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Early adversity, brain development and emotion processing in monozygotic twins

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RÉSUMÉ

Il existe actuellement de nombreuses preuves démontrant que des facteurs génétiques et environnementaux interagissent pendant des périodes spécifiques du développement pour rendre une personne vulnérable aux troubles psychologiques via diverses adaptations physiologiques. Cette thèse porte sur l'impact de l'adversité prénatale (représentée par le petit poids à la naissance, PPN) et de l'adversité postnatale précoce (symptômes dépressifs maternels et comportements maternels négatifs), sur le développement du cerveau, particulièrement les régions fronto-limbiques impliquées dans le traitement des émotions, pendant l'enfance et l'adolescence. Des jumeaux monozygotes (MZ) sont utilisés, lorsque possible, afin de contrôler pour les effets génétiques. Les chapitres 1 et 2 présentent les résultats de la vérification de l'hypothèse que l'adversité prénatale et postnatale précoce sont associées à une altération du fonctionnement des régions fronto-limbique tels que l'amygdale, l'hippocampe, l'insula, le cortex cingulaire antérieur et le cortex préfrontal, en réponse à des stimuli émotifs chez des enfants et des adolescents. On observe que les symptômes dépressifs maternels sont associés à une activation plus élevée des régions fronto-limbiques des enfants en réponse à la tristesse. Les résultats de l'étude avec des adolescents suggèrent que le PPN, les symptômes dépressifs et les comportements maternels négatifs sont associés à une fonction altérée des régions fronto-limbiques en réponse à des stimuli émotionnels. Chez les jumeaux MZ on observe également que la discordance intra-paire de PPN et de certains comportements maternels est associée à une discordance intra-paire du fonctionnement du cerveau et que ces altérations diffèrent selon le sexe. Le chapitre 3 présente les résultats de la vérification de l'hypothèse que l'adversité prénatale et postnatale précoce sont associées à un volume total réduit du cerveau et de l'hypothèse que les comportements maternels peuvent servir de médiateur ou de modérateur de l'association entre le PPN et le volume du cerveau. Avec des jumeaux MZ à l'adolescence on observe a) que le PPN est effectivement associé à une diminution du volume total du cerveau et b) que la discordance intra-paire de PPN est associée à une discordance du volume du cerveau. En somme, cette thèse présente un ensemble de résultats qui soutiennent deux hypothèses importantes pour comprendre les effets de l'environnement sur le développement du cerveau : que l'environnement prénatal et postnatal précoce ont un impact sur le développement du cerveau indépendamment du code génétique et

que les mécanismes impliqués peuvent différer entre les garçons et les filles. Finalement, l'ensemble de ces résultats sont discutés à la lumière des autres travaux de recherche dans ce domaine et des avenues à explorer pour de la recherche ultérieure sont proposées.

Mots clés: adversité précoce, fonction du cerveau, jumeaux monozygotes, traitement des émotions, environnement, imagerie cérébrale

ABSTRACT

There is now increasing evidence that both genetic and environmental factors interact together during specific periods of development to render an individual vulnerable to mental health disorders through multiple physiological adaptations. This thesis focuses on the impact of in utero adversity (indexed by low birth weight, BW) and early postnatal adversity (maternal depressive symptoms and negative maternal parenting behaviours) for brain development, particularly of fronto-limbic regions involved in emotion processing during childhood and adolescence. We utilize monozygotic (MZ) twins whenever possible to control for genetics. Chapters 1 and 2 present results of work testing the hypothesis that in utero and early postnatal adversity is associated with altered functioning of fronto-limbic regions including the amygdala, hippocampus, insula, anterior cingulate cortex and prefrontal cortex, in response to emotional stimuli in children and adolescents. We detect that greater maternal depressive symptomatology is associated with altered activation of fronto-limbic regions in their children in response to sadness. Results of the study in adolescents suggest that low BW, maternal depressive symptoms and negative maternal parenting behaviours are associated with altered function of fronto-limbic regions in response to emotional stimuli. In MZ twins we observe that within-pair discordance in BW and maternal parenting behaviours is associated with within-pair discordance in brain function, and that these alterations are sex-specific. Chapter 3 presents results of work testing the hypothesis that in utero and early postnatal adversity is associated with reduced total brain volume, and the hypothesis that maternal parenting habits may mediate or moderate the association between BW and brain volume. With MZ twins during adolescence, we observe that a) lower BW is indeed associated with decreased total brain volume and b) that within-pair discordance in BW is associated with within-pair discordance in brain volume. Together, this thesis presents a set of results that reinforce two important hypotheses to understand the effects of the environment on brain development: that the in utero and early postnatal environment impact brain development independent of genetics and that mechanisms involved may differ in boys and girls. Finally, these results are discussed in light of other research projects in this area and avenues for future research are proposed.

Keywords: early adversity, brain function, monozygotic twins, emotion processing, environment, neuroimaging

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ABBREVIATIONS

5-HT: 5-hydroxytryptamine or serotonin

5-HTT: serotonin transporter

5-HTTLPR: serotonin-transporter-linked polymorphic region

AAL: automated anatomical labeling

ACC: anterior cingulate cortex

ADHD: attention deficit hyperactivity disorder

BA: broadmann area

BDNF: brain derived neurotrophic factor

BOLD: blood oxygenation level dependent

BW: birth weight

dACC: dorsal anterior cingulate cortex

DA: dopamine

DARTEL: Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra

DF: degrees of freedom

DLPFC: dorsolateral prefrontal cortex

DNA: deoxyribonucleic acid

DSM-IV: Diagnostic and Statistical Manual of Mental Disorders, 4th edition

DTI: diffusion tensor imaging

DZ: dizygotic

EPI: echoplanar image

FA: fractional anisotropy

fMRI: functional magnetic resonance imaging

FOV: field of view

FWE: familywise error

FWHM: full width half maximum

GM: gray matter

G x E: gene by environment

HD: higher depressive symptomatology

HLA: human leukocyte antigen

HPA: hypothalamic-pituitary-adrenal

JEPQ: Junior Eysenck Personality Questionnaire

Kcals: kilocalories

KSADs: Kiddie Schedule for Affective Disorders and Schizophrenia

LD: lower depressive symptomatology

MARINA: MAsks for Region of INterest Analysis

LOBFC: lateral orbitofrontal cortex

MD: major depression

MLM: multilevel modeling

MNI: Montreal Neurological Institute

MOBFC: medial orbitofrontal cortex

MZ: monozygotic

NE: norepinephrine

OBFC: orbitofrontal cortex

PACOTIS: Parent's Cognition and Conduct Toward the Infant Scale

PFC: prefrontal cortex

PTSD: post-traumatic stress disorder

PVN: paraventricular nucleus

QNTS: Quebec Newborn Twin Study

rACC: rostral anterior cingulate cortex

ROI: region of interest

SCL-90: Symptom Checklist 90-items

SD: standard deviation

sgACC: subgenual anterior cingulate cortex

SES: socioeconomic status

SPM5: Statistical Parametric Mapping version 5

SPM8: Statistical Parametric Mapping version 8

SPSS: Statistical Package for the Social Sciences

SSRI: selective serotonin reuptake inhibitor

TE: time-echo

TPH2: tryptophan hydroxylase 2

TR: time repetition

VAS: visual analog scale

VBM: voxel-based morphometry

VLPFC: ventrolateral prefrontal cortex

WM: white matter

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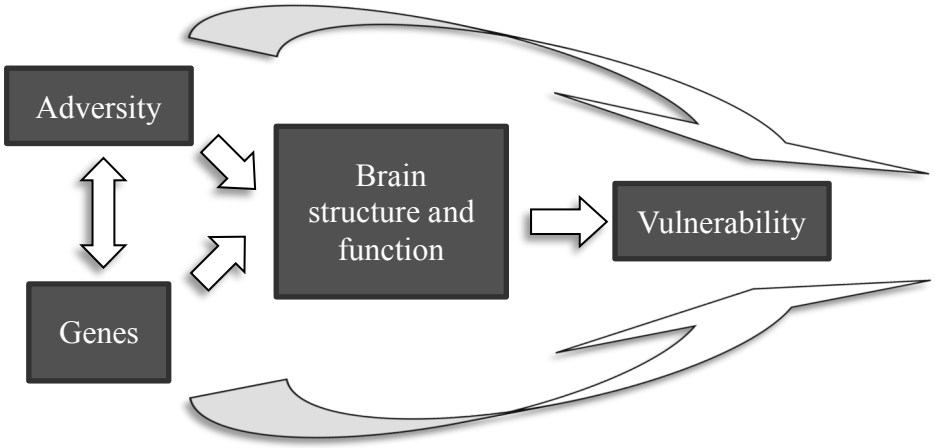
INTRODUCTION

Mental health disorders are common but little understood. It is estimated that 20% of Canadians will personally suffer from a mental health disorder sometime during their lifetime, and that all Canadians are personally or indirectly affected (Public Health Agency of Canada, 2012). Mental health disorders affect people of all ages, cultures, and education levels and account for 51 billions of dollars per year in Canada from loss of productivity and health care costs (Lim et al, 2008b). In fact, mental health disorders constitute the second leading cause of disability and premature death after heart disease in Canada (Lim et al, 2008a). Mood disorders are particularly prevalent, accounting for 62-76% of short-term disability due to mental health disorders (Government of Canada, 2006). Approximately 5% of individuals 15 years and older have suffered at least one episode of major depression in the past year and more than 50% of those who have suffered one episode will experience at least one more (Government of Canada, 2006). Women are particularly vulnerable to mood disorders, being nearly twice as likely as men to experience a major depressive episode in their lifetime (Government of Canada, 2006). Despite years of study, we still do not fully understand vulnerability to depression and mental health disorders in general. Why do some individuals go on to develop a mental health disorder and others don't? The overarching hypothesis of this thesis is that in combination with genes, early adversity during critical periods of development is associated with altered brain development and later function of fronto-limbic regions associated with emotion processing, and that these neural alterations are associated with vulnerability for mental health disorders, including depression (see figure 1). In this introduction, I focus on work to date that has assessed the impact of in utero and early postnatal adversity on brain development. If we can gain a better understanding of the mechanisms involved in conveying vulnerability to mental health disorders, we may eventually be able to develop targeted interventions, with the goal of decreasing both the distress and economic burden associated with mental health disorders.

Figure 1. Physiological changes mediating the association between early adversity and increased vulnerability for mental health disorders.



Working model of posited physiological changes leading to increased vulnerability following early adversity.

There is much support for both genetics and the environment being implicated in conveying vulnerability. Various studies have found associations between polymorphisms of specific genes such as the serotonin transporter (5-HTT) [reviewed in (Booij et al, 2013; Kendler et al, 2005)] and tryptophan hydroxylase 2 (TPH2) (Berger et al, 2012; Chen & Miller, 2013; Ottenhof et al, in prep) and mental health disorders, including depression. Mental health disorders tend to run in families and twin studies have shown that genetics contribute to most if not all mental health disorders (van Belzen & Heutink, 2006). However, even monozygotic (MZ) twins, who share 100% of their genes, can be discordant for mental health disorders. Indeed, no single gene appears to be sufficient in and of itself to cause mental health disorders (Prathikanti & Weinberger, 2005). Following Bronfenbrenner's ecological model of development which posits that a person's personality is influenced not only by his or her own characteristics but also his/her immediate and more distant environment (Bronfenbrenner, 1979), numerous studies have demonstrated associations between adverse environmental events [i.e. (Pechtel & Pizzagalli, 2011)] such as low socioeconomic status

(SES) (van Oort et al, 2011) and maternal stress (Walker et al, 2011), and mental health disorders. However, two people can be exposed to the same stressor and fare quite differently. It is thus becoming increasingly obvious that vulnerability is quite complex, with multiple factors and mechanisms interacting in conveying risk. The gene by environment (G x E) model has been proposed (Karg & Sen, 2012; Prathikanti & Weinberger, 2005), which postulates that the impact of a specific environment on brain development and later behaviour varies depending on genetics or, conversely, that the effect of a specific genotype depends on the environment (Karg & Sen, 2012). One of the most well known such association is the link between the short allele of the serotonin-transporter-linked polymorphic region (5-HTTLPR) and depression, which usually surfaces when combined with life stress such as abuse during childhood (Caspi et al, 2003). Results are inconsistent with some studies replicating this association and others not (Fergusson et al, 2011; Karg & Sen, 2012), although a meta-analysis supports these findings (Karg et al, 2011). It has also been proposed that timing of adversity may be highly relevant for conveying vulnerability. Adverse events occurring during the prenatal and early postnatal period may have particularly significant impacts on development, in effect increasing vulnerability for later mental health disorders (Danese & McEwen, 2012; Shonkoff et al, 2009; Shonkoff & Garner, 2012). A better understanding of these critical periods may enable us to target interventions when and in whom they may be the most useful.

EARLY ADVERSITY

Much research supports the association between prenatal adversity and increased vulnerability for mental health disturbances. This is well demonstrated by studies of the Dutch Famine of 1944-45, a brief naturalistic period of starvation in some cities during the winter months where rations were limited to 900 kcals/day for 24 weeks. Offspring of mothers who were pregnant during the famine as well as controls from other cities were followed longitudinally (Susser et al, 1998), and these naturalistic follow-up studies have demonstrated increased rates of depression and schizophrenia (Roseboom et al, 2011), addictive disorders (Franzek et al, 2008) and lower quality of life (Stein et al, 2009) in children exposed to famine

in utero, particularly those exposed during the first trimester of pregnancy (Franzek et al, 2008), relative to children not exposed to famine during gestation. Other studies have shown that various forms of in utero adversity, including maternal exposure to medications, alcohol and other drugs (Dunkel Schetter, 2011; Fergusson et al, 1998), obstetric complications (Allen et al, 1998; Arseneault et al, 2002; Batstra et al, 2004) and high levels of stress, are associated with impairments in executive function, memory and sociability, particularly when exposure occurs during the first half of the pregnancy (Charil et al, 2010). Moreover, low birth weight (BW), an index of global in utero adversity (Allin et al, 2004; Dunkel Schetter, 2011; Himpel et al, 2006), has also been associated with impairments in executive function and memory (Luu et al, 2011) as well as alterations in brain volume and cortical thickness (Martinussen et al, 2005).

An important challenge of studying the environment is the intertwined influence of genes. One of the best ways to assess the specific impact of the environment while controlling for genetic confounds is to utilize MZ twins. Since they share 100% of their genes (Vitaro et al, 2009), divergent phenotypes in MZ twins must be due to unique environmental experiences (Vitaro et al, 2009). Using the MZ twin paradigm, researchers have found that discordance in BW is associated with discordance in externalizing behaviours in childhood (Asbury et al, 2006; Ficks et al, 2013; Mankuta et al, 2010; van Os et al, 2001), attention deficit hyperactivity disorder (ADHD) (Lehn et al, 2007), anxiety (Asbury et al, 2006), social skills (Asbury et al, 2006), cognitive function and mathematical skills (Torche & Echevarria, 2011) as well as academic achievement (Asbury et al, 2006), further supporting the impact of the in utero environment for development. Interestingly, the impact of in utero adversity may differ across the sexes, with some studies suggesting that boys are more affected than girls (Dancause et al, 2011; Eriksson, 2009; Lazinski et al, 2008). On the other hand, increased prevalence of several mental health disorders such as mood and anxiety disorders is found in women (Government of Canada, 2006). More work is thus needed to assess gender effects.

Early life adversity factors including malnutrition, low SES, maternal depression, child institutionalisation and negative parenting behaviours (e.g., hostility), have also been associated with negative cognitive, emotional and/or behavioural outcomes later in life,

including poor emotional regulation strategies and subclinical symptoms of mental health disturbances (e.g., internalizing symptoms) as well as alterations in neural circuits involved in emotion regulation (Bayer et al, 2011; Cote et al, 2009; Gonzales et al, 2011; Lansford et al, 2006; Manian & Bornstein, 2009; Melchior et al, 2010; Silk et al, 2006; Taylor, 2010; van Oort et al, 2011; Walker et al, 2011). Using the MZ difference score method, several researchers have demonstrated that within-pair discordance in parenting behaviours are associated with discordance in self-control (Cecil et al 2012), internalizing (Schermerhorn et al, 2011) and externalizing behaviours (Hou et al, 2013; Schermerhorn et al, 2011), social skills (Guimond et al, 2012) as well as depression (Shields & Beaver, 2011) in childhood and adolescence. Furthermore, Cath and colleagues found that twins who had experienced more adverse life events, particularly sexual abuse, demonstrated greater obsessive-compulsive symptoms than twins with fewer adverse life events (Cath et al, 2008). Together, these findings support the importance of the early postnatal environment independent of genetic factors.

BRAIN DEVELOPMENT

Adverse events occurring during the in utero and early postnatal period can alter development of the brain, including regions of the fronto-limbic system such as the hippocampus, amygdala, thalamus and prefrontal cortex (PFC). Given the varied rate and progression of development of these regions, it is hypothesized that an adverse event will have the most impact on the region undergoing greatest development at the time (Pechtel & Pizzagalli, 2011). Thus, taking into account timing of adversity may help to explain the long-lasting impact of specific adverse events.

Anatomy

Brain development begins in utero, in a carefully controlled pattern of cell growth and migration, with the third trimester presenting a period of intense and accelerated synaptogenesis, peaking at gestational week 34 with almost 40 000 new synapses formed per

second (Tau & Peterson, 2010). At birth, the human brain is approximately ¼-⅓ of its adult size (Gilmore et al, 2007; Gilmore et al, 2012; Toga et al, 2006) then more than doubles between 0 and 2 years, at which point it has reached 80-90% of its adult size (Alexander-Bloch et al, 2013; Tau & Peterson, 2010). It peaks around age 5, although progressive and regressive events continue thereafter (Durston et al, 2001; Tau & Peterson, 2010).

Postnatal development of gray matter (GM) progresses in a precisely programmed sequence consisting of progressive and regressive events, rapid growth and later degradation of inefficient or unused connections. These synaptic overproduction and pruning mechanisms progress differently across regions (Toga et al, 2006). GM development proceeds in a posterior to anterior direction (Blakemore, 2012) and appears to occur first in phylogenetically older areas concerned with basic functions of sensing and movement, then move on to areas involved in more complex functions including spatial orientation and language around ages 11-13, and end with phylogenetically newer areas involved in advanced cognitive and integrative functions in late adolescence (Toga et al, 2006). The overall peak of GM occurs around age 5-10 (Toga et al, 2006), and is followed by a decrease thereafter (Groeschel et al, 2010; Tau & Peterson, 2010). Pruning does not equate to loss, but rather represents normative fine-tuning processes and improves connectivity and efficiency of brain networks (Tau & Peterson, 2010). Finally, GM growth correlates with motor, social and cognitive abilities (Durston et al, 2001; Gogtay & Thompson, 2010).

Premyelinating oligodendrocytes begin to develop prenatally (Tau & Peterson, 2010) and by birth, the proportion of the brain containing myelinated white matter (WM) represents 1-5%. Postnatally, WM continues to develop rapidly through early childhood, and then decreases in speed to reach a slow, steady pace during late childhood and adolescence, peaking in adulthood (Durston et al, 2001; Gogtay & Thompson, 2010; Groeschel et al, 2010; Toga et al, 2006). Similarly to GM, WM advances in a posterior to anterior direction, beginning with sensory and motor pathways before reaching association areas (Tau & Peterson, 2010). Within any functional circuit, subcortical structures are myelinated prior to cortical structures (Tau & Peterson, 2010).

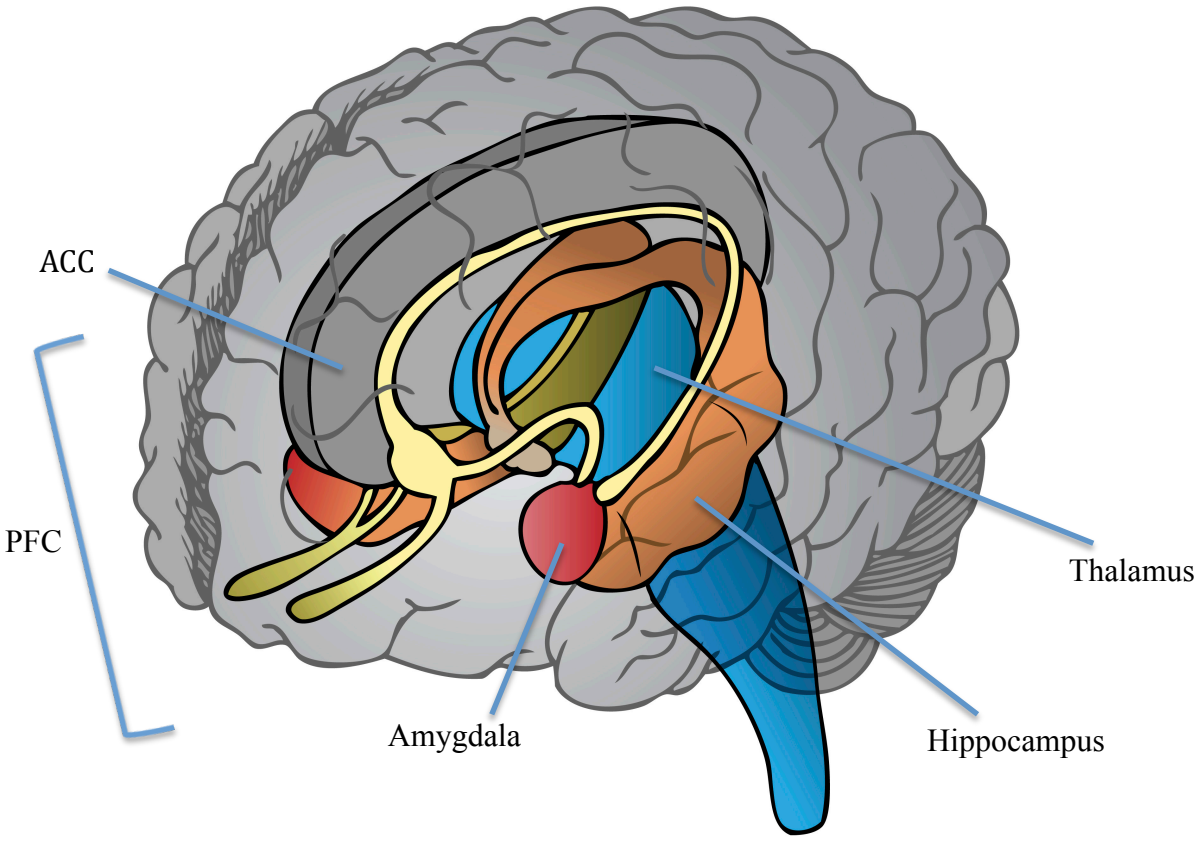
Some heterogeneity in brain development exists with, for instance, structural growth differing across the sexes. Total gray matter volume peaks earlier in girls than in boys, at approximately 7 and 10 years, respectively (Lenroot et al, 2007), as does total brain volume, during adolescence (Lenroot et al, 2007), although total GM and WM volumes are greater in boys at maturity (Groeschel et al, 2010). In fact, the male brain is 10% larger at maturity even after correcting for body size (Durston et al, 2001; Sacher et al, 2013), although sulcation is equivalent across the sexes (Vasung et al, 2013). Women, on the other hand, have a greater GM to WM ratio than do men (Groeschel et al, 2010; Sacher et al, 2013), greater overall cortical connectivity (Sacher et al, 2013) as well as larger caudate nuclei and possibly larger hippocampi and globi pallidi, while men have larger amygdala and thalami (Durston et al, 2001; Koolschijn & Crone, 2013). Thus, a certain amount of structural heterogeneity may be normal and expected.

Function

In addition to brain volume and structure, it is also important to consider brain function throughout development, particularly in fronto-limbic regions implicated in processing emotional information. These regions include the amygdala, hippocampus, insula, anterior cingulate cortex (ACC) and PFC (Adolphs, 2002; van der Werff et al, 2013) (see figure 2). Experimental studies have shown that the amygdala is activated in response to emotional stimuli, particularly fear (Adolphs, 2002; Fusar-Poli et al, 2009; Herba & Phillips, 2004; Lindquist et al, 2012; van der Werff et al, 2013). It is also involved in the attribution of emotional valence to stimuli (Bechara, 2004) and activates the hypothalamic-pituitary adrenal (HPA) axis when faced with stress. The hippocampus, in addition to its crucial role in memory, regulates HPA axis activation, and together with the insula, is implicated in processing the context of a potential threat (van der Werff et al, 2013). The insula is also involved in higher-level cognitive control and attention, is implicated in awareness of bodily signals and is particularly responsive to disgust (Bechara, 2004; Eugene et al, 2003; Hennenlotter & Schroeder, 2006). The ACC, which can be divided into three subregions: the dorsal ACC (dACC), rostral ACC (rACC) and subgenual ACC (sgACC) (van der Werff et al, 2013), is implicated in the evaluation, reappraisal and suppression of emotions (Eugene et al,

2003; Herba & Phillips, 2004; Lindquist et al, 2012; Ochsner & Gross, 2005). It is particularly active in response to sadness (Lindquist et al, 2012). The PFC is likewise implicated in cognitive control of emotions, by inhibiting amygdala activity (Lindquist et al, 2012; van der Werff et al, 2013). PFC subregions with different functions can be identified. Briefly, the medial PFC has been implicated in attribution of mental states (Ochsner & Gross, 2005) while the lateral PFC is implicated in top-down executive processes including monitoring and controlling incoming information in order to produce voluntary action (Eugene et al, 2003). The ventral PFC is involved in the evaluation of the appropriate emotional response to a stimulus and is necessary for experiencing and expressing emotions (Bechara, 2004), while the dorsal PFC is involved in evaluation and reappraisal of emotional states through integration of visceromotor information (Eugene et al, 2003; Phillips et al, 2008). Finally, the orbitofrontal cortex (OBFC) has been suggested to be responsible for the integration of bottom-up automatic bodily processes with top-down executive cognitive processes (Beauregard et al 2003) and the selection of appropriate emotional responses (Ochsner & Gross, 2005). Globally, the frontal limbic circuitry subserving emotion processing can also be divided into two parallel neural streams: a ventral stream comprising subcortical and ventral frontal cortical regions involved in the identification of emotional cues and generation of emotional states, and a dorsal stream comprising dorsal frontal cortical regions important for regulation of emotion and behaviour (Herba & Phillips, 2004).

Figure 2. Fronto-limbic system



ACC= anterior cingulate cortex; PFC= prefrontal cortex

Adapted from http://thereesewellnesssystem.com/wp-content/uploads/2013/06/HiRes.jpg

Through development, regions implicated in emotion processing change as the brain matures. Emotional stimuli activate the earlier-developing ventral regions including the amygdala and hippocampus in children and the later-maturing dorsal cognitive areas (ACC/PFC) beginning in adolescence (Hogan & Park, 2000; Hung et al, 2012; Perlman & Pelphrey, 2010; Vink et al, 2014). This increase in use of prefrontal regions also parallels increased functional coupling between the amygdala and hippocampus with the OBFC and ventrolateral PFC (VLPFC) with age (Gee et al, 2013; Vink et al, 2014). This increased

coupling is associated with greater inhibition of the limbic system by the PFC and may explain why adults are better able to control their affective states then adolescents (Vink et al, 2014). Moreover, activation appears to be less diffuse and more focalized as children mature, potentially indicating greater efficiency of function (Poldrack, 2010). As the brain develops, efficiency in emotion processing and regulation increases in parallel.

