

**ENVIRONMENTAL INFLUENCES AND EPIGENETIC MECHANISMS IN
RISK FOR DEPRESSION**

by

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Abstract

Genetic and environmental factors interact to influence vulnerability for internalizing psychopathology, including Major Depressive Disorder (MDD). The mechanisms that account for how environmental stress can alter biological systems are not yet well understood yet are critical to develop more accurate models of vulnerability and targeted interventions. Epigenetic influences, and more specifically, DNA methylation, may provide a mechanism by which stress could program gene expression, thereby altering key systems implicated in depression, such as frontal-limbic circuitry and its critical role in emotion regulation. This thesis investigated the role of environmental factors from infancy and throughout the lifespan affecting the serotonergic (5-HT) system in the vulnerability to and treatment of depression and anxiety and potential underlying DNA methylation processes.

First, we investigated the contributions of additive genetic vs. environmental factors on an early trait phenotype for depression (negative emotionality) in infants and their stability over time in the first 2 years of life. We provided evidence of the substantial contributions of both genetic and shared environmental factors to this trait, as well as genetically- and environmentally- mediated stability and innovation. Second, we studied how childhood environmental stress is associated with peripheral DNA methylation of the serotonin transporter gene, SLC6A4, as well as long-term trajectories of internalizing behaviours. There was a relationship between childhood psychosocial adversity and SLC6A4 methylation in males, as well as between SLC6A4 methylation and internalizing trajectory in both sexes. Third, we investigated changes in emotion processing and epigenetic modification of the SLC6A4 gene in depressed adolescents before and after Mindfulness-Based Cognitive Therapy (MBCT). The alterations from pre- to post-treatment in connectivity between the ACC and other network regions and SLC6A4 methylation suggested that MBCT may work to optimize the connectivity of brain networks involved in cognitive control of emotion as well as also normalize the relationship between SLC6A4 methylation and activation patterns in frontal-limbic circuitry. Our results from these three studies strengthen the theory that environmental influences are critical in establishing early vulnerability factors for MDD, driving

epigenetic processes, and altering brain processes as an individual undergoes treatment, or experiences relapse.

Co-Authorship

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List of Abbreviations

5-HT – Serotonin; 5-hydroxytryptamine

5-HTT or SERT – Serotonin transporter

5-HTTLPR - Serotonin transporter-linked polymorphic region

ACC - Anterior cingulate cortex

ACE – Model of the additive genetic, shared environment, and unique environment contributions

ADE – Model of the additive genetic, non-additive genetic, and unique environment contributions

AIC – Akaike information criterion

BA – Brodmann area

BIC – Bayesian information criterion

BOLD – Blood-oxygen-level dependent

BYI-II – Beck Youth Inventory-II

CpG – Cytosine-phosphate-guanosine

CSF – Cerebro-spinal fluid

DLPFC – Dorsolateral-prefrontal cortex

DNA – Deoxyribonucleic acid

DSM-IV – Diagnostic and Statistical Manual-IV

DZ – Dizygotic

EPI – Echo planar imaging

FDR – False discovery rate

FIML – Full information maximum likelihood

fMRI – Functional magnetic resonance imaging

FWHM – Full-width half-maximum

G X E – Gene by environment

GR – Glucocorticoid receptor

GM – Grey matter

GRIP – Group for research and psychosocial intervention

h – Heritability factor

HPA axis – Hypothalamic-Pituitary-Adrenal axis

K-SADS – Kiddie Schedule for Affective Disorders

MBCT – Mindfulness-Based Cognitive Therapy

MDD – Major Depressive Disorder

MNI – Montreal Neurological Institute

MRI - Magnetic Resonance Imaging

MZ – Monozygotic

PFC – Prefrontal cortex

ROI – Region of interest

sgACC – subgenual anterior cingulate cortex

SES – Socioeconomic status

SGM – Semi-parametric group based modeling

SLC6A4 gene – Human serotonin transporter gene (Solute Carrier Family 6 Member 4)

SPM8 – Statistical Parametric Mapping Program

SSRI – Selective serotonin reuptake inhibitor

WM – White matter

Chapter 1

Introduction

Theoretical Context

Depression describes a state characterized by a combination of elements: persistent depressed mood, loss of interest, cognitive impairments, feelings of hopelessness or worthlessness, as well as disturbances in bodily processes (American Psychiatric Association, 2013; Segal, Williams, & Teasdale, 2012). The highly prevalent Major Depressive Disorder (MDD) is also associated with a significant degree of disability, and is projected to inflict the second-largest burden on worldwide health by the year 2020 (Lopez and Murray, 1998; Ustun, 2001). With approximately one half of those affected by a first major depressive episode going on to have a second episode in their lifetime, individuals with recurrent depression represent a major public health problem (American Psychiatric Association, 2013; Frank et al., 1990; Kupfer, Frank, & Wamhoff, 1996). Furthermore, since individuals with chronic depressive histories are also more likely to have had their first episode during adolescence, early prediction of vulnerable individuals as well as treatment of early-onset MDD in this group are important priorities for treatment research (Segal et al., 2012).

