly, in line with this finding the study also revealed a main effect of novelty during the visual scanning of previously seen affective stimuli. More specifically, the study showed that subjects exhibited a decrease on the preferential looking in response to emotions (independently of the valence) when the same/previously seen affective stimuli was presented on the second block matched with novel neutral stimuli. This suggested that children as young as 4 years old, were able to recognise the previously seen positive or negative affective stimuli and recruit the appropriate regulatory abilities to switch their eye gaze away from the familiar stimuli and explore the novel neutral stimuli. Taken together, the study suggests that children early in life are able to shift their eye gaze towards novel stimuli, and conversely to avoid explore the fearful stimuli especially the one associated with animals at the later stage of processing (2001-3000 ms).

Limitations

Taking into account the absence of previous empirical studies in the field, the purpose of the current study was to examine the contribution of normal genotype variations in the visual scanning of aversive stimuli, aiming to fill an existing gap in the extant literature. Therefore, it was beyond the remit of the present thesis to delineate the same patterns affectivity in children diagnosed with affective disorders. Future studies, however, that will investigate the visual scanning of fear in atypically developing populations, such as children with anxiety disorders would be necessary for the field. To this end, the results of the present empirical study can act as a springboard for future research in other populations and ages of children, to achieve a holistic perspective for the unveiling of the neurobiological underpinnings of early emotional reactivity. Especially considering populations of children that are affected from profound social interaction deficits, such as children with autism, anxiety, and rare genetic syndromes (e.g. Williams Syndrome) the investigation of the early manifestation of atypicalities on preferential looking, and whether these are disorder specific or not, will further delineate the development of early affectivity. To this end, taken that the study sample was relatively underpowered, further research using a larger sample is required to further delineate the complex interactions between genes, neural structures of emotional processing, and their importance as precursors of later maladaptive behaviour.

Future longitudinal investigations, that will focus on the long-term effects of the susceptibility patters for better and for worse outcomes, would be critical to be conducted in the future. In line with this claim, the cognitive models of child anxiety suggest that threat avoidance may maintain anxiety traits in children, since they are not developing the critical evaluation abilities for the formation of effective emotion regulation (Hudson & Rapee, 2004; Rapee, 2001).To this end, taken the absence of direct evidence coming from young populations in the field, the present findings fill an existing gap in the literature on the effect of 5-HTTLPR in preferential looking towards threat as early as the age of four, that may account as a first-stage contribution on the field that may inform future research on the role of gene-mediated risk mechanisms of affectivity in determining differential behavioural outcomes in the later life.

CHAPTER 6

General Discussion

6.1. Preface

The study in Chapter 5 investigated the effects of serotonin transporter genotype on visual scanning pathways in response to aversive scenes in a group of typically developing children aged between 4 and 7 years. The results uncovered associations between serotonin-related normal genetic variations and visual scanning patterns of non-social threat that may account as a first stage contribution on the neurobiological basis of early reactivity. In the current chapter, the results of all of the empirical studies presented in this thesis will be discussed and synthesised with the existing literatures, with a view to highlighting the impact of the current work as well asimplications for future research and directions in the area of vulnerability for affectivity and early behavioural problems.

6.2. Introduction

The development of emotion regulation is a critical ability for an individual's later psychological functioning, which is involved in the establishment of positive and negative affectivity patterns early in life (Fonagy & Target, 2008). In a similar vein, the developmental nature of affective disorders has been previously highlighted, where the early behavioural manifestation of affectivity in children has been identified as a reliable predictor of later psychological maladjustment (Leonardo & Hen, 2008). During recent years, there has been a line of research examining the complex neural, behavioural and genetic mechanisms involved in the manifestation of affective problems and psychopathology early in life (for a review see Moffitt, 2005; Caspi & Moffitt, 2006; Cummings, Davies & Campbell, 2000).

The literature review presented in this thesis highlighted that variations in experimental designs and samples characteristics (i.e., age, ability) can impede the development of a single, reliable, and conclusive framework in the field of early behavioural problems. Similarly, confusion surrounding the various terms and associated definitions used in the field to describe the constructs of early affectivity has further challenged this area of inquiry. Therefore, the broader aim implemented throughout this thesis was to review and carefully consider the existing theoretical concepts and relevant methods and measurements to most effectively examine early markers for behavioural problems. For example, the most significant theoretical proposals were selected to drive and frame measures of early affectivity. This was pursued through an effort to delineate potentially key neural, behavioural, and genetic constructs that might be involved on the early manifestation of behavioural problems.

Consistent with the overreaching aim of the thesis, four empirical studies were conducted. As part of the first two studies, EEG was employed in an effort to derive neurophysiological signatures of early behavioural problems (Chapter 2), and to investigate novel associations between genes, brain, and behaviour (Chapter 3) that may suggest the existence of gene-brain mechanisms which may relate to increased sensitivity for behavioural problems in early childhood. In the third and fourth studies, eye-tracking technology was utilized to investigate the genetic underpinnings of early reactivity in response to facial emotions and features (Chapter 4) and aversive emotional scene information (Chapter 5). Collectively, these empirical studies, described in the thesis, elucidate putative associations that may help delineate the nature of the manifestation of behavioural problems, and allow a first stage contribution towards the identification of novel neurobiological mechanisms that may drive early affectivity. Below, I discuss the main findings, strengths, limitations, and clinical implications of this research, with reference to the existing literature. Furthermore, implications for future research directions in the field of behavioural problemsand developmental psychopathology will be presented.

6.3. Main Findings

The present thesis had a broad research aim, where a range of methods and techniques were employed. To this end, the key results and implications will be considered within three domains: (1) neurophysiology of early behavioural problems, (2) serotonin influences of affective patterns of frontal activation, and (3) putative genetic markers of early emotional reactivity during early childhood.

6.3.1. Neurophysiological signatures of behavioural problems in early childhood

One of the key aims of this thesis was to critically review the existing empirical research on the development of behavioural problems, and the early neurophysiological underpinnings of behavioural problems, in both typical and atypical development. Most notably, in Chapter 2 it was highlighted that frontal EEG studies have classified the same clusters of behavioural problems differently in terms of negative or positive affectivity. For instance, externalizing behaviourshave been seen as a component of positive reactivity in some studies (Baving *et al.,* 2003) where in others has been perceived as a negative component of behaviour (Fox, 1991). This documented inconsistency in the definition of the different constructs of affectivity may generate a major definition issue in the field, and subsequently a considerable discrepancy in the literature that investigates brain-behaviour associations.

In light of these issues in the literature, the first empirical study of the thesis aimed to investigate the putative brain-behaviour associations that are present early in life by utilizing a novel frontal EEG experiment and by employing well-structured standardised parent-filled measures of early behavioural problems. Frontal EEG has been widely utilized in the past as a reliable index of affectivity in children, adolescents, and adults. More specifically, right asymmetry has been consistently associated with negative affectivity, whereas more left asymmetry with positive affectivity (for a review see Coan & Allen, 2003).

The results of the study confirmed the literature that suggests negative behavioural and neurophysiological affectivity (i.e., greater right frontal EEG asymmetry) is not only associated with internalizing problems (e.g., Fox, 1991), but may also infer the presence of externalizing problems (Baving *et al.,* 2003; Santesso *et al.,* 2006). Most notably, it was highlighted that the context in which aggression is expressed may be a key mediator of the negative or positive component of the manifested aggressive behaviour (Smits & Kuppens, 2005; Cooper *et al.,* 2007). Consistent with previous evidence with children, the study suggested that children exhibiting increased approach behaviours (who traditionally were associated with higher right frontal activation) are more likely to develop aggressive behaviours. This may be due to their difficulties in regulating their negativity-related affectivity that results from their approach-related aggressive behaviours (Smith & Bell, 2010).

In addition to the investigation of behavioural measures as a predictor of frontal brain activation, the utilization of frontal EEG as a trait versus state measure of affectivity was also examined in this thesis, by investigating the frontal asymmetries in response to social and non-social experimental conditions. To this end, it was hypothesised that negativity/withdrawn-related patterns of greater right asymmetry would be specific to social stimuli (as a way of inhibiting the emotional arousal of the social situation) and would be associated with elevated rates ofbehavioural problems in young children. In contrast to the expectations, the results indicated that frontal activation was independent of whether children were observing videos with social or non-social component, providing support for a utilization of the EEG as a trait measure. The patterns of these results also suggest that EEG activation early in life may indicate a non-disorder specific endophenotype of affectivity (Burnette *et al.,* 2011).

A number of methodological issues may also account for the findings in this study. In contrast to previous studies that utilized EEG as an index of temperament and used a similar, but more ecological valid social stimuli (Hane & Fox, 2006; Marshall *et al.,* 2002), the present study did not uncover a significant effect of the type of videos viewed on the modulation of frontal EEG activation. This pattern of findings is in favour of the literature suggesting a reliable trait, instead of state, utilization of frontal EEG activation. However, it is worth highlighting the evidence supporting that an EEG procedure itself can be experienced as an affective situation for some individuals, which may influence brain asymmetries accordingly (Blackhart *et al.,* 2006). To this end, children's patterns of brain activation that relate with negative and positive affectivity may be influenced by minimum environmental stimulation, when compared to adults.