In addition to age, sex is also responsible for some heterogeneity in emotion processing. Indeed, sex differences are found in brain function as they are in brain volume and structure, and are particularly important to consider in the context of emotion processing (Sacher et al 2013). In a meta-analysis, greater peaks of activation were found in men in several regions including the amygdala, hippocampus, parahippocampal gyrus, insula, caudate, putamen, thalamus, ACC, posterior cingulate cortex, superior frontal cortex, inferior OBFC and dorsolateral PFC (DLPFC), while greater peaks of activation were found in women in the amygdala, hippocampus, insula, thalamus, rostral ACC, OBFC and caudal DLPFC [reviewed in (Sacher et al, 2013)]. These sex differences in brain function in response to emotional stimuli may explain, at least in part, why women are 2-3 times more likely than men to suffer from mental health disorders such as depression (Kessler et al, 1993; Wade et al, 2002).

Neural alterations following adversity

Following early stress exposure, alterations are seen in several fronto-limbic regions (Davidson & McEwen, 2012; Ulrich-Lai & Herman, 2009). For instance, studies in rats and monkeys (Davidson & McEwen, 2012; Lupien et al, 2009) have shown that early stress such as that caused by repeated maternal separation is associated with alterations in brain volume and/or structure in many regions involved in stress and emotion regulation, including the PFC, amygdala, hippocampus, dentate gyrus and hypothalamus. In humans, Tomalski and Johnson have shown that institutionalization is associated with altered brain development. Specifically, children adopted later than 15 months (but not earlier) show smaller amygdala volumes than non-institutionalised children (Tomalski & Johnson, 2010). Exposure to early maternal depression has been associated with decreased hippocampal volume bilaterally (Chen et al, 2010) and functional alterations in the insula, ACC and caudate nucleus in children, even

when maternal depressive symptoms were below threshold for clinical depression (Levesque et al, 2011). Furthermore, changes in brain structure and function have been associated with cognitive biases and/or impairments, maladaptive behaviours, altered emotion processing and symptoms of mental health disorders such as depression, following early adversity including prenatal exposure to cigarettes (Lotfipour et al, 2009), and prenatal brain injury (Tomalski & Johnson, 2010). However, findings are inconsistent, possibly due to heterogeneity in adversity timing, type and magnitude (Danese & McEwen, 2012).

Studies have shown that some of the effects of early stress may be reversible. For instance, the administration of the selective serotonin reuptake inhibitor (SSRI) fluoxetine starting on postnatal day 1 in rodents, which corresponds to the third trimester in humans (Romijn et al, 1991), reversed the decrease in hippocampal cell proliferation and neurogenesis as well as anxiety-like behaviours found in maternally stressed offspring not exposed to fluoxetine (Rayen et al, 2011). Moreover, positive parenting in the form of parental warmth (Gonzales et al, 2011) as well as formal childcare (Cote et al, 2007; Geoffroy et al, 2010; Giles et al, 2011; Lee et al, 2006), have been shown to moderate the negative impact of early adversity. These results could inform the development of interventions to protect vulnerable children at risk following exposure to early stress.

Although the specific regions and direction of change (e.g., increased or decreased volume) vary depending on the nature, timing, chronicity and magnitude of the adversity, multiple changes are occurring in fronto-limbic regions involved in emotion processing. These appear to be persistent through life (Danese & McEwen, 2012; Tomalski & Johnson, 2010). This stability of alterations in stress regulation may very well underlie a general vulnerability consisting of impaired emotional and behavioural regulation in the face of stress and increasing susceptibility to mental health disorders.

OBJECTIVE

The overarching aim of this thesis is to test the hypothesis that early adversity impacts brain development and later function of fronto-limbic regions involved in emotion processing, including the amygdala, hippocampus, insula, ACC and PFC, in childhood and adolescence. Early adversity is defined as biological or psychosocial stressors occurring in utero, at birth or during the first two years of life. In chapter 1, we test the hypothesis that early adversity, notably maternal depressive symptoms, is associated with altered function of fronto-limbic regions during childhood in response to an emotional task. In chapter 2, we test the hypothesis that early adversity factors including low BW, maternal depressive symptoms and negative maternal parenting behaviours, are associated with altered function of fronto-limbic regions in adolescent monozygotic twins, and that this altered function is associated with current measures of altered mood regulation, notably neuroticism and internalizing symptoms. We assess brain function in both genders together as well as separately. Finally, in chapter 3, we test the hypothesis that early adversities including low BW, maternal depressive symptoms and negative maternal parenting behaviours are associated with altered brain volumes and assess whether early postnatal maternal parenting behaviours may mediate or moderate the association between BW and total brain volume. This thesis ends with a discussion of the implications of the results and of current methodological challenges of this type of research, and avenues for future research are proposed.

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CHAPTER 1 – Early adversity and brain function in childhood

Foreword

In this first chapter we assess brain function in the Quebec Newborn Twin Study (QNTS) cohort at 8 years of age. The fMRI data collected when the twins were 8 years old belong to Mario Beauregard. Richard Tremblay, Mara Brendgen, Daniel Pérusse, Ginette Dionne, Frank Vitaro and Michel Boivin are responsible for the twin cohort. Emilie Fortier taught me how to analyse these data and performed all pre-processing. Koen Ottenhof performed some of the 2nd level analyses under my supervision as part of an internship at the Sainte-Justine Hospital Research Centre. I performed 2nd level analyses, wrote up the paper and submitted it for publication. Mario Beauregard edited the paper, and all co-authors reviewed and approved it. Linda Booij supervised data analysis and provided feedback on earlier versions of the manuscript. It was accepted and published by the *Journal of Affective Disorders* (Lévesque et al. J Affect Disord. 2011; 135(1-3):410-413).

Unfortunately, many scans could not be used due to excessive motion, and within-twin pair considerations were not possible. For this reason, they are considered as singletons.

Altered Patterns of Brain Activity during Transient Sadness in Children at Familial Risk for Major Depression

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ABSTRACT

Introduction: We used functional magnetic resonance imaging to investigate the neural correlates of sadness, the prevailing mood in major depression (MD), in a prospective, well-documented community sample followed since birth. **Methods:** The children, comprising 136 children (65 boys and 71 girls) of mothers with varying levels of depressive symptomatology, were scanned - using a 1.5-Tesla system - while they watched five blocks of both sad and neutral film excerpts. Following scanning, they rated the emotions they experienced, and if they identified sadness, they were also asked to rate its intensity. **Results:** In children whose mothers exhibited higher depressive symptomatology, compared to children whose mothers displayed lower depressive symptomatology, altered neural responses to sad film excerpts were noted in brain regions known to be involved in sadness and MD, notably the insula, anterior cingulate cortex and caudate nucleus, even though the children did not differ in current mood. **Limitations:** Whether this represents genetic vulnerability or a consequence of exposure to maternal depressive symptoms at a young age is unknown. **Discussion:** The results are consistent with the results of studies in healthy adults and MD patients. The present study suggests that an altered pattern of regional brain responses to sad stimuli are already present in childhood and might represent vulnerability for MD later in life.

INTRODUCTION

Findings from a number of neuroimaging studies indicate that the activity of brain regions involved in emotional processing, such as the amygdala, insula and lateral orbitofrontal cortex (LOBFC), is altered in individuals with major depression (MD) during a transient state of sadness (Beauregard et al, 2004; Beauregard et al, 2006; Levesque et al, 2003). Such functional alterations may be related, at least in part, to a dysfunction of the neural circuitry underlying emotion regulation. In accordance with this view, there is considerable evidence that an inability to regulate emotions effectively plays a pivotal role in MD (Kring, 1999). Emotion regulation is mediated by various prefrontal cortical areas, including the medial orbitofrontal cortex (MOBFC) and the ACC (Beauregard et al, 2004). The functioning of these prefrontal areas is known to be altered in individuals with MD (Beauregard et al, 2006; Phillips et al, 2008).

Early childhood adversities may influence the risk for MD later in life. Regarding this issue, it has been shown that offspring of parents with MD have a threefold greater risk for this disorder than offspring without such family histories (Weissman et al, 2006). A familial risk for MD may exert a negative impact on brain development, with deleterious consequences for the cerebral structures implicated in emotional processing and emotion regulation (Beauregard et al, 2004; Forbes et al, 2006; Maughan et al, 2007). Nothing is known yet with respect to this important question. Given that MD often develops in adolescence (Kessler et al, 2001), it is of further relevance to explore this issue in younger children, to investigate whether brain activation can predict later depressive symptoms.

This study investigated neural correlates of sadness, the prevailing mood in MD, in a prospective, community sample comprising children of mothers with varying levels of depressive symptoms who have been regularly followed since birth. Neural activation was measured during a mood induction task, which consists of neutral and sad film extracts utilized in previous studies (Cote et al, 2007; Eugene et al, 2003; Levesque et al, 2003) to induce temporary sadness. We hypothesized that greater levels of depressive symptoms in the mother would be associated with altered functioning of regions associated with emotion

during a mood induction task during childhood, notably the OBFC, ACC, insula, amygdala, hippocampus and caudate nucleus.

METHODS

Participants

Participants were 136 children (65 boys and 71 girls) from the QNTS (Brendgen et al, 2005; Forget-Dubois, 1997), who were scanned using functional magnetic resonance imaging (fMRI) when they were 8 years and 4 months of age (Cote et al, 2007). The research protocol was approved by the appropriate ethics committees. Written consent was obtained from parents of all participants and oral assent from participants. In some cases, scans from both twins were used; in others one was excluded due to head movement (64 single twins; 36 twin pairs or 72 individuals).

Measures

The Dominic-R interview based on the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) was used to evaluate the mental health of the participants (Valla et al, 2000). The different groups scored below clinical cut-off scores for all mental health problems assessed with this measure, including MD, anxiety, ADHD and conduct problems. In regards to depressive symptomatology, the children's scores were within a healthy range and not statistically different between the two groups (HD: mean = 6, s.d. = 4.30; LD: mean = 5.30, s.d. = 3.50). Maternal depressive symptoms were assessed using the Symptom Checklist (SCL-90) (Derogatis, 1983, 2000) when the children were 5, 18, 30 and 48 months, and averaged over these time points. Children whose mothers exhibited higher depressive symptomatology (Group HD, highest third of sample, n = 45; mean = 61.6, s.d. = 4.2) were compared to children whose mothers displayed lower depressive symptomatology (Group LD, lower two thirds, n = 91; mean = 48.6, s.d. = 5.1). The decision to compare the highest third of the sample in depressive symptoms to the lower two thirds was made given that we used a healthy, non-clinical sample, in order to approximate depressive symptomatology in the high

group. Members of the HD group also had significantly lower family income (defined as 30 000 $ or less) than members of the LD group, which could indicate an interaction or an additive effect of these two adversity factors.

Following Fortier and colleagues (Fortier et al, 2010), T2* weighted functional images were acquired on a 1.5 Tesla system (Magnetom Vision, Siemens Electric, Erlangen, Germany), using an echoplanar image (EPI) pulse sequence (time repetition (TR) = 0.8ms, time-echo (TE) = 54ms, flip=90°, field of view (FOV) = 215mm, matrix = 64 x 64, voxel size=3.36mm x 3.36mm x 5mm). Twenty-eight 5 mm slices were acquired every 2.65 seconds in an inclined axial plane, aligned with the anterior commissure-posterior commissure axis. High-resolution data were then acquired via T1-weighted 3-D volume acquisition using a gradient echo pulse sequence (TR=9.7mm, TE=4ms, flip=12°, FOV=250mm, matrix=256x256, voxel size=0.94mm^3).

Procedure

Participants underwent a functional scan while they watched five 39-second blocks of emotionally neutral film excerpts (television news interview) followed by five 39-second blocks of sad film excerpts (clip depicting death of a father) (Gross, 1995). This order was used in order to avoid contamination of neutral stimuli by emotional stimuli. Blocks were separated by 15-second resting periods consisting of fixation of a cross. After scanning, participants identified the primary emotions they felt during the sad and neutral excerpts using a visual analog scale (VAS). If participants identified sadness, they were asked to rate its degree (1: sad, 2: very sad, 3: extremely sad, 4: saddest ever) (Cote et al, 2007).

Analyses

Pre-processing steps were done using Statistical Parametric Mapping version 5 (SPM5; Wellcome Department of Cognitive Neurology, London UK) in accordance with Fortier (Fortier et al, 2010). Images of all participants were realigned to correct for small head movements and spatially normalized into an EPI stereotactic space (Montreal Neurological Institute template). This template was then used to derive Talairach and Tournoux (1988)

coordinates defining the regions of interest (ROIs). Next, images were convolved with a 3D isotropic Gaussian kernel at 12 mm full width half maximum in order to improve the signal-to-noise ratio and accommodate for small inter-individual neuro-anatomical differences.

Statistical analyses were performed using Statistical Parametric Mapping version 8 (SPM8), which allows correction of non-independence of observations. This rendered possible the inclusion of the scans of pairs of twins when available. The general linear model was utilized to estimate voxel-level effects. A one-sample *t*-test was performed for each group to measure blood oxygenation level dependent (BOLD) brain activity during transient sadness (Sad minus Neutral contrast). In addition, a "random-effects model" was implemented, and a two-sample *t*-test was carried out to compare brain activity across groups. An a priori search strategy was used and a small volume correction was performed in the following brain ROIs: ACC (Brodmann areas [BA] 24 and 32), MOBFC (BA 11), LOBFC (BA 47), anterior temporal pole (BA 21 and 38), insula (BA 13), amygdala, hippocampus and caudate nucleus. These regions have been consistently activated in previous neuroimaging studies of sadness or emotion regulation (Beauregard et al, 2006; Levesque et al, 2003). The search volumes corresponding to the ROIs were defined by creating an inclusive mask using the MAsks for Region of INterest Analysis (MARINA) program (Bender Institute of Neuroimaging). A probability threshold for multiple comparisons of $P < 0.05$ corrected was used. Only clusters showing a spatial extent of at least 10 contiguous voxels were kept for image analysis (Fortier et al, 2010). Sex was added as a covariate in all analyses given that sex differences have been found in emotion processing irrespective of mood and depressive symptoms (Domes et al, 2010).

RESULTS

Participants from mothers with higher levels of depressive symptoms had significantly lower family income than participants from mothers with lower levels of depressive symptoms ($p<0.01$). There were no other demographic or behavioral differences between the two groups (Table I).

The viewing of the sad film excerpts induced a transient state of sadness in all participants. The mean level of reported sadness was not statistically different in the HD group (mean = 3.19, SD = 0.94; range 1–4), compared to the LD group (mean = 2.99, SD = 1.05; range 1–4). In addition, the viewing of the sad film excerpts did not produce significant changes in emotional state other than sadness.

Relative to the LD group, the HD group showed significantly greater activity, during sad mood induction, in the left insula (BA 13; x=-39 y=-15 z=15; T=3.40; $p < 0.05$). Greater activity in response to sad stimuli was also found in the right ACC (BA 24; x=9 y=-6 z=28; T=4.89; k=814; $p < 0.001$), but in a low income group only. Greater activity was also noted for the HD group, compared to the LD group, in the right caudate nucleus (x=18 y=18 z=15; T=3.40; k=10; $p < 0.05$) (Fig. 1).

DISCUSSION

In children whose mothers exhibited higher depressive symptomatology, compared to children whose mothers displayed lower depressive symptomatology, our results revealed an alteration in neural responses to sad stimuli in brain regions involved in sadness and MD. Notably, the children did not differ in mood.

Previous neuroimaging studies suggest that the insula (BA 13) supports a representation of somatic and visceral responses associated with the subjective experience of sadness (Beauregard et al, 2004). It is therefore possible that children whose mothers demonstrated greater depressive symptomatology at a young age were more aware of their bodily responses to sadness. In regards to the caudate nucleus, it has been speculated that it might be implicated in the coupling of visceromotor and somatomotor activity in the context of emotional responses (Carretie et al, 2009). Finally, evidence suggests that the ACC plays a key role in the regulation of the autonomic aspect of primary emotions such as sadness (Beauregard et al, 2004), therefore suggesting that regulation was higher in children of the HD group compared to children of the LD group.

Limitations include the distinction of HD and LD groups, which were not divided according to a specifically defined cut off. As previously mentioned, this study was conducted in a healthy sample. We chose to compare a more symptomatic group in a community sample to a more "normal" group, and validity of the group division is supported by the comparability of our results with those using clinical diagnoses. Furthermore, whether results demonstrating altered functioning in children of mothers with higher levels of depressive symptoms represent genetic vulnerability or a consequence of exposure to maternal depressive symptoms at a young age is unknown.

The altered pattern of regional brain responses to sad stimuli detected here, in children whose mothers displayed higher levels of depressive symptomatology, might represent an index of vulnerability for MD later in life. As low socioeconomic status in childhood can contribute to the eventual development of mood disorders (Mazza et al, 2010), this risk factor might also have been conducive to the altered neural responses noted in the present study.

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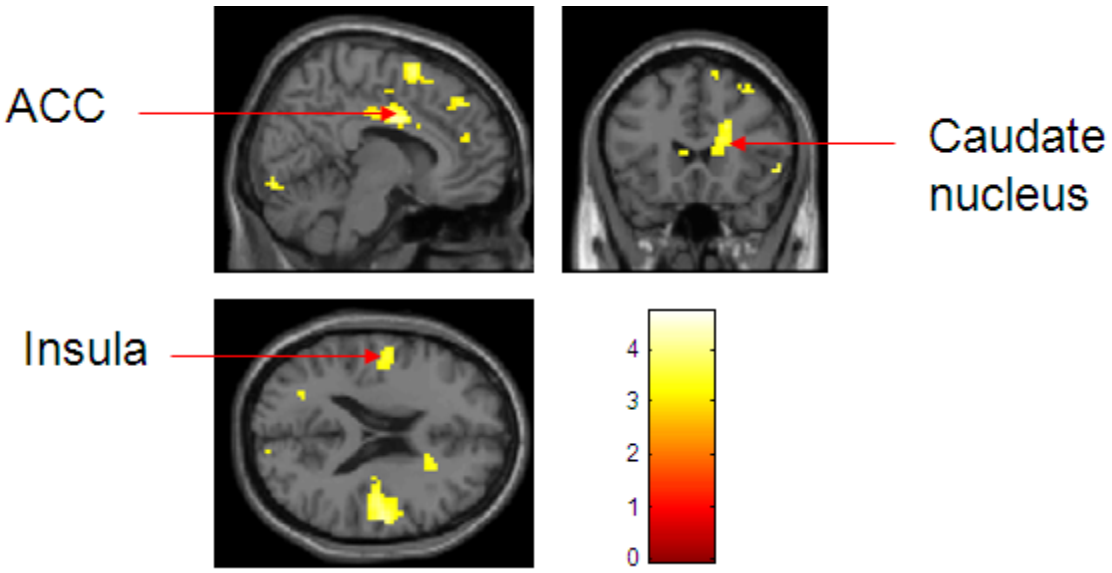
Table I. Sample Characteristics

Variable	Mean ± SD / Frequency		F, χ^2 (df)
	Low maternal depression	High maternal depression	
N	91	45	
Maternal depressive symptoms score	48.6 ± 5.1	61.6 ± 4.2	**F** (134/136) = 215.40*
Boys / Girls	41 / 50	24 / 21	χ^2 (1) = 0.83
Low / High income	17 / 74	18 / 27	χ^2 (1) = 7.16^
Dominic depression score (child)	5.3 ± 3.5	6.0 ± 4.3	**F** (126/128) = 1.02
Prenatal exposure to nicotine (yes / no)	60 / 25	35 / 8	χ^2 (1) = 1.74
Hostile parenting scores	1.4 ± 1.6	2.0 ± 1.5	**F** (116/118) = 3.49
Birth weight in kg	2.5 ± 0.4	2.5 0.6	**F** (62.8) = 0.39
Intensity of sad mood induction (on a 4 pt. scale)	3.0 ± 1.1	3.2 ± 0.9	**F** (112/114) = 1.08

Descriptive statistics are contrasted for low and high maternal depressive symptoms groups. Both the level of maternal depressive symptoms scores and income are significantly different across groups.

* $p < 0.001$; ^ $p \leq 0.01$

Fig. 1. HD group > LD group (Sad minus Neutral contrast)



Statistical activation maps showing greater regional activity in the HD group relative to the LD group. Significant loci of activation were measured in the right ACC, the right caudate nucleus, and the left insula. ACC: anterior cingulate cortex.

CHAPTER 2 – Early adversity and brain function in adolescent monozygotic twins

Foreword

In this second chapter, we assess the association between early adversity factors and brain function in adolescence in the QNTS. Between-pair analyses were performed on one twin per pair and within-pair analyses were performed using the difference score method. These analyses were performed on data that I collected with the help of other lab members, which included neuroimaging, saliva sampling for extraction of DNA methylation levels, questionnaires and computer-testing. I performed the analyses, with the help of a colleague, Kris Marble, who provided matlab scripts for first level modelling steps. These scripts are included in Annex 1. Elmira Ismaylova, Marie-Pier Verner and Kevin Casey helped with recruitment and data collection. Marjolein van der Wal performed the analyses regarding neuroticism and internalizing disorder symptoms during her internship at CHU Sainte-Justine as part of the requirements of her Master's thesis, which I checked for quality and precision. I drafted the manuscript. Mara Brendgen, Ginette Dionne, Frank Vitaro, Michel Boivin and Richard Tremblay are responsible for the twin cohort and approved the final version of the manuscript, which will be submitted for publication shortly following the submission of this thesis. Linda Booij and Richard Tremblay came up with the idea for this project and secured funding, supervised data collection and analysis and provided feedback on earlier versions of this manuscript.

Early adversity and neural responses to emotions in adolescence: a longitudinal analysis of monozygotic twins

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ABSTRACT

Introduction. In utero and early postnatal adversity may negatively impact brain development, with consequences for brain regions implicated in emotion processing. This study tested the hypothesis that the adverse in utero and early postnatal environment would be associated with greater neural responses of fronto-limbic regions to sad and fearful stimuli. We also tested whether alterations in fronto-limbic responses to sad and fearful stimuli would be associated with risk factors for depression. **Methods.** Hypotheses were tested in 52 pairs of 15-year old adolescent monozygotic twins followed longitudinally since birth, allowing us to control for genetics and providing us with prospective measures of the early environment. **Results.** Results show an association between the in utero/early postnatal environment and neural responses to sadness and fear in fronto-limbic regions including the amygdala, anterior cingulate cortex and prefrontal cortex in adolescence. We also show that these effects are independent of DNA sequence and sex-specific. Moreover, we show that in the absence of current psychopathology, higher levels of neuroticism and internalizing symptoms contribute to the variation of neural activity to emotional stimuli in fronto-limbic regions. **Conclusion.** By identifying at-risk individuals, it may eventually be possible to target preventive interventions in whom they may be most beneficial.

INTRODUCTION

Studies have shown that the early environment may be partly responsible, in combination with genetics, for altered emotion processing representing vulnerability for development of mood disorders. BW can be used as an index of global in utero adversity (Allin et al, 2004; Dunkel Schetter, 2011; Himpel et al, 2006; Lazinski et al, 2008), and has been associated with emotion processing difficulties during childhood and adolescence (Aarnoudse-Moens et al, 2009; Allen MC, 2008; Allen NB et al, 1998; Bohnert & Breslau, 2008; Delobel-Ayoub et al, 2009; Field, 2011; Lazinski et al, 2008; Luu et al, 2011). Even when in the normal range, it is correlated with later cognitive and behavioural functions so that children born with low BW show greater prevalence of cognitive and behavioural problems (Barker, 2004; Kramer, 1987; Newcombe et al, 2007; van Os et al, 2001). Early postnatal adversity factors including maternal depression and negative parenting behaviours have also been associated with negative emotional outcomes later in life (Bayer et al, 2011; Cote et al, 2009; Gonzales et al, 2011; Lansford et al, 2006; Manian & Bornstein, 2009; Melchior et al, 2010; Silk et al, 2006; Taylor, 2010; van Oort et al, 2011; Walker et al, 2011). In fact, Pechtel and Pizzagalli estimated that early adversity accounts for as much as 32% of psychiatric disorders and 44% of childhood-onset disorders (Pechtel & Pizzagalli, 2011), supporting the importance of studying the impact of early adversity. This impact can also be seen through early mental health indicators such as neuroticism and depressive symptoms, or their precursors, including altered emotion processing.

Many regions are involved in emotion processing and include the amygdala, insula, hippocampus, thalamus, caudate nucleus, ACC and PFC. A common way to assess emotion processing is to investigate brain regions activated by human faces with different facial expressions. Studies suggest that the amygdala is particularly involved in the response to fearful stimuli (Fusar-Poli et al, 2009; Lindquist et al, 2012) and that the ACC is highly responsive to sadness (Lindquist et al, 2012). What's more, emotion processing varies by age and sex. During childhood, emotion processing activates mostly early-maturing limbic regions such as the amygdala and hippocampus, but a shift occurs during adolescence to later-maturing ACC and PFC (Hogan & Park, 2000; Hung et al, 2012; Perlman & Pelphrey, 2010;

Vink et al, 2014). In regards to sex, emotion processing in adults is associated with peaks of activation in the amygdala, hippocampus, insula, caudate, putamen, thalamus, ACC, posterior cingulate cortex superior frontal cortex, inferior orbitofrontal cortex (OBFC) and DLPFC in men, and peaks in the amygdala, hippocampus, insula, thalamus, rostral ACC, OBFC and caudal DLPFC in women [reviewed in (Sacher et al, 2013)]. Thus, both men and women activate fronto-limbic regions when processing emotional information, but peaks differ. Sex differences in activation to emotion processing may explain, at least in part, the greater prevalence of mood disorders in women (Kessler et al, 1993; Wade et al, 2002) but more work is needed; even more so in non-adult populations.

Adolescence is a transitional period characterised by heightened experience of emotions as well as increased independence and important psychosocial (Blakemore, 2012; Casey et al, 2008; Yurgelun-Todd, 2007) and physiological change (Blakemore, 2012), correlating with an increase in symptoms of psychopathology such as depression. Indeed, prevalence of depression increases from 1% in children below age 12 to 17-25% by the end of adolescence (Andersen & Teicher, 2008). Even if psychopathology is absent, risk factors may be present and render an individual susceptible to later development of psychopathology. High neuroticism may be such a risk factor, since it is often found in association with psychopathology, particularly internalizing disorders (Barrantes-Vidal et al, 2009; Bienvenu et al, 2007; Hansell et al, 2012; Khan et al, 2005) and individuals high in neuroticism react more strongly to negative emotions in emotion induction paradigms (Canli, 2004). Assessing emotion processing in association with the early environment, neuroticism and depressive symptomatology during adolescence is thus of particular interest.

Disentangling the influence of the early environment from genetic confounds represents an important challenge, which can be solved utilizing a MZ twin paradigm. Since MZ twins are genetically identical, it follows that phenotypic differences between them must be due to unique environmental influences. We thus tested, in adolescent MZ twins, the following hypotheses: Lower BW and early adverse parenting would be associated with greater peaks of activation to emotional stimuli in fronto-limbic circuitry (i.e., amygdala, hippocampus, insula, ACC and PFC); and, among MZ twin pairs, discordance in BW and

parenting behaviours would be associated with discordance in neural activation to emotional stimuli in fronto-limbic circuitry. Furthermore, since symptoms of internalizing disorders often manifest in adolescence, we tested the hypothesis that current internalizing symptoms and high neuroticism would be associated with greater responses to emotional stimuli in fronto-limbic circuitry; and discordance in internalizing symptoms and neuroticism would be associated with discordance in neural activation to emotional stimuli in these regions. We also examined sex differences. In all conditions, our primary focus was on neural activation in response to sad and fearful stimuli.