Though distinct disorders, depression and anxiety, are commonly grouped together under the broader term of internalizing disorder based on their intropunitive nature (Tandon, Cardeli, & Luby, 2009). Moreover, longitudinal studies have shown that internalizing problems are highly stable from very early infancy and have enduring patterns of behavioural inhibition, sadness, and anxiety throughout the lifespan (for review, see Tandon et al., 2009). Although they have been the topic of research for decades, the underlying mechanisms for development of these disorders

and how multiple multi-level systems, as well as genetic, cognitive, physiological, developmental, and social risk and resilience factors are involved, are not yet well established (Belmaker & Agam, 2008). Notably, we know very little about the mechanism of recurrent depression (Willner, Scheel-Krüger, & Belzung, 2012). The aim of this dissertation was to investigate environmental influences on vulnerability to internalizing disorders throughout the lifespan and a possible underlying biological mechanism for this influence, with an emphasis on the role of the neurotransmitter serotonin. This research was informed by, and aimed to help enrich, recent neurodevelopmental theories of psychopathology, focusing on the early origins of health and disease. With regard to the serotonin system and its role in development and psychopathology (see below), a neurodevelopmental stress-diathesis framework has been proposed (Booij, Tremblay, et al., 2015). According to this framework, early gene by environment (G X E) interactions lead to disruption of the 5-HT system driving subtle structural and functional alterations in brain development that establish vulnerability for psychopathology. A model of depression based on this framework is presented in Figure 1.1.

The model also provides a basis for integrating the cognitive phenomena and neural mechanisms of depression (for review, see De Raedt & Koster, 2010). The brain's frontal-limbic circuitry consists of key structures and pathways involved in cognitive-emotional regulation. Recent theories postulate that the interaction between prefrontal and subcortical regions (e.g. the amygdala, hippocampus, anterior cingulate cortex, or ACC) plays a critical role in risk for depression (Disner, Beevers, Haigh, & Beck, 2011). The prefrontal cortex (PFC) exerts guiding control to regulate activations in other regions of the brain in order to enhance goal-oriented processes and inhibit extraneous ones (De Raedt & Koster, 2010). Decreased prefrontal control of subcortical brain regions involved in emotion regulation, including the amygdala, results in the

prolonged activation of these limbic structures (De Raedt & Koster, 2010; Disner et al., 2011). This pattern leads to sustained negative affect and attentional biases, and is associated with increased negative cognitive response style such as rumination and self-focus in individuals with MDD (Carballedo et al., 2011; De Raedt & Koster, 2010; Disner et al., 2011). Still, the way in the environment may influence these neural processes to alter vulnerability to depression is not yet clear.

The brain regions underlying cognitive phenomena of MDD (and emotion regulation in general) are widely connected to other brain regions and biological systems. Of particular interest in the context of MDD is the modulation by the neurotransmitter serotonin (5-HT). Moreover, the development and functioning of the 5-HT system is largely dependent on serotonin itself, and the aforementioned brain regions are dense in serotonin transporters and receptors (Booij, Tremblay, et al., 2015). Our knowledge of the role of the 5-HT system in models of depression pathogenesis continues to be refined by ongoing research on the neurophysiology, development, heritability, and environmental factors of MDD.

In this introduction, we chart the changing biological (5-HT) models of depression vulnerability in order to see how the neurodevelopmental stress-diathesis model of depression synthesizes the research from the last 40 years, as well as introduce a possible underlying epigenetic mechanism, DNA methylation. In doing so, we aim to highlight the need for developmentally-informed, longitudinal research that integrates different approaches to elucidate the processes of environmental influence on depression vulnerability throughout the lifespan.

Early biological theories of depression

Following reports of low 5-HT function (reflected in low levels of the serotonin metabolite 5-HIAA) in MDD and the introduction of tricyclic antidepressants that inhibited 5-HT reuptake in the 1970s, scientists believed that a straight-forward deficit in 5-HT was the primary cause of MDD (for review, see Booij, Tremblay, Szyf, & Benkelfat, 2015). However, not all patients showed deficits in 5-HT, nor did they all benefit from serotonin-based antidepressant medication. Moreover, serotonergic alterations were also present in patients who had recovered from a depression, and in never-depressed individuals with a strong family history of depression, providing evidence that low serotonin is not causally linked to depression (Albert, Benkelfat, & Descarries, 2012; Benkelfat, Ellenbogen, Dean, Palmour, & Young, 1994; Booij, Tremblay, et al., 2015). Hence, research from the past two decades has shown that the direct causal link between low 5-HT and depression began to appear unlikely.

Diathesis-stress models

As researchers gradually agreed upon the idea that low 5-HT may play a role in depression but is not a direct cause of depression, continued exploration of the genetic underpinnings of MDD evidenced its substantial heritable component: estimates of the heritability of depression range from 31 to 42% (Sullivan et al., 2000; Willner et al., 2012). However, these estimates are much lower than the heritability estimated for schizophrenia or bipolar disorder (Belmaker & Agam, 2008). In any case, failed attempts to discover a “depression gene” required to researchers to look beyond the DNA sequence (Booij et al., 2013).

Subsequent models strove to integrate the influence of environmental stress, such as childhood adversity and maternal depression, following evidence that these stressors contributed

to depression vulnerability (or diathesis) in tandem with biological factors such as 5-HT (Côté et al., 2009; Donaldson et al., 2014; Holmes & Hariri, 2003; Karg, Burmeister, Shedden, & Sen, 2011; Risch et al., 2009; Sullivan, Neale, & Kendler, 2000; Uher & McGuffin, 2010). In this way, G X E interaction models of internalizing disorders conceptualized external factors (e.g., childhood adversity, stress) influencing a specific genetic vulnerability or feature (candidate gene or polymorphism) associated with the function of the physiological system resulting in deregulation in the specified system(s) (Booij, Tremblay, et al., 2015; McGowan et al., 2009).