Furthermore, it is known that resting EEG effects and associations are strongest with eyes closed, and a proportion of EEG studies employ this kind of baseline resting state condition, which helps draw better comparisons and conclusion across conditions. However, the fact children as young as 4 years old experience difficulties sitting with their eyes closed during the EEG assessment, the employment of a baseline condition would potentially result in increased risk for data loss, as well as in a final sample consisting by a group of children with specific abilities. Moreover, alternative reasons to explain the null effect of the social versus non-social videos in frontal brain activation may relate to the information included in the videos that may elicit more eye movement artefacts or activate more memories that may interfere with the passive viewing of videos. To this end, it is possible the information included on the videos to elicit specific memories in children that may affect frontal EEG activation. These cognitive processes have not been controlled here for their role in mediating frontal EEG activation; therefore, the role of other cognitive processes in frontal EEG activation during the processing of social versus non-social information requires further research.

6.3.1.2. Summary of the EEG signatures of early behavioural problems

The results of the study presented in Chapter 2 demonstrated proof of principle that early markers of emotional affectivity may be predicted from measures of relatively higher right frontal EEG activation. Whilst this index alone may not entail vulnerability for negative outcomes later in life, the evident associations between behaviour and frontal EEG activation that were demonstrated in the study prompt further utilization of frontal EEG in investigating the constructs of affectivity early in life. As shown later in Chapters 3, 4 and 5, the existence of complex gene, neurophysiology and behaviour-mediated mechanisms may further account for an index of later affectivity that can help to determine psychological outcomes later in life.

6.3.2. Serotonin influences on affective patterns of frontal activation

Previous studies with children (Santesso *et al.,* 2006; Baving *et al.,* 2003) and the results of the study in Chapter 2, have highlighted the patterns of frontal EEG asymmetries as putative endogenous markers for negative and positive affectivity in the later life (e.g., Schmidt *et al.,* 2009; for a recent discussion see Schmidt & Moscovic, 2013). More specifically, relatively more right frontal asymmetry has been associated with withdrawn-related behaviours and negative affectivity, whereas left frontal asymmetry with approach-related behaviours and positivity (e.g., Coan & Allen, 2003). A separate line of research has highlighted that normal genotype variations on the serotonin transporter polymorphism are also associated with the manifestation of a range of affective disorders; particularly depression. More specifically, the presence of the Short allele on 5-HTTLPR has been shown to be associated with the depressogenic effects of stressful events (Caspi *et al.,* 2003) and increased risk for affective disorders. Conversely, children with at least one Long allele have been found to manifest behavioural resilience against affective disorders, when compared to children homozygous for the 5-HTTLPR Short allele (L/S, L/L; Bogdan *et al,* 2014).

Interestingly, variations on the 5-HTTLPR polymorphism have not only been associated with risk (diathesis-stress model), but have been also associated with plasticity for both positive and negative outcomes depending on the context (differential susceptibility; Belsky *et al.,* 2009; Belsky & Pluess, 2009) as well as with unilateral positive outcomes (vantage sensitivity; Eley *et al.,* 2012). Based on the later account, it has been shown that the presence of the Short 5-HTTLPR allele was associated with low levels of neuroticism in face of positive events, when compared to other genotypes (Kuepper *et al.,* 2012). The individual differences in this increased possibility to benefit from positive experiences has been associated with behavioural, physiological and genetic variables, which have been previously described as per se “risk” or “vulnerability” factors in the literature (for a review see Pluess & Belsky, 2013). To this end, there is an increasing consensus in the field to support that these factors may be required to reconceptualised as plasticity markers (Belsky *et al.,* 2009; Belsky & Pluess, 2009).

Taking into account the above evidence, a second key aim of the thesis was to investigate the genetic influences of children’s behavioural and neurophysiological patterns of early affectivity, as recorded from frontal EEG activation and parent reports of early behavioural problems. The novel findings that resulted from these investigations contribute to further delineating the complex neurobiological mechanisms that may be associated with the development of positive and negative affectivity in early childhood.

The study in Chapter 3 showed that the presence of two copies of the 5-HTTLPR Short allele, associated with low availability of serotonin uptake, were also strongly associated with the negativity-related greater right frontal asymmetry. Interestingly, control analyses with the COMT Val^{158}Met genotype or the parietal region did not show significant patterns of findings.The findings suggest the existence of an associative mechanism that may relate to the early manifestation of affectivity and behavioural problems early in life. These findings extended preliminary fMRI studies in adolescence suggesting a link between serotonin availability and activation over the dorsolateral frontal region (Wiggins *et al.,* 2012). Similar pattern of findings was also found in a recent EEG study with adults (Papousek *et al.,* 2012). Especially, considering the developmental stage of the study’s sample; these results may suggest the existence of vulnerability patterns of affectivity well before the trait or risk for a psychopathology may be diagnosable, which can act as a unique prognostic value for future research.

Together with the existing findings in adolescents (Wiggins *et al.,* 2012) and adults (Papousek *et al.,* 2013), the outcomes of this investigation open up a possibility for the existence of early mechanisms that may place an individual in higher vulnerability for affective disorders through the existence of neurobiologically determined cognitive and behavioural tendencies that are present early in life. However, taken the increased consensus in the literature suggesting the existence of 5-HTTLPR by environment interactions with differential behavioural outcomes (e.g.., Belsky & Pluess, 2009), it would be necessary for the documented gene-brain associations here to be considered with caution. It is possible that complex gene by brain interactions during sensitive periods of development form plasticity mechanisms that in combination with positive and negative environmental exposures later in life may generate differential behavioural outcomes. The findings of the study can act as a springboard for further investigation and replication of this novel hypothesis early in life.

6.3.2.2. Summary of the serotonin variation influences on frontal EEG

The results of the study have identified novel mechanistic relationships between normal genetic variations that determine serotonin uptake and patterns of affectivity of frontal EEG activation early in life. These findings have important implications for both the theoretical and clinical understanding of early manifestation of behavioural problems during early childhood. The study provided proof of principle that neurobiological markers that have been previously independently associated with plasticity for negative and positive behavioural outcomes are actually associated to one another in a mechanistic context. To this end, the study is consistent with the evidence that underscored frontal EEG asymmetries as a critical endogenous factor (Schmidt *et al.,* 2009). This factor may interact with the genetic mechanisms of plasticity and depending in contextual contributions to lead to better or worse outcomes later in life.

6.3.3. Genetic markers of emotional reactivity in young children

A final aim of this thesis was to investigate the genetic underpinnings that may relate to increased reactivity in response to different types of emotional stimuli in a group of typically developing young children.Two separate eye-tracking investigations were conducted aiming to unveil the effects of normal variations in the BDNF Val^{66}Met and 5-HTTLPR polymorphisms that may influence the processing of faces as well as the visual scanning of affective/aversive scenes.

6.3.3.1. Genetic influences of face processing early in life

Eye-tracking measures have been widely employed in the last two decades, with research showing that recording of eye-movements in response to affective stimuli may be a reliable neuropsychological index related with the presence or increased risk for psychopathology (e.g., Armstrong *et al.,* 2010). More specifically, biased visual scanning of emotional stimuli from faces has been widely reported as a reliable neuropsychological index for affective disorders (e.g., Calvo & Avero, 2005; Rohner, 2002), such as depression and anxiety. In a similar vein, by employing eye-tracking technologies, research has examined the role of early atypicalities of visual scanning of emotions as an index for risk versus resilience for psychological problems during development (e.g., Pine *et al.,* 2009).

From a neurobiological perspective, previous research with children and adolescents has suggested heightened reactivity in carriers of the neuroplasticity low activity Met BDNF Val^{66}Met allele in response to negative environmental stressors (Scharinger *et al.,* 2010; Gerritsen *et al.,* 2011; Montag *et al.,* 2008; Schofield *et al.,*2009; Lau *et al.,* 2010). The BDNF Val^{66}Met polymorphism has been widely associated with modulation of emotional regulation and affective processing (e.g., Joffe *et al.,* 2009; Lang *et al.,* 2007). The study presented in Chapter 4, by employing a novel eye-tracking investigation of preferential looking of emotional faces (i.e. angry versus happy versus neutral), provided evidence for the existence of visual scanning pathways that relate to increased reactivityin response to facial expressions of anger determined by variations on the BDNF Val^{66}Met polymorphism.

More specifically, the findings of the study confirmed the role of the Met allele as a moderator of affectivity-related behaviours early in life (Beevers *et al.,* 2009; Gatt*et al.,* 2009; Kretschmer *et al.,* 2014). Met allele carriers were found to look away from the negative facial expressions, probably as a way to inhibit the arousal that the stimuli generated to them. In a methodological progression that allowed the reliable measurement of the time course of preferential looking across different emotional facial expressions, the patterns of these findings provided support for the vigilance-avoidance model of visual scanning. According to this model, individuals initially spend more time scanning the affective stimuli, but later look away as a way to inhibit the arousal that the stimuli provoke to them. This is the first study with children to show that early behavioural patterns of reactivity-related visual scanning pathways may be modulated by normal variations in genetic mechanisms related to neuroplasticity.