METHODS

Participants

Participants were 52 fifteen-year old MZ twin pairs (22 male and 30 female pairs) from the Quebec Newborn Twin Study (QNTS) (Boivin et al, 2005; Brendgen et al, 2005), followed longitudinally since their birth, in the area of Montreal, Canada, between April 1995 and December 1998. All participants were healthy, free of any medication liable to affect brain function and free of current depression and substance dependence. The research protocol was approved by the appropriate ethics committees. Written assent was obtained from all participants and written consent from parents of all participants.

Measures

BW was extracted from hospital records and measured on a continuous scale. BW is commonly used as an index of in utero adversity. In order to calculate BW discordance, one twin's BW was subtracted from the other twin's (Vitaro et al, 2009). Maternal hostile parenting habits were assessed using a subscale of the Parental Cognitions and Conduct toward the Infant Scale [PACOTIS; (Boivin et al, 2005)], a 23-item self-report questionnaire rated by the mother at 5 months. The hostile-reactive subscale consists of items such as "I have raised my voice or shouted at my baby when he/she is particularly fussy". This scale was continuous for the between-pair analysis, and within-twin pair discordance scores were

calculated for the within-twin pair analyses. Maternal depressive symptoms were assessed using the Symptom Checklist (SCL-90; (Derogatis & Melisaratos, 1983) in accordance with (Levesque et al, 2011), when the twins were 5, 18, 30 and 48 months and averaged over these four time points.

At age 15, in addition to neuroimaging, we measured neuroticism using a subscale of the Junior Eysenck Personality Questionnaire [JEPQ; (Eysenck & Eysenck, 1975)], a 97-item self-report questionnaire rated on a dichotomous scale. In addition, we measured internalizing symptoms using the Dominic, a computerized questionnaire designed for children and adolescents (Scott et al, 2006) to screen for a range of psychopathologies, namely specific phobias, generalized anxiety, depression, opposition, conduct problems, inattention and hyperactivity, as well as substance use (Hamilton & Gillham, 1999). We also administered the Kiddie Schedule for Affective Disorders and Schizophrenia (KSADs), a semi-structured diagnostic interview based on DSM-IV criteria (Endicott & Spitzer, 1978; Kaufman et al, 1997) as a more extensive assessment of potential psychopathologies. These tests were performed with the aim of identifying exclusion criteria, but we also retained the internalizing disorders subscale of the Dominic (Scott et al, 2006), consisting of 48 items rated dichotomously (yes/no), as a measure of current internalizing symptoms.

Neuroimaging

All participants were scanned on a 3T Siemens TIM Trio Scanner located at the Montreal Neurological Institute (MNI). The scan included a brief localizer, a 9-minute anatomical scan, and a 15-minute functional scan during which an event-related emotion processing task was performed. For fMRI, 40 functional whole-brain images (multi-slice gradiant echo EPI with 3.5 mm isotropic resolution and TR/TE = 2100/30ms) were acquired using a 32-channel head-coil. The emotion processing task was adjusted from the task used in (Canli et al, 2005) and consisted of a series of 120 Ekman faces with different emotions (happy, sad, angry, fearful and neutral) from the Pictures of Facial Affect series (P & WV, 1975). Faces were presented randomly and followed by a fixation cross and a question asking whether the face belonged to a man or a woman. Neural activation to sadness and fear relative to neutral stimuli were the main outcomes of interest.

Analyses

Pre-processing steps were performed in SPM8 (Wellcome Department of Cognitive Neurology, London UK). Images of all participants were corrected for slice timing differences in acquisition, realigned to correct for small head movements, co-registered with their anatomical scan, spatially normalized into an EPI stereotactic Space (MNI template) and convolved with a 3-D isotropic Gaussian kernel at 7mm FWHM in order to improve the signal-to-noise ratio and correct for minor heterogeneity in neural anatomy across participants.

Intra-individual first level analyses were performed in order to calculate contrasts (emotion minus neutral) for each emotion at each voxel. Additionally, we calculated within-pair discordance by subtracting the activation of one twin from the other. These contrast and discordance files were then used for group comparisons across participants both between- and within-twin pairs, respectively.

Regression analyses were performed between-pairs, by regressing brain activation in response to specific emotion contrasts independently onto early adversity measures as well as neuroticism and internalizing disorder symptoms at each voxel, in one randomly selected twin per pair. Then, we regressed within-twin pair brain activation discordance in response to each emotion onto within-twin pair discordance in early adversity measures, neuroticism and internalizing disorder symptoms to control for genetics (Vitaro et al, 2009). These analyses were performed first in both sexes together, then in boys and girls separately in order to assess the effect of sex. These analyses were conducted using SPM8. Images were created with xjview (www.alivelearn.net/xjview). We set the threshold of activation in the whole brain at $p<0.001$ and only considered clusters showing a spatial extent of at least 20 contiguous voxels. We corrected for multiple comparisons using the familywise error rate (FWE) (Shaffer, 1995).

Small volume corrections were performed in region of interests (ROIs) defined a priori: the amygdala, hippocampus, ACC, insula, as well as superior, medial and orbital PFC. These regions have been consistently activated in neuroimaging studies of emotion processing (Adolphs, 2002; Beauregard et al, 2006; Eimer & Holmes, 2007; Fusar-Poli et al, 2009; Hennenlotter & Schroeder, 2006; Herba & Phillips, 2004; Hogan & Park, 2000; Hung et al,

2012; Levesque et al, 2011; Straube & Miltner, 2011) Masks were made with the ImCalc function in SPM8 based on the Automated Anatomical Labeling (AAL) atlas (Quantitative Neuroscience Laboratory 2011-2012). The threshold p value used was again $p<0.001$. For all ROIs except the amygdala, only clusters showing a spatial extent of at least 20 contiguous voxels were considered, and for the amygdala, that threshold was set at five contiguous voxels.

RESULTS

Descriptives

Table I describes the characteristics of the sample. There was a significant correlation between maternal depressive symptoms and maternal parenting behaviours ($r=0.46$; $p<0.01$), as well as between maternal depressive symptoms and internalizing symptoms ($r=0.29$; $p<0.05$) in adolescents. There was also a significant correlation between discordance in neuroticism and internalizing symptoms ($R=0.56$; $p<0.01$).

Early adversity

Birth weight. Although BW was not found to be significantly associated with brain activation in any of the regions of interest between pairs, within-pair discordance in BW was associated with within-pair discordance in neural responses to internalizing disorder-related stimuli (see table II), but the effects were sex-specific. In girls, greater discordance in BW was associated with greater discordance in the left middle frontal gyrus in response to sad faces ($pFWE=0.009$; MNI coordinates: -30 50 16) (see figure 1). In boys, there was a negative association between BW discordance and brain activation discordance in response to fearful faces in the right mid cingulate gyrus ($pFWE=0.047$; MNI coordinates: 4 2 30) and the left insula ($pFWE=0.032$; MNI coordinates: -32 -14 18).

Maternal hostile parenting behaviours. We found that the activation patterns depended on sex and emotion (see table II). In girls, greater maternal hostility at 5 months was associated

with greater activation in the left medial superior frontal cortex in response to sad faces (pFWE=0.047; MNI coordinates: -12 54 16). In boys, maternal hostile parenting behaviours were negatively associated with brain activation in the left medial frontal gyrus in response to fearful faces (pFWE=0.024; MNI coordinates: -24 52 14). No association was found between within-twin pair discordance in maternal hostile behaviours at 5 months and within-pair discordance in brain activation.

Maternal depressive symptoms. Greater maternal depressive symptoms were associated with greater activation in the right medial superior frontal cortex (pFWE=0.032; MNI coordinates: 8 60 28) in response to sadness in girls (right: pFWE<0.001; MNI coordinates: 4 -32 30 and left: pFWE=0.048; MNI coordinates: -14 36 22). See figure 2 and table II. There was no association with maternal depressive symptoms in boys.

Neuroticism and subclinical depressive symptoms

Neuroticism. Between-pairs, greater neuroticism was associated with greater activation in the right middle frontal gyrus in response to fearful faces (pFWE=0.043, MNI coordinates: 48 10 44), irrespective of sex (see figure 3). Additionally, in girls, greater neuroticism was associated with greater activation in the left amygdala (pFWE=0.041, MNI coordinates: -22 0 -20) and the right middle frontal gyrus (pFWE=0.029, MNI coordinates: 48 6 44) in response to fearful faces. In boys, there was a negative association between neuroticism discordance and brain activation discordance in the left thalamus in response to sad faces (pFWE<0.001, MNI coordinates: -22 -30 0). See table II.

Internalizing symptoms. We found no significant association between internalizing symptoms as assessed by the Dominic and brain activation between-pairs. Discordance in internalizing symptoms was negatively associated with discordance in activation in the left hippocampus (pFWE=0.041, MNI coordinates: -36 -20 -16; see figure 4) and left amygdala (pFWE=0.041, MNI coordinates: -25, -4, -22) in response to sad faces. A separate analysis for boys and girls showed that these effects appear to be specifically driven by boys (see table II).

Anger and happiness

Although neural activation in response to anger and happiness was outside our primary interest, we explored whether it would be associated with early adversity factors and current symptoms of internalizing disorders. In girls, we found a within-pair association with BW discordance in the right PFC and ACC in response to angry faces, as well as between-pair associations with maternal hostility and depression in the ACC and PFC in response to angry and happy faces. In boys, we found a within-pair association with neuroticism discordance in the right amygdala in response to angry faces, as well as a between-pair association with maternal hostility in the left PFC in response to angry faces (see table II).

DISCUSSION

This study assessed the association between mild in utero and early postnatal adversity and neural responses to emotional stimuli using a MZ twin design, with an emphasis on stimuli relevant for (vulnerability to) depression. BW, hostile parenting and maternal depressive symptomology were used as indices of the in utero and early postnatal environment, while within-MZ twin pair discordances (BW, parenting practices) allowed us to control for genetic influences. The main findings of the present study were that both the in utero and early postnatal environment were associated with fronto-limbic neural processing of depressive-relevant emotions, irrespective of genetics. Furthermore, these associations were sex-specific.

In regards to sex, we mostly found positive associations in girls and negative associations in boys. This was unexpected, and seems to suggest that the in utero and early postnatal environment plays a greater role for some aspects of brain development in girls than in boys, or that when faced with adversity, genetics contribute to brain development more strongly in boys than in girls. This notion is in line with one of our recent studies, in which we assessed genetic and environmental contributions to negative emotionality in MZ and dizygotic (DZ) infant twins. We found that at 18 months, negative emotionality was mostly attributed to the shared environment in girls, while in boys, the genetic sequence was the greatest contributor (Schumann et al, in prep). Furthermore, BW has been associated with

hippocampal volume in those with low maternal care in adult women but not men (Bock et al, 2014). On the other hand, studies have found greater vulnerability to maternal stress during pregnancy for boys compared to girls (Dancause et al, 2011; Eriksson, 2009). Nevertheless, the findings are in accordance with research showing divergent functioning of frontal regions in response to emotional stimuli across the sexes (Sacher et al, 2013). The impact of the early environment on males and females may depend on the type, timing and severity of environmental events; and specific investigated outcome measure. Overall, the present study is the first to show that the in utero environment affects neural processing of depressive-relevant stimuli in adolescence when controlling for genetic factors.

Of note, no within-pair association between early maternal hostile parenting behaviours and brain function was found. This is mostly likely due to the similarity in how mothers treat or report treating their children. Indeed, Boivin and colleagues assessed parenting behaviours in this sample and a related sample of singletons and found that the intra-class correlation for maternal hostile parenting behaviours was 0.83 in MZ twins (Boivin et al, 2005). Some within-pair variability was found, which is why we found it worthwhile to assess, but between-pair variability was greater than within-pair variability, as expected.

Overall, this study demonstrated associations between neural functioning of various fronto-limbic regions in response to different emotions and both early adversity and current manifestations of susceptibility to depressive disorders. According to previous work (Etkin et al, 2011; Fusar-Poli et al, 2009; Hariri et al, 2002; Lindquist et al, 2012), we expected to find mainly altered functioning in response to negative emotions, particularly fear and sadness. We did find associations with fear and sadness, but associations with happiness and anger were also found. This was surprising (Adolphs, 2002; Fusar-Poli et al, 2009; Herba & Phillips, 2004; Lindquist et al, 2012; van der Werff et al, 2013), particularly since activation to positive and negative emotions were in the same direction. Perhaps given that this is a young and healthy sample, particular response patterns to specific emotions have not yet been set in stone. Indeed, studies have shown a shift in brain regions processing emotions during development (Hogan & Park, 2000; Hung et al, 2012). In a review, Adolphs reports that some studies have failed to find amygdala activation in response to fearful faces in children, while

others report that activation is most robust prior to adolescence and a general shift to more frontal activation post adolescence, particularly in females (Adolphs, 2002). More work is needed to distinguish the impact of differing emotion processing paradigms in boys and girls across development.

Aside from a limited sample size, our study could have benefited from an emotion processing task that includes dynamic faces as well as static faces. As reported by (Kilts et al, 2003), brain regions processing information from static vs. dynamic faces may differ, and dynamic faces are more realistic. It would thus be of interest to compare neural responses to static vs. dynamic faces. However, that would have lengthened the task substantially, which would not have permitted the assessment of other brain modalities (e.g. brain structure) due to the limited time participants can be expected to lie still in the scanner. We thus chose to limit the task to static faces. In addition, although it would have been interesting to assess whether brain activation to sadness and/or fear mediates the association between early adversity and current internalizing symptoms and neuroticism, we could not perform a formal mediation analysis since brain activation to emotional stimuli, internalizing symptoms and neuroticism were assessed at the same time. It will certainly be interesting however, to assess whether brain activation in response to emotional stimuli at age 15 predicts internalizing symptoms and neuroticism at a later time point.

Strengths of the study include the use of a prospective sample of monozygotic twins followed longitudinally. By assessing discordance in brain function within twin pairs and thus controlling for genes, we demonstrate the importance of the early unique environment for brain function during adolescence implicated in emotion processing. To our knowledge, no fMRI studies have used this design. Moreover, since the sample is followed longitudinally, all of our measures were prospective and thus avoid retrospective biases.

Overall, this study supports the association between early adversity factors and altered brain development, and builds on previous work by demonstrating that discordant environmental factors contribute to phenotypic discordance while controlling for genetics. Moreover, we show that even in the absence of current psychopathology, potential risk factors for psychopathology, including higher levels of neuroticism and mild internalizing disorder

symptoms, contribute to the variation in neural activity to emotional stimuli in fronto-limbic brain regions. By identifying at-risk individuals, it may eventually be possible to target preventive interventions when and in whom they may be the most beneficial and hopefully prevent development of psychopathology in vulnerable individuals.

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Table I. Sample descriptives.

	N	Range	Mean (SD) or frequency
Sex (girls/boys)	52		30/22
Birth weight (1 twin; in kg)	52	1.36 to 3.73	2.58 (0.53)
BW discordance (in kg)	52	-1.07 to 1.04	0.03 (0.39)
Maternal depressive symptoms (SCL-90)	50	34 to 70	55.82 (7.66)
Maternal hostility (1 twin; PACOTIS)	42	0 to 7.5	1.58 (1.68)
Maternal hostility discordance	41	-3.75 to 2.0	-0.05 (1.03)
Neuroticism (1 twin)	52	0 to 18	7.94 (4.91)
Neuroticism discordance	52	-11 to 11	-0.40 (5.01)
Dominic internalizing symptoms score (1 twin)	51	1 to 27	9.82 (6.74)
Dominic internalizing symptoms score (discordance)	51	-11 to 15	-0.63 (5.51)

Descriptives for the 52 pairs of MZ twins assessed in this study.

SD = standard deviation; SCL-90 = Symptom Checklist; PACOTIS = Parental Cognitions and Conduct toward the Infant Scale

Table II. Significant associations between predictors and neural activation in response to emotion processing.

Predictor	**Sex**	**Emotion**	**Contrast**	**Whole-brain or ROI**	**T**	**k**	**pcorr**	**MNI Coordinates**	**Region**
Sex	Both sexes	Happy	Girls - boys	ROI	3.87	48	0.034 (FWE)	14 38 8	R ACC
BW (1 twin)	Both sexes	Angry	Negative	WB	4.86	202	0.042 (FWE)	-30 -56 16	L temporal lobe (sub-gyral WM)
BW discordance	Boys	Angry	Negative	WB	5.11	149	0.050 (FWE)	-16 -42 18	L lateral ventricle/corpus callosum
	Boys	Fearful	Negative	WB	5.94	157	0.047 (FWE)	4 2 30	R mid-cingulate gyrus
	Boys	Fearful	Negative	WB	4.79	665	0.000 (FWE)	-30 -48 16	L temporal lobe (sub-gyral WM)
	Boys	Fearful	Negative	WB	4.72	211	0.014 (FWE)	-18 -38 16	L lateral ventricle
	Boys	Fearful	Negative	ROI	4.24	59	0.032 (FWE)	-32 -14 18	L insula
	Girls	Sad	Positive	ROI	5.15	127	0.009 (FWE)	-30 50 16	L middle frontal cortex
	Girls	Angry	Positive	WB	5.00	211	0.013 (FWE)	22 38 8	R superior frontal cortex extending to ACC
Maternal hostile behaviours (1	Boys	Angry	Negative	WB	5.14	181	0.047 (FWE)	-14 56 6	L medial frontal cortex

twin)									
		Fearful	Negative	WB	5.86	178	0.024 (FWE)	-24 52 14	L medial frontal cortex
	Girls	Happy	Positive	WB	5.10	128	0.042 (FWE)	-30 -54 46	L inferior parietal lobule
				ROI	5.30	77	0.016 (FWE)	-44 6 50	L medial frontal cortex
				ROI	5.16	47	0.021 (FWE)	20 42 -14	R superior orbitofrontal cortex
		Sad	Positive	ROI	4.45	50	0.047 (FWE)	-12 54 16	L medial superior frontal cortex
		Angry	Positive	WB	5.30	142	0.039 (FWE)	-38 2 50	L PFC
Maternal depression	Girls	Happy	Positive	WB	4.57	404	0.000 (FWE)	4 -32 30	R ACC
				ROI	4.13	31	0.048 (FWE)	-14 36 22	L ACC
				ROI	4.47	49	0.039 (FWE)	-14 42 24	L medial superior frontal cortex
				ROI	5.06	97	0.011 (FWE)	-46 6 48	L PFC
		Sad	Positive	ROI	3.99	59	0.049 (FWE)	36 0 42	R PFC
				ROI	4.55	95	0.017 (FWE)	26 -16 66	R PFC
				ROI	3.88	61	0.032 (FWE)	8 60 28	R medial superior frontal cortex
Neuroticism	Both	Happy	Positive	ROI	4.36	48	0.035 (FWE)	12 36 14	R ACC

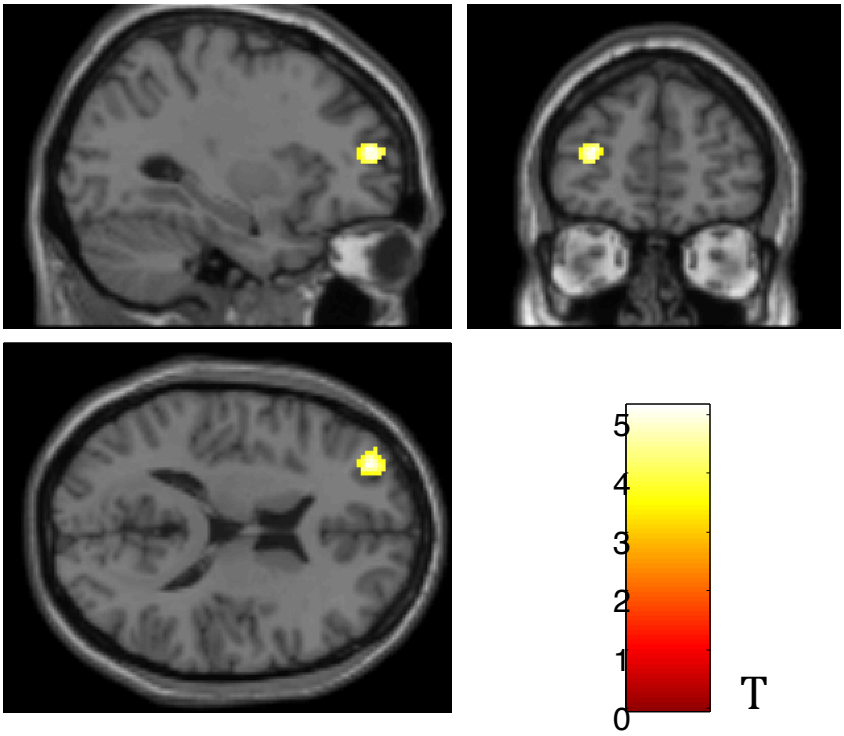
(1 twin)	sexes								
		Fear	Positive	WB	3.75	71	0.043 (FWE)	48 10 44	R Precentral gyrus
	Girls only	Happy	Positive	WB	4.86	177	0.019 (FWE)	-5 18 56 & 4 18 58	L & R Superior frontal gyrus
		Fear	Positive	ROI	4.12	9	0.041 (FWE)	-22 0 -20	L Amygdala
		Fear	Positive	ROI	4.96	69	0.029 (FWE)	48 6 44	R middle frontal gyrus
Neuroticism (discordance)	Both sexes	Angry	Positive	WB	4.35	222	0.017 (FWE)	-4 -70 -22 & 6 -72 -22	L & R Cerebellum
	Boys only	Sad	Negative	WB	5.55	805	0.000 (FWE)	-22 -30 0	L Thalamus
			Negative	WB	5.58	805	0.000 (FWE)	44 -56 -6	R Inferior temporal lobe
		Angry	Negative	ROI	4.78	9	0.041 (FWE)	26 -4 -16	R Amygdala
Dominic internalizing symptoms subscale (discordance)	Both sexes	Happy	Negative	ROI	4.0	54	0.048 (FWE)	-42 -4 -6	L insula
		Sad	Negative	WB	4.65	198	0.041 (FWE)	-36 -20 -16	L hippocampus
			Negative	ROI	4.02	6	0.046 (FWE)	-26 -2 -22	L amygdala
			Negative	WB	4.48	263	0.014 (FWE)	2 -54 -12	R cerebellum

	Boys only	Sad	Negative	ROI	7.39	71	0.005 (FWE)	-28 0 -18	L Amygdala
			Negative	WB	5.73	225	0.023 (FWE)	-40 2 -14	L Temporal
			Negative	WB	6.36	1591	0.000 (FWE)	-8 -50 -14	L Cerebellum
			Negative	WB	5.73	1591	0.000 (FWE)	-34 -14 -20	L Hippocampus

Complete list of results.

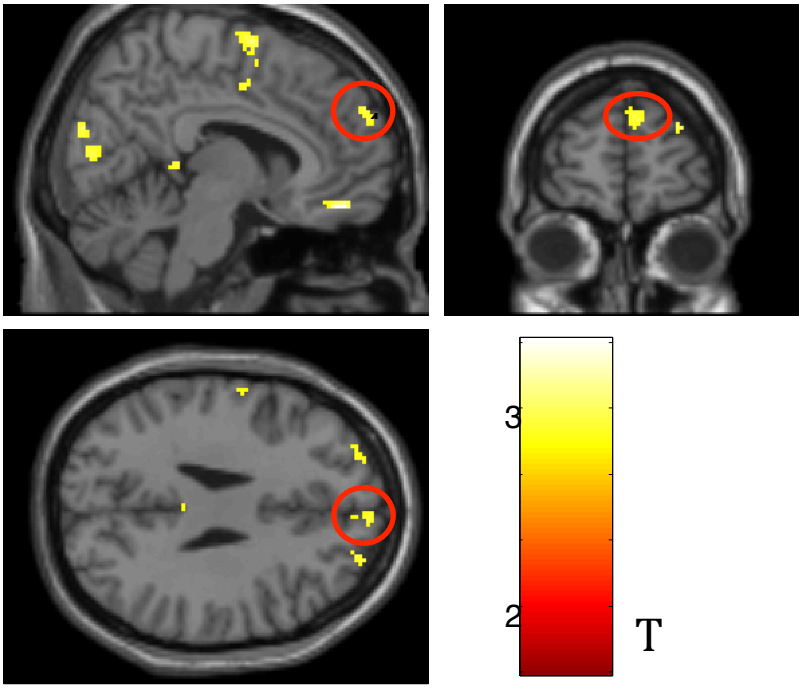
WB=whole brain; ROI=region of interest; k=number of voxels L=left; R=right; FWE=familywise error rate.

Figure 1. Association between BW and neural activation to an emotion processing task.



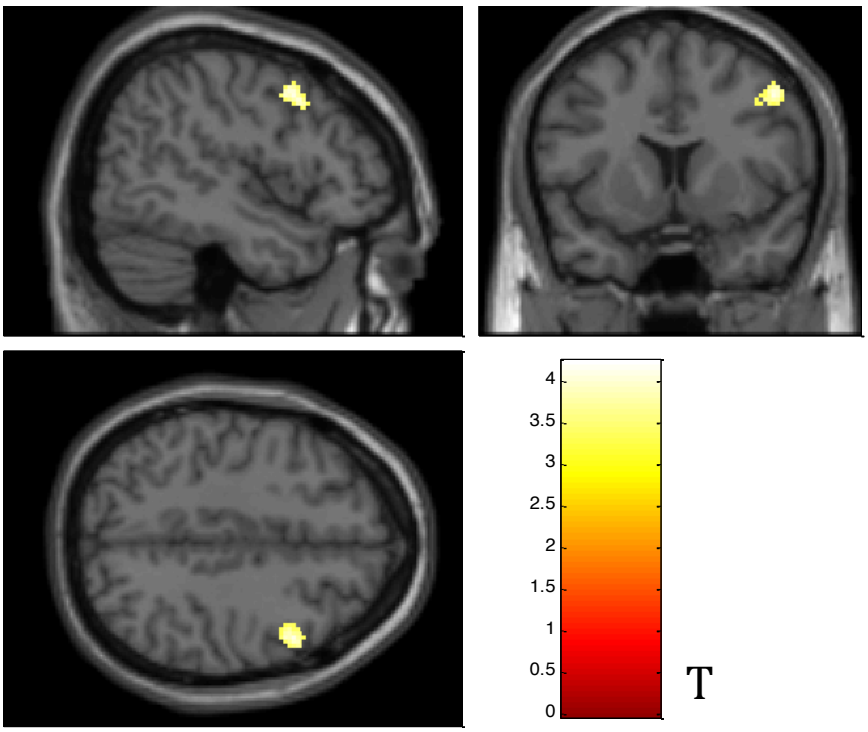
Greater BW discordance is associated with greater activation discordance in the left middle frontal cortex in response to sadness in girls only.

Figure 2. Association between maternal depressive symptoms and brain activation to an emotion processing task.