The most well-studied of these candidate genes for MDD is the 5-HTTLPR polymorphism of the serotonin transporter gene, SLC6A4. The short, or *s*, allele is associated with some reduced 5-HTT expression relative to the long, or *l* allele (Heils et al., 1996; Hranilovic et al., 2004), as well as a heightened sensitivity or reactivity to stress as measured by increased levels of cortisol (Gotlib, Joormann, Minor, & Hallmayer, 2008). Importantly, the short 5-HTTLPR polymorphism has been found by some researchers to be associated with an increased risk for depression, especially in the context of early life adversity (Caspi et al., 2003; Holmes & Hariri, 2003; Jans, Riedel, Markus, & Blokland, 2007; Uher & McGuffin, 2010). The *s* allele have also been linked to anxious-depressive personality types in non-clinical samples (Lesch, 1999). Evidence from a meta-analysis suggested that the effect of the polymorphism on vulnerability to depression was small, but robust (Clarke et al., 2010). Thus, just as researchers concluded that there was no single “depression gene,” the evidence also suggested that there was no single gene conferring stress sensitivity leading to vulnerability for depression or anxiety (Belmaker & Agam, 2008; Booij, Tremblay, et al., 2015; Booij et al., 2013; Hankin, 2012; Karg et al., 2011; Levesque, Szyf, & Booij, 2016; Uher & McGuffin, 2010). Rather than a risk allele, the *s* allele of the 5-HTTLPR polymorphism may represent a plasticity factor that confers

increased susceptibility to positive and negative influences from the environment on its bearer (Belsky et al., 2009; Belsky and Pluess, 2009; Hankin, 2012). With increasing focus on the early environment's role in pathogenesis of psychopathology including MDD, researchers also began to see the merit of fully integrating developmental concepts into these diathesis-stress models (Booij, Tremblay, et al., 2015).

Development-informed biological models of depression

Aside from the potential role of 5-HT in depression risk, 5-HT plays a crucial role in early brain development (Booij, Tremblay, et al., 2015). Interestingly, the 5-HT system undergoes rapid and critically-important changes in utero and early life, with the most prominent changes stabilized by the time an individual reaches adolescence (Booij, Tremblay, et al., 2015; Chugani et al., 1999). The development and maturation of the 5-HT system occur much earlier than many other neurotransmitters (Booij, Tremblay, et al., 2015). Early alterations in serotonergic function have been shown to have downstream effects on key aspects of brain development, including neuronal growth, differentiation, and pathway development (Booij, Tremblay, et al., 2015). Animal research provides evidence that alterations in 5-HT homeostasis due to environmental stress during rodent development can cause permanent change to adult rodent stress dysregulation, emotion regulation, psychopathology, and behaviour dysregulation (Booij, Tremblay, et al., 2015; Esaki et al., 2005; Gaspar, Cases, & Maroteaux, 2003). Knockout studies have also documented 5-HT alterations in SERT knockout mice (Esaki et al., 2005; Gingrich & Hen, 2001). Although these studies cannot be replicated in humans, molecular imaging studies have shown that, consistent with animal models, the *s* allele has been linked to greater neural

responses to negative stimuli (Booij, Tremblay, et al., 2015; Holmes & Hariri, 2003) and lower hippocampal and amygdala volume (Frodl et al., 2008; Selvaraj et al., 2011).

As such, much of the vulnerability for MDD (reflected in a vulnerable 5-HT system) may be established in pregnancy and early childhood, yet we know relatively little about how environmental influences interact with neurodevelopment processes at critical periods during development (Booij, Tremblay, et al., 2015; Uddin et al., 2011; Willner et al., 2012). As well, it has become clear that understanding the biological mechanisms underpinning the early dynamics of G X E interactions from infancy in the 5-HT system stands to substantially contribute to an comprehensive developmental model of MDD vulnerability (Donaldson et al., 2014; Hasler, Drevets, Manji, & Charney, 2004).

A comprehensive, mechanistic, risk model of MDD

Thus, focus has shifted away from looking for deterministic causes of depression toward integrating genetic and environmental factors and developmental concerns within one model, which is also informed by research on the neural mechanisms underlying the cognitive aspects of depression (Disner et al., 2011). The integration of genetic and environmental factors in the model necessitates a closer examination of how stress triggers long-lasting changes in biological systems implicated in MDD. The physiological mechanism by which the environment interacts with genes, thereby programming biological processes through altered gene expression is still not known, and represents a potentially crucial missing piece in comprehensive models of depression vulnerability, especially one that also incorporates mechanisms of recurrent or chronic depression (Booij, Tremblay, et al., 2015; Booij et al., 2013; Willner et al., 2012). One explanation is that epigenetic (“above-genetic”; functional alterations without changes in genotype) modifications

affecting monoamine neurotransmitter expression (Booij et al., 2013; Weaver et al., 2004) serve as this environmental programming mechanism in addition to direct and interactive effects of the genotype. Epigenetic changes in 5-HT related genes may be an important regulator of 5-HT function but may also be dependent on genotype (Philibert et al., 2007), although research so far has not been consistent (Booij, Szyf, et al., 2015; Dongsha Wang et al., 2012; Wankerl et al., 2014).

DNA methylation

Epigenetic modifications such as DNA methylation are said to be heritable, stable, self-perpetuating, reversible and able to mediate changes in gene expression in response to environmental events and behavioural experiences (Booij, Tremblay, et al., 2015; Booij et al., 2013; Vialou, Feng, Robison, & Nestler, 2013). Epigenetic regulation continues throughout life and is critical in the programming and regulation of various systems, including the 5-HT system (Vialou et al., 2013).