However, this pattern of findings may not be only interpreted in the basis of vigilance-avoidance for the low plasticity Met-, as it can be argued that the effects may be driven from the high plasticity Val/Val genotype group which was shown to exhibit an increased interest towards exploring the negative facial expressions, without switching their eye gaze away to explore the neutral stimuli in the trial. Although there is evidence to suggest the involvement of the BDNF Val^{66}Met polymorphism in modulating responses to environmental stressors (Scharinger *et al.,* 2010; Gerritsen *et al.,* 2011; Montag *et al.,* 2008; Schofield *et al.,* 2009; Lau *et al.,* 2010), it is not yet clear from the present findings how the increased time spent looking the angry faces relative to the neutral in the high plasticity Val/Val allele relates with the modulation of neural pathways that involved in emotion reactivity. In a similar vein, taking into account evidence highlighting a differential involvement of the BDNF genotype on both positive and negative outcomes (e.g., Drury *et al.,* 2012) it is not yet clear from the current investigation, or other available evidence in the literature, whether the spending of more versus less time exploring the affective stimuli may link with a neuropsychological behaviour associated with per se risk or resilience for affective problems. Future research is needed to investigate how environmental influences may account for the manifestation of better and worse outcomes later in life.

In addition to the effects of the BDNF Val^{66}Met genotype in the processing of emotional faces, an additional investigation of the study revealed effects of the 5-HTTLPR genotype on children's eye gaze towards neutral facial features. The 5-HTTLPR polymorphism is part of the promoter region of the serotonin 5-HTT gene that is involved in serotonin uptake with recent meta-analytical studies highlighting the polymorphism's involvement in modulating amygdala reactivity in response to negative or arousing environmental conditions (Munafo *et al.,* 2009; Murphy *et al.,* 2013; Walsh *et al.,* 2012). The study showed that carriers of at least one low serotonin uptake-related Short allele spent significant less dwell time looking at the eyes region of neutral faces and more time looking at the mouth region. In contrast, children homozygous for the Long allele spent more time looking at the eyes region and less on the mouth region.One possible explanation for the observed pattern of looking behaviour is that Short allele carriers diverted their eye gaze away from the eye region of neutral faces, and turned their attention away to looking the mouth region of the face, perhaps as a compensatory mechanism to down-regulate heightened reactivity when processing the eyes region. Conversely, Long allele homozygotes may be less reactive to socially demanding stimuli, and therefore have less of an urge to switch their eye gaze towards the mouth region of the face (see also Beevers *et al.,* 2011).

The possibility that 5-HTTLPR Short allele carriers, known to experience higher vulnerability for poor reactivity to distressing negative emotional cues, may help to link with the literature that suggests that reduced looking to the eye region is evident in individuals with social anxiety (Crawford *et al.,* 2015; Farzin *et al.,* 2009). However, although the sample size and size of effects is similar to the ones previously reported, the present pattern of genetic findings needs to be interpreted cautiously. It has been previously showed that the 5-HTTLPR Short allele can act as a plasticity factor (e.g., for a review see Pluess & Beslky, 2013), which in conjunction with other context-specific factors, such as life events, may differentially increase the risk versus resilience for later affective problems. This hypothesis requires further investigation, which will potentially incorporate the longitudinal measurement of behavioural outcomes.

6.3.3.2. Genetic influences of processing of aversive scenes early in life

Reactivity in response to threatening stimuli of the environment is a critical component of affectivity, with research suggesting the existence of a specific evolutionary component in relation to threat and the immediate responses, which is provoked in humans. Compared to the emotional reactivity that is related to visual scanning of emotional faces (Chapter 4), the processing of affective stimuli may inform for a separate aspect of affectivity that is related with the facilitation of immediate reactive response when an individual is exposed in a threatening environmental condition. In line with this claim, existing literature in children suggests the existence of atypical vigilance-avoidance patterns of visual scanning of affective pictures in children with separation anxiety compared to controls (In-Albon et al., 2010). However, the common or differing neurobiological constructs that may be involved in the affectivity concerning social (facial emotions and features) or non-social aversive processing are not yet known. To this end, the empirical study conducted in Chapter 5 aimed to investigate the neurobiological underpinnings of the visual scanning patterns of reactivity in response to threat. Moreover, to further clarify and investigate whether the documented neurobiological patterns of affectivity that are related to facial processing, as observed in Chapter 4, represent face-related reactivity or whether distinct fear-related neuropsychological pathways of reactivity exist, the study conducted in Chapter 5 was done with the same population of children.

Similar to the patterns of recent studies highlighting the effects of serotonin transporter-linked 5-HTTLPR polymorphism in early affectivity in young populations (Bogdan *et al.,* 2014) and adults (Beevers *et al.,* 2010), the study presented in Chapter 5 showed associations between the plasticity-related 5-HTTLPR genotype and visual scanning of aversive scenes with non-social component (i.e., aggressive animals). Interestingly, contrary to the emotional face processing investigation, a control analysis with the BDNF Val^{66}Met genotype groups for the aversive processing investigation did not provide significant differences for visual scanning for any time of the affective stimuli used. More specifically, carriers of at least one Short allele, when compared to homozygotes for the high uptake Long allele, spent significantly less time looking for the aversive non-social stimuli. This is probably because of a gene-influenced behaviour pattern, which serves to suppress the arousal that the exposure to the negative stimuli elicits. Conversely, Long allele homozygotes were found to spend significantly more time processing the non-social aversive scenes that suggests the existence of a serotonin-induced visual scanning of threat through the detailed exploration (instead avoidance) of the negative stimuli.

However, it is not yet understood whether the documented genetic influences in early reactivity affect the behavioural arousal response, or if there is an influence that is specific to thenature or the size of this behavioural arousal. Future research that will employ the same experiment, but will manipulate the stimuli’s presentation time in groups of participants at different developmental stages, may shed light on this issue. Moreover, it not yet clear why the effect of the 5-HTTLPR genotype emerge in response to non-social, but not social threatening stimuli. A possible explanation may relate to the subcortical neural pathways such asamygdala function that has been shown to be a key component for social processing (Adolphs, 2009; Lieberman, 2007; Vuilleumier & Pourtois, 2007). Detection of threat has an evolutionary component that may be critical for survival (Green & Phillips, 2004). Taken together, the evidence effect of 5-HTTLPR genotype on the processing of non-social threat early in life may relate with complex, but poorly understood, serotonin-induced neural pathways. This area of inquiry requires further delineation.

Although behavioural evidence exists on the differential role of non-social fear in uniquely generating affective responses (Kirsch *et al.,* 2005; Prather *et al.,* 2001; Meyer-Lindenberg *et al.,* 2005), this is the first known study in child, adolescent, and adult psychopathology to show the moderating effect of 5-HTTLPR polymorphism in the visual scanning of non-social fear. The study adds to the existing evidence of genetic influences of preferential looking towards and away from aversive non-social stimuli are present early in life, suggesting a genetic mechanism that may act as a precursor for increased vulnerability for later emotional and psychological maladjustment. However, taken the increasing literature to describe 5-HTTLPR polymorphism as a plasticity variable, where depending on the context to be involved in both better and worse outcomes, it would be critical for future research to examine how the early neuropsychological mechanisms of reactivity as documented on the present study may interact with positive versus negative environmental influences to predict outcomes later in life.

6.3.3.3. Summary of the genetic markers of early emotional reactivity

The findings of the study on the serotonin effects on visual scanning of aversive scenes are of particular interest when compared to the findings of the earlier face processing study. While the first face processing study provided novel insights in the existing adult literature for the involvement of the BDNF Val^{66}Met genotype in the processing of angry versus happy faces early in life, it has also provided an involvement of the 5-HTTLPR genotype in looking behaviour towards facial features important for effective social interaction. Moreover, although this second finding itself is of significant importance for the field, the second study on the processing of aversive stimuli adds that the same plasticity-related polymorphism may also modulate fear-related reactivity in a different context. This is important for the field of child, adolescent and adult psychopathology, highlighting for the first time that related, in terms of valence, yet differing experimental stimuli may contribute to the manifestation and potentially establishment of social-related difficulties and non-social threat-related reactivity. Likewise, the alongside investigation of the processing of emotional faces and aversive scenes in the same population of children strengthens the reliability of the outcomes suggesting the putative existence of common serotonin-mediated neural pathways for regulation of the reactivity in response to different types of experimental stimuli.

The results of the two eye-tracking studies provide first-stage contributions on the neurobiological influences of early reactivity in response to environmental stressors. From a developmental perspective, taking into account the previous behavioural data that indicate atypical patterns of preferential looking of emotional faces in children at increased risk for affective disorders (Battagglia *et al.,* 2004; 2005), additional research on the potential for a mechanistic interaction between neuropsychological measures of emotional reactivity through eye-tracking may aid in the identification of the early cognitive, behavioural, and genetic precursors and potential markers for maladaptation. Interestingly, a recent eye-tracking study has shown that children with separation anxiety disorder reduced their vigilance pattern of visual scanning of negative facial expressions after they received Cognitive Behavioural Therapy (CBT; In-Albon & Schneider, 2012). Therefore, the early manifestation of atypicalities in visual scanning of emotions may be a significant neuropsychological marker of affectivity that may aid not only in the early identification of the individuals at increased risk for affective disorders, but also facilitate the development and implementation of early therapeutic interventions.