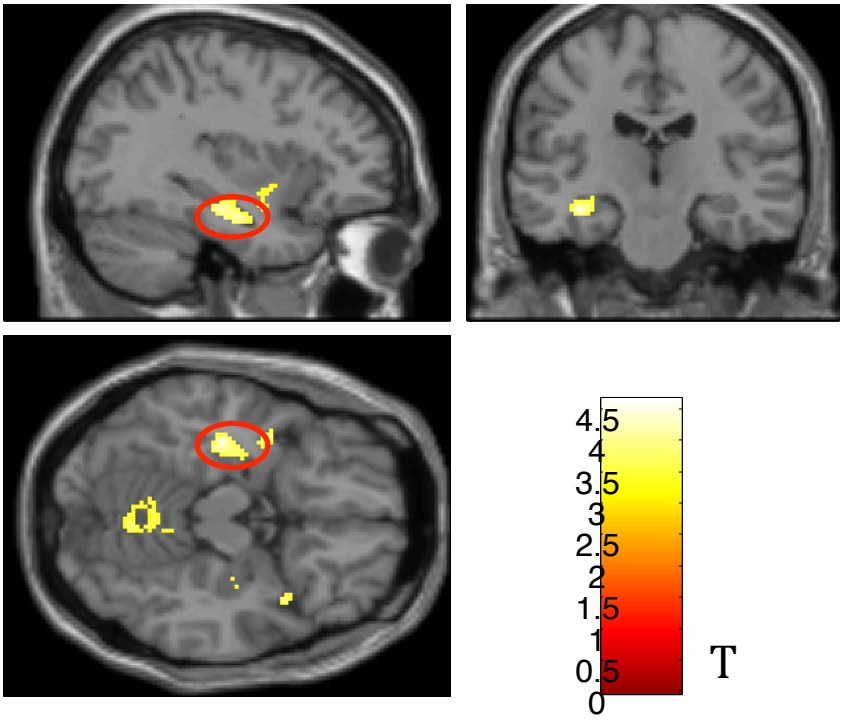
In girls, greater maternal depressive symptoms were associated with greater activation to sadness in the right medial superior cortex.

Figure 3. Association between neuroticism and brain activation to an emotion processing task.



Greater neuroticism is associated with greater activity in the right middle frontal cortex in response to fearful faces in both genders.

Figure 4. Association between internalizing disorder symptoms and brain activation in response to an emotion processing task.



There was a negative association between internalized symptoms assessed by the Dominic and brain activation in the left hippocampus in response to sad faces.

CHAPTER 3 – Early adversity and brain volume in adolescent monozygotic twins

Foreword

In this third chapter we assess the association between early adversity factors and brain volume in adolescence in the QNTS. These analyses were performed on data that I collected with the help of other lab members, which included neuroimaging, saliva sampling for extraction of DNA methylation levels, questionnaires and computer-testing. This is the same sample as the previous chapter. I performed all SPSS analyses including mediation/moderation and multilevel analyses and Cherine Fahim performed the analysis in SPM assessing in which regions BW was associated with brain volume. Elmira Ismaylova, Marie-Pier Verner and Kevin Casey helped with recruitment and data collection. I drafted the manuscript. Frank Vitaro, Michel Boivin, Richard Tremblay, Mara Brendgen and Ginette Dionne are responsible for the twin cohort and approved the final version of the manuscript, which we will submit for publication shortly following the submission of this thesis. Linda Booij and Richard Tremblay came up with the idea for this project and secured funding, supervised data collection and analysis and provided feedback on earlier versions of this manuscript. A special thanks goes to Mara Brendgen for contributing to the interpretation of the findings.

The impact of the in utero and early postnatal environments on grey and white matter volume: A study with adolescent monozygotic twins

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ABSTRACT

Introduction. Prenatal and early postnatal adversities have been shown to be associated with brain development. However, we do not know how much of this association is confounded by genetics, nor whether the postnatal environment can mediate or moderate the impact of in utero adversity. This study used a monozygotic (MZ) twin design to assess 1) the association between birth weight (BW) and brain volume in adolescence; 2) the association between within-twin-pair BW discordance and brain volume discordance in adolescence; and 3) whether the association between BW and brain volume in adolescence is mediated or moderated by early negative maternal parenting behaviours. **Methods.** These associations were assessed in a sample of 108 MZ twins followed longitudinally since birth and scanned at age 15. Total grey (GM) and white matter (WM) volumes were obtained using the DARTEL toolbox in SPM8. **Results.** We found that BW was significantly associated with total GM and WM volumes, particularly in the superior frontal gyrus and thalamus. Within-twin-pair discordance in BW was also significantly associated with within-pair discordance in both GM and WM volumes, supporting the hypothesis that the specific in utero environment has an impact on brain development independent of genetics. Early maternal hostile parenting behaviours and depressive symptoms were associated with total GM volume, but not WM volume. Finally, greater early maternal hostility may moderate the association between BW and GM volume in adolescence, since the positive association between BW and total GM volume appeared stronger at higher levels of maternal hostility (trend). **Conclusion.** Together, these findings support the importance of the in utero and early postnatal environment for brain development.

INTRODUCTION

In utero events can affect brain development and thus can have long-term effects on global functioning. BW can be used as an overall index of the in utero environment (Allin et al, 2004; Dunkel Schetter, 2011; Himpel et al, 2006; Lazinski et al, 2008). Research has shown that individuals born with very low BW (<1500g) have smaller brains than controls from infancy to early adulthood (Kesler et al, 2004; Kesler et al, 2008; Martinussen et al, 2005; Ment & Vohr, 2008; Nagy et al, 2009; Nosarti et al, 2008; Parikh et al, 2013; Schlotz et al, 2014; Taylor et al, 2011). Structural associations with BW are found throughout the brain, even when BW is within a normal range, as shown in various populations and age groups (Haukvik et al, 2014; Raznahan et al, 2012; Schlotz et al, 2014; Walhovd et al, 2012). However, findings are inconsistent, with other studies finding no association between BW and brain volume (Kesler et al, 2008; Ordaz et al, 2010; van Soelen et al, 2010). This may be due in part to differing definitions of low BW and confounds including prematurity, genetics, and neural injury.

Adversity occurring during the first two years of life can also affect brain development. Examples of stressors that have been associated with altered brain volumes include maltreatment (Hanson et al, 2010; Kelly et al, 2013; McCrory et al, 2010), low SES (Hackman et al, 2010; Noble et al, 2012; Tomalski & Johnson, 2010), maternal depression (Lupien et al, 2011), and institutionalization (Tottenham et al, 2010). What's more, recent studies are showing that the later environment may mediate or moderate the impact of early stressors on brain development (Garner, 2013). For instance, the impact of SES on brain development, as well as emotional and cognitive outcomes, has been shown to be at least partially mediated by parental care [reviewed in (Hackman et al, 2010; Luby et al, 2013; Tomalski & Johnson, 2010)], parental education (Noble et al, 2012), stimulation from the environment and diet (Tomalski & Johnson, 2010). However, the moderating effects of early environmental factors including maternal parenting on in utero adversity have not been studied extensively.

Distinguishing environmental influences on brain development from genetic effects can be difficult since the two are so tightly intertwined (Gatt et al, 2012). MZ twins provide the ideal method to assess environmental factors given that they share 100% of their gene

sequence. Associations found between low BW and brain volume in MZ twins should therefore be attributable to the unique environment. However, to our knowledge, the only study to have assessed the association between BW and brain structure in a MZ twin sample is by Raznahan and colleagues (Raznahan et al, 2012). They assessed brain development longitudinally in MZ and DZ twin pairs, as well as in singletons, and found that lower BW, even when in the normal range, is associated with decreases in brain volume in several regions implicated in mental health problems. In addition, these findings were replicated within MZ twin pairs. The twin with lower BW had a comparatively smaller brain volume than his/her co-twin, providing further support for the importance of the in utero environment for brain development into adulthood (Raznahan et al, 2012). However, the sample included participants aged 3 to 30, making it difficult to assess the specific impact of BW during particular developmental time periods. Adolescence, a period of great physiological and psychosocial change, as well as brain maturation, is a particularly important time period to assess brain development following early adversity. Furthermore, the impact of the postnatal environment was not assessed.

In the present study we aimed to assess 1) the association between the in utero environment (as indexed by BW) and total GM and WM volumes in adolescent MZ twins, and 2) the association between BW discordance and discordance in total GM and WM volumes. We also assessed 3) whether early negative maternal parenting behaviours mediate or moderate the association between BW and total brain GM and WM volumes.

METHODS

Participants

Participants were 108 fifteen-year-old adolescents (54 pairs of MZ twins: 23 male and 31 female) recruited from the QNTS (Boivin et al, 2005; Brendgen et al, 2005). The QNTS used the Quebec Ministry of Health and Social Services registry of new births occurring in the Province of Quebec, between April 1, 1995 and December 31, 1998 to recruit participants and

followed them longitudinally. All participants who underwent scanning at age 15 were healthy and free of psychotropic medications, neurological disease, as well as current depression and substance use disorders. Out of 96 participants with reported gestation length, 20 (10 pairs) were born with gestation less than 36 weeks. See Table I for the sample characteristics. Written informed consent and assent was obtained from the parents and twins, respectively, and the study protocol was approved by the appropriate ethics committees.

Measures

BW was obtained from medical records and measured on a continuous scale. Maternal parenting behaviours were assessed using the PACOTIS (Boivin et al, 2005), a 23-item self-report questionnaire rated by the mother when the twins were 5, 18 and, 30 months. We retained the hostility subscale, measured by items such as "I have raised my voice or shouted at my baby when he/she is particularly fussy." Scores were continuous and averaged over time points. Maternal depressive symptoms were assessed on a continuous scale with the SCL-90 (Derogatis & Melisaratos, 1983; Derogatis et al, 1976) when the twins were 5, 18, 30, and 48 months and averaged over time points in accordance with (Levesque et al, 2011).

Scans were acquired at the Montreal Neurological Institute (MNI) Brain Imaging Centre, with a Siemens Magnetom 3T Tim Trio scanner (www.medical.siemens.com), using a magnetization-prepared rapid acquisition gradient-echo (MPRAGE) 9 min sequence (176 slices; 1 mm thickness, TR=2300 ms, TE=2.98ms, TI=900 ms, flip-angle=9°, FOV=240x256mm).

Pre-processing

SPM8 (Wellcome Department of Cognitive Neurology) implemented in MATLAB R2010a (Mathworks, Sherborn, MA) was used for image analyses. Images were pre-processed and analyzed using the Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra (DARTEL) toolbox in SPM8. The DARTEL toolbox uses a high dimensional warping process that increases the registration between individuals, which results in improved localization and increased sensitivity in analyses (Ashburner, 2007). Smoothing was

performed with an 8mm FWHM Gaussian kernel. We used voxel-based morphometry (VBM) to assess the voxel-wise comparison of the local concentration of GM and WM within the MZ twins. Total GM, WM, and brain volumes were obtained using SPM8.

Analyses

To assess whether the in utero environment, as indexed by BW, is associated with total brain volume, we regressed total GM and WM volumes onto BW in the 108 participants, using the Statistical Package for the Social Sciences (SPSS). Then, we conducted an exploratory VBM whole-brain analysis in SPM8, in order to assess whether there was an association between BW and brain volume in specific brain regions. All reported brain regions were examined at a threshold corrected for multiple comparisons (corrected using FWE at cluster-level, $p<0.05$). To assess the specific effect of the discordant in utero environment within twin pairs, we also regressed within-twin-pair total GM and WM volume discordance onto discordance in BW in SPSS (see Figure 1 for a histogram of BW discordances in our sample). Although GM and WM volumes were significantly lower in girls [$F(1,106)=44.89$, $p<0.001$ for GM; $F(1,106)=41.24$, $p<0.001$ for WM] compared to boys (which is consistent with the literature), since BW did not interact with sex, we collapsed the sexes together for the main analyses. However, we also report on the associations between BW and total GM and WM volumes separated by sex.

We then regressed total GM and WM volumes onto early maternal hostile parenting behaviours and maternal depressive symptoms using SPSS. Then, we conducted multilevel-modelling (MLM) to confirm the associations while accounting for both between- and within-pair variability. For each outcome (total GM and WM volumes), we first assessed the null model with fixed and random effects for the intercept. We then added 1st level predictors (BW, maternal hostile parenting behaviours) one by one, first as fixed effects only, then as fixed and random effects; we kept them in the model if they contributed significantly to the model. We then added 2nd level predictors (sex, maternal depressive symptoms) one by one as fixed factors (Tabachnick & Fidell, 2013). Finally, we assessed whether maternal hostile parenting behaviours might independently mediate or moderate the association between BW and total brain volume, in accordance with (Baron & Kenny, 1986) and (Aiken & West, 1991).

RESULTS

BW and brain volume

We found that BW was significantly associated with both total GM (standardized beta=0.32, t(106)=3.46; p=0.001) and WM (standardized beta=0.30, t(106)=3.22; p=0.002) volumes at age 15; these findings were confirmed using MLM (see Table II) and in a sample consisting of only twins with gestation length greater than or equal to 36 weeks. Using SPM8, we found a significant positive association between BW and the right superior frontal cortex GM volume (MNI coordinates 26 9 60; k=118; peak T(103)=4.55; pFWE=0.008) and a significant negative association with the left thalamus GM volume (MNI coordinates -15 -30 0; k=389; peak T(103)=4.76; pFWE<0.001), as well as a significant negative association between BW and the right superior frontal WM volume (MNI coordinates 30 41 30; k=61; peak T(103)=3.74; pFWE=0.004). See Figures 2–4. When we repeated analyses in boys and girls independently, only the association between BW and total WM volume in girls remained significant, likely due to reduced sample size. Furthermore, within-pair analyses showed that greater within-pair BW discordance was significantly associated with both greater within-pair discordance in GM (standardized beta=0.32, t(52)=2.43; p=0.02) and WM (standardized beta=0.52, t(52)=4.41; p<0.001) volumes, and this finding held in a subsample consisting of only twin pairs with gestation length of 36 weeks or more. When analyzing boys and girls separately, the associations between BW discordance and both GM and WM discordance were still significant in girls, but not in boys.

The postnatal environment and brain volume

In regards to the postnatal environment, there was a significant negative association between maternal hostile parenting behaviours and total GM volume (standardized beta=-0.21, t(96)=-2.11; p=0.04), but not WM volume. There was also a trend for a positive association between maternal depressive symptoms and total GM volume (standardized beta=0.18, t(102)=1.85; p=0.067), but not for WM volume. Results were very similar in MLM (see Table II). Discordance in hostile parenting behaviours was not associated with discordance in GM or WM volumes.

Mediation/moderation

We then assessed whether early maternal hostile parenting behaviours might mediate or moderate the association between BW and total GM volume. Maternal hostile parenting behaviours did not mediate the association between BW and total GM volume. We did, however, find a trend for an interaction between BW and maternal hostile parenting behaviours ($p=0.07$); it appears that the positive association between BW and total GM volume tends to get stronger at higher levels of maternal hostile parenting behaviours (see Figure 5).

DISCUSSION

The present study aimed to assess the association between the in utero and early postnatal environment and total GM and WM volumes in a sample of adolescent monozygotic twins. We found that BW was associated with total GM and WM volumes, particularly in the superior frontal cortex and thalamus. Importantly, greater discordance in BW was associated with greater discordance in total GM and WM volumes within twin pairs, which highlights the importance of the unique environment, independent of genetics. Early maternal depressive symptoms, as well as maternal hostile parenting behaviours, were also associated with total GM volume, but not with total WM volume. Together, these results highlight the importance of the in utero and early postnatal environment for brain volume in adolescence.

Our finding that lower BW is associated with lower total GM and WM volumes is in accordance with previous studies that found altered GM and WM volumes in infants, children, and adolescents born with low BW (Abernethy et al, 2002; Allin et al, 2004; Bjuland et al, 2014; Lowe et al, 2011), and particularly with those studies that used a sample with normative BW variation (Haukvik et al, 2014; Raznahan et al, 2012; Schlotz et al, 2014; Walhovd et al, 2012). At a regional level, we found both increases and decreases in volumes in association with lower BW, which is also in accordance with previous reports [e.g., (Nosarti et al, 2002)]. However, results across studies are somewhat conflicting in regards to direction of change.

Thus, further studies that carefully control for confounds and assess volumes of subregions will be necessary.

We then assessed the association between BW discordance and brain volume discordance within MZ twin pairs during adolescence, in order to control for DNA sequence and assess the specific impact of the unique in utero environment. The study by Raznahan and colleagues is the only other study that has used such a design; however, they used a sample with a wide range of ranges (i.e., from 3 to 30 years of age) (Raznahan et al, 2012). The present study thus corroborates that the in utero environment has a significant impact on brain development above and beyond genetic effects in a sample of adolescent MZ twins followed longitudinally since birth. Together, the findings of our study suggest that those born with low BW do develop differently from their heavier peers, even in the absence of neurodevelopmental impairments.

We did not find any association between discordance in early hostile parenting behaviours and brain volume of either GM or WM. This could be due to low within-family variability in maternal parenting behaviours (Boivin et al, 2005). Alternatively, it could be that maternal parenting behaviours do not affect brain volume independent of genetics. Brain development shows high heritability (Thompson P et al, 2002; Thompson PM et al, 2001), and, genetics and parenting practices are tightly intertwined and influence one another in a bi-directional manner (Pike et al, 1996). It is possible that genetic effects simply outweigh non-shared environmental effects.

We found that early maternal hostile parenting behaviours are associated with GM volume, but not WM. Previous diffusion tensor imaging (DTI) studies have found an association between early adversity, including a positive family history of major depressive disorder, parental verbal abuse, witnessing domestic violence, as well as childhood neglect and maltreatment, with lower fractional anisotropy (FA) values in several WM tracts during adolescence and young adulthood (Choi et al, 2012; Choi et al, 2009; Huang et al, 2011; Huang et al, 2012). It should be noted, however, that these all represent severe forms of adversity and that these studies were most often conducted using participants with post-traumatic stress disorder (PTSD), thereby making it difficult to distinguish the impact of early

adversity from that of having PTSD (Hart & Rubia, 2012). Our sample was exposed to mild forms of early postnatal adversity, and we assessed WM volume using a whole-brain VBM approach as opposed to WM integrity using DTI. It could be that more severe forms of early postnatal adversity have an impact on WM, or that WM integrity is affected to a greater degree than WM volume.

The main limitation of this study was the limited number of participants born with very low BW; however, our findings confirm the importance of BW even within a normal range. Moreover, with a greater sample size, the trend for the moderation of the association between BW and brain volume in adolescence by maternal hostile parenting behaviours may have been significant. Nonetheless, this is one of the few studies that controlled for genetics and isolated the impact of the unique environment on brain development by using a within-MZ-twin design. Furthermore, since this cohort has been followed longitudinally since birth, all information on the early environment is prospective.

Overall, these findings demonstrate that the in utero environment can have an impact on brain volume during adolescence, independent of genetics. Given these results, it may be beneficial to target children born with low BW for preventive interventions early in life.

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Table I. Characteristics of the sample.

Variable	N	Range	Mean (SD)
Birth weight, in kilograms	108	1.0 – 3.73	2.55 (0.5)
BW difference score, in kilograms	54	-1.07 – 1.04	0.02 (0.4)
Gestation length, in weeks	96	30 – 40	36.92 (2.4)
Maternal hostile parenting, assessed with the PACOTIS	98	0 – 8.67	3.49 (2.0)
Maternal depressive symptoms, assessed with the SCL-90	104	34 – 70	55.44 (7.8)
Total GM volume	108	514.06 – 928.87	700.85 (63.6)
Total WM volume	108	380.53 – 672.62	479.16 (57.0)

Descriptive statistics in the sample of 108 fifteen-year-old adolescents.

SD = standard deviation; BW = Birth Weight; SCL-90 = Symptom Check List-90 items; GM = grey matter; WM = white mater.

Table II. Within- and between-family associations with total brain volume as assessed using MLM.

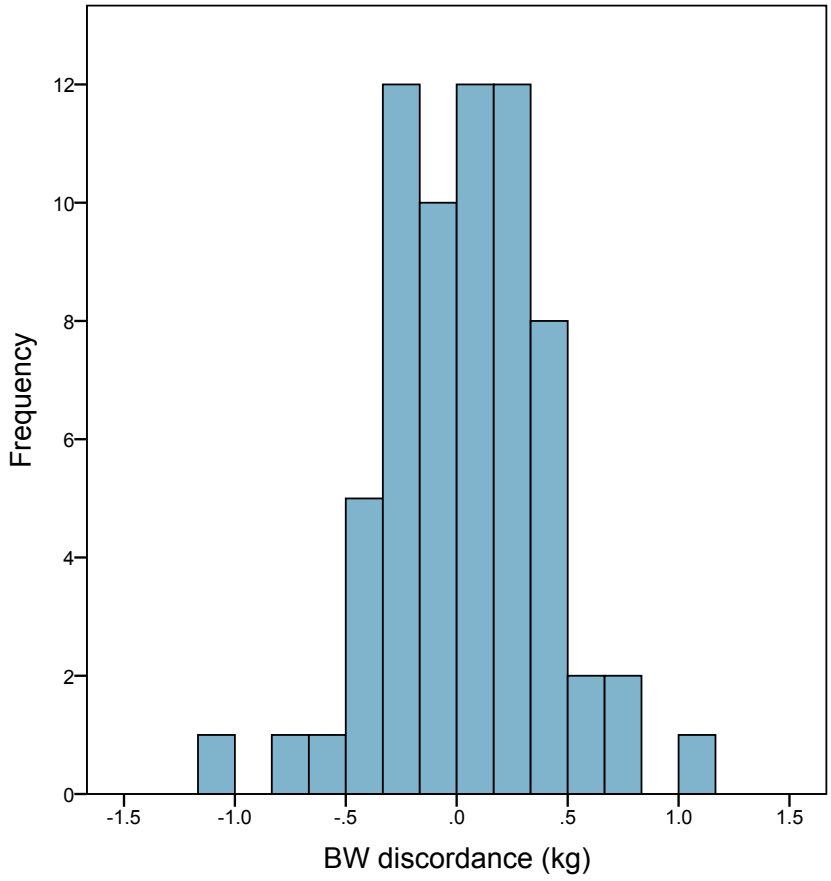
Parameters	Total GM volume	Total WM volume
Fixed effects		
Intercept	737.44 (10.9)****	509.23 (10.3)****
Level 1		
BW	23.39 (8.5)***	22.30 (4.9)****
Level 2		
Sex	-64.58 (14.2)****	-54.12 (13.3)****
Maternal depressive symptoms	1.77 (0.9)**	1.43 (0.8)*
Random effects		
Intercept	2174.79 (471.9)****	2117.03 (426.6)****
Residuals	435.00 (85.4)****	109.02 (21.4)****

The best fitting models for both total GM and WM volumes included BW, sex, and maternal depressive symptoms as fixed factors, and the intercept as a random factor. There is greater variability between- than within-families, after controlling for BW, sex, and early maternal depressive symptoms.

Values for fixed and random effects represent estimates (standard error)

*p<0.1 (trend); **p<0.05; ***p<0.01; ****p<0.001

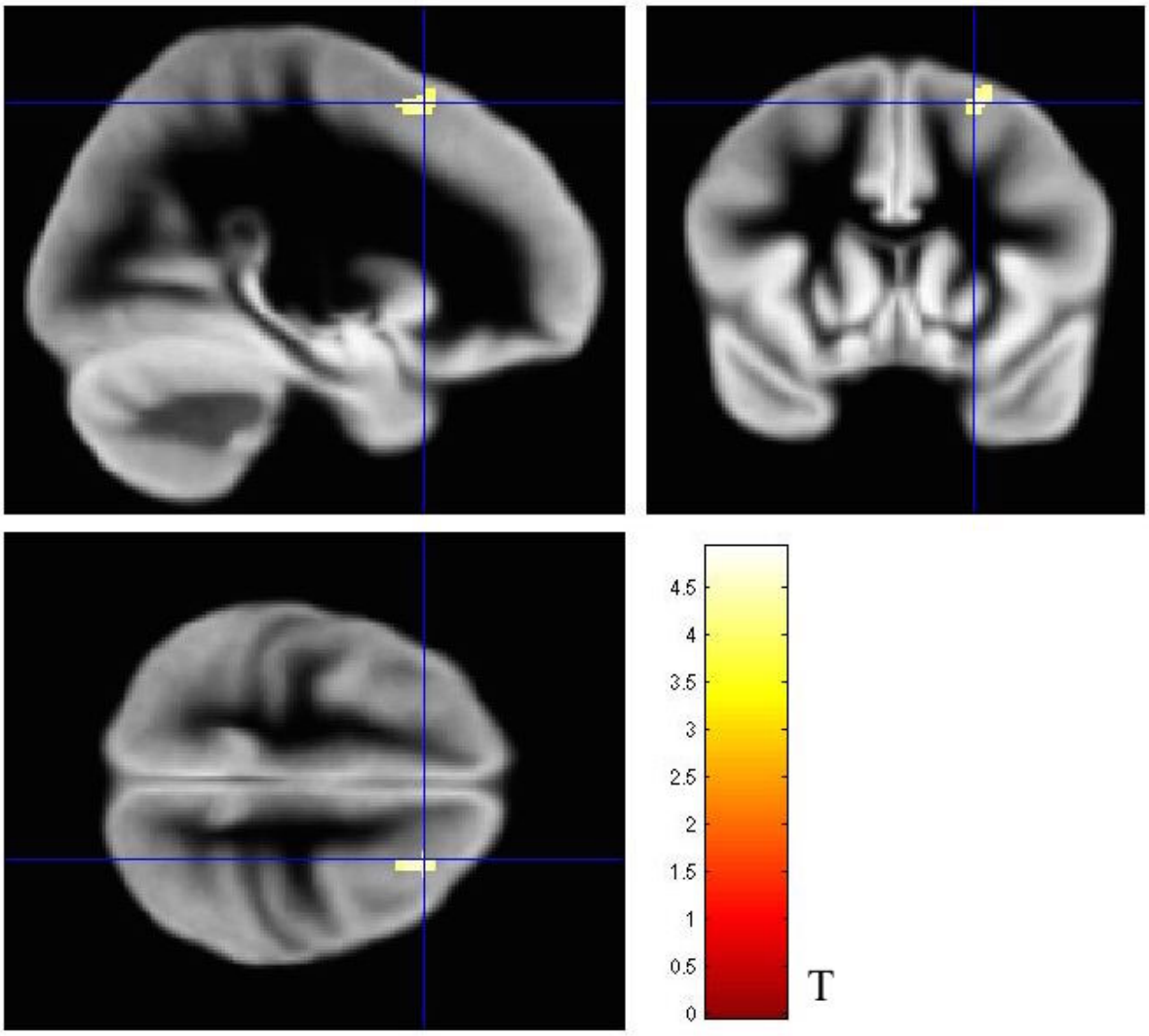
Figure 1. Intra-pair birth weight discordance.



Frequency of BW discordances in MZ twin pair, in kg.

BW = birth weight; MZ = monozygotic; GM = grey matter; WM = white matter.