DNA methylation, the physical addition of a methyl group to a portion of DNA sequence in order to alter its expressive function, is of great interest to health scientists who seek to understand how environmental factors increase risk of cancer (Ehrlich, 2002; Esteller & Herman, 2002; Szyf, 2003). Investigation of DNA methylation as a mediator allowing environmental factors to affect risk of psychopathology rapidly expanded following Weaver et al.'s (2004) work with rat pups in their first weeks of life. In response to early life stress (conceptualized as lack of maternal grooming), rat pups showed an increase in DNA methylation at the glucocorticoid receptor (GR) gene promoter, leading to decreased expression of hippocampal GR and increased stress response (Weaver et al., 2004). Moreover, cross-fostering of low maternal care rat pups in

the previously mentioned study by high maternal care mothers resulted in a reversal of the heightened stress reactivity and methylation differences (Weaver et al., 2004).

Weaver's experiments illustrate the ability of DNA methylation to be both stable and reversible, and to reflect a global response to environmental stress. These are the characteristics that suggest that DNA methylation may be a proxy for long-lasting changes in brain processes that affect vulnerability to emotion and behavioural dysregulation (Booij et al., 2013; Vialou et al., 2013).

Following these results, numerous studies have found an association between early life stress and neurotransmitter system DNA methylation in humans (Beach, Brody, Todorov, Gunter, & Philibert, 2010, 2011; Devlin, Brain, Austin, & Oberlander, 2010; Essex et al., 2013; van der Knaap, Riese, et al., 2015). However, the magnitude of the associations differs largely between studies, and most efforts have focused on the glucocorticoid receptor (GR) gene, which is involved in the hypothalamic-pituitary-adrenal (HPA) axis system.

Still, an increasing number of studies have studied methylation in the SLC6A4 gene in the context of G X E models, including its link with MDD (for review, see Januar, Saffery, & Ryan, 2015). Although SLC6A4 DNA methylation level has been positively associated (or positively trending) with MDD symptoms or lifetime incidence in community samples in some studies (Kang et al., 2013; Philibert et al., 2008; Uddin et al., 2011; Zhao, Goldberg, Bremner, & Vaccarino, 2013), others have found no association (Okada et al., 2014) or genotype dependent associations only (s-allele carriers only; Kim et al., 2013; Olsson et al., 2010).

One issue that complicates the measurement of DNA methylation status is the inaccessibility of methylation levels in the human brain (Dongsha Wang et al., 2012). Researchers have negotiated this challenge by measuring the DNA methylation of brain tissue in

deceased persons (McGowan et al., 2009), yet this has obvious constraints. Another option favored by many researchers is to study the DNA methylation of peripheral tissues. Although there is extensive evidence that DNA methylation is tissue specific, an increasing number of studies provide support for the idea that peripheral methylation may be informative for the brain. For instance, evidence suggests that white blood cell methylation of the SLC6A4 gene promoter is associated with lower in vivo human brain 5-HT synthesis (Dongsha Wang et al., 2012), as well as decreased hippocampal volume and neural responses to negative stimuli in the insula (Booij, Szyf, et al., 2015; Frodl et al., 2015). Furthermore, Nikolova et al. (2014) found that increased peripheral methylation of the SLC6A4 gene promoter was associated with greater amygdala reactivity in response to fearful stimuli. Finally, Muehlhan et al. (2015) found a significant positive association between peripheral SLC6A4 promoter methylation and resting state functional connectivity between the amygdala and key nodes of the salience network, including the insula and ACC. Together, these findings indicate the relevance of peripheral methylation for human brain processes implicated in depression and support the notion that SLC6A4 methylation may be an underlying mechanism of how genes and environment physiologically interact to affect brain processes and development.

Nevertheless, significant gaps exist in our understanding of how peripheral DNA methylation status and MDD symptoms are related, what mediates this relationship (i.e., early stress/abuse) and how it may respond to environmental influences targeted at treating MDD. Notably, almost all studies have focused on adults and very few studies have been performed in adolescence; a critical time period when those with chronic MDD most often develop their first episode (Segal et al., 2012). In particular, the developmental relationship between DNA

methylation and symptoms of MDD in a clinically-depressed adolescent population and how it is affected by treatment for MDD is not yet understood (Olsson et al., 2010).

The aim of this dissertation was to investigate the early environment's influence on vulnerability to internalizing disorders throughout the lifespan and an epigenetic mechanism (DNA methylation) by which environmental stress may program the 5-HT system. Before we investigate an epigenetic mechanism (DNA methylation) whereby environmental events and behavioural experiences could trigger alterations in SLC6A4 gene expression, we need to first establish the influence of genetic and environmental factors underlying depressive vulnerability at the beginning of life. Therefore, study one (Chapter 2 of this thesis) employed a longitudinal twin study design to estimate the changes in genetic and environmental contributions to individual differences in infant negative emotionality (244 monozygotic and 394 dizygotic twin pairs). Here, our twin approach allowed us to disentangle the genetic vs. shared vs. unique environmental influences, while the longitudinal approach allowed for an evaluation of the gene-environment underpinnings of the phenotypic stability and innovation in this early risk factor for internalizing disorders. The research described in Chapters 3 and 4 focuses on early environment and serotonin and behavioural/brain outcome. Specifically, study two (Chapter 3) investigated different types of adversity and risk for internalizing trajectories and their association with SLC6A4 methylation in a large longitudinal sample of adults (N=300) followed regularly since childhood for the past 21 years. This second project not only contributes to a deeper understanding of the potential epigenetic mechanism for the G X E interaction involved in vulnerability for internalizing patterns over time, but also of the nature of different types early adversity in environmental influence over a long period of time. Study three (Chapter 4) explored alterations in brain functional connectivity and concurrent epigenetic differences in response to a

therapeutic psychological (environmental) intervention. Specifically, we employed a pilot project consisting of a cognitive-emotional fMRI task and measure of SLC6A4 DNA methylation in depressed adolescents to compare functional connectivity within the frontal-limbic network and methylation before and after MBCT.