The amygdala is a core neural mechanism that is involved on the modulation of reactivity. To this end, the employment of eye-tracking as a neuropsychological index of the activation of neural structures, such as amygdala, may be critical on the identification of those at increased risk for the development of psychological problems early in life. Especially considering scientific approaches that employ multimodal measures such as brain, genome, and behaviour, future research may be of vital importance for the effective identification of individuals that are at familiar risk for the development of a particular set of symptoms. Moreover, such scientific approaches may be able to inform about the effectiveness of particular interventions that target the treatment of behaviour with pre- and post- intervention eye-tracking assessments, and how this impacts upon neural structures and neuropsychological reactivity.

6.3.4. Overall summary

As part of the present thesis, four empirical studies were conducted. The studies investigated the neural, behavioural and genetic underpinnings of affectivity in early childhood. Although the individual studies had a unique design and scope, it would be also interesting to have an overall overview of the importance and significance of the thesis research outputs.

Most importantly, the present thesis highlighted the particular role of the variations in 5-HTTLPR on various neuropsychological aspects that link to early reactivity and affectivity. More specifically, 5-HTTLPR has been shown to modulate individual differences in frontal brain activation, and in a sub-group of the same population to modulate eye gaze duration in response to angry faces as well as non-social affective stimuli, in a separate investigation. Examining the neurobiological underpinnings of reactivity using a variety of techniques in the same population may be a useful tool to delineate the exact nature of early susceptibility for affective problems. The evidence of the present thesis highlights that common serotonin-mediated neural pathways, may produce the same neurophysiological reactions under different experimental conditions. Collectively, future research will answer whether the observed effects of the 5-HTTLPR polymorphism with frontal brain and eye gaze patterns of reactivity may account as reliable endophenotype markers for later behavioural outcomes.

While the EEG study in Chapter 3 examined frontal brain patterns associated with negative and relative affectivity, the observations in Chapters 4 and 5 where looking for the same patterns of affectivity as reflected in eye gaze in response to affective stimuli. To this end, if a need existed to collapse the research outputs across the different observations, it would require great caution, as different methodologies, timing and analytical procedures where employed for each investigation. Moreover, for the later eye-tracking observations only a sub group of children was studied after an average period of 6 months from the original EEG study. To this end, it is not yet clear, how these overlapping effects can be solely attributed to the genotype effect, as other environmental event may have contributed on the multiple 5-HTTLPR effects. Future cross-sectional studies with consistent sizes of young samples would be required to further delineate this area of inquiry.

The evident gene-related effects on brain functioning and reactivity may suggest that early mechanisms of affectivity may exist that may place some individuals in higher risk versus resilience for psychopathological problems. Taking into account previous evidence conceptualising both the 5-HTTLPR and BDNF Val^{66}Met polymorphism as a plasticity variable, it would be useful the current research outputs to be replicated in longitudinal studies that involve the measurement of context-specific influences on the development of better and worse outcomes. Therefore, while the documented EEG by Genotype or Eye gaze by Genotype associations may suggest a type of behaviour that can be linked with existing models of reactivity, it would be critical future studies to include longitudinal investigation of environmental influences into generating behavioural outcomes for better and for worse.

In addition, across the four empirical studies of the thesis, standardised parent reports of children's behavioural problems were employed. The results show that elevated rates of behavioural problems were associated with patterns of frontal brain activation (Chapter 2), which was not replicated in a larger sample (Chapter 3) and did not provided associations with eye gaze patterns towards types of emotional stimuli (Chapters 4 and 5). It is possible that early in life behavioural associations with reactivity may exist but were not documented in the present thesis. This may relate to the behavioural measures employed, or other potential sample characteristics, e.g. typically developing children (instead of children with a particular set of symptoms), ethnical homogeneity of the sample (instead of a more culturally diverse). Future research is required to delineate this area of inquiry, that ideally will include various methods of measuring behavioural manifestations, such as a range of standardised parent and self-report measures, as well as observations. To this end, it is likely that other behavioural characteristics, such as parental well-being, or life events (e.g. school transitions) to account as confounding variables in this area of research, but here have not been tested. There is research that needs to be conducted to test this novel hypothesis.

It is anticipated that the empirical evidence describedin this thesis will act as a springboard for the direct testing of several novel research hypotheses related to potential mechanistic interactions between genes, brain, and behaviour early in life that may contribute to the current understanding and determination of the neurobiological basis of the manifestation of early reactivity and psychological maladjustment. Together, this and future research on this topic may also lead to the development of new theoretical understandings related to vulnerability and protection for psychopathological problems. Furthermore, similar investigations employing the methods developed and utilised in this thesis for the study of atypical populations may aid in the development of targeted interventions for the treatment of those at increased risk for, or experiencing, affective disorders. For this to be effective, it is vital the measurement of the effect of positive versus negative environmental contexts on better and worse outcomes to be incorporated. Future research that will implement novel therapeutic approaches in individuals that are identified as vulnerable early in life may be a reliable approach for tackling the prevalence of affective disorders in the society. For these approaches to be effective, the complex interactions among behaviour, environment, and neurobiology need to be carefully considered and studied.

6.4. Limitations and Strengths of the Research

Whilst the main findings of the thesis provide novel and experimentally valuable information and insights on the neurobiological basis of early emotion reactivity and psychological maladjustment, a number of limitations also need to be acknowledged. Most notably, throughout the thesis children's early manifestation of behavioural problems was measured via parents' reports of early affectivity rather through direct and structured observations of children's behaviour. Thus, this aspect of all of the studies described may lack part of the ecological validity that an observational method can provide. Moreover, throughout the thesis,the main evidence that was examined as affectivity-related from the empirical studies of the thesis was quantified through the investigation of indexes of endophenotypic diversity, by employing EEG and eye-tracking technologies.Longitudinal observations of the mechanistic associations between neurophysiology, genes, behaviour and the environmental context would be necessary to be conducted in the future, to inform about the validity of such associations in predicting behavioural outcomes. Likewise, in combination with the very limited available evidence in child literature, the comparison and replication of the findings of the present thesis with previous studies is made difficult. However, through the integration of genetic, behavioural and neurophysiological investigations across the thesis, a comprehensive examination of the field was allowed by suppressing the possibility for false-positive effects. The consistent utilization of this approach may further ensure the future high levels replication validity of the empirical studies denoted in this thesis.

Moreover, although the two separate eye-tracking investigations provided evidence for the involvement of 5-HTTLPR genotype on patterns of early affectivity, a potential alternative explanation for the differentiated 5-HTTLPR effects between the two studies is necessary to be acknowledged. More specifically, it was shown that 5-HTTLPR genotype effects were evident for the processing of the eyes region of neutral faces and for the processing of non-social aversive scenes, but not for the time-course of processing emotional faces. Although this pattern of findings may be due to the previously documented increased amygdala reactivity in carriers of the short 5-HTTLPR allele (Munafo *et al.,* 2008; Murphy *et al.,* 2013; Walsh *et al.,* 2012), variations in the experimental structure and stimuli used between the two eye-tracking experiments may also have critical contribution to the manifestation of young children's reactivity. In addition, it is possible that maturational factors may drive the differential response of the same genotype group across different types of stressors. Due to the absence of developmental evidence in the field, it is difficult to infer conclusions of why children at the age of the sample may provide this differential response. This hypothesis requires further investigation.

Finally, across the thesis there was a consistent weakness of sample size. However, compared to previously published neurophysiological and behavioural studies examining the role of candidate genes in youth, the empirical studies utilized larger or equal samples. Moreover, through the employment of a fine grained and hypothesis-driven statistical strategy for each investigation, including comparison control analyses, the validity of the results has been further enhanced. It would be important to highlight, however, that sample sizes for genetic studies of the kind that conducted in the present thesis, is a consistent issue in the field. More specifically, replication problems have been previously highlighted in candidate gene studies (e.g. Gillespie, Whitfield, Williams, Heath, & Martin, 2005; Surtees *et al.,* 2006) that may contribute to slowing down the delineation of the biological underpinnings of human affectivity. To this end, and taking into account the underpowered sample sizes across the empirical studies conducted in the present thesis, it would be vital to increase the sample size in future research in the field. Moreover, in keeping the methodological procedures and the sample characteristics consistent (e.g. age, ancestry, cognitive abilities), this may also help to tackle replication difficulties in the field.To this end, throughout the thesis attempts were made to delineate the theoretical background in the field, where various empirical evidence, techniques and approaches were taken into account. This allowed the generation of novelinsights, but also novel research questions for the field.