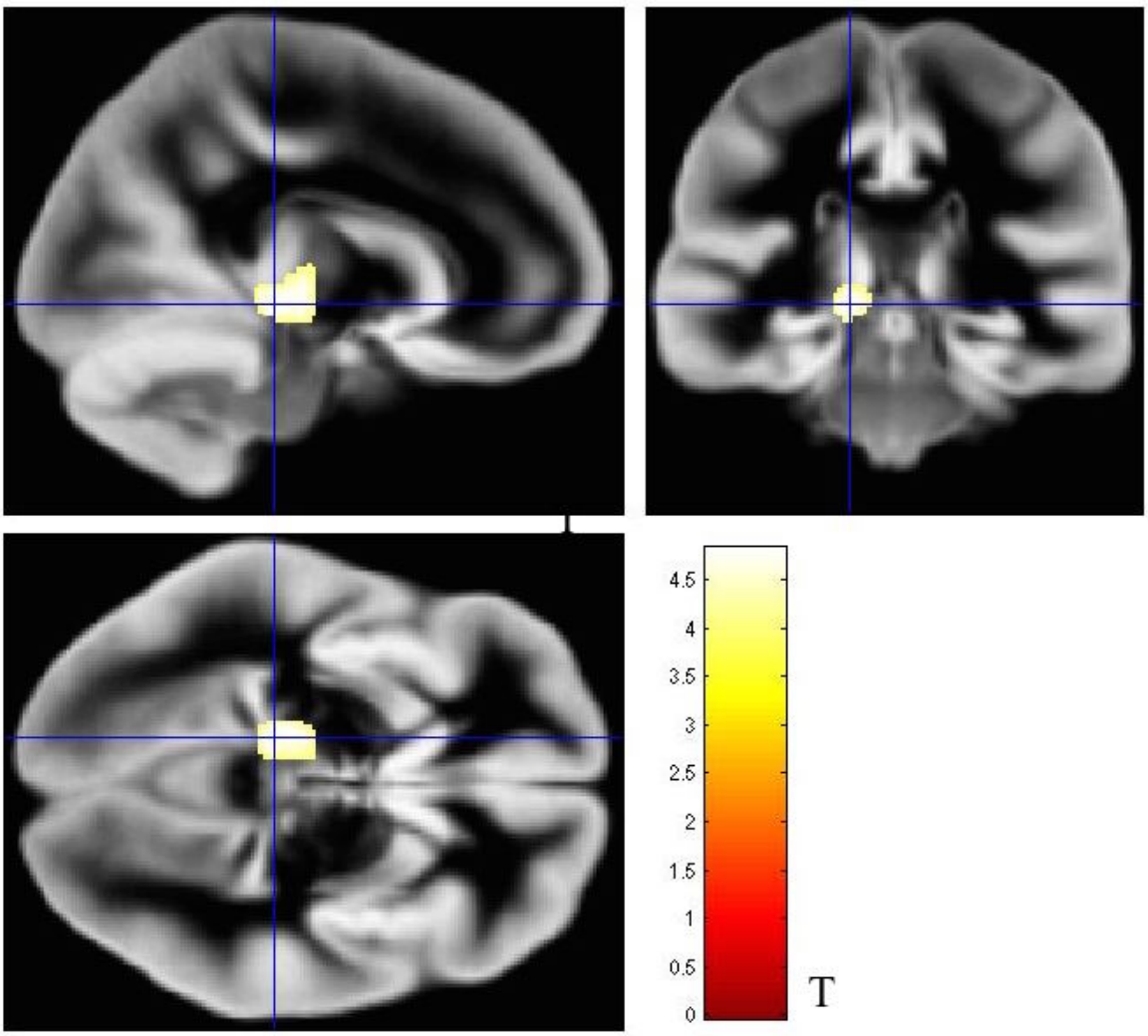
Figure 2. Birth weight is associated with total grey matter volume in the superior frontal cortex.



T-Statistic map of the positive association between regional superior frontal GM volume and BW (pFWE=0.008). Hot and yellowish colors indicate volume increases are correlated with BW.

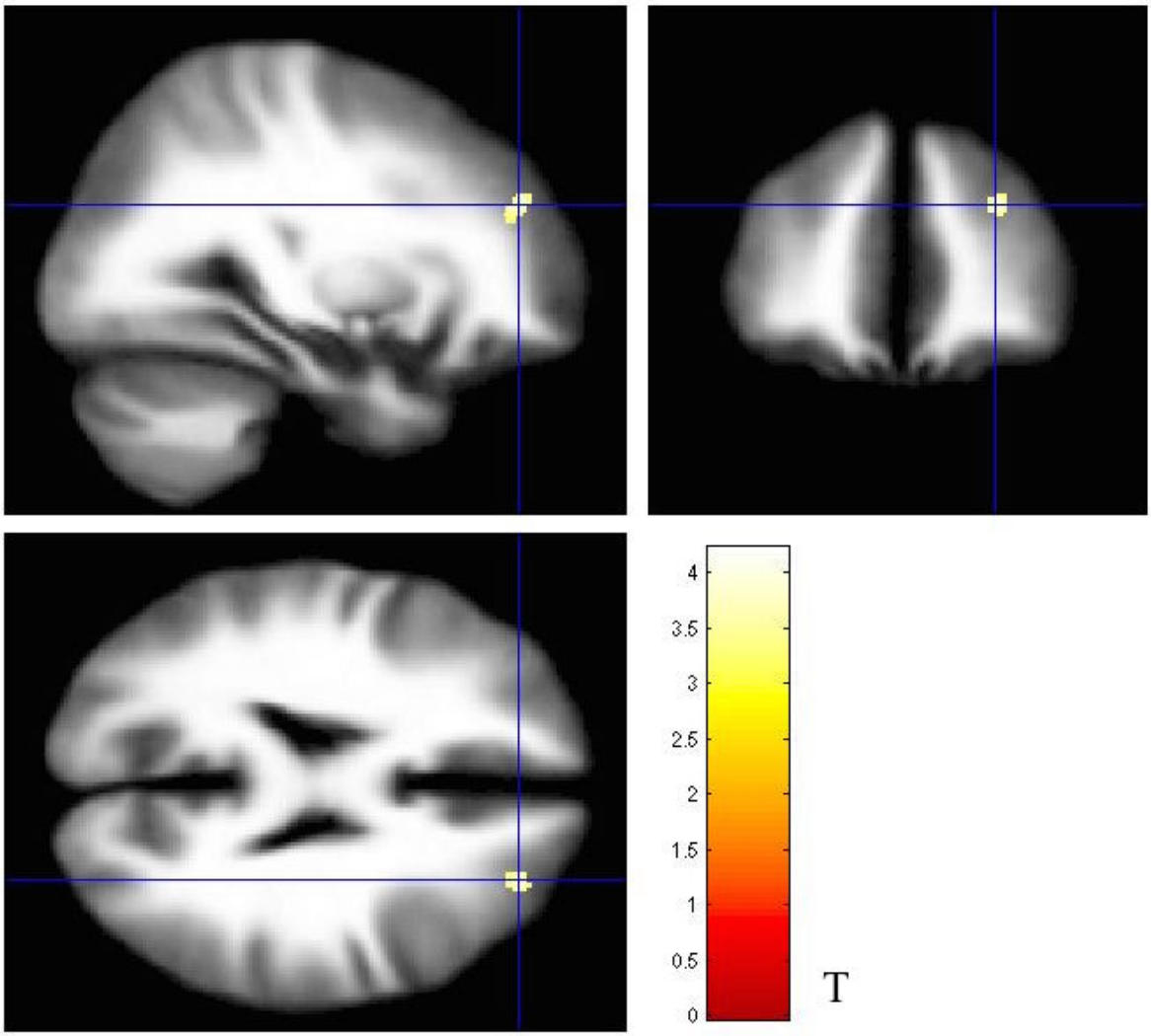
GM = grey matter; BW = birth weight

Figure 3. Birth weight is associated with total grey matter volume in the thalamus.



T-Statistic map of the negative association between regional thalamus GM volume and BW (pFWE<0.001). Hot and yellowish colors indicate volume decreases are correlated with BW. GM = grey matter; BW = birth weight

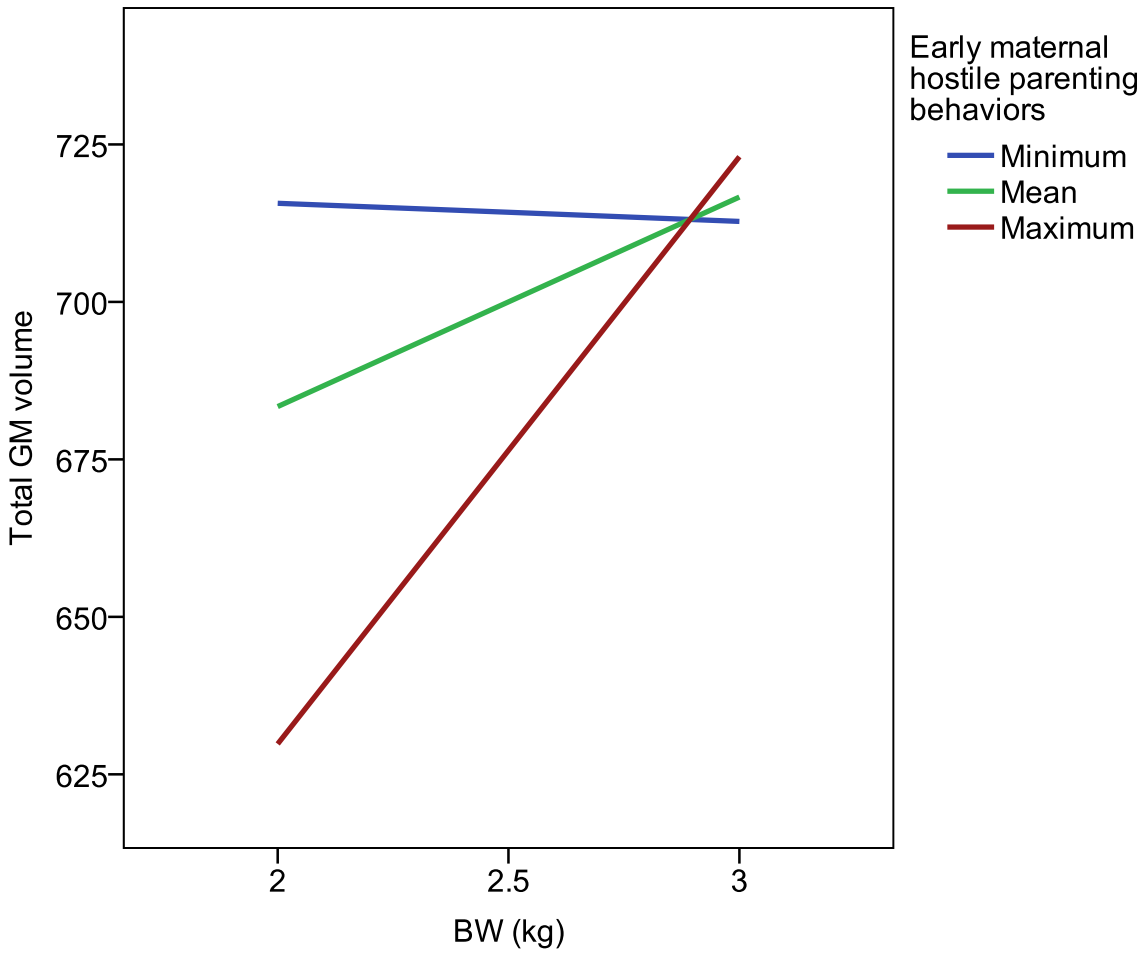
Figure 4. Birth weight is associated with total white matter volume in the superior frontal cortex.



T-Statistic map of the negative association between regional superior frontal WM volume and BW (pFEW=0.004). Hot and yellowish colours indicate volume decreases are correlated with BW.

WM = white matter; BW = birth weight

Figure 5. Association between birth weight and total grey matter volume as moderated by early maternal hostile parenting behaviours.



Graph depicting results showing the trend for maternal hostile parenting behaviours moderating the association between BW and total GM volume.

Horizontal axis represents BW in kg and vertical axis represents total GM volume in adolescence.

BW = birth weight, GM = grey matter

DISCUSSION

The overarching goal of this thesis was to test the association between the early environment and brain development. Specifically, we tested the hypothesis that in combination with genes, early adversity during critical periods of development would be associated with brain volume and function of fronto-limbic regions associated with emotion processing. We also tested the hypothesis that these neural alterations would be associated with vulnerability for mental health disorders, including depression. In chapter 1, we tested the hypothesis that brain activation in response to sadness during childhood would be altered following early adversity and found that children of mothers with higher depressive symptoms (when the children were 0-2 years) showed greater neural activity in the insula, ACC and caudate nucleus to sadness, compared to children of mothers with lower depressive symptoms. Although genetic factors cannot be entirely ruled out, the data indicate that, in combination with genetics, minor early postnatal adversity (i.e., exposure to maternal depressive symptomatology) can affect neural activity during emotion processing in middle childhood.

In chapter 2, we extended the research to the adolescent period. Specifically, we tested the hypothesis that in utero adversity indexed by low BW and early postnatal adversity including maternal depressive symptoms and hostile parenting behaviours would be associated with altered activation of fronto-limbic regions in response to emotional stimuli in adolescence, particularly in response to sad and fearful stimuli. We found that maternal hostile parenting behaviours and maternal depressive symptoms during the early postnatal period were associated with brain function in the ACC and PFC in adolescence. Furthermore, within-twin pair discordance in BW and maternal hostile parenting behaviours, thus controlling for genetics, were also associated with discordance in activation to emotional stimuli in the PFC. These associations were sex-specific, which is not surprising given that there are known sex effects in emotion processing and vulnerability for mood disorders. To assess the relevance of alterations in processing of emotional stimuli for susceptibility to developing psychopathology such as internalizing disorders, we then tested the hypothesis that functioning of fronto-limbic regions would be associated with levels of neuroticism and internalizing symptoms. We found that neuroticism was positively associated with activation in the ACC, PFC and amygdala in

girls, while within-pair discordance in neuroticism was negatively associated with within-pair discordance in the thalamus and amygdala in boys. Finally, within-pair discordance in internalizing symptoms was negatively associated with within-pair discordance in brain activation to emotion processing in the insula, hippocampus and amygdala, particularly in boys. We thus show that a) the early environment is associated with fronto-limbic activation in response to emotion processing in adolescents, b) that current neuroticism and internalizing symptoms in adolescence are correlated with neural activity in response to emotions in fronto-limbic regions including the amygdala, c) that within-pair discordance in fronto-limbic function is associated with within-twin pair discordance in early adversity factors and current neuroticism and internalization, and that d) these effects are sex-specific. Together, these results support the importance of the early environment for fronto-limbic functioning, even when independent of DNA sequence.

In chapter 3, we tested, in the same adolescent twin sample, the hypothesis that total brain volume would be lower in adolescent MZ twins following in utero and early postnatal adversity. In addition, we assessed whether early maternal hostile parenting behaviours may mediate or moderate the association between mild in utero adversity as indexed by BW and total brain volume in adolescence. We found that lower BW was associated with total brain volume, particularly in the PFC and thalamus. Notably, greater within-pair discordance in BW was associated with greater within-pair discordance in both total GM and WM volumes, supporting the importance of the in utero environment independent of DNA sequence. Furthermore, although a trend, it appears that maternal hostile parenting behaviours may moderate the association between BW and total GM volume: the positive association between BW and total GM volume was greater at higher levels of maternal hostile parenting behaviours. Overall, these findings suggest that both the in utero and early postnatal environments can influence structural brain development into adolescence.

Overall, we found that mild in utero and early postnatal adversity is associated with brain function in childhood and adolescence and brain volume in adolescence, in fronto-limbic regions including the ACC and prefrontal cortex, in monozygotic twins. Our results support previous findings of associations between early adversity and brain development in singletons,

but take it a step further by demonstrating the importance of the unique environment for brain development while controlling for genetics in a prospective longitudinal monozygotic twin design. Furthermore, we show that the association holds with relatively minor adversity, extending results showing associations between brain structure and very low (<1500g) or extremely low (<1000g) BW [i.e. (Abernethy et al, 2002; Bjuland et al, 2014; de Kieviet et al, 2012; Nosarti et al, 2002; Nosarti et al, 2008; Taylor et al, 2011)] as well as maternal clinical depression and maltreatment [i.e. (Dubin et al, 2012; Kelly et al, 2013; Sheridan et al, 2010; Tottenham et al, 2010)]. Our results thereby provide evidence that even relatively minor adversity can have a significant impact on brain development through childhood and adolescence. These findings are relevant for intervention efforts to prevent the development of mental health disorders. Although we do not suggest that all children exposed to mild adversity factors be targeted for intervention, we suggest that support offered to parents of twins, and particularly of low BW twins, may be beneficial.

We also extended previous investigations of BW to brain function. BW has been studied in association with brain volume and structure, but to our knowledge, no study to date has investigated the association between BW and brain function in response to emotion processing, even though associations have been found between BW and brain volume, cognitive development, emotion regulation and behaviour (Aarnoudse-Moens et al, 2009; Allen, 2008; Allen et al, 1998; Bohnert & Breslau, 2008; Delobel-Ayoub et al, 2009; Field, 2011; Lazinski et al, 2008; Luu et al, 2011). Our results are not entirely consistent with our original hypothesis of an association between BW and neural activation in the amygdala, hippocampus, insula, ACC and PFC, in response to sadness and fear. Within pairs, we show that brain function is associated with BW in several fronto-limbic areas implicated in emotion processing in response to sadness and fear, but also anger and happiness. What's more, neural activation to positive and negative emotions is in the same direction. These results may be due to the immaturity of fronto-limbic circuitry. In adolescence, development of limbic regions is mostly complete, but frontal regions are still maturing (Toga et al, 2006). Functionally, we also see a shift in regions implicated in emotion processing from the earlier-developing ventral regions during childhood to the later-maturing dorsal cognitive areas during adolescence (Hogan & Park, 2000; Hung et al, 2012; Perlman & Pelphrey, 2010; Vink et al, 2014). It could

be that brain circuits involved in processing negative vs. positive emotions are not fully differentiated at this age.

Furthermore, contrary to our hypothesis, we did not find that BW was associated with fronto-limbic activation between pairs in our sample. However, this hypothesis was based on studies finding associations between BW and decreased brain volume (Abernethy et al, 2002; Bjuland et al, 2014; de Kieviet et al, 2012; Lowe et al, 2011; Martinussen et al, 2005; Nagy et al, 2009; Nosarti et al, 2008; Taylor et al, 2011), as well as brain activation during working memory and response inhibition tasks (Frye et al, 2009; Nosarti et al, 2006). Since we did find that within-pair BW discordance was associated with within-pair discordance in brain activation in response to emotion processing, the in utero environment does appear to be associated with brain development and function into adolescence.

Our childhood and adolescence samples were recruited from the same cohort of MZ twins. However, there was little overlap in the samples at the different time points. In addition, different machines were used to assess brain function and different fMRI tasks were used. Thus, we could not perform a longitudinal analysis. Nonetheless, we can still comment on similarities between results in neural activation patterns in children and adolescents exposed to maternal depressive symptomatology. Specifically, we found that both children and adolescents of mothers with greater depressive symptomatology showed greater activation in the ACC. This finding suggests that altered functioning of the ACC may indicate vulnerability for depression. Consistent with this hypothesis, studies in populations considered to be at risk for depression, children of depressed mothers and people not currently depressed but having suffered from major depression in the past, show altered activation in fronto-limbic regions including the ACC in response to criticism (Hooley et al, 2009) and a memory task (Mannie et al, 2014). It would be interesting to assess whether patterns of activation in the ACC can predict later development of depressive disorders.

Several mechanisms have been proposed to underlie the association between early adversity and altered brain structure and function. One of the systems affected by early stress and a potential mechanism underlying vulnerability for mental health disturbances is the HPA

axis, one of the main systems involved in stress reactivity. When excessively activated, life-long alterations in its function can occur, leading to altered basal HPA activity as well as increased HPA reactivity to acute stressors and altered production and release of glucocorticoids (De Kloet et al, 1998; Gunnar & Quevedo, 2007; Heim et al, 2000; Miller et al, 2007; Murgatroyd et al, 2009). During prenatal and early postnatal development, the fetus is particularly sensitive to glucocorticoid concentrations, and the level can have an effect on BW and later stress sensitivity (Harris & Seckl, 2011). In animal models (Glover et al, 2010; Harris & Seckl, 2011; Murgatroyd & Spengler, 2011a; Pryce et al, 2011; Veenema, 2009; Weaver, 2007), repeated prenatal and early postnatal stress leads to excessive exposure to glucocorticoids, which is associated with altered functioning of the HPA axis. In humans, investigations have found that preterm birth, smoking during pregnancy and exposure to maternal depression early in life are associated with higher baseline cortisol during infancy (Grunau et al, 2007; Varvarigou et al, 2006). Fronto-limbic regions containing high concentrations of glucocorticoid receptors such as the hippocampus are especially vulnerable to excessive HPA activation, and indeed, studies have shown that early adversity is associated with altered development of fronto-limbic regions including the hippocampus, PFC, amygdala and hypothalamus (Booij et al, 2013). What's more, alterations in the HPA axis are often found in the context of psychopathology including post-traumatic stress disorder and depression (Booij et al, 2013; de Kloet et al, 2006). Thus, there is convincing evidence that the HPA axis is implicated in the association between early adversity and brain development as well later susceptibility to psychopathology such as depression.

Proper brain development and functioning also requires the delicate balance of several excitatory and inhibitory neurotransmission systems, which include the monoamines (5-HT, dopamine [DA], norepinephrine [NE]), neurotransmitters containing one amino group and implicated in arousal, cognition and emotion. Given that these neurotransmitters are developing during the prenatal and early postnatal period, early adverse events may modify the course of their development and thereby long-term functioning, just as with the HPA axis (Herlenius & Lagercrantz, 2004). As described in (Herlenius & Lagercrantz, 2004) and (Huppertz-Kessler et al, 2012), early stress may disturb the timing or level of expression of monoamine neurotransmitters, and this may in turn lead to changes in brain development.

Indeed, studies report that prenatal stress alters levels and turnover of 5-HT, NE and DA in the adult brain, particularly in fronto-limbic regions involved in stress reactivity and emotion regulation, although direction of change varies (Arborelius & Eklund, 2007; Welberg & Seckl, 2001). Furthermore, these alterations in neurotransmitter systems are associated with behavioural and emotional problems as early as childhood (Holmes et al, 2003; Lira et al, 2003), which likely represent a manifestation of vulnerability for mental health disturbances.

Accumulating evidence is also suggesting that alterations to the immune system may be associated with mental health disorders including mood disorders (Christian, 2012; Felger & Lotrich, 2013; Irwin & Miller, 2007; Mills et al, 2013). Cytokines, signalling molecules with immune modulating activity that can be either pro- or anti-inflammatory depending on their target, may be at the base of this association (Mills et al, 2013). Increased levels of circulating cytokines have been found in the context of stress and/or depression and antidepressants appear to modify cytokine levels (Dowlati et al, 2010; Hassanain et al, 2005; Pesce et al 2011). The association appears to involve activation of inflammatory signalling pathways in the brain, which results in changes in neurotransmitters, particularly monoamines, glutamate and neuropeptides as well as the HPA axis and growth factors such as brain derived neurotrophic factor (BDNF) (Felger & Lotrich, 2013; Mills et al, 2013), following early adversity (Felger & Lotrich, 2013). What's more, cytokine levels during pregnancy appear to be particularly relevant to the outcome of the pregnancy. Healthy pregnancies are associated with mild elevations of both pro- and anti-inflammatory cytokine levels, and exaggerated increases are associated with risk of spontaneous preterm delivery (Christian, 2012). Thus, there is increasing evidence that alterations to the immune system may contribute to increased vulnerability for mental health disorders such as depression.

Epigenetic mechanisms represent another potential mechanism underlying the association between early adversity and altered brain development. Epigenetic mechanisms encompass any long-term change to gene expression, the epigenome, that persists past the end of the trigger without a change in gene sequence or structure (McGowan & Szyf, 2010). The epigenome consists of DNA, chromatin and other chemical compounds which bind to chromatin or DNA (Razin, 1998). Briefly, DNA wraps around chromatin and a number of

modifications to chromatin or DNA itself affect how DNA is expressed. DNA methylation is one such epigenetic mechanism, and affects the DNA molecule itself through enzymatic addition of a methyl group to DNA (McGowan & Szyf, 2010; Murgatroyd & Spengler, 2011b). When occurring within CpG islands (DNA patches of approximately 1000 base pairs rich in CpGs often associated with genes, particularly promoters and enhancers), methylation leads to gene inactivation (Bird, 2002; Jones et al, 1998; Nan et al, 1998a; Nan et al, 1998b; Suzuki & Bird, 2008). DNA methylation patterns are shaped during gestation (Benvenisty et al, 1985; Razin & Cedar, 1993) and are highly vulnerable to the environment. For instance, a study has shown that individuals who were exposed to the Dutch famine in the perinatal period had, six decades later, altered DNA methylation patterns compared to their siblings (Heijmans et al, 2007). Furthermore, several studies have found associations between DNA methylation and brain structure and/or function. In animal models, studies have shown that early stress in the form of maternal separation in mice and rearing by surrogates in rhesus monkeys, is associated with altered mRNA levels of the glucocorticoid receptor NR3C1 in the hippocampus, paraventricular nucleus (PVN) and pituitary (Weaver et al, 2004), as well as DNA methylation patterns in the medial PFC (Provencal et al, 2012). In human post-mortem studies, suicide victims with a history of early life abuse had enhanced DNA methylation in the NR3C1 promoter and decreased NR3C1 mRNA expression in the hippocampus (McGowan et al, 2009). No change was found in suicide and depressed patients with no history of early life abuse (Murgatroyd & Spengler, 2011b). In addition, Alt and colleagues found decreased NR3C1 expression in the amygdala, cingulate gyrus and inferior PFC of depressed patients compared to controls (Alt et al, 2010). Furthermore, studies have also found an association between methylation of the 5-HTT gene promoter and brain structure or function. For instance, methylation levels were associated with hippocampal GM volume (Booij et al, Submitted; Dannlowski et al, 2014), amygdala activity in response to threat (Nikolova et al, 2014), and in vivo measures of brain 5-HT synthesis in the lateral OBFC bilaterally and childhood aggression (Wang et al, 2012). Finally, high within-MZ twin pair variability in methylation was found in genes associated with development [(Levesque et al, 2014); see Annex 2]. Taken together, these findings support the hypothesis that epigenetic mechanisms, particularly DNA methylation, underlie the association between the early

environment and brain development. Of course, these findings are correlational and further work is necessary in order to assess causal mechanisms.

More work will also be necessary to address certain challenges concerning measurement of behaviour and internal states. First is the enduring question concerning the validity of self-report measures. For instance, there is concern regarding desirability of responses, particularly when addressing parenting behaviours. Will mothers admit to mistreating their children or treating their twins differently? Is a child able to understand his or her own mental state? Laboratory measures of parenting and structured interviews can be used; however this increases session length and can potentially overtax and thereby discourage participants from continuing with future assessments. Some compromise is always necessary, and the nature of the compromise will depend on the objectives of the study.

Future work should also assess the impact of specific stressors. Duration, intensity, type and timing of stressors may significantly influence their impact, and stressors shouldn't be studied out of context. In accordance with both the cumulative stress and match/mismatch hypotheses (Daskalakis et al, 2013), the impact of a stressor will depend on the presence/absence of other risk and protective factors. Several studies have shown that some individuals suffer no negative consequences from adversity, potentially due to various protective factors. These include the presence of social support and the use of non-maternal care services, which have been shown to mitigate the impact of adversity such as maternal depression and low SES, on vulnerability for mental health disorder symptoms during childhood (Cote et al, 2007; Giles et al, 2011; Lee et al, 2006). This thesis focused on early adversity but most people are exposed to a myriad of both positive and negative experiences. Future studies will need to assess both adverse and protective factors in conjunction. Large-scale prospective longitudinal research from pregnancy to adulthood should be favoured, as it allows for the investigation of vulnerability over the years. The presence/absence of multiple risk and protective factors can be identified, measured and correlated with neural, cognitive and mental health outcomes, and the timing of adversity and consequences can be delineated.

The identification of buffering factors promoting resilience is of particular interest for the development of interventions in at risk individuals. Findings suggest that targeting children and adolescents having faced some adversity and/or manifesting symptoms of internalizing disorders or high neuroticism, prior to the onset of psychopathology, may be most beneficial. Indeed, there is increasing support for the hypothesis that sub-threshold internalizing symptoms can lead to the development of MD later on (Bertha & Balazs, 2013; Hill et al, 2014; Luby et al, 2014). Early interventions may prevent the development of clinically significant psychopathology. Family-based interventions teaching effective parenting skills as well as non-maternal childcare programs appear particularly beneficial (Cote et al, 2009; Engle et al, 2011; Geoffroy et al, 2010).