The results of this research may increase our understanding of the dynamics and influence of environmental factors occurring throughout development, as well as provide evidence for epigenetic processes as a key biological mechanism allowing for environmental programming of gene expression in systems implicated in MDD. It is not yet known how DNA methylation is associated with Cognitive Therapy, as well as how methylation status is then associated with concurrent changes in frontal limbic-circuitry in the brain. Moreover, the role of possible epigenetic processes in recurrent depression is not well understood. If altered DNA methylation levels underlie pathogenesis of depression, and can be affected by life events, it follows that they may also be amenable to pharmaceutical and psychotherapeutic intervention (Moylan et al., 2013).

Objectives and Hypotheses

1. Determine the genetic and environmental contributions to individual differences in infant negative emotionality and their stability over time in a longitudinal twin sample in the first years of life.

We expect that both genetic and environmental factors are associated with negative emotionality, but this study was designed to estimate the specific proportions of genetic, shared environment and unique environment contributions.

2. Investigate different types early adversity and risk for internalizing trajectories and their association with peripheral SLC6A4 methylation in a large longitudinal sample of adults followed since childhood for 21 years.

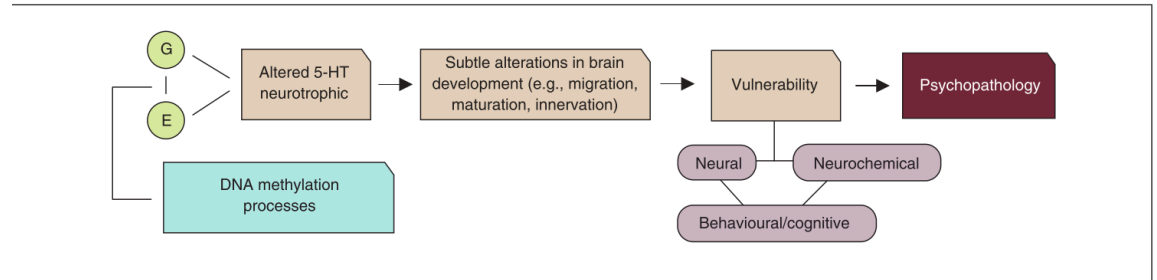
We expect that adversity is positively associated with peripheral SLC6A4 methylation. We examine the relative contributions of psychosocial adversity and different types of abuse accounting for this association. Additionally, we hypothesize a positive association between trajectories of internalizing behaviors in childhood-adolescence and peripheral SLC6A4 methylation.

3. Investigate the functional connectivity in emotion processing and cognitive control in frontal limbic circuitry before and after 6 weeks of Mindfulness-Based Cognitive Therapy in a clinically depressed sample of adolescents, including associations between connectivity and SLC6A4 methylation changes.

We hypothesize that during emotional regulation, we would observe increased functional connectivity in the frontal limbic circuitry (in particular ACC, insula and the PFC) in the post-MBCT treatment condition relative to the pre-MBCT treatment condition. We also expect that

the peripheral SLC6A4 DNA methylation state was significantly associated with fMRI responses in various frontal-limbic brain regions responsible for emotional processing and cognitive control.

Figure 1.1: Neurodevelopmental stress-diathesis model of psychopathology (Booij, Tremblay, et al., 2015)



Chapter 2

Stability and innovation in genetic and environmental factors in negative emotionality during infancy: A twin study

Abstract

Difficult temperament in infancy is a risk factor for forms of later psychopathology, including depression and anxiety. A better understanding of the roots of difficult temperament requires assessment of its early development with a genetically informative design. The goal of this study was to estimate genetic and environmental contributions to individual differences in infant negative emotionality and their stability over time. Participants were 244 monozygotic and 394 dizygotic twin pairs (49.7% male) recruited from birth. Mothers rated their twins for negative emotionality at 5 and 18 months. Longitudinal analysis of stability and innovation between the two time points was performed in Mplus. There were substantial and similar heritability (approximately 31%) and shared environmental (57.3%) contributions to negative emotionality at both 5 and 18 months. The trait's interindividual stability across time was both genetically- and environmentally- mediated. Evidence of innovative effects (i.e., variance at 18 months independent from variance at 5 months) indicated that negative emotionality is developmentally dynamic and affected by new genetic and environmental factors at 18 months. In the first two years of life, ongoing genetic and environmental influences support temperamental negative emotionality but new genetic and environmental factors also indicate dynamic change of those factors across time. A better understanding of the source and timing of factors on temperament in early development, and role of sex, could improve efforts to prevent related psychopathology, such as anxiety and depression.