A key strength of the research conducted in this thesis is that it was driven from the critical need to the field to unveil the complex neurobiological pathways and associations that may relate to the manifestation of affectivity-related patterns of behaviour later in life. Despite the increasing evidence in normal and affected adult populations, little focused research with young populations had been conducted in the field so far. The empirical studies presented in the present thesis were conducted in an ethnically homogenous sample of young children. Previous evidence have highlighted that the absence of ethnical homogeneity in the studied samples in genetic study, may have generated discrepancy in the field (for a recent protocol review see Culverhouse *et al.,* 2013). Together, the findings of this thesis offer valuable and exciting first-stage contributions to our understanding on the putative effects of the complex associations between genes, brain, and behaviour and generate novel research questions on the individual differences that may drive risk versus resilience for affective problems later in life.

6.5. Future Directions

As a result of the research in this thesis, a number of future key research areas can be identified. Firstly, a longitudinal study that begins early in life, where the temperamental formation takes place would be necessary to be conducted in the future. This research should evaluate the developmental trajectories that may contribute to the complex G×E interactions of positive and negative affectivity the earliest possible in life. Moreover, future studies in this area would require careful selection of various methodologies and the recruitment of substantial samples of both typically, but also atypically, developing young populations. To this end, the utilization of EEG and eye-tracking methods in a comprehensive framework that accounts for phenotypic, endophenotypic and genotypic diversity would aid in the development of interventions for those at increase vulnerability for affective disorders.

Linking neuropsychological data with biological or other behavioural data may be a very useful tool not only for the early identification of young children at increased risk for psychological problems, but also for understanding the nature of different psychopathologies and designing tailored interventions. These tailored interventions may target the atypical behaviours that are identified as precursors of a particular psychopathology and apply a therapeutical approach to modify these behaviours early in life by applying cognitive and behavioural approaches. If this protocol can be applied in both pre- and post-interventionbasis, the putative differential responsiveness of the applied intervention in differing populations can be effectively and reliably determined. For instance, previous evidence has shown differential susceptibility in institutionalised young children, where children carrying at least one copy of the low uptake BDNF Met allele and two copies of the low serotonin uptake 5-HTTLPR Short allele exhibited most indiscriminate behaviour when placed in the usual care but the least indiscriminative in enhanced caring environment (e.g., Drury *et al.,* 2012). This evidence further suggests the importance of the genetic influences and their impact on other constructs of affectivity (i.e., neurophysiology, behaviour) in response to environmental modifications.To this end, future research would be critical to further delineate the complex G×E interactions or even the complex interaction among different genetic systems (i.e. Gene×Gene interactions; epistasis) when aiming to aiming to delineate what works for whom.

From a methodological perspective, there are specific aspects of employing EEG and eye-tracking technologies in clinical practice that may further enhance the usefulness of the current empirical evidence of the study. Most notably, EEG and eye-tracking equipment are relatively inexpensive compared to fMRI that makes large-scale studies possible to be conducted in the future. In line with this claim, with the most hospitals and clinical settings nowadays to have EEG equipment available, future inexpensive investigations of early affectivity may be possible to be conducted across multiple sites. Moreover, despite the temporal and inferential weaknesses of both EEG and eye-tracking methodologies, the employment of these methods has been shown to be reliable indexes of individual neurophysiological variation, providing recording of brain activation or eye gaze swifts in milliseconds. Taking into account recent studies that highlighted the usefulness of using methods that measure blood-flow to access brain-derived mechanisms of affective disorders, such as fMRI (e.g., Savitz, Rauch & Drevets, 2013), the employment of multiple methods in the investigation of human affectivity may help to minimize the weaknesses of each of these methods and assist to measure more reliably the neural mechanisms that relate to human affectivity. For example compared to fMRI, EEG provides the ability to measure brain activation with the necessary temporal resolution, but it has limited spatial resolution. Conversely, fMRI provides highly accurate location of brain activity but with poor temporal accuracy (for a review see Menon & Crottaz-Herbette, 2005). In line with this claim, recent studies with youth have started to incorporate different methods under the same multimodal investigation. For instance, recent studies have combined fMRI with EEG (Schelenz *et al.,* 2013) or eye-tracking methods (Fan, Chen, Chen, Decety & Cheng, 2013) to inform about affectivity in response to environmental emotional stressors. Future developmental studies will be important to incorporate multimodal neurophysiological mapping of affectivity by employing large samples to delineate the particular constructs in neurophysiological and behavioural diversity.

From a genetic perspective, advancements in the field have highlighted the recent years that alterations in the biochemical process of DNA methylation may lead to alterations in the baseline transcription procedures of multiple genes and infer susceptibility for affective disorders (e.g., McGowan *et al.,* 2009; Booij *et al.,* 2013). Most notably, as Booij et al. (2013) highlight, DNA methylation is the most reliable epigenetic modification that can be observed in brain during development (e.g. in the case of childhood abuse), and therefore may be accounted for as a robust predictor for the manifestation of affective disorders later in life. There are still a lot to be discovered in this respect in the near future, and the research community need to pay extra attention on the outcomes of future advancement in the field and their importance in understanding individual variation in brain, mind and psychopathology.

Finally, given the significant implications that the early identification of the psychopathology precursors has for both social policy and clinical practice, research must focus on evaluating the strengths and weaknesses of the existing interventions that target early manifestation of affective disorders. The findings of this thesis have identified novel neural, behavioural, and genetic mechanisms that may provide a first-stage contribution towards understanding early affectivity and markers for behavioural problems. To this end, if a pragmatic argument is accepted that intervention for the treatment for behavioural problems is more effective during the early years of life, the present data may potentially contribute, in the near future, to the successful reduction of early affectivity through eventual application of individualised early therapeutic interventions.

6.6. Closing Summary

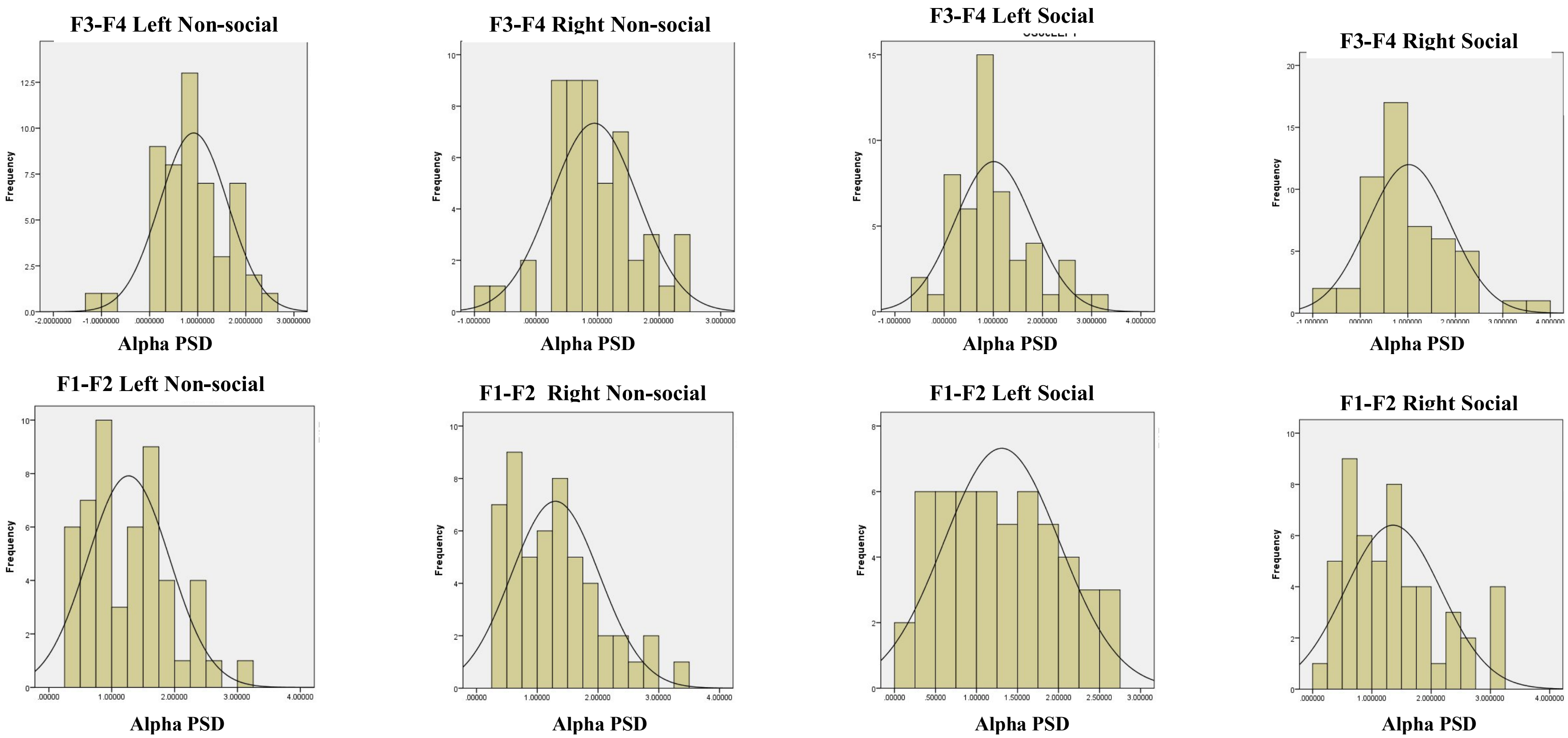
Whilst the present thesis has generated many potential new research inquiries in the neurobiological basis of early emotion reactivity and early onset behavioural problems, it has also directly given novel, direct, and robust answers to critical questions on the neurophysiological and genetic mechanisms involved on the individual differences in affectivity and early problematic behaviour.These findings show that differing neurobiological and behavioural precursors of affectivity exist early in life, and suggest that complex interactions among them may be critical for advancing our understanding of the manifestation of psychopathologies and affective-related behaviours later in life. Given that this thesis was broadly motivated by a critical need to further delineate the nature of early affectivity during early childhood, the current results suggest that it is important for the future direction of research to remain focused on examining the developmental constructs of vulnerability versus protection, acknowledging at the same time that further theoretical questions may still need to be addressed in the field. Once both empirical and theoretical components of research are investigated alongside, successful advances in the field of developmental psychopathology can be made in the near future with an ultimate goal to tackle the prevalence of affective disorders in the society.