Efficient interventions to prevent and/or treat psychopathology will however benefit from a greater understanding of the mechanisms underlying pathology. Future studies will need to examine systems as they truly function, that is, functioning, interacting and influencing one another, as networks. An ideal study would follow large amounts of people beginning before birth, through development and into adulthood and encompass many biological and psychosocial measures, including but not limited to assessments of the in utero and early postnatal environment, mental states and behaviour using multiple informants and methods, brain development both structurally and functionally, neurotransmitter and hormonal levels, whole-genome genetics and epigenetics. Of course, in addition to being logistically complex, with the necessity of following large groups of people and trying to keep attrition rates low, the costs of equipment and personnel, the analysis of such a wealth of data is quite complex. Methods are still being developed for combining these types of complex multimodal data. This type of research will thus require increased collaboration between disciplines, with neuroscientists, psychologists, biologists, geneticists working together with statisticians and bioinformatics experts.

In line with these ideas, although outside the scope of one thesis, there is still work to be done with the collected data. Indeed, data collection has now been completed in 67 MZ twin pairs and includes DNA methylation as well as measures of perceived stress, personality, reward processing and motor impulsivity. In addition to anatomical and functional scans, DTI

and resting state scans were also acquired in a subsample of 54 pairs. These data are currently being analyzed. Moreover, future analyses will assess some of these multimodal data together. For instance, data-driven exploratory analyses will be performed on whole-brain structural MRI and whole-genome DNA methylation data together in order to assess the association between early adversity, molecular adaptations and neural outcomes.

Limitations and strengths

Since this study is a longitudinal prospective study in well-characterized MZ twins, it is unfortunate that there is little overlap in twins assessed at 8 and 15 years old. We therefore could not assess longitudinal changes in brain function in response to emotion processing. In addition, we did not have information on current maternal depressive symptoms and/or parenting behaviours. It could have been interesting to assess the impact of early vs. current maternal factors on brain development. Finally, this study could not distinguish whether the neural changes that we find are present from birth or surface later during childhood/early adolescence. Future studies following participants from before birth and through development into adulthood would benefit greatly from regular measurements of brain structure and function. Our knowledge of the timing of the effects of early adversity on brain development is still limited and could be improved with regular assessments throughout development.

The fact that the investigated sample was healthy and free of current depressive and substance abuse disorders could be construed as both a limitation and strength. On the one hand, the exclusion of participants with current depressive and/or substance use disorders eliminates a potential confound in our data. Current depression would have invariability affected emotion processing, and, given our emotion processing task, we would not have been able to distinguish the effects of current, state-like depression from underlying trait-like risk. Moreover, substance use is well known to affect brain function (Guerri & Pascual, 2010; Rubino et al, 2012; Squeglia et al, 2009). A disadvantage of excluding current depressive and substance use disorders however, is that our findings may not be generalizable to individuals with psychopathology. Furthermore, we could not evaluate whether levels of current neuroticism and internalizing symptoms truly represent vulnerability for psychopathology in

this particular sample. Future follow-ups of the same cohort will be of interest in order to further assess the clinical relevance of the current findings.

Strengths of this study include the use of a prospective, well-characterised longitudinal cohort. By extracting BWs from medical records and assessing parent and child behaviours throughout the children's development, not only did we acquire a wealth of data but we also avoided retrospective biases. What's more, testing during adolescence included both structural and functional MRI, as well as measures of personality and mental health symptomatology, which allowed and will continue to allow us to test many potential associations in this well-characterized sample. Finally, our use of MZ twins in adolescence allowed us to investigate the association between early adversity factors and outcomes both across and within families. Given that MZ twins share 100% of their genes as well as a large part of their environment, discordance in outcomes must be associated with discordance in the unique environment (Vitaro et al, 2009), and if we can identify the characteristics of those unique environmental experiences, we can hope to gain a greater understanding of brain development in the context of risk and resilience.

Conclusion

Overall, we demonstrated the importance of the in utero and early postnatal environments for development of brain function and structure while controlling for the gene sequence. Our research contributes to our understanding of mechanisms linking early adversity to later vulnerability and eventually to psychopathology. Future work will be necessary to further our understanding of risk and resilience, with the underlying goal of identifying at risk individuals and developing interventions to promote resilience and prevent development of psychopathology in developing children and adolescents.

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ANNEXES

ANNEX 1 – Matlab scripts

ANNEX 2 – Paper on variability of DNA methylation patterns in adolescent MZ twins

ANNEX 1 – Matlab scripts

ANNEX 1.1 – Pre-processing

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anatomicals={
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}

for i=1:length(anatomicals)
```

```
    TwinFMRIPreprocess(functionals{i},anatomicals{i})
end
```

```
function TwinFMRIPreprocess(fmri_path, anatomical_path)

%write spm jobfiles for twins study fMRI startign with 3D nii files
%usage batchfile=MakeTwinJobFileFMRI('fmri_path','anatomical_path','batchfile_path')

functional=SurfStatListDir(fmri_path);
for i=1:length(functional);
   scanstring{i}=sprintf('%s,1',functional{i});
end

anatomical=SurfStatListDir(anatomical_path);
if length(anatomical)>1
   echo "found more than one anatomical: make sure to use a wildcard e.g o*nii"
end

matlabbatch{1}.spm.temporal.st.scans = { scanstring };
matlabbatch{1}.spm.temporal.st.nslices = 40;
matlabbatch{1}.spm.temporal.st.tr = 2.11;
matlabbatch{1}.spm.temporal.st.ta = 2.05725;
matlabbatch{1}.spm.temporal.st.so = [39 37 35 33 31 29 27 25 23 21 19 17 15 13 11 9 7 5 3 1
40 38 36 34 32 30 28 26 24 22 20 18 16 14 12 10 8 6 4 2];
matlabbatch{1}.spm.temporal.st.refslice = 20;
matlabbatch{1}.spm.temporal.st.prefix = 'a';
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1) = cfg_dep;
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).tname = 'Session';
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).tgt_spec{1}(1).name = 'filter';
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).tgt_spec{1}(1).value = 'image';
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).tgt_spec{1}(2).name = 'strtype';
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).tgt_spec{1}(2).value = 'e';
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).sname = 'Slice Timing: Slice Timing
Corr. Images (Sess 1)';
```

```
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).src_exbranch = substruct('.','val',
'{}',{1}, '.','val', '{}',{1}, '.','val', '{}',{1});
matlabbatch{2}.spm.spatial.realign.estwrite.data{1}(1).src_output = substruct('()',{1},
'.','files');
matlabbatch{2}.spm.spatial.realign.estwrite.eoptions.quality = 0.9;
matlabbatch{2}.spm.spatial.realign.estwrite.eoptions.sep = 4;
matlabbatch{2}.spm.spatial.realign.estwrite.eoptions.fwhm = 5;
matlabbatch{2}.spm.spatial.realign.estwrite.eoptions.rtm = 1;
matlabbatch{2}.spm.spatial.realign.estwrite.eoptions.interp = 5;
matlabbatch{2}.spm.spatial.realign.estwrite.eoptions.wrap = [0 0 0];
matlabbatch{2}.spm.spatial.realign.estwrite.eoptions.weight = '';
matlabbatch{2}.spm.spatial.realign.estwrite.roptions.which = [2 1];
matlabbatch{2}.spm.spatial.realign.estwrite.roptions.interp = 5;
matlabbatch{2}.spm.spatial.realign.estwrite.roptions.wrap = [0 0 0];
matlabbatch{2}.spm.spatial.realign.estwrite.roptions.mask = 1;
matlabbatch{2}.spm.spatial.realign.estwrite.roptions.prefix = 'r';
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1) = cfg_dep;
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).tname = 'Reference Image';
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).tgt_spec{1}(1).name = 'filter';
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).tgt_spec{1}(1).value = 'image';
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).tgt_spec{1}(2).name = 'strtype';
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).tgt_spec{1}(2).value = 'e';
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).sname = 'Slice Timing: Slice Timing Corr.
Images (Sess 1)';
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).src_exbranch = substruct('.','val', '{}',{1},
'.','val', '{}',{1}, '.','val', '{}',{1});
matlabbatch{3}.spm.spatial.coreg.estimate.ref(1).src_output = substruct('()',{1}, '.','files');
matlabbatch{3}.spm.spatial.coreg.estimate.source = anatomical;
matlabbatch{3}.spm.spatial.coreg.estimate.other = {''};
matlabbatch{3}.spm.spatial.coreg.estimate.eoptions.cost_fun = 'nmi';
matlabbatch{3}.spm.spatial.coreg.estimate.eoptions.sep = [4 2];
```

```
matlabbatch{3}.spm.spatial.coreg.estimate.eoptions.tol = [0.02 0.02 0.02 0.001 0.001 0.001
0.01 0.01 0.01 0.001 0.001 0.001];
matlabbatch{3}.spm.spatial.coreg.estimate.eoptions.fwhm = [7 7];
matlabbatch{4}.spm.spatial.preproc.data = anatomical;
matlabbatch{4}.spm.spatial.preproc.output.GM = [0 0 1];
matlabbatch{4}.spm.spatial.preproc.output.WM = [0 0 1];
matlabbatch{4}.spm.spatial.preproc.output.CSF = [0 0 1];
matlabbatch{4}.spm.spatial.preproc.output.biascor = 1;
matlabbatch{4}.spm.spatial.preproc.output.cleanup = 0;
matlabbatch{4}.spm.spatial.preproc.opts.tpm = {
                                        'C:\Users\levmel00\Documents\spm8\tpm\grey.nii,1'
                                        'C:\Users\levmel00\Documents\spm8\tpm\white.nii,1'
                                        'C:\Users\levmel00\Documents\spm8\tpm\csf.nii,1'
                                        };
matlabbatch{4}.spm.spatial.preproc.opts.ngaus = [2
                                          2
                                          2
                                          4];
matlabbatch{4}.spm.spatial.preproc.opts.regtype = 'mni';
matlabbatch{4}.spm.spatial.preproc.opts.warpreg = 1;
matlabbatch{4}.spm.spatial.preproc.opts.warpco = 25;
matlabbatch{4}.spm.spatial.preproc.opts.biasreg = 0.0001;
matlabbatch{4}.spm.spatial.preproc.opts.biasfwhm = 60;
matlabbatch{4}.spm.spatial.preproc.opts.samp = 3;
matlabbatch{4}.spm.spatial.preproc.opts.msk = {''};
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1) = cfg_dep;
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).tname = 'Parameter File';
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).tgt_spec{1}(1).name = 'filter';
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).tgt_spec{1}(1).value = 'mat';
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).tgt_spec{1}(2).name = 'strtype';
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).tgt_spec{1}(2).value = 'e';
```

```
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).sname = 'Segment: Norm Params Subj->MNI';
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).src_exbranch = substruct('.','val', '{}',{4}, '.','val', '{}',{1}, '.','val', '{}',{1});
matlabbatch{5}.spm.spatial.normalise.write.subj.matname(1).src_output = substruct('()',{1}, '.','snfile', '()',{':'});
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1) = cfg_dep;
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).tname = 'Images to Write';
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).tgt_spec{1}(1).name = 'filter';
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).tgt_spec{1}(1).value = 'image';
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).tgt_spec{1}(2).name = 'strtype';
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).tgt_spec{1}(2).value = 'e';
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).sname = 'Realign: Estimate & Reslice: Resliced Images (Sess 1)';
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).src_exbranch = substruct('.','val', '{}',{2}, '.','val', '{}',{1}, '.','val', '{}',{1}, '.','val', '{}',{1});
matlabbatch{5}.spm.spatial.normalise.write.subj.resample(1).src_output = substruct('.','sess', '()',{1}, '.','rfiles');
matlabbatch{5}.spm.spatial.normalise.write.roptions.preserve = 0;
matlabbatch{5}.spm.spatial.normalise.write.roptions.bb = [-78 -112 -50
                                                          78 76 85];
matlabbatch{5}.spm.spatial.normalise.write.roptions.vox = [2 2 2];
matlabbatch{5}.spm.spatial.normalise.write.roptions.interp = 5;
matlabbatch{5}.spm.spatial.normalise.write.roptions.wrap = [0 0 0];
matlabbatch{5}.spm.spatial.normalise.write.roptions.prefix = 'w';
matlabbatch{6}.spm.spatial.smooth.data(1) = cfg_dep;
matlabbatch{6}.spm.spatial.smooth.data(1).tname = 'Images to Smooth';
matlabbatch{6}.spm.spatial.smooth.data(1).tgt_spec{1}(1).name = 'filter';
matlabbatch{6}.spm.spatial.smooth.data(1).tgt_spec{1}(1).value = 'image';
matlabbatch{6}.spm.spatial.smooth.data(1).tgt_spec{1}(2).name = 'strtype';
matlabbatch{6}.spm.spatial.smooth.data(1).tgt_spec{1}(2).value = 'e';
```

```
matlabbatch{6}.spm.spatial.smooth.data(1).sname = 'Normalise: Write: Normalised Images (Subj 1)';
matlabbatch{6}.spm.spatial.smooth.data(1).src_exbranch = substruct('.','val', '{}',{5}, '.','val', '{}',{1}, '.','val', '{}',{1}, '.','val', '{}',{1});
matlabbatch{6}.spm.spatial.smooth.data(1).src_output = substruct('()',{1}, '.','files');
matlabbatch{6}.spm.spatial.smooth.fwhm = [7 7 7];
matlabbatch{6}.spm.spatial.smooth.dtype = 0;
matlabbatch{6}.spm.spatial.smooth.im = 0;
matlabbatch{6}.spm.spatial.smooth.prefix = 's';



%%% Runs the Jobfile %%%
nrun = 1; % enter the number of runs here
% jobfile = { batchfile_path };
% jobs = repmat(jobfile, 1, nrun);
inputs = cell(0, nrun);
for crun = 1:nrun
end
spm('defaults', 'FMRI');
spm_jobman('serial',  matlabbatch, '', inputs{:});

end
```

ANNEX 1.2 – Read targets, onsets, durations

```
% This script creates the condition .mat files from the e-prime output .txt files.
%The condition file will be named according to the "Subject: " line in the e-prime file.
% Manual setup section
onset_offset=15000; % This number will be subtracted from all onset times
eprimedir='G:\TWINS fMRI\e-prime files';
file_filter='Task*Order* FINAL *-*.txt'; % Find files with names matching this

% These are used to determine the conditions based on the .bmp files listed in the e-prime %
% output. Their order determines the condition numbering for the rest of the analyses.
names={'Neu' 'Hap' 'Sad' 'Ang' 'Fea'};

%%
list=dir(fullfile(eprimedir,file_filter));
fnames={list(:).name}';
onsets=cell(1,length(names));
durations=cell(1,length(names));

for i=1:length(fnames) % For each filename that matched the filter
    fname=fnames{i};
    % Load and read the file
    fid=fopen(fullfile(eprimedir,fname),'r');
    text=textscan(fid,'%c');
    text=text{1}';
    text(~text)=[];
    fclose(fid);

    if isempty(text)
        warning('File %s was empty. Skipping.',fname);
        continue;
```

```
    end

    subj=regexp(text,'Subject:\s*(.+?)\s','tokens'); % Find the subject number in the file contents
    subj=subj{1};
   subjname=regexp(fname,'(?:ENGLISH|FRANÇAIS)-(.*?)[-\.]','tokens'); % Get subj number
   % from filename
    subjname=subjname{1};
    if ~strcmp(subj,subjname) % Check for match between file contents and filename
        warning('Subject identifier of filename (%s) does not match that in the file
(%s).',subjname{1},subj{1});
    end

    % Find all text following "Target:"
    targets=regexp(text,'Target:\s*(.+?)\s','tokens');
    targets=[targets{:}]';

    % Find all text following "ImageDisplay1.OnsetTime:"
    ons=regexp(text,'ImageDisplay1\.OnsetTime:\s*(.+?)\s','tokens');
    ons=[ons{:}]';
    ons=cellfun(@str2num,ons);

    % Find all text following "ImageDisplay1.Duration:"
    dur=regexp(text,'ImageDisplay1\.Duration:\s*(.+?)\s','tokens');
    dur=[dur{:}]';
    dur=cellfun(@str2num,dur);

    % Check for matching array lengths
    if any(length(targets)~=[length(ons) length(dur)])
        error('Couldn''t find the same number of targets, onsets, and durations in %s.',fname);
    end
```

```
    ons=ons-onset_offset; % Subtract the offset value
    ons=ons/1000; % Convert to sec from millisec

    dur=dur/1000;

    % Extract the three letters in targets that match emotions (e.g. Fea)
    emot=regexp(targets,'_(\S)+?\.bmp','tokens');
    emot=[emot{:}]';
    emot=[emot{:}]';
    [~,emot]=ismember(emot,names);
    for j=length(names):-1:1
        % Get all the onsets and durations for emotion j
        onsets{j}=ons(emot==j);
        durations{j}=dur(emot==j);
    end
    save(fullfile(eprimedir,sprintf('conditions-%s',char(subj))),'names','onsets','durations');
end
```

ANNEX 1.3 – First level modeling

```
% This script sets up the design matrix for each subject, in preparation for the estimation step.
% Manual setup section
conddir='G:\TWINS fMRI\e-prime files'; % Directory containing the condition .mat files
datadir='G:\TWINS fMRI\Raw data';
firstdir='1st level';
do_new_only=false; % "new" is determined by the absence of SPM.mat in the 1st level folder

%%
clear matlabbatch;
list=ls(datadir); % Get all files and folders in the directory
list=list(list(:,1)=='0',:); % Assumes all subject IDs start with a zero
%list=list(cellfun(@isempty,strfind(cellstr(list),'struct only')),:);
% Remove subjects containing 'struct only'

for i=size(list,1):-1:1 % Create an element in matlabbatch for each subject
   subj=deblank(list(i,:)); % Subject ID

   matlabbatch{i}.spm.stats.fmri_spec.timing.units = 'secs';
   matlabbatch{i}.spm.stats.fmri_spec.timing.RT = 2.11;
   matlabbatch{i}.spm.stats.fmri_spec.timing.fmri_t = 16;
   matlabbatch{i}.spm.stats.fmri_spec.timing.fmri_t0 = 1;

   % Find all scans for this subject
   imgdir=fullfile(datadir,subj,'niftii','Functional');
   imgs=dir(fullfile(imgdir,'swra*'));
   imgs=cellfun(@(x) fullfile(imgdir,x),{imgs(:).name}','UniformOutput',false);
   if isempty(imgs)
      warning('Subject %s: no image files found',subj);
      matlabbatch(i)=[];
```

```
        continue;
    end
    matlabbatch{i}.spm.stats.fmri_spec.sess.scans = imgs;

    %%
    matlabbatch{i}.spm.stats.fmri_spec.dir = {fullfile(datadir,subj,firstdir)};
    pd=pwd;
    cd(fullfile(datadir,subj,firstdir));
    if exist('SPM.mat','file') && do_new_only
        matlabbatch(i)=[];
        continue;
    end
    spm_unlink('SPM.mat'); % Delete old SPM.mat
    cd(pd);
    matlabbatch{i}.spm.stats.fmri_spec.sess.cond = struct('name', {}, 'onset', {}, 'duration', {},
'tmod', {}, 'pmod', {});
    matlabbatch{i}.spm.stats.fmri_spec.sess.multi = {fullfile(conddir,sprintf('conditions-
%s.mat',subj))};
    matlabbatch{i}.spm.stats.fmri_spec.sess.regress = struct('name', {}, 'val', {});
    matlabbatch{i}.spm.stats.fmri_spec.sess.multi_reg = {''};
    matlabbatch{i}.spm.stats.fmri_spec.sess.hpf = 128;
    matlabbatch{i}.spm.stats.fmri_spec.fact = struct('name', {}, 'levels', {});
    matlabbatch{i}.spm.stats.fmri_spec.bases.hrf.derivs = [0 0];
    matlabbatch{i}.spm.stats.fmri_spec.volt = 1;
    matlabbatch{i}.spm.stats.fmri_spec.global = 'None';
    matlabbatch{i}.spm.stats.fmri_spec.mask = {''};
    matlabbatch{i}.spm.stats.fmri_spec.cvi = 'AR(1)';
end

spm('defaults', 'FMRI');
spm_jobman('serial', matlabbatch, '', cell(0,1));
```

ANNEX 1.4 – Estimation

```
% This script does the first-level model estimation for each subject
% It creates the beta_####.img files in the 1st level analysis folders
% Manual setup section
datadir='G:\TWINS fMRI\Raw data';
firstdir='1st level';
do_new_only=false; % "new" is determined by the absence of SPM.mat in the 1st level folder

%%
clear matlabbatch;
list=ls(datadir); % Get all files and folders in the directory
list=list(list(:,1)=='0',:); % Assumes all subject IDs start with a zero
%list=list(cellfun(@isempty,strfind(cellstr(list),'struct only')),:);
% Remove subjects containing 'struct only'

for i=size(list,1):-1:1 % Create an element in matlabbatch for each subject
   subj=deblank(list(i,:)); % Subject ID

   matlabbatch{i}.spm.stats.fmri_est.spmmat = {fullfile(datadir,subj,'1st level','SPM.mat')};
% Location of SPM.mat file
   matlabbatch{i}.spm.stats.fmri_est.method.Classical = 1;
   if exist(fullfile(datadir,subj,firstdir,'mask.img'),'file') && do_new_only
      matlabbatch(i)=[];
      continue;
   end
end

spm('defaults', 'FMRI');
% Run all the jobs in matlabbatch
spm_jobman('serial', matlabbatch, '', cell(0,1));
```

ANNEX 1.5 – Contrasts

```
% This script does the first-level contrasts for each subject
% Manual setup section
% Define contrasts here. Any changes should also be done in twindifference_step2.m
contrasts(1)=struct('contrast',[-1 1 0 0 0 0],'name','Hap-Neu','type','T');
contrasts(2)=struct('contrast',[-1 0 1 0 0 0],'name','Sad-Neu','type','T');
contrasts(3)=struct('contrast',[-1 0 0 1 0 0],'name','Ang-Neu','type','T');
contrasts(4)=struct('contrast',[-1 0 0 0 1 0],'name','Fea-Neu','type','T');

datadir='G:\TWINS fMRI\Raw data';
firstdir='1st level';
do_new_only=false; % "new" is determined by the absence of ANY con_####.img files in the
% 1st level folder

%%
list=ls(datadir); % Get all files and folders in the directory
list=list(list(:,1)=='0',:); % Assumes all subject IDs start with a zero
% list=list(cellfun(@isempty,strfind(cellstr(list),'struct only')),:);
% Remove subjects containing 'struct only'

for i=1:size(list,1) % Loop through each subject
   subj=deblank(list(i,:)); % Subject ID
   try
      load(fullfile(datadir,subj,firstdir,'SPM.mat'));
   catch
      warning('SPM.mat file not found for subject %s.',subj);
      continue;
   end
   % Skip this subject if we're only doing new ones and there are con images already there
   if do_new_only && exist(fullfile(datadir,subj,firstdir,'con_0001.img'),'file')
```

```
        continue;
    end

    % Set up the information for each contrast
    for cn = length(contrasts):-1:1
        P(cn) = spm_FcUtil('Set',...)
                contrasts(cn).name,...
                contrasts(cn).type,...
                'c',...
                contrasts(cn).contrast', ...
                SPM.xX.xKXs);
    end

    SPM.xCon = P;
    spm_contrasts(SPM);  % Do contrasts
end
```

ANNEX 1. 6 – Twin difference step 1

```
% This script creates the diff_####.img files, by subtracting one twin's con_####.img from the
% other's.
% Manual setup section
datadir='G:\TWINS fMRI\Raw data';
firstdir='1st level';

%%
list=ls(datadir); % Get all files and folders in the directory
inds=list(:,1)=='0';
list=list(inds,:); % Assumes all subject IDs start with a zero
list=list(cellfun(@isempty,strfind(cellstr(list),'struct only')),:); % Remove subjects containing
% 'struct only'

subjectnums=str2num(list);
% Find twins of each subject
for i=size(list,1):-1:1
   numdiff=subjectnums-subjectnums(i);
   twin(i)=find(numdiff>-5 & numdiff<5 & numdiff~=0);
end
twin1=list(twin>1:length(twin),:);
twin2=list(twin<1:length(twin),:);

fprintf('Working on pair:      ,      \n');
for i=1:size(twin1,1) % Loop through each pair
   subj1=deblank(twin1(i,:));
   subj2=deblank(twin2(i,:));
   fprintf('%s%5s, %5s\n',repmat(char(8),1,13),subj1,subj2);
   cons=dir(fullfile(datadir,subj1,firstdir,'con_*.img'));
   ncontrasts=length(cons);
```

```
    for cn=1:ncontrasts % for each contrast
        % Load header info of both twin's con image
        V=spm_vol(fullfile(datadir,subj1,firstdir,sprintf('con_%04u.img',cn)));
        V(2)=spm_vol(fullfile(datadir,subj2,firstdir,sprintf('con_%04u.img',cn)));
        % Read the image data
        img1=spm_read_vols(V(1));
        img2=spm_read_vols(V(2));
        % Reuse the header info for the output diff images, but with the following changes:
        V(1).fname=strrep(V(1).fname,'con','diff');
        V(2).fname=strrep(V(2).fname,'con','diff');
        V(1).descrip=strrep(V(1).descrip,'SPM contrast','Difference (this minus other twin)
contrast');
        V(2).descrip=strrep(V(1).descrip,'SPM contrast','Difference (this minus other twin)
contrast');
        % Write the diff images
        spm_write_vol(V(1),img1-img2);
        spm_write_vol(V(2),img2-img1);
    end
end
fprintf('Done.\n');
```

ANNEX 1.7 – Twin difference step 2

```
% This script performs the 2nd-level analysis on the con_#### and diff_#### images from the
% 1st-level analysis.
% Manual setup section
datadir='G:\TWINS fMRI\Raw data';
anadir='G:\TWINS fMRI\Pair analysis';
firstdir='1st level';
anaPrefix='BW_discor';
anaSuffix='';

clear regressors
% Set up extra regressors here:
regressors(1)=struct('name','BW_discor',... % Name of regressor
                'file','G:\TWINS fMRI\2nd level\BW_discor.txt',... % Filename of column of
                data, % in text format
                'type','diff',... % Apply this regressor to which type of 2nd-level analysis (con or
                % diff)?
                'data',[]); % Leave this empty
%regressors(2)=struct('name','Gender',...
%                'file','G:\TWINS fMRI\2nd level\Gender.txt',...
%                'type','diff',...
%                'data',[]);

% This should be the same as in contrasts_all.m:
contrasts(1)=struct('contrast',[-1 1 0 0 0 0],'name','Hap-Neu','type','T');
contrasts(2)=struct('contrast',[-1 0 1 0 0 0],'name','Sad-Neu','type','T');
contrasts(3)=struct('contrast',[-1 0 0 1 0 0],'name','Ang-Neu','type','T');
contrasts(4)=struct('contrast',[-1 0 0 0 1 0],'name','Fea-Neu','type','T');

types={'con' 'diff' 'diff'};
```

```
out={'con' 'diff1-2'};
run_types=[1 2]; % Which analyses to do?  **[1 2] means just con and diff1-2**
run_contrasts=1:4; % Which contrasts to do?