Introduction

Temperament describes the individual differences in behavioural style and emotional functioning that appear in early infancy. Investigation of the biological basis of temperament with twins has provided evidence supporting the moderate heritability of individual differences in temperament (Briley & Tucker-Drob, 2012; Goldsmith, Buss, & Lemery, 1997; Lemery-chalfant, Kao, Swann, & Goldsmith, 2013; Rowe & Plomin, 1981). Variability in temperament is also accounted for by shared and non-shared environmental factors (Goldsmith et al., 1997; Rowe & Plomin, 1981; Silberg et al., 2005). However, little is known about how key aspects of temperament, such as tendency toward negative emotionality, develop in the first two years of life, and whether the same or new genetic or environmental influences play a role in this development. A deeper understanding of the stability and change of genetic and environmental sources of variance could help alleviate potential risks posed by difficult temperament in early life.

Longitudinal studies suggest that a difficult temperament may indicate vulnerability for the development of anxious and depressive symptomatology in children (Bates, Freeland, & Lounsbury, 1979; Lonigan, Phillips, & Hooe, 2003; Silberg et al., 2005; Watson, Clark, & Carey, 1988). Difficult temperament is a constellation of behaviours, including unadaptability, lack of soothability, and negative emotionality. In this paper, we focus on negative emotionality, the disposition to experience negative emotional states, since it represents an important aspect of difficult temperament in the context of vulnerability to psychopathology (Watson & Clark, 1984). This temperament dimension is expressed as an externalized response motivated by frustration

and/or a lack of stimulation and observed in infants as fussiness, crying, and negative emotional behaviour (Rhee et al., 2012; Vitaro, Dylan, Michel, Brendgen, & Tremblay, 2006; Watson & Clark, 1984).

In particular, negative emotionality is one key temperamental characteristic associated with depression and anxiety (Lonigan et al., 2003; Sayal, Heron, Maughan, Rowe, & Ramchandani, 2014; Watson & Clark, 1984). Furthermore, infant negative reactivity has been shown to predict (pre)frontal cortical morphology in adulthood (Schwartz et al., 2010). Accordingly, negative emotionality may be a marker in the developmental pathway of psychopathology. Children's heritable difficultness has also been shown to evoke a negative parenting response in mothers (Boivin et al., 2005; Forget-Dubois et al., 2007). Thus, an early temperament marked by high negative emotionality, particularly one that remains stable, may increase the risk of later psychopathology or other adverse outcomes. Despite its importance, the early development of negative emotionality, including its qualitative and quantitative sex differences and gene-environment (G X E) underpinnings, remains unknown.

The origins of child temperament were a focus of many studies in the 1980s and 1990s (Gjone & Stevenson, 1997; Lemery, Goldsmith, Klinnert, & Mrazek, 1999; Plomin et al., 1993; Rowe & Plomin, 1981; Wilson & Matheny, 1986). Analyses of the G X E underpinnings of negative emotionality highlight a substantial genetic component (Baker, Cesa, Gatz, & Mellins, 1992; Goldsmith et al., 1997; Krueger, Markon, & Bouchard, 2003; Kupper, Denollet, de Geus, Boomsma, & Willemsen, 2007; Neiss, Almeida, & David, 2004; Rhee et al., 2012; Silberg et al., 2005; Tackett, Waldman, Van Hulle, & Lahey, 2011). Estimates of heritability (h^2) typically range from 13% to 62%, with most of the estimates falling between 40% and 48% (Goldsmith et al., 1997; Kupper et al., 2007; Lemery-chalfant et al., 2013; Smith, Rhee, Corley, & Robinson,

2012; Tackett et al., 2011). However, the extent to which early developmental factors are translated into variability in genetic and environmental etiology for negative emotionality is not yet known. For example, we know little about the change and stability of negative emotionality heritability from infancy, a period during which many brain changes occur (Brown & Jernigan, 2012). Few studies have tested both ACE model (the additive genetic, shared environment, and unique environment contributions) and ADE model (the additive genetic, non-additive genetic, and unique environment contributions), or the stability and change of these contributions to negative emotionality, in populations under the age of three.

Two cross-sectional studies investigated negative emotionality/affectivity in a toddler and pre-school sample (55 Monozygotic, or MZ pairs and 65 Dizygotic, or DZ pairs aged 33-99 months; Goldsmith et al., 1997), as well as an infant sample (121 MZ pairs and 181 DZ pairs aged 3-16 months old; Lemery et al., 1999). A third cross-sectional study explored negative emotionality (used interchangeably with infant difficultness) in 865 infant twins (zygosity determined using probability estimation) ranging in age from 1 to 32 months born in Puerto Rico (Silberg et al., 2005). In all three studies, negative emotionality had substantial additive genetic and non-shared environment components that most closely fit an AE model (the effect of C was non-significant). The proportion of variability in negative emotionality attributed to genetic effects was estimated at 42% in the toddler and preschool sample (Goldsmith et al., 1997), 64% in infancy (Goldsmith, Lemery, Buss, & Campos, 1999), and 75.2% overall from birth to 32 months (Silberg et al., 2005). Sex differences in the models were either not investigated (Goldsmith et al., 1997) or not significant (Silberg et al., 2005). There was, however, a sibling interaction contribution (B) in the direction of a possible contrast effect (sibling interaction that magnifies intra-pair differences) found in the 2005 study performed by Silberg and colleagues.

However, all three studies were cross-sectional in design and thus do not help us understand the stability and change in the genetic and environment sources of these traits.