APPENDIXES

Appendix 2. 1 CBCL 1 ½ -5 items for internalizing and externalizing scales.

Internalizing				Externalizing		
Emotionally Reactive	**Anxious/ Depressed**	**Somatic Complaints**	**Withdrawn**	**Sleep Problems**	**Attention problems**	**Aggressive behaviour**
Disturbed by change	Clings	Aches	Acts too Young	Not Sleep Alone	Concentrate	Can't Wait
Twitching	Feelings Hurt	Can't stand things out of place	Avoids Eye	Sleep Problems	Can't Sit Still	Defiant
Shows Panic	Upset by Separation	Constipated	Doesn't Answer	Nightmares	Clumsy	Demanding
Rapid Shifts	Looks Unhappy	Diarrhoea	Refuses active games	Resists Bed	Shifts Quickly	Destroys others'
Mood Change	Nervous	Doesn't Eat well	Unresponsive	Sleeps Less	Wanders	Disobedient
Sulks	Self-Conscious	Headaches	Little Affection	Talks in Sleep		No Guilt
Upset by new	Fearful	Nausea	Little Interest	Wakes Often		Frustrated
Whining	Sad	Painful BM	Withdrawn			Fights
Worries		Stomach aches				Hits Others
		Too Neat				Hurts accidentally
		Vomiting				Angry Moods
						Attacks
						Punishment
						Screams
						Selfish
						Stubborn
						Temper
						Uncooperative
						Wants attention

Appendix 2.2. Histograms illustrating the PSD values for each condition, hemisphere and region.



F3-F4 Left Non-social

F3-F4 Right Non-social

F3-F4 Left Social

F3-F4 Right Social

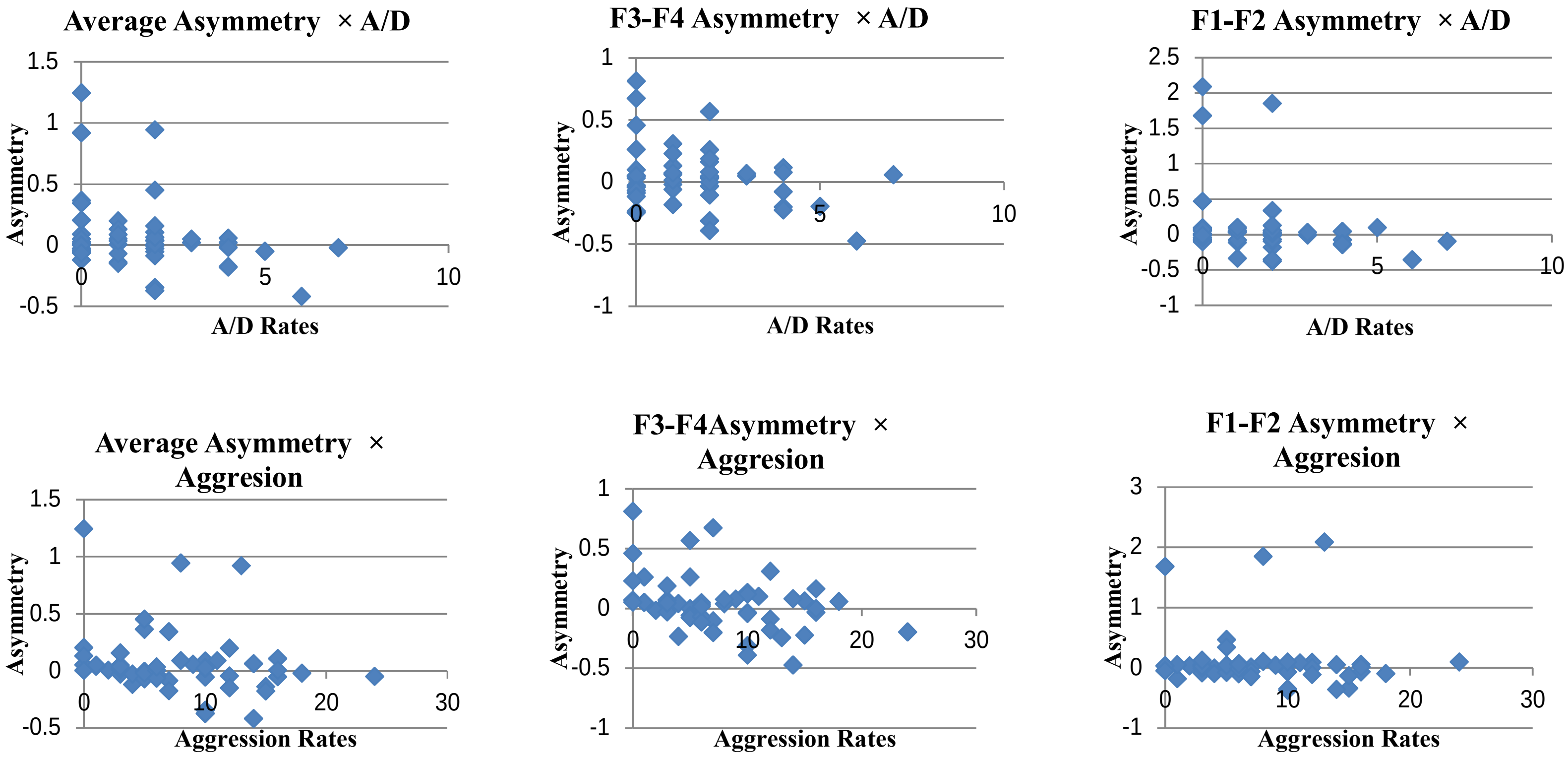
F1-F2 Left Non-social

F1-F2 Right Non-social

F1-F2 Left Social

F1-F2 Right Social

Appendix 2.3. Scatter plots illustrating correlations coefficients between behavioural problems and asymmetry ratios



Appendix 3.1. Artefact-free EEG data ,asymmetry frequencies and demographics per 5-HTTLPR and COMT Val^{158}Met genotype groups.

Table 1. Time (in minutes) of artefact-free EEG data after bad channel replacement per 5-HTTLPR and COMT Val^{158}Met genotype and condition.

SNP	**Social**	**Non-Social**
	Mean *(SD)*	Mean *(SD)*
5-HTTLPR		
L/L	3.43 *(0.13)*	3.09 *(0.16)*
S/L	*3.30 (0.13)*	3.09 *(0.14)*
S/S	*3.72 (0.15)*	3.44 *(0.22)*
COMTVal^{158}Met		
V/V	3.30 *(0.61)*	3.31 *(0.88)*
M/V	3.47 *(0.71)*	3.14 *(0.81)*
M/M	3.44 *(0.77)*	3.03 *(0.85)*

Table 2. Asymmetry frequencies in each genotype group.

SNP	**Asymmetry**	
	Left Asymmetry N*(%)*	**Right Asymmetry N*(%)***
5-HTTLPR		
L/L	17*(24.28)*	7 *(10)*
S/L	21 *(30)*	12 *(17.14)*
S/S	4 *(5.71)*	9 *(12.85)*
COMTVal^{158}Met		
V/V	7 *(10)*	8 *(11.42)*
M/V	26 *(37.14)*	15 *(21.42)*
M/M	8 *(11.42)*	6 *(8.57)*

Table 3. Participants' demographic characteristics by 5-HTTLPR genotype.

		5-HTTLPR Genotype			**ANOVA**		
		S/S	**S/L**	**L/L**	***F***	**df**	***P***
N		13	33	1			
Gender	% Male*(N)*	8.5 *(6)*	25.7 *(18)*	20.0 *(14)*	*.244*	2	*.784*
	% Female *(N)*	10.0 *(7)*	21.4 *(15)*	14.2 *(10)*			
Handedness	% Right *(N)*	14.2 *(10)*	37.1 *(26)*	31.4 *(22)*	*.955*	2	*.375*
	% Left *(N)*	4.2 *(3)*	10 *(7)*	2.8 *(2)*			
SCQ Total Score	Mean*(SD)*	4.76 *(3.34)*	3.96*(3.47)*	4.37*(2.55)*	*.323*	2	*.725*

Table 4. Participants' Cognitive abilities and developmental ages by 5-HTTLPR genotype.