% Get list of all subjects
list=ls(datadir); % Get all files and folders in the directory
list=list(list(:,1)=='0',:); % Assumes all subject IDs start with a zero
list=list(cellfun(@isempty,strfind(cellstr(list),'struct only')),:); % Remove subjects containing
% 'struct only'

% Get list of first twins and second twins
subjectnums=str2num(list);
for i=size(list,1):-1:1
   numdiff=subjectnums-subjectnums(i);
   twin(i)=find(numdiff>-5 & numdiff<5 & numdiff~=0);
end
twin1=list(twin>1:length(twin),:);
twin2=list(twin<1:length(twin),:);

% Load regressors
if exist('regressors','var')
   for r=length(regressors):-1:1
      regressors(r).data=load(regressors(r).file);
      if isempty(regressors(r).data)
         error('Unable to load file %s.',regressors(r).file);
      end
   end
else
   regressors=struct('data',[],'type','placeholder','name',[]);
end
% Loop through each contrast and each type of analysis
```

```
ncontrasts=length(contrasts);
for cn=run_contrasts
   for t=run_types
      clear SPM;
      if t==1
         subs=list;
      elseif t==2
         subs=twin1;
      elseif t==3
         subs=twin2;
      end
      anaName=sprintf('%s%s_%s%s',anaPrefix,contrasts(cn).name,out{t},anaSuffix);
      fprintf('\nWorking on %s\n',anaName);
      nsub=size(subs,1);
      for i=nsub:-1:1 % Get image locations

SPM.xY.P{i}=fullfile(datadir,deblank(subs(i,:)),firstdir,sprintf('%s_%04u.img',types{t},cn));
         if ~exist(SPM.xY.P{i},'file')
            warning('Image file not found: %s\nSkipping this subject in
analysis.',SPM.xY.P{i});
            SPM.xY.P(i)=[];
            nsub=nsub-1;
         end
      end
      VY = spm_vol(SPM.xY.P);
      for v=length(VY):-1:1
         SPM.xY.VY(v)=VY{v}; % Convert from cell to struct array
      end

      % Switch to appropriate directory (make it if it doesn't exist)
      condir=fullfile(anadir,anaName);
```

```
if ~exist(condir,'dir')
   mkdir(condir);
end
cd(condir);

apply_regs=ismember({regressors(:).type},types(t));
regs=regressors(apply_regs); % Select only those regressors that apply to this analysis
try
   reg_matrix=[ones(nsub,1) regs(:).data]; % Check the length of each regressor
catch
   error('All regressors must have length %u.',nsub);
end

% Build SPM data structure
SPM.xX = struct('X', reg_matrix,...
   'iH',1,'iC',zeros(1,0),'iB',zeros(1,0),'iG',zeros(1,0),...
   'name',{{'mean' regs(:).name}},...
   'I',[(1:nsub)' ones(nsub,3)],'sF',{{'obs'  ''  ''  ''}});

SPM.xC = [];

SPM.xGX = struct(...
   'iGXcalc',1,    'sGXcalc','omit',              'rg',[],...
   'iGMsca',9, 'sGMsca','<no grand Mean scaling>',...
   'GM',0,     'gSF',ones(nsub,1),...
   'iGC',  12, 'sGC',  '(redundant: not doing AnCova)',    'gc',[],...
   'iGloNorm',9,   'sGloNorm','<no global normalisation>');

SPM.xVi = struct('iid',1,'V',speye(nsub));

Mdes    = struct(   'Analysis_threshold',   {'None (-Inf)'},...
```

```
    'Implicit_masking', {'Yes: NaNs treated as missing'},...
    'Explicit_masking', {'Yes: SPM2 Brain Mask'});

SPM.xM  = struct('T',-Inf,'TH',ones(nsub*2,1)*-Inf,...
    'I',1,'VM',[],'xs',Mdes);

Pdes    = {{'1 condition, +0 covariate, +0 block, +0 nuisance'; '1 total, having 1 degrees of
freedom'; 'leaving 8 degrees of freedom from 9 images'}};

SPM.xsDes = struct( 'Design',       {'One sample t-test'},...
    'Global_calculation',   {'omit'},...
    'Grand_mean_scaling',   {'<no grand Mean scaling>'},...
    'Global_normalisation', {'<no global normalisation>'},...
    'Parameters',       Pdes);

% Estimate parameters
spm_unlink(fullfile('.', 'mask.img')); % avoid overwrite dialog
SPM = spm_spm(SPM); % model estimation

% Do contrast(s)
P = spm_FcUtil('Set',...
    contrasts(cn).name,...
    'T',...
    'c',...
    [1 zeros(1,length(regs))]', ...
    SPM.xX.xKXs);
for rn=nnz(apply_regs):-1:1 % Set up contrasts for the extra regressors, if any
    P(rn+1) = spm_FcUtil('Set',...
        sprintf('%s_%s',contrasts(cn).name, regs(rn).name),...
        'T',...
        'c',...
```