Scant longitudinal design research has been performed to examine the magnitude of genetic and environmental contributions to negative emotionality in toddler-aged twin populations (Plomin et al., 1993; Rhee et al., 2012; Saudino & Cherny, 2001). Rhee et al. assessed negative emotionality at 14, 20, and 24 months (224 MZ pairs and 179 DZ pairs) through behavior observations during experimental tasks and found heritability estimates of 62% at 14 months, 29% at 20 months and lower still at 24 months (13%). Conversely, the magnitude of shared environmental contribution (c^2) increased over the same time period, from 5% to 51%. No sex differences were found at any time point. Similarly, Saudino and Cherny found that negative emotionality heritability estimates initially increased from 14 to 20 months (37% to 47%), and then decreased from 20 to 36 months (47% to 11%). The role of the non-shared environment followed the opposite pattern (initially decreasing over 14 to 20 months, then increasing from 20 to 36 months). Neither study provided any information about ACE contributions in infancy (i.e., before 12 months of age) or their stability and/or change up to this point.

A few longitudinal twin studies have also provided evidence to indicate that stability in certain traits and behavior patterns is largely due to genetic factors (Lacourse et al., 2014; Petitclerc, Boivin, Dionne, Pérusse, & Tremblay, 2011; Saudino, 2005). Furthermore, there is also evidence to indicate that behavior patterns are also “developmentally dynamic,” or that genetic effects vary over time through genetic innovation (previously inactive genes coming online) (Lewis & Plomin, 2015). Additionally, environment factors appeared to drive some of the change in temperament during this time (Lacourse et al., 2014; Saudino, 1996, 2005). It is

postulated that genetic factors may be largely responsible for continuity in temperament, whereas environmental factors are largely responsible for change in temperament (Saudino, 2005).

However, genetic and environmental factors may operate or develop differently according to age- or sex-specific genetic innovations, occurring in early childhood (Gabory, Attig, & Junien, 2009; Goldsmith et al., 1997). As such, our understanding of the continuity and change of genetic and environment contributions to negative emotionality's in infancy is limited.

The goal of the present study was to use a twin birth cohort to estimate stability and innovation in genetic and environmental contributions to negative emotionality between 5 and 18 months after birth using a longitudinal design. We also explored sex differences. Sex differences in negative emotionality heritability have yielded mixed results, with some studies finding no significant sex differences (Rhee et al., 2012; Silberg et al., 2005). Other studies have found sex differences necessitating separate models (Tackett et al., 2011), or significantly higher correlations between same sex, dizygotic (DZ) twins than opposite sex DZ twins (Goldsmith et al., 1997). The study aimed to investigate these in a large longitudinal cohort of twins (244 monozygotic, 200 same-sex dizygotic, and 194 opposite-sex dizygotic twin pairs) with measurements at 5 and 18 months.

Method

Participants

The twins were recruited at birth in the province of Quebec (Canada) between November 1995 and July 1998, as part of a longitudinal cohort study in newborn twins in Quebec (Quebec Study of Newborn Twins, QSNT) in the Research Unit on Children's Psychosocial Maladjustment (GRIP) in Montreal. The original study included 1334 children (667 twin pairs) at

5 months whose parents agreed to participate and for whom zygosity was determined. Participants were 634 boys and 642 girls. Zygosity was assessed using the Zygosity Questionnaire for Young Twins, and confirmed through DNA testing in 30% of the sample (Dionne, Tremblay, Boivin, Laplante, & Pérusse, 2003; Forget-Dubois et al., 2007). The sample was representative of Quebec in terms of family characteristics, with very similar parental education, yearly household income, age of parents at child's birth, and marital status.

Measures

Children's behaviour was assessed with the Bates Infant Characteristics Questionnaire (Bates et al., 1979) at 5 and 18 months during home visits. The mother of each twin pair filled out a 37-item checklist for one twin during the visit, and filled out another for the second child two weeks after.

Negative emotionality scale. The negative emotionality scale was composed of seven items from the Bates Infant Characteristics Questionnaire (item composition similar to the Fussy/difficult scale). The term negative emotionality was used by both Vitaro et al. in their study (Vitaro et al., 2006) of difficult temperament using the Bates Infant Characteristics Questionnaire, as well as by Rhee et al. (Rhee et al., 2012) in their study of infant temperament to describe assessment of trait difficultness, anger, fussiness, and distress. The items measured how often the baby was fussy per day, amount of fuss/crying, intensity of protest, how easily the baby became upset, amount of attention the baby requires, how often the baby plays with self when alone, and overall degree of difficulty the baby presents for the parent on a seven-point scale. A score of 1 represented low negative emotionality and a score of 7 represented high negative emotionality.

An average of these seven negative emotionality items was used in the analyses. Cronbach's alpha was previously found to be .71 for mother's ratings (Vitaro et al., 2006). Phenotypic stability was computed to be $r = .50$ for boys and $r = .51$ for girls, indicating moderate stability in the trait from 5 to 18 months.

Data Analyses

The relative contributions of genetic and environmental factors to negative emotionality were determined using structural equation models based on the biometric model (Neale & Maes, 2004; Posthuma et al., 2003). First, sex-limited cross-sectional models were fit at 5 months and 18 months where we explored both qualitative and quantitative sex differences. Second, a multivariate correlational model (McArdle & Hamagami, 2003; Neale, Røysamb, & Jacobson, 2006) was used to estimate the additive genetic, shared environment and non-shared environment components of negative emotionality at 5 and 18 months. To interpret the results in terms of stability and innovation of genetic and environmental influences, we transformed the correlation matrix using classical algebra.