		5-HTTLPR Genotype			**ANOVA**		
		S/S	**S/L**	**L/L**	***F***	**df**	***P***
Chronological Age	Mean*(SD)*	58.15*(11.38)*	60.78*(10.94)*	62.33 *(12.84)*	.537	2	.587
Overall Ability	Mean*(SD)*	103.71 *(8.91)*	105.90 *(9.10)*	106.81 *(8.11)*	*.533*	2	*.589*
Verbal Ability	Mean*(SD)*	100.53 *(12.55)*	110.81*(12.96)*	104.95*(11.52)*	*1.16*	2	*.318*
Non-verbal Ability	Mean*(SD)*	106.30 *(12.27)*	99.63*(14.69)*	109.50*(13.47)*	*5.60*	2	*.574*
Developmental Age (Months)	Mean*(SD)*	61.09 *(15.32)*	64.81 *(11.80)*	65.02 *(13.26)*	*.457*	2	*.635*
Developmental Verbal Ability	Mean*(SD)*	60.23 *(18.35)*	60.96 *(14.71)*	63.68 *(14.71)*	*.306*	2	*.737*
Developmental Non Verbal Ability	Mean*(SD)*	61.84 *(16.12)*	69.36 *(14.24)*	66.50 *(15.47)*	*1.28*	2	*.312*

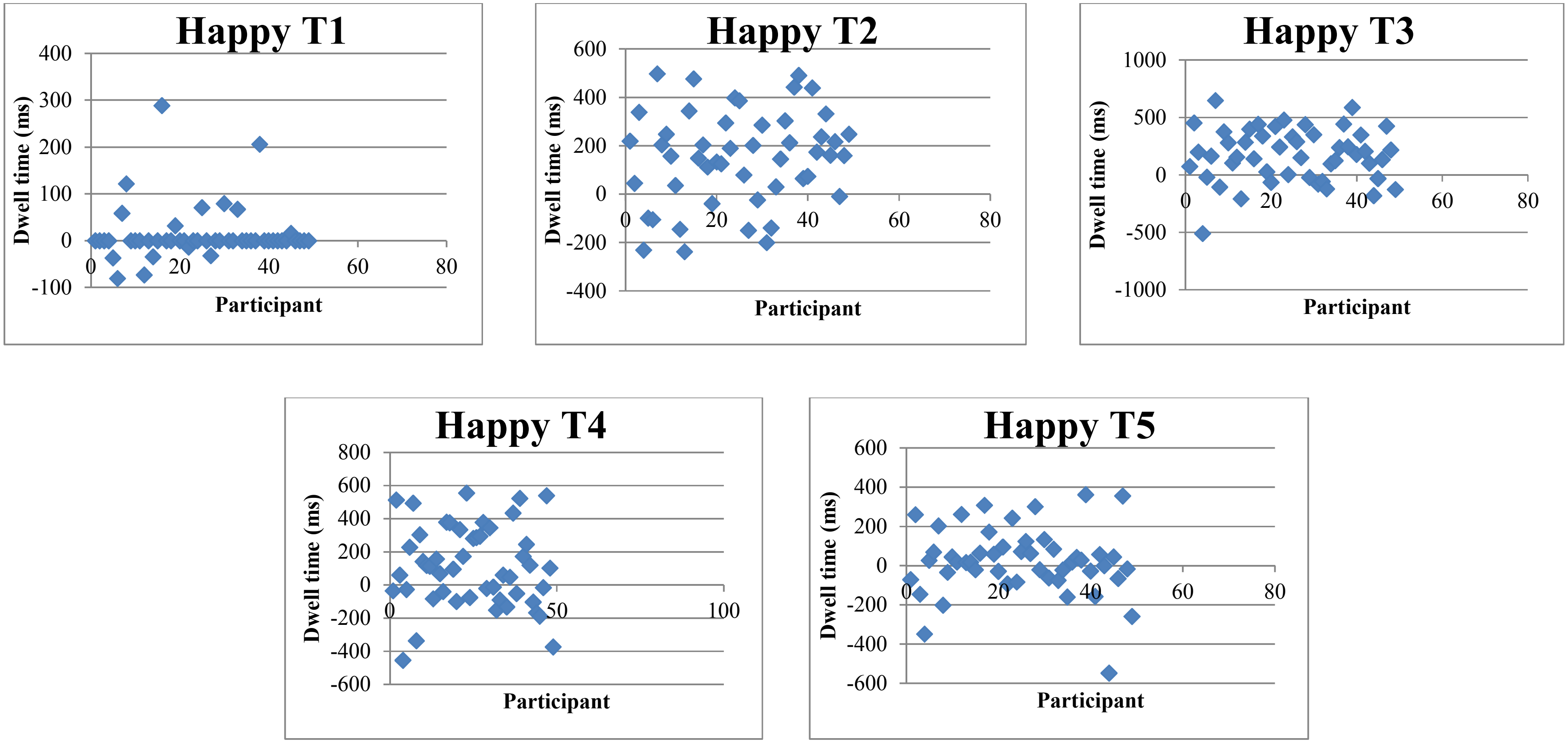
Table 5. Participants' Cognitive abilities and developmental ages by COMT Val^{66}Met genotype.

		COMT Val^{66}Met Genotype			**ANOVA**		
		M/M	**M/V**	**V/V**	***F***	**df**	***P***
Chronological Age	Mean*(SD)*	58.15*(11.38)*	60.73*(11.83)*	61.06 *(12.43)*	.555	2	.577
Overall Ability	Mean*(SD)*	109.35 *(10.09)*	104.54 *(8.69)*	105.97 *(6.59)*	*1.63*	2	*.202*
Verbal Ability	Mean*(SD)*	106.21 *(10.53)*	99.85 *(14.47)*	102.20 *(12.00)*	*.550*	2	*.304*
Non-verbal Ability	Mean*(SD)*	112.71 *(13.41)*	108.97*(12.54)*	108.06*(13.96)*	*5.60*	2	*.579*
Developmental Age (Months)	Mean*(SD)*	63.57 *(13.70)*	63.68 *(12.98)*	66.17 *(12.58)*	*.457*	2	*.802*
Developmental Verbal Ability	Mean*(SD)*	61.57 *(15.22)*	60.75 *(15.60)*	64.70 *(13.45)*	*.221*	2	*.688*
Developmental Non Verbal Ability	Mean*(SD)*	67.71 *(14.52)*	66.54 *(15.12)*	67.50 *(16.36)*	*.041*	2	*.960*

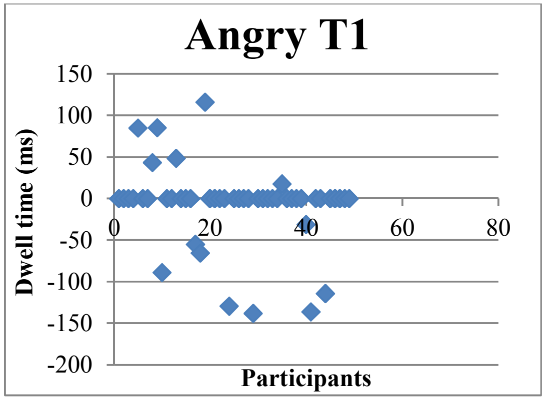
Appendix 3.2. Time (in minutes) of artefact-free EEG data after bad channel replacement per 5-HTTLPR genotype and condition.

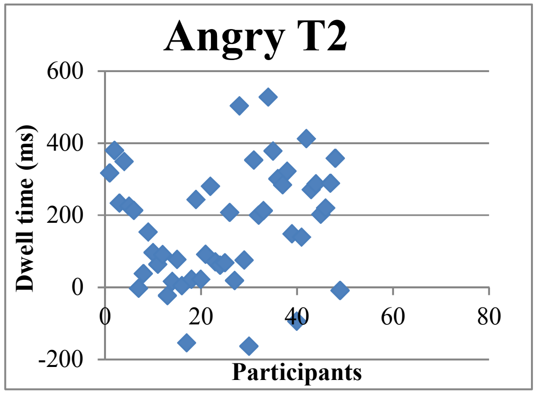
SNP	**Social**	**Non-Social**
	Mean *(SD)*	Mean *(SD)*
5-HTTLPR		
L/L	3.43 *(0.13)*	3.09 *(0.16)*
S/L	*3.30 (0.13)*	3.09 *(0.14)*
S/S	*3.72 (0.15)*	3.44 *(0.22)*
COMTVal^{158}Met		
V/V	3.30 *(0.61)*	3.31 *(0.88)*
M/V	3.47 *(0.71)*	3.14 *(0.81)*
M/M	3.44 *(0.77)*	3.03 *(0.85)*