```
            (1:length(regs)+1==rn+1)'+0, ... % Define appropriate contrast vector
            SPM.xX.xKXs);
        end
        SPM.xCon = P; % Add contrast information to SPM structure
        spm_contrasts(SPM); % Do the contrasts
    end
end
```

ANNEX 2 – Paper on variability of DNA methylation patterns in adolescent MZ twins

Genome-wide DNA methylation variability in adolescent monozygotic twins followed since birth

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ABSTRACT

DNA methylation patterns are characterized by highly conserved developmental programs, but allow for divergent gene expression resulting from stochastic epigenetic drift or divergent environments. Genome-wide methylation studies in monozygotic (MZ) twins are providing insight into the extent of epigenetic variation occurring irrespective of genotype. However, little is known about the variability of DNA methylation patterns in adolescence, a time period involving significant and rapid physical, emotional, social, and neurodevelopmental change. Here, we assessed genome-wide DNA methylation using the 450K Illumina BeadChip in a sample of 37 MZ twin pairs followed longitudinally since birth to investigate: 1) the extent of variation in DNA methylation in identical genetic backgrounds in adolescence and 2) whether these variations are randomly distributed or enriched in particular functional pathways. We also assessed stability of DNA methylation over 3–6 months to distinguish stable trait-like and variable state-like genes. A pathway analysis found high within-pair variability in genes associated with development, cellular mechanisms, tissue and cell morphology, and various disorders. Test-retest analyses performed in a sub-sample of eight twin pairs demonstrated enrichment in gene pathways involved in organismal development, cellular growth and proliferation, cell signalling, and particular disorders. The variability found in functional gene pathways may plausibly underlie phenotypic differences in this adolescent MZ twin sample. Furthermore, we assessed stability of methylation over 3–6 months and found that some genes were stable while others were unstable, suggesting that the methylome remains dynamic in adolescence and that dynamic sites tend to be organized in certain gene pathways.

INTRODUCTION

Although every individual's genome is fixed throughout life and from one cell type to another, epigenetic mechanisms are implicated in gene expression. Cell type DNA methylation patterns emerge during development[1, 2] and are postulated to play a role in gene expression by directing the configuration of inactive chromatin[3] or interfering with the binding of transcription factors.[4] The involvement of DNA methylation in controlling cell identity implies that DNA methylation patterns should show little variation during the life span. However, emerging evidence suggests that DNA methylation is responsive to both physical and social environments during pregnancy[5] and early in life.[6, 7] Indeed, many studies have shown that environmental events are associated with epigenetic modifications, including DNA methylation.[7-10] For example, a study by Heijmans and colleagues demonstrated that individuals who were exposed to famine in the perinatal period had, six decades later, altered DNA methylation patterns compared to their siblings.[11] Furthermore, Borghol and colleagues[8] found an association between methylation levels in key cell-signalling pathways and low socioeconomic status during childhood.

These data raise the questions of how much variability is present in DNA methylation and whether this variation is stochastic or reveals some level of functional organization. Most of the epigenome must be well conserved for an organism to be viable, but some variability is possible.[12,13] Since genetics can influence DNA methylation, genetically identical monozygotic (MZ) twins have been examined in order to differentiate between genetically and externally driven DNA methylation variation.[14-24] Several studies have shown high genome-wide within-twin pair similarity in DNA methylation, although the level of similarity varies depending on tissue, gene, and twin pair.[14,16,17,19-22,25] More specifically, Gordon and colleagues[17] found that the most discordant DNA methylation sites across co-twins were associated with genes that are associated with the immune system and responding to the environment. Similar findings of DNA methylation discordance on genes associated with immune function were found in MZ twins discordant for psoriasis[22] and autoimmune inflammatory diseases.[26] Moreover, both Gordon[17] and Saffery[27] found that the most discordantly methylated genes from cord blood mononuclear cells (CBMCs) and human

umbilical vascular endothelial cells (HUVECs) were those shown to be involved in responding to the environment. Additionally, studies have found within-pair DNA methylation discordance in association with autism,[28,29] bipolar disorder,[30] risk taking behaviour,[31] Alzheimer's disease,[32] intestinal disease,[23] diabetes,[33-35] and even birth weight.[25] See supplemental table 1 for a summary of epigenetic findings in MZ twins. Overall, studies show high similarity within twin pairs across tissues; however, differences are also found, particularly when phenotypes diverge across twins.

In addition to within-twin pair variability in methylation, a related question is whether DNA methylation is responsive to external factors throughout the lifespan. If the methylome is indeed dynamic throughout life, then differences in DNA methylation profiles in identical twins should increase through life. As expected, studies demonstrate that although within-pair discordances are present from birth,[36] they increase with age,[37-45] particularly when twins experience divergent medical histories/environments.[46-49] In fact, Novakovic and colleagues[50] found differences during gestation, which increased with gestational age. The direction of change is complex: DNA methylation increased with age at some loci and decreased at others.[37, 51] Furthermore, methylation does not vary with age in all genes equally, suggesting some specificity.[52,53] Genes found to be associated with age are enriched for functions including DNA binding and regulation of transcription,[44] molecular and cellular characteristics of skin tissue development,[42] and aging-related conditions including Alzheimer's disease, cancer, tissue degradation, DNA damage and oxidative stress.[43] See supplemental table 2 for a summary of findings associating epigenetic marks with age.

Notably, little is known about methylation patterns in adolescence, even though this developmental transition is a time of increased independence and physiological maturation, and therefore may potentially be a period of increased variability in epigenetic mechanisms within twin pairs. The few studies that have examined DNA methylation during adolescence have produced similar findings. Kaminsky and colleagues[54] assessed DNA methylation in MZ and DZ twin pairs aged 12–15 and found significant within-twin pair variability across different tissues (e.g., white blood cells, buccal epithelial cells, and gut biopsies). Variability was greater within DZ than MZ twin pairs, and among MZ twins, variability was higher

among dichorionic than monochorionic twins. Furthermore, Essex and colleagues[9] assessed methylation in buccal epithelial cells and found an association between parental stress in children's early lives and methylation of several genes involved in biosynthetic and metabolic processes during adolescence. However, to our knowledge, no study has used saliva to assess genome-wide methylation differences in adolescence—which may be a useful non-invasive means to acquire DNA in this population—and only one study has assessed the hypervariability across the widest range of CpG sites currently possible (> 480,000 methylation sites) in adolescents. By specifically examining DNA methylation from buccal cells in monozygotic twin preadolescents (8–10 y.o.) and young adults (18–19 y.o.), van Dongen and colleagues[55] found that most twin pairs clustered together. However, the short-time stability of hypervariable genes is still unknown. Distinguishing stable genes from those that are highly dynamic among MZ twins is necessary in order to identify genes that may be responsive in a stable trait-like manner to the immediate environment.

The present study examined MZ twin pairs through a whole-genome approach to determine: (1) whether within-twin pair differences in DNA methylation are present during adolescence, and (2) whether these differences reflect a level of functional organization. We then assessed (3) whether the DNA methylation pattern in adolescence exhibits dynamic features independently of their genetic background. By limiting ourselves to short time intervals, we were able to directly examine how dynamic the methylome is in adolescence.

RESULTS

We used the 450K Illumina BeadChip to assess whole-genome DNA methylation profiles from saliva on one or two time points in a sample of 37 adolescent MZ twin pairs. Following data filtering (see methods section), we were left with a final dataset of 179,408 temporally stable probes and 241,211 temporally unstable probes. Z-scores of absolute twin differences were calculated and probes more than three standard deviations above the mean were considered to be hypervariable. This resulted in 258 temporally stable probes, which mapped to 226 unique genes and 47 temporally unstable probes, which mapped to 46 unique

genes. For a list of these genes along with locations and types, please see supplementary table 3 for trait-like sites and table 1 for state-like sites.

Twin similarity

Correlations among mean methylation levels across all samples were very high, suggesting conservation of DNA methylation states in humans. Each individual predicted at least 95.6% of the variability in every other individual ($r > 0.978$). Twins were the best predictors of each other's mean methylation. Indeed, twins predicted between 95.85% and 99.57% (r values ranged from 0.958 to 0.998) of the variance in one another's DNA methylation patterns. Twin correlations were also assessed using hierarchical clustering. In almost every case, an individual's data was best predicted by their twin's data. This association is displayed in a clustergram (figure 1).

Pathway analyses of hypervariable genes stable in time

In spite of the strong conservation of DNA methylation states across individuals, hypervariable DNA methylation sites were observed between genetically identical twins, indicating that these differences are not genetically predetermined. A question that has remained unanswered is whether these differences are functionally organized or randomly distributed in the genome. We first focused on the most variable, but also temporally stable, DNA methylation sites within twin pairs, as potential representatives of "early life" differences in DNA methylation that remain stable throughout life. Ingenuity Pathway Analysis (IPA) of the 226 genes showing highly variable DNA methylation sites identified enrichment of 16 networks or pathways involved in several diseases and disorders: neurological, metabolic, reproductive system and hematological diseases, as well as psychological, developmental, hereditary, and endocrine system disorders. Developmental networks, including organismal, embryonic, cellular, tissue, skeletal, muscular, and cardiovascular system, were also prominent, as were cellular mechanisms involving cell-to-cell signalling, cellular assembly and organization, cell cycle, small molecule biochemistry, cell death and survival, as well as cell morphology. The top 11 networks with a score of 15 or greater are presented in table 2 and the top network (cell-to-cell signalling and interaction,

tissue development, cardiovascular system development and function) is visually represented in figure 2. Scores were used to rank networks according to fit between the biological pathway and the number of eligible molecules found in our analyses, and were calculated using the right-tailed Fisher's Exact Test using the following formula: Network score = -log(Fisher's Exact Test result). A greater score represents a better fit. Finally, sites can also categorized into top diseases and functions. Here, the top diseases and functions of hypervariable genes were cancer, with 162 molecules (p = 2,81E-06), and organismal survival, with 54 molecules (p = 2,41E-05). See supplementary table 4 for a complete list.

Pathway analyses of hypervariable genes unstable in time

We then examined whether DNA methylation states are fixed early in life and remain stable during adolescence onwards, or whether certain DNA methylation states remain dynamic later in life. By examining the most variable sites within eight adolescent twin pairs at two time points 3–6 months apart we were able to discover dynamic DNA methylation changes during adolescence that are independent of genetics. We identified 47 such sites. We then examined whether these dynamic DNA methylation sites in adolescence were functionally organized or whether they were randomly scattered across the genome. Our analysis revealed three significant networks. Network 1 (score of 43) contained 18 molecules involved in organismal development, cellular growth and proliferation, as well as digestive system development and function. Network 2 contained 15 molecules (score of 34) involved in connective tissue disorders, dental disease, and developmental disorders. Finally, network 3 contained 8 molecules (score of 15) involved in cancer, organismal injury and abnormalities, as well as reproductive system disease. See table 3 for details and figure 3 for a visualization of the top network (organismal development, cellular growth and proliferation, digestive system development and function). See supplementary table 5 for a complete list of top diseases and functions.

Sex

Sex effects are not reported in this paper because MZ twins cannot vary in sex within a pair. While sex differences between twin pairs are certainly possible and scientifically interesting,

in this paper we focused on the discordance between twin pairs. It is also possible that discordance would vary between male and female twin pairs, but we found no evidence for this proposition. In examining discordance at 174202 probes used to assess the trait-like probes, t-tests for the effect of sex revealed 8152 probes where the difference has an unadjusted $p < 0.05$. This represents 4.68% of the sample, slightly less than the 5% of the sample that would be expected by random chance. Adjusting these p-values for the false discovery rate suggested that only two of these probes should be considered significant, and they do not occur at probes with high discordance and do not affect our reported results. Results for the state-like probes were similar, with 4.91% of the sample showing sex effects at an unadjusted threshold of $p = 0.05$ and none of these probes surviving correction for the false discovery rate.

DISCUSSION

We used the 450K Illumina BeadChip Kit to profile DNA methylation states across the genome in saliva at one or two time points in a sample of 37 adolescent MZ twin pairs. Similar to previous studies using other Illumina BeadChips that cover fewer sites,[14, 16, 17, 19-22,25] we found that DNA methylation profiles were highly conserved across unrelated individuals and that this conservation was enhanced in MZ twins, presumably because of both their identical genome and their similar environment. This finding suggests high conservation of DNA methylation states during human evolution, which is consistent with the critical role of DNA methylation in defining cellular identities. In addition to the use of a beadchip with greater coverage, we focused on methylation during adolescence, an under-studied period of great change with significant consequences for the rest of our lives. Furthermore, we demonstrated the convergence in DNA methylation in saliva, which may be sampled non-invasively and at lower cost in a greater number of people. Finally, our test-retest samples across a short period of time allowed us to assess the state- vs. trait-nature of specific genes, the results of which will be highly relevant for future studies.

The high conservation of DNA methylation in humans and the fact that identical twins revealed a high level of conservation is consistent with the view of "innate" evolutionary conserved and predetermined factors delineating DNA methylation states. At the same time, if DNA methylation is implicated in physiological responses to the environment, there should be sites in the genome where the state of methylation varies within an identical genetic background. We addressed this question by examining sets of identical twin pairs and identified genetically independent variability in DNA methylation in a subset of 226 genes. The following functional analysis suggested that these variations were not randomly distributed across the genome but were rather associated with various diseases (i.e., neurological, reproductive system, hematological, and metabolic); developmental, hereditary, and psychological disorders; tissue and cell morphology; development (i.e., organismal, embryonic, cellular, tissue and cardiovascular, muscular and skeletal system); and cellular mechanisms involving cellular movement, cell-to-cell signalling, and cell death and survival. Hypergeometric tests indicated that the assignments of the variable DNA methylation sites to particular genomic pathways were not random. This supports the idea that the human genome contains sites that are responsive to different extraneous signals that are particularly involved in nodal regulatory pathways, and is thus consistent with the view that the DNA methylome is adapted to signals from the environment.[56] The fact that these variable sites were common to many twin pairs and stable over time may mean that many of these changes occurred early in life and were then maintained throughout life. These kinds of DNA methylation changes are hypothesized to play a role in stable phenotypes that emerge in response to early life exposures.

Another critical question is whether this putative process of DNA methylation variation is stable or dynamic over short periods of time. In the present twin study, those genes that were the most dynamic or unstable over time were associated with similar, albeit fewer, networks involved in organismal development and developmental disorders, cellular growth and proliferation, as well as cell signalling and different diseases. This provides support for the hypothesis that the DNA methylome is highly responsive in adolescence to experience and extraneous signals. It should be emphasized that the stable and dynamic sites identified in our study were likely a conservative estimate of such variation in adolescence, given the limited

environmental variation within twin pairs. It stands to reason that the variation in DNA methylation would be larger in the general population given the wider range of environmental exposures and life course experiences. Nevertheless, the present study distinguished genetic-innate variations from others and thus established the plausibility of this hypothesis.

In regards to hypervariable genes that were stable over time, it is of particular interest that we found multiple sites on several major histocompatibility complex genes (MHC), also known as the human leukocyte antigens (HLA) in humans. These genes are involved in immune functions,[57] and may be divided into three different classes (MHC Class I, II, and III). Among our hypervariable genes, we specifically found the HLA-C from Class I and the MHC complex II, HLA-DQA1, HLA-DQB1, HLA-DRB1, and HLA-DRB5 from Class II. All are implicated in presenting foreign antigens to the immune system.[57] Other studies have found DNA methylation of such genes in association with gastric cancer (HLA-C;[58]) and type 1 diabetes (HLA-DQB1 and HLA-DRB1;[59]). Interestingly, Ye and colleagues[58] found that HLA-C promoter methylation patterns were also associated with age and gender (higher methylation rates negatively associated with age in males). The present twin study suggests that environmental epigenetic processes may drive some of the variation in HLA functions (irrespective of DNA sequence) that are already associated with inter-individual differences in susceptibility to disease in adolescence.

Remarkably, the HLA-DQB1 gene came up as both variable in a stable manner and responsive in adolescence, although different sites were associated with stability (trait-like) and variation in time (state-like). A member of the MHC Class II, HLA-DQB1 provides instructions for making a protein with a critical role for the immune system and assists the immune system in distinguishing foreign invaders from the body's own proteins (RefSeq, Sep 2011) and has been involved in both celiac disease[60] and narcolepsy,[61] again pointing to putative epigenetic-environmental origins for some of these vulnerabilities.

In addition to its strong design and the fact that this study had a very narrow age range specifically focused at the mid-adolescent period, a strength of this study is the assessment of variability in epigenetic patterns over a short period of time, thereby allowing for the identification of state vs. trait epigenetic marks. As shown in Ziller and colleagues,[13] it appears

that parts of the epigenome may be quite stable, whereas others may be much more dynamic across short periods of time. This is relevant information when designing a study of epigenetic mechanisms, particularly through time.

A limitation of this study is the use of only one tissue type. It is now well known that epigenetic patterns differ across tissues.[25,62] Is it worthwhile to assess methylation in a peripheral tissue as a marker of less accessible tissue such as the brain? If it is shown that epigenetic patterns can be assessed non-invasively using saliva, this will increase the feasibility of doing methylation studies on a large scale, particularly in samples in which obtaining blood samples would be difficult (e.g., youth, newborns). Research to date suggests that some epigenetic variation may be found across tissues. For instance, Gordon and colleagues[17] found that the most discordant genes across MZ twins are consistently discordant across both HUVECs and CBMs, but more work is needed to replicate and extend this finding. What's more, even one tissue type can contain different cell types that may contain divergent epigenetic patterns. A study by Talens and colleagues[63] assessing whether cellular heterogeneity in whole blood might explain inter-individual variability in DNA methylation patterns found no effect of monocyte percentage, but this issue of assessing methylation from peripheral tissue merits further consideration.

In line with this intra-tissue heterogeneity, the saliva samples we obtained contained a mixture of buccal epithelial cells and leukocytes, and DNA was extracted from both cell types. The proportion of these cell types can vary between individuals and over time, which introduced a known confound into our data. We attempted to remove this variability by comparing the methylation at each probe with a probe known to reliably distinguish the cell types, and to statistically remove the distinctive methylation patterns of the buccal epithelial cells, leaving us with methylation data that primarily represents the methylation of leukocytes.

We required that the variability of these probes be at least three standard deviations above the mean variability of the sample. Although another threshold could equally well have been used, the appeal of our chosen cutoff is twofold: 1) a threshold of three standard deviations is often used as a rule-of-thumb when assessing outliers in a dataset, and 2) this threshold yields a list of hypervariable probes that are suitable for pathway analysis. A well-

known limitation of pathway software is that overlong lists of genes, even if they are selected at random, generate highly significant associations which are likely spurious. However, we think that our chosen cutoff maximized our chances of finding biologically relevant results.

Notwithstanding these limitations, this study extended observations from previous studies that DNA methylation patterns are highly similar in MZ twin pairs in the mid-adolescent period. It also demonstrated that this similarity is variable across pairs during adolescence, a period of great physiological maturation and psychosocial change. Furthermore, this study identified networks of genes that show the greatest discordance in adolescent MZ twin pairs, both in a trait-like (stable over a period of 3–6 months) and a state-like (variable across 3–6 months) pattern. Ideally, future studies should repeat this type of analysis across a range of tissues in order to simultaneously assess stability across tissue types.

Our study findings are consistent with the hypothesis that the human methylome evolved to consist of at least three classes of DNA methylation profiles. First, there were stable DNA methylation sites across individuals and time, which may be innately determined and are most probably involved in establishing cellular identity. Second, there were highly variable sites even in identical genetic backgrounds that may be responsive to external signals, but that remained stable through short periods of time and are presumably involved in establishing trait-like phenotypes. Third, there were highly variable sites in time that may respond to changes in external signals and experiences throughout the life course. These results are relevant for future studies assessing methylation variation in association with environmental events, as they identified stable sites that are likely to be of relevance and others that should be regarded with caution due to their dynamic nature.

METHODS

Participants

Seventy-four MZ twins (37 pairs) who have been followed since birth as part of the Quebec Newborn Twin Study (QNTS;[64]) cohort were recruited. Participants were 15 years old and

consisted of 38 males and 36 females (19 and 18 same-sex twin pairs, respectively). All reported good current health, denied any history of medical or neurological illness, and were determined to be free of any current psychopathology. Presence or absence of current psychopathology was determined using the Dominic, a 15–20 minute computerized diagnostic interview designed for children and adolescents,[65] and the Kiddie-Schedule for Affective Disorders and Schizophrenia (K-SADS), a version of the semi-structured interview assessing DSM IV disorders designed for school-age children of 6–18 years.[66] The appropriate institutional ethics committees approved the study and all participants and parents signed informed assent and consent forms, respectively.

Saliva samples

Whole saliva was collected using the Oragene™ DNA self-collection kit following the manufacturer's instructions (DNA Genotek Inc., 2004, 2006). Participants were asked not to eat, chew gum, or drink anything but water for 30 minutes before the samples were taken. Each participant was asked to provide 2 ml of saliva, which was mixed with 2 ml of the oragene solution, beginning the initial stage of DNA isolation and stabilizing the sample until extraction could be performed. Extraction was accomplished using the Promega Genomic DNA Purification kit, and sent to Genome Quebec for whole-genome analysis using Illumina. In a sub-sample of eight twin pairs, we took a second saliva sample 3–6 months following the first in order to perform a test-retest analysis.

Illumina

We made use of the Illumina Infinium HumanMethylation450 BeadChip Kit, which covers more than 480,000 methylation sites per sample, including 96% of CpG islands, as well as additional coverage in island shores and surrounding regions, again at single-nucleotide resolution. Briefly, DNA was analyzed using the 450K Illumina BeadChip Kit at the Genome Quebec Innovation Centre. The manual protocol supplied by Illumina was followed for all steps except for Single Base Extension and Staining, which were conducted using the automated protocol. Briefly, the isolated DNA was first checked for quality with picogreen and then bisulfite-converted using the Zymo EZ-96 DNA Methylation-Gold Kit. Samples

were transferred to BCD and then MSA4 plates, and neutralized before overnight amplification. MSA4 plates were fragmented, precipitated, and re-suspended before hybridization and transfer to Multi BeadChips. The Multi BeadChips then underwent washing, single-base extension, and staining, before imaging using the HiScan array scanner.

Data Analysis

The raw Illumina output was processed using the R package minfi, a part of biocLite (http://bioconductor.org). The data were first read in and preprocessed (preprocessIllumina) by background correcting and normalizing the data. The main outcome measures were beta-values at each probe, and a number ranging from zero to one, which represents the proportion of methylated samples, was detected. Next, each CpG was associated with a particular chromosome and gene based on the manifest files provided by Illumina (http://support.illumina.com/downloads/humanmethylation450_15017482_v1-2_product_files.ilmn). The beta values and their positional information were then exported to MATLAB (http://mathworks.com, version 13a).

Cellular composition of saliva

In this protocol, DNA samples were collected from saliva. This has the advantage of being non-invasive, particularly in an adolescent population. However, the resulting DNA comes from two major cell types, leukocytes and buccal epithelial cells, and these cell types may differ in DNA methylation. Importantly, individual samples may differ in the proportions of these two cell types, which can bias results. A method for removing this confound has recently been proposed.[19] Briefly, Souren and colleagues identified CpGs, which differentiated whole blood samples (including leukocytes) from samples of buccal epithelial cells, and found that the two cell types were best discriminated by methylation at cg18384097 in the PTPN7 gene. They then used methylation at that site as an index of the cell-type proportion, and fit a regression model between that probe and every other probe on the chip. In cases where the correlations were high, the probe values were replaced by the regression residuals, giving a dataset that is linearly independent of this index of cell-type proportion. In our dataset we fit a regression *model 1* at every probe.

1) CpG β values = $\beta_1 + \beta_2$cg18384097

In cases where β_2 significantly contributed to the model ($p < 0.05$), the values at that probe were replaced by the raw regression residuals. This fitting was done twice. In the first case, the dataset included a set of technical replicates (three samples processed three times each) and the values for these replicates could be more accurately estimated by developing the regression equation in a larger dataset. In the second case, only one sample per subject was included, and this data set was used for further analysis.

Assessment of test-retest variability

After the data was adjusted for the ratio of leukocytes to buccal epithelial cells, the values from the replicated samples were isolated and test-retest differences were calculated for each pair of samples for a given individual (sample A - sample B, sample A - sample C, sample B - sample C). This allowed us to calculate both the maximum observed pairwise difference, and a standard deviation for this difference distribution. These numbers were used in the data filtering steps below.

Data filtering

After removing the replicates and technical control samples from our dataset, we had a matrix of 482,421 probes by 74 participants (37 twin pairs). As a first step we replaced or removed missing values. There were a total of 955 missing values in the dataset; whenever possible, an individual's missing value was replaced with the value of their twin. In cases where data from both twins was missing the mean of the entire sample was used. This could introduce a slight bias, and cause us to over-estimate twin-similarity, but as the main purpose of the study was to assess within-twin pair variability, this method tends to weight against finding effects, and allowed us to keep those probes in the dataset (0.2% of probes). We next removed data from probes where the maximum observed test-retest difference was larger than the maximum difference between data points at that probe (max(probe)-min(probe)). This excluded 13,642 probes from further analysis. We then removed the 11,135 probes on the X chromosome and the 416 probes on the Y chromosome. Because our aim was to examine highly variable

pathways and networks, we restricted our search to probes associated with known genes according to the Illumina manifest. This removed 117,778 probes from the dataset. Because methylation is not necessarily stable over time, we took advantage of test-retest in 16 individuals (8 twin pairs), in which a second saliva sample was collected approximately 3–6 months following the first. After correction for differences in the buccal epithelial cell content of the sample, we compared the temporal stability of each probe. We were interested in distinguishing between temporally stable probes that might contribute to more trait-like phenotypes, and temporally unstable probes that might be more closely associated with state-like phenotypes. This analysis identified 179,408 temporally stable probes (37% of the original dataset) and 241,211 temporally unstable probes (50% of the original dataset). Although it is common in analyses of Illumina microarrays to omit probes whose hybridization could be disrupted by common SNPs, our experimental design based on monozygotic twin pairs excludes that possibility, so these probes were not excluded.

Z-scores

The Z-scores of absolute twin differences were calculated, and probes more than 3 standard deviations above the mean were considered to be hypervariable. This yielded a list of 250 mapped probes, associated with 226 unique genes. These gene names were further processed using Ingenuity Pathway Analysis. The core analysis procedure was used with default options.

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Table 1. Genes hypervariable across MZ twins and time.

Gene Symbol	Gene Name	Location	Type(s)
ADAM3A	ADAM metallopeptidase domain 3A (pseudogene)	Other	other
ADORA3	adenosine A3 receptor	Plasma Membrane	G-protein coupled receptor
AGAP1	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	Cytoplasm	enzyme
AMACR	alpha-methylacyl-CoA racemase	Cytoplasm	enzyme
APITD1/APITD1-CORT	apoptosis-inducing, TAF9-like domain 1	Nucleus	other
B4GALNT3	beta-1,4-N-acetyl-galactosaminyl transferase 3	Other	enzyme
BAIAP3	BAI1-associated protein 3	Extracellular Space	other
BRD2	bromodomain containing 2	Nucleus	kinase
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	Plasma Membrane	ion channel
CDH20	cadherin 20, type 2	Plasma Membrane	other
CLDN11	claudin 11	Plasma Membrane	other

CNNM4	cyclin M4	Plasma Membrane	other
DDR2	discoidin domain receptor tyrosine kinase 2	Plasma Membrane	kinase
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	Nucleus	transcription regulator
EGFR	epidermal growth factor receptor	Plasma Membrane	kinase
ENPP7	ectonucleotide pyrophosphatase/phosphodiesterase 7	Plasma Membrane	enzyme
FAM20C	family with sequence similarity 20, member C	Extracellular Space	enzyme
GALNT9	polypeptide N-acetylgalactosaminyltransferase 9	Cytoplasm	enzyme
GNA12	guanine nucleotide binding protein (G protein) alpha 12	Plasma Membrane	enzyme
HBE1	hemoglobin, epsilon 1	Cytoplasm	transporter
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	Plasma Membrane	Other
HLA-DRB6	major histocompatibility complex, class II, DR beta 6 (pseudogene)	Other	Other
KCTD2	potassium channel tetramerization domain containing 2	Other	ion channel
KDM1A	lysine (K)-specific demethylase 1A	Nucleus	enzyme

LPP	LIM domain containing preferred translocation partner in lipoma	Nucleus	Other
LRWD1	leucine-rich repeats and WD repeat domain containing 1	Nucleus	Other
MCF2L	MCF.2 cell line derived transforming sequence-like	Cytoplasm	Other
MCF2L	MCF.2 cell line derived transforming sequence-like	Cytoplasm	Other
METTL9	methyltransferase like 9	Other	Other
mir-548	microRNA 548c	Cytoplasm	microRNA
OR52N5	olfactory receptor, family 52, subfamily N, member 5	Plasma Membrane	G-protein coupled receptor
PCGF3	polycomb group ring finger 3	Nucleus	other
PKDCC	protein kinase domain containing, cytoplasmic	Cytoplasm	kinase
PLCH2	phospholipase C, eta 2	Cytoplasm	enzyme
PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	Plasma Membrane	phosphatase
RANBP6	RAN binding protein 6	Cytoplasm	other
RTN2	reticulon 2	Cytoplasm	other
SCAMP1	secretory carrier membrane protein 1	Cytoplasm	transporter
SLC45A4	solute carrier family 45, member 4	Other	other

TP63	tumor protein p63	Nucleus	transcription regulator
USP42	ubiquitin specific peptidase 42	Other	peptidase
UVSSA	UV-stimulated scaffold protein A	Nucleus	other
VGLL2	vestigial-like family member 2	Nucleus	transcription regulator
VPS13B	vacuolar protein sorting 13 homolog B (yeast)	Nucleus	transporter
YTHDF3	YTH domain family, member 3	Cytoplasm	other
ZNF155	zinc finger protein 155	Nucleus	transcription regulator
ZNF665	zinc finger protein 665	Other	other

List of 47 genes, locations and types that were found to be hypervariable both across individuals and across time points 3-6 months apart.

Table 2. Networks of hypervariable genes (stable in time).

ID	Molecules	Score	# Focus molecules	Top Diseases and Functions
1	**ALOX12**, amylase, **ATF1**, CaMKII, Caveolin, **CCAR2**, Creb, **DEPTOR**, **DMBT1**, ERK1/2, **GALNT2**, **HTATIP2**, **ITPR3**, **JPH3**, LDL, **MCF2L**, Mek, **MGRN1**, Mlc, **MTA1**, **NTN1**, **PCGF3**, PDGF BB, PI3K (family), Pkg, PP1 protein complex group, PP2A, **PROCR**, **RCAN1**, **RPS6KA2**, **SALL4**, **SLC6A3**, **TRIM9**, **VASP**, **YAF2**	38	22	Cell-To-Cell Signaling and Interaction, Tissue Development, Cardiovascular System Development and Function
2	**ABCC1**, **ADAP1**, Akt, Alp, **ALPPL2**, **AMPD2**, AMPK, **BRSK2**, Collagen type I, **CUX1**, Cyclin A, estrogen receptor, **EXOC7**, Fgf, Fgfr, **FGFR1**, growth factor receptor, GTPase, **IGSF9B**, Integrin, Laminin, **LPCAT1**, **MAGI2**, N-cor, **NCF2**, **PBX1**, PLC gamma, PRKAA, Proinsulin, **PTPRN2**, **RARB**, **SCD**, **TBL1XR1**, **TGFA**, **WNT5A**	31	19	Organismal Development, Embryonic Development, Skeletal and Muscular System Development and Function
3	ADCY, Ap1, **APOBEC3G**, **ARHGAP26**, Calcineurin protein(s), calpain, Collagen type	25	16	Drug Metabolism, Glutathione Depletion In Liver, Cellular Development

	IV, **CSK**, **DUSP22**, **FBL**, Fibrinogen, FSH, G protein alphai, **GNA12**, **GNAS**, GST, **GSTM1**, **GSTT1**, **HBP1**, **IGF2BP3**, Igm, Lh, MAP2K1/2, Mapk, **MT1L**, **NFIC**, NFkB (complex), Pdgf (complex), PLC, **PRKCA**, Rac, Sos, **SPIB**, **TFRC**, VAV			
4	**AKR1C1/AKR1C2**, CD3, **CHMP3**, **CLDN4**, **COL17A1**, Cpla2, ERK, **FBLN2**, **FCGR2C**, **HLA-C**, **HLA-DQA1**, **HLA-DQB1**, **HLA-DRB1**, **HLA-DRB5**, Hsp70, Hsp90, IgG, IgG1, IgG2a, IL1, IL12 (complex), **IL4I1**, Immunoglobulin, Interferon alpha, MHC, MHC Class II (complex), P38 MAPK, **PDIA6**, **PRX**, SRC (family), STAT5a/b, TCR, Tgf beta, **VIPR2**, **XCL1**	25	16	Neurological Disease, Psychological Disorders, Developmental Disorder
5	26s Proteasome, Actin, **AGAP1**, Alpha catenin, **ANO1**, **ASAP1**, Calmodulin, caspase, Clathrin, **DNAJC6**, Focal adhesion kinase, G-protein beta, **GGA1**, Gpcr, **HCN2**, Hdac, **HMOX2**, Insulin, **IQGAP2**, **ITGA8**, Jnk, **LPHN1**, **MOV10**, NMDA Receptor, PI3K (complex), Pka, Ras, Ras homolog, **RFX4**,	25	16	Cellular Assembly and Organization, Cell Morphology, Cell-To-Cell Signaling and Interaction

	RIMBP2, **SHANK2**, Shc, **SLC9A3R2**, **SRC**, voltage-gated calcium channel			
6	ATP10D, ATP11B, ATP11C, **ATP4B**, ATP8A1, ATP8B2, ATP8B3, **ATP9B**, **CHTF18**, DENR, **FNDC3B**, IBA57, **MARCH5**, **MRPL3**, **NCLN**, NDUFA11, NDUFA12, NDUFAF2, NDUFB4, NDUFB11, **NDUFS6**, NDUFV3, **NXN**, **PCDHA6**, **PLEKHA7**, **POTEM (includes others)**, **RBMS1**, SLC4A10, **SLC4A11**, TST, **TUT1**, TXNRD3, UBC, **WDR37**, ZCCHC6	25	16	Metabolic Disease, Developmental Disrder, Hereditary Disorder
7	**ABCA6**, ALKBH6, ARL17A/ARL17B, **BAI2**, **BTBD11**, BTN2A1, **C20orf195**, **C5orf30**, **CCDC33**, CCND1, **FHOD1**, FUK, FUT5, GPR137, **GRIK3**, HHLA2, HNF1A, HNF1α dimer, HNF4A, HRAS, Ins1, **LHX4**, MDFI, MRO, **PAMR1**, **RPH3AL**, SLC38A4, **TBC1D16**, TRAF2, TSH, VN1R1, **VPS54**, **ZAN**, ZNF155, **ZNF707**	23	15	Energy Production, Cell Cycle, Cellular Development
8	ACE, **ACSF3**, ADA, **AIFM3**, ANKRD37, AUP1, **B3GNT6**, **B3GNTL1**, **C1orf52**, CREB3L1,	21	14	Cell-To-Cell Signaling and Interaction, Cellular Development, Tissue

	DALRD3, **DDHD1**, DDIT4, **DENND3**, **DIP2C**, FBXL18, FEM1B, HIF1AN, **KIAA0319**, LRR1, **LUZP1**, MARCKS, MUC2, PPM1F, RAD9A, SIM2, SLC27A2, SLC27A3, SYT2, UBC, UBE3B, USP9Y, **ZFYVE28**, **ZMAT2**, **ZNF506**			Development
9	**AATK**, AHR, Ahr-aryl hydrocarbon-RelA, APP, **ARL6IP6**, BCL2L1, CASR, **CLDN14**, CLDN20, **COL6A1**, DGCR6/LOC102724770, **DNALI1**, **EBF3**, EZH2, **FMO2**, **GALNT10**, GIMAP5, GPR35, GPR61, GPR78, ITK, LEP, **LRRC8D**, **LYPD6B**, MRAP2, **OPLAH**, POPDC2, **PYDC2**, RELA, Slpi (includes others), **ST8SIA2**, TNF, **TRIM35**, TTPA, TWIST1	21	14	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
10	AIM1, ALB, **AMN**, ANKRD32, ARHGAP11A, **CCDC57**, CROT, **DNAJC7**, **GABRP**, **GALNTL5**, GMNN, GPR83, GSTP1, Gstt3, HSPA12B, **KIAA1324**, **KIAA1804**, KRTAP1-3, **LOC100133315**, MAPK1, **MYADML2**, **MYT1L**, NEIL3, NUMA1, NUPR1, OSBPL6, PCYOX1, PDX1,	17	12	Endocrine System Disorders, Organismal Injury and Abnormalities, Reproductive System Disease

	PLA2G4F, SCAF1, **SHPK**, SMARCA4, **SPATA24**, TNNC2, TP53BP1			
11	C1q, **DEAF1**, Gpcr, **GPR37**, GPR62, GPR82, **GPR85**, GPR97, **GPR111**, GPR112, GPR128, **GPR133**, GPR139, GPR144, GPR149, GPR150, GPR152, GPR157, GPR162, GPR174, GPR137C, GPRC5D, **HCRTR1**, **HTR1D**, IFNB1, **LAIR2**, MAPK14, MAS1L, **MCOLN1**, **MYOM2**, **NMUR1**, OXGR1, RAC1, VN1R5, **ZDHHC14**	17	12	Cell-To-Cell Signaling and Interaction, Cell Signaling, Cell Death and Survival

Computed by ingenuity. Genes in bold are genes found to be hypervariable in our sample. Genes not in bold are related genes implicated in the network but not found to be hypervariable in our sample. Networks with scores greater than 15 are presented here.

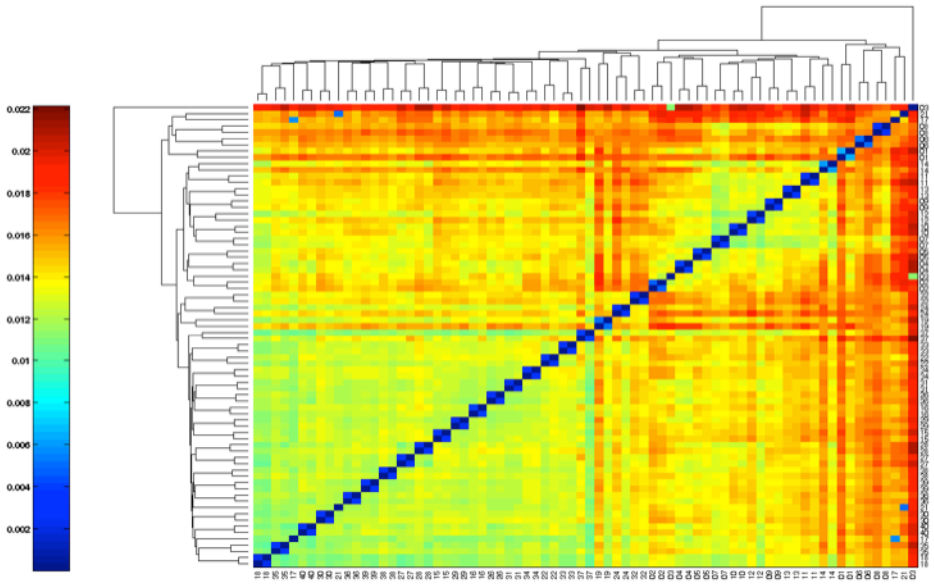
Table 3. Networks of hypervariable genes (hypervariable in time).

ID	Molecules	Score	# Focus molecules	Top Diseases and Functions
1	**ADORA3**, **AGAP1**, ART1, **BRD2**, **CACNA1A**, Calmodulin, Calmodulin-Camk4-Ca2+, caspase, **DDR2**, **DNAJB6**, **EGFR**, Focal adhesion kinase, **GALNT9**, **GNA12**, GPR55, **HBE1**, Hdac, Histone h3, Histone h4, **KDM1A**, **LPP**, **LRWD1**, **MCF2L**, P38 MAPK, PI3K (complex), **PKDCC**, PLC, Plcd2, **PLCH2**, PLCZ1, Ras homolog, **SCAMP1**, TAAR5, **TP63**, Vegf	43	18	Organismal Development, Cellular Growth and Proliferation, Digestive System Development and Function
2	**APITD1/APITD1-CORT**, **BAIAP3**, Basp1, CECR5, **CLDN11**, **CNNM4**, COX11, **ENPP7**, FAM213A, FBRSL1, GIMAP1, GOLGA7, **HLA-DQB1**, HLA-DQB2, HRAS, **KCTD2**, **METTL9**, MGME1, Olfr1508, **PCGF3**, PRELID1, **RANBP6**, RASIP1, RPL39, RT1-A3 (includes others), SLC39A6, **SLC45A4**, TENM3, TOX2, UBC, **UVSSA**, **VPS13B**, **YTHDF3**, ZDHHC9, **ZNF665**	34	15	Connective Tissue Disorders, Dental Disease, Developmental Disorder
3	ADCK3, Alp, **AMACR**, AS3MT, CES2, ECD, ELMOD3, ETFDH, ETNK2, **FAM20C**, FASTKD2, FCAMR, GLIPR1, HNF4A, HPN, INSR, **mir-548**, miR-548c-3p (miRNAs w/seed	15	8	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease

	AAAAAUC), MIS18BP1, MOCOS, MRPS14, MSRB1, NR3C1, PANK1, **PTPRN2**, **RTN2**, SH3BGRL2, SLC38A1, SLC43A1, TP53, USP29, **USP42**, **VGLL2**, **ZNF155**, ZNF175			

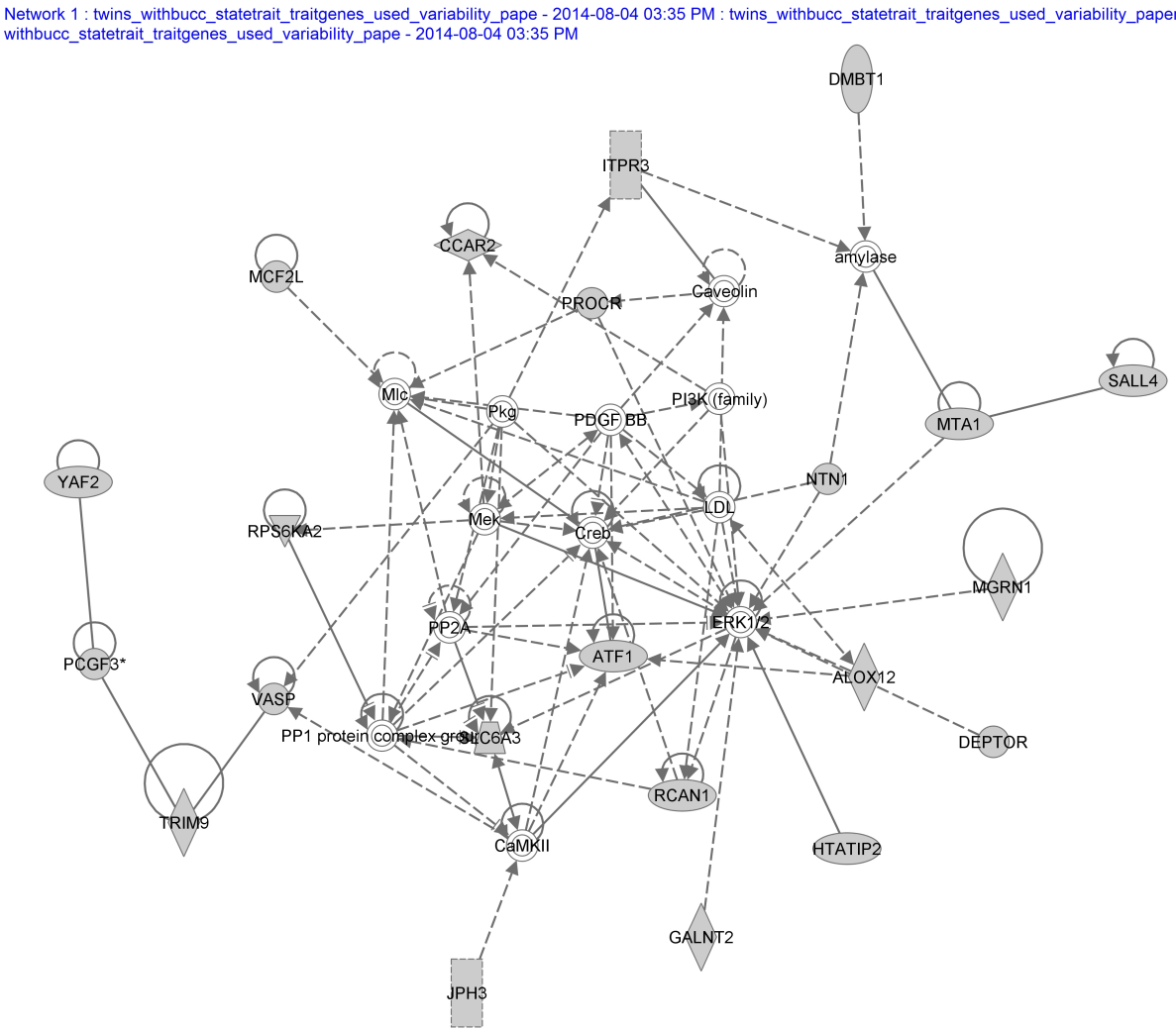
Computed by ingenuity. Genes in bold are genes found to be hypervariable in our sample. Genes not in bold are related genes implicated in the network but not found to be hypervariable in our sample. The top three networks with scores of 15 and greater are presented.

Figure 1. Twin correlations as assessed by hierarchical clustering



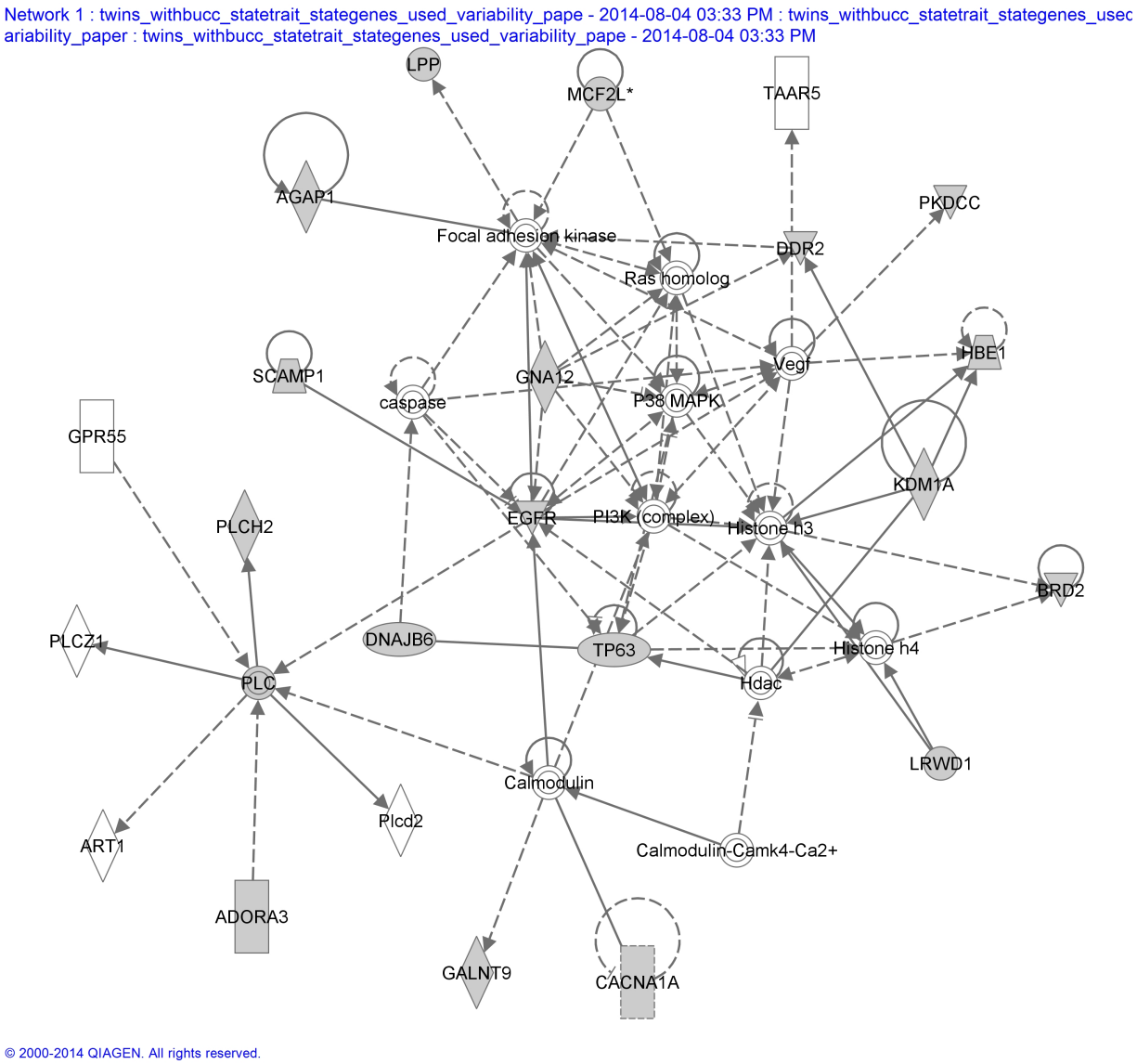
Clustergram representing the discordance between all participants. Blue represents the least amount of discordance, and red, the greatest amount. This demonstrates that in almost every case, an individual's DNA methylation was best predicted from his or her twin's DNA methylation.

Figure 2. Top trait-like network: Cell-to-cell signalling and interaction, tissue development, and cardiovascular system development and function



Network 1 as assessed by IPA. Trait-like genes are genes whose state of methylation are hypervariable within twin pairs but remain stable over time. Genes in gray are those found as hypervariable in our analyses. Genes in white are genes that are part of the network but not hypervariable in our data.

Figure 3. Top state-like network: Organismal development, cellular growth and proliferation, and digestive system development and function



Network 1 as assessed by IPA. State-like genes are genes whose state of methylation varies among twin pairs within 3-6 months during adolescence. Genes in gray are those found as hypervariable in our analyses. Genes in white are genes that are part of the network but not hypervariable in our data.