Model-fitting within time points

Estimates of the additive genetic (A), dominant genetic (D), shared environment (C), and non-shared environment (E) components were based on comparison of the theoretical relationship between MZ or DZ twins reared together and the measured concordance between twins in a pair. The quantitative genetic approach cannot test for the presence of both shared environment (C) and dominant genetic (D) effects at the same time due to under-identification. The researcher usually has to choose between ACE models or ADE models. The ratio of MZ/DZ intra-pair correlations can be used as an indicator of the presence of a dominant genetic effect. If the DZ

correlation is less than half the MZ, it is an indication of a dominant genetic effect. For a more precise estimation, one can also compare the two models (ACE and ADE) against a saturated model (i.e., model that accounts for all parameters) to assess their respective fit. Another way to consider the small DZ correlation relative to the MZ and negative DZ correlations is by thinking of a contrast effect (sometimes referred to as a sibling interaction or a competition effect; Eaves 1976). A contrast effect represents a situation where siblings influence each other in a dynamic process of continual feedback leading to a greater difference between them. In this situation, part of the difference between siblings is due to active environmental effects in the form of the sibling behaviour. As others have reported, a third option to interpret the small DZ correlation in relation to the MZ as a rater bias (Ebejer et al., 2014; Silberg et al., 2005; Simonoff et al., 1998).

By looking at the ratio of intra-pair correlations, we suspected dominant genetic effects or sibling interactions. We tested for the presence of dominant genetic effects (D) in place of shared environment (C) and found that the ACE model with sibling interaction fit better than the ADE. The ACE models used included a contrast effect (parameter S) by the addition of two direct effects between the twins (a first from twin 1 to twin 2 and a second from twin 2 to twin 1). The contrast effects in all ACE models were significant and were necessary components to obtain adequate estimates of heritability of temperamental measures (Carey, 1986; Neale & Maes, 2004; Silberg et al., 2005). In all models, the contrast effects coefficients had a negative value and showed no signs of being affected by zygosity or sex. Thus, the S parameter was set to be equal for MZ and DZ twins, as well as for girls and boys.

In order to further explore sex differences, we used sex-limited models to estimate sex differences in the etiology of negative emotionality (Neale & Maes, 2004; Neale et al., 2006). We explored the sex differences in cross-sectional models as well as in longitudinal models. We first

tested for qualitative sex differences at each time point to assess whether the different sets of genes were associated with the trait for boys and girls. To test for qualitative sex differences, in addition to the ACE factors, a fourth factor (G) is modeled to represent an additive genetic effect specific for boys. The paths to this sex-specific genetic factor were not significant indicating that, for each time point, the same set of genes was influencing both boys and girls. We then executed quantitative sex difference models to assess if the magnitude of the genetic factors were the same for females and males. The quantitative sex difference model significantly improved the fit for the 18 month measure, indicating possible sex differences in the magnitudes of genetic effects for females and males. As these analyses are important, yet underpowered, these results are presented as exploratory.

Data analyses were performed with Mplus 7.1 (Muthen & Muthen, 1998-2012) using full information Maximum Likelihood (FIML). All available cases were used for estimation of each parameter. Statistical significance of the individual parameter estimates for the paths in the model was determined by dividing the estimates by their respective standard errors (result is the t-value). The confidence intervals of the A, C, and E variance components were obtained through 10 000 bootstrapped samples.

The goodness of fit was evaluated using Akaike Information Criterion (AIC), the Bayesian Information Criterion (BIC) as well as χ^2 difference tests comparing twice the difference between the log-likelihood of a model and the saturated model. Degrees of freedom were calculated as the difference in the number of estimated parameters between the two models. A non-significant χ^2 indicated that the model with fewer parameters fit the data as well as the saturated model and thus had acceptable model fit (Schumacker & Lomax, 2004). For AIC and

BIC, a smaller value indicates better fitting models (Akaike, 1987; Schwarz, 1978). The AIC also reflects the balance between fit and parsimony (fewer parameters; Akaike, 1987).

Longitudinal model fitting analyses

Since it was found that the sex-limited model for the 18 month data had the best fit, a sex-limited biometric correlational model was applied to negative emotionality scores at 5 and 18 months (Neale et al., 2006). The biometric part of the model allowed for estimates of the additive genetic (A), shared environment (C), and non-shared environment (E) influences respectively at 5 months and 18 months, as well as the correlation between those sources of influences (r_A , r_C , r_E). Since we were primarily interested in stability and innovation of the gene-environment influences, we transformed the resulting matrices to a Cholesky decomposition form (Figure 2.1). The correlational model specifies that the genetic factors are the same for boys and girls by imposing a single genetic correlation parameter for both sexes (see Neale et al., 2006). Statistical significance and goodness of fit tests were performed in the same way as in cross-sectional models.

We first computed the ACE ratios for each time point as well as the proportion of each type of effects at 18 months that were explained by influences already present at 5 months. The stability of negative emotionality was assessed using the phenotypic correlation. The phenotypic correlation can be obtained from the Cholesky covariance matrix by using standard procedure to compute a correlation from a covariance matrix (Tuvblad, Raine, Zheng, & Baker, 2009). We then computed the proportion of genetic and environmental influences in the phenotypic correlation. Lastly, the extent of innovative genetic and environmental effects were computed using the ratio of each specific effect at 18 months to the total innovation effects at 18 months.

For example, the proportion of innovation due to genetic factors was computed using: $a_{22}^2 / a_{22}^2 + c_{22}^2 + e_{22}^2$ (Tuvblad et al., 2