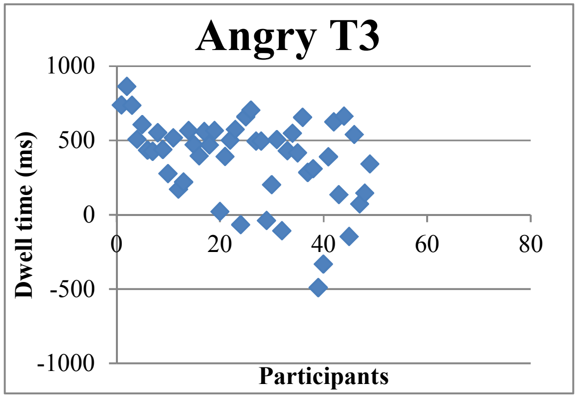
Appendix 4.1. Scatter plots illustrating the dwell time data for each face emotion and time point

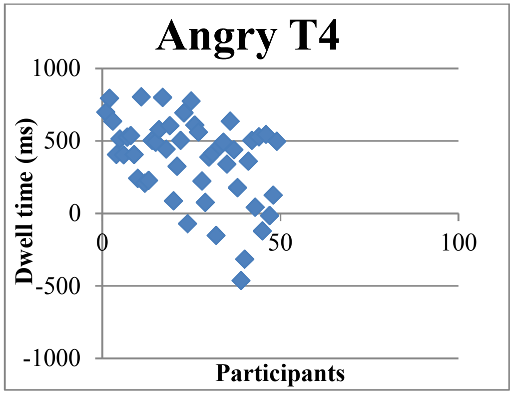


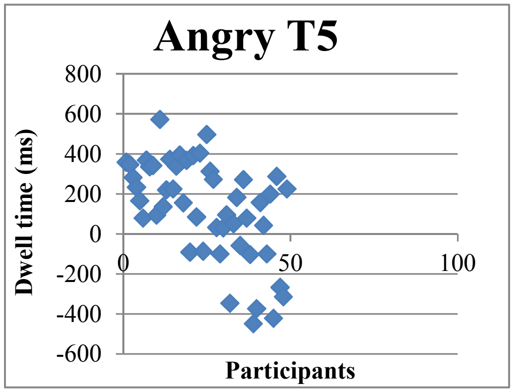
Appendix 4.1. (Continuing)











Appendix 4.2. Overall dwell time (in ms) and standard deviations (in brackets) viewing angry and happy faces among BDNF Val^{66}Met and 5-HTTLPR genotype groups, showing an aggression-specific vigilance-avoidance patterns of attention allocation in carriers of at least one Met allele.

Time Interval	**BDNF Val^{66}Met**			**5-HTTLPR**		
	M/M (N=3)	**M/V (N=18)**	**V/V (N=28)**	**S/S (N=10)**	**S/L (N=17)**	**L/L (N=22)**
Facial expressions of Anger						
T1	0.00	-9	-7	20	-13	-16
	(0.00)	*(53)*	*(49)*	*(43)*	*(43)*	*(55)*
T2	197	223	134	237	146	164
	(138)	*(142)*	*(168)*	*(92)*	*(172)*	*(173)*
T3	135	319	438	455	373	332
	(545)	*(298)*	*(233)*	*(210)*	*(270)*	*(343)*
T4	92	286	465	460	387	314
	(482)	*(286)*	*(237)*	*(220)*	*(276)*	*(336)*
T5	13	51	191	171	136	94
	(427)	*(217)*	*(236)*	*(199)*	*(255)*	*(270)*
Facial expressions of Happiness						
T1	0.00	53	18	-9	24	60
	(0.00)	*(195)*	*(63)*	*(28)*	*(56)*	*(204)*
T2	-3	189	174	87	185	196
	(173)	*(301)*	*(175)*	*(214)*	*(180)*	*(290)*
T3	309	159	199	187	165	228
	(348)	*(342)*	*(218)*	*(202)*	*(209)*	*(376)*
T4	280	78	135	180	87	136
	(271)	*(309)*	*(251)*	*(238)*	*(244)*	*(331)*
T5	130	29	23	68	15	33
	(215)	*(237)*	*(181)*	*(161)*	*(124)*	*(294)*

Appendix 4.3. Means of dwell time (in ms) and standard deviations (in brackets) of the BDNF Val^{66}Met and 5-HTTLPR genotype groups in attentional patterns towards eye and mouth region on neutral faces. Carriers of at least one Short 5-HTTLPR allele are spending significantly less time looking the eyes region, whereas spend more time fixating the mouth region of neutral faces.

RoI	**BDNF Val^{66}Met**			**5-HTTLPR**		
	M/M (N=3)	**M/V (N=18)**	**V/V (N=28)**	**S/S (N=10)**	**S/L (N=22)**	**L/L (N=17)**
Eyes Region	0.27 *(0.10)*	0.25 *(0.10)*	0.28 *(0.09)*	0.24 *(0.12)*	0.24 *(0.06)*	0.32 *(0.10)*
Mouth Region	0.04 *(0.04)*	0.06 *(0.09)*	0.04 *(0.04)*	0.09 *(0.12)*	0.05 *(0.04)*	0.02 *(0.02)*

Appendix 5.1 Participants' mean time (in ms) and standard deviations (in brackets) spent per emotion, condition and block, averaged across time points.

	Social		**Non-Social**	
	Block 1	**Block 2**	**Block 1**	**Block 2**
Positive	1851*(334)*	1105 *(238)*	1836 *(383)*	1247*(269)*
Negative	1416 *(231)*	1008*(205)*	1295 *(134)*	575 *(227)*

Appendix 5.2. 5-HTTLPR genotype groups dwell time (in ms) and standard deviations (in brackets) per Emotion, Block, Condition and Time Points. Carriers of at least one Short allele are spending less time fixating negative stimuli overall, across blocks, different which is more pronounced for the non-social threat stimuli.

		Block 1						**Block 2**					
		Social			**Non-Social**			**Social**			**Non-Social**		
		T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
L/L	Negative	80	905	719	129	945	617	-7	368	117	8	296	178
		(149)	*(448)*	*(586)*	*(207)*	*(602)*	*(626)*	*(140)*	*(500)*	*(514)*	*(74)*	*(401)*	*(543)*
	Positive	44	925	680	79	1065	877	23	501	274	35	479	225
		(163)	*(398)*	*(403)*	*(228)*	*(331)*	*(516)*	*(116)*	*(385)*	*(434)*	*(110)*	*(428)*	*(499)*
S/L	Negative	97	743	387	91	478	69	-50	334	77	-19	-95	-186
		(140)	*(541)*	*(680)*	*(116)*	*(632)*	*(806)*	*(87)*	*(534)*	*(703)*	*(128)*	*(525)*	*(634)*
	Positive	97	879	468	48	941	769	-6	353	144	9	513	408
		(113)	*(425)*	*(391)*	*(140)*	*(516)*	*(606)*	*(92)*	*(495)*	*(549)*	*(62)*	*(435)*	*(467)*
S/S	Negative	-18	576	439	39	522	215	0	336	107	39	156	-35
		(112)	*(481)*	*(468)*	*(183)*	*(417)*	*(553)*	*(98)*	*(538)*	*(558)*	*(86)*	*(415)*	*(592)*
	Positive	-9	740	560	-3	804	538	-38	258	290	-34	218	81
		(114)	*(416)*	*(593)*	*(130)*	*(298)*	*(492)*	*(83)*	*(405)*	*(541)*	*(112)*	*(311)*	*(488)*

Appendix 5.3. BDNF genotype groups mean dwell time (in ms) and standard deviations (in brackets) per Emotion, Block, Condition and Time Points. No significant variations between the two genotypes observed.

		Block 1						Block 2					
		Social			Non-Social			Social			Non-Social		
		T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
V/V	Negative	83	825	527	132	669	295	-40	398	149	5	181	86
		(134)	*(458)*	*(503)*	*(171)*	*(597)*	*(701)*	*(94)*	*(494)*	*(492)*	*(116)*	*(387)*	*(451)*
	Positive	111	966	546	85	938	762	-5	399	198	10.59	472	338
		(111)	*(433)*	*(481)*	*(160)*	*(427)*	*(617)*	*(101)*	*(466)*	*(525)*	*(95)*	*(330)*	*(438)*
M/V	Negative	59	668	440	55	546	221	12	268	-44	-3	-27	-113
		(168)	*(605)*	*(795)*	*(161)*	*(663)*	*(814)*	*(114)*	*(586)*	*(775)*	*(85)*	*(635)*	*(790)*
	Positive	6	733	624	17	1007	820	-10	420	312	26	437	252
		(134)	*(343)*	*(417)*	*(194)*	*(426)*	*(497)*	*(107)*	*(439)*	*(489)*	*(78)*	*(539)*	*(543)*
M/M	Negative	15	760	750	-25	804	309	-114	313	376	910	0	-485
		(63)	*(289)*	*(574)*	*(60)*	*(363)*	*(482)*	*(198)*	*(329)*	*(285)*	*(172)*	*(323)*	*(529)*
	Positive	-102	580	274	-63	828	317	64	46	-175	-101	58	-308
		(177)	*(188)*	*(207)*	*(130)*	*(567)*	*(213)*	*(41)*	*(214)*	*(403)*	*(137)*	*(124)*	*(144)*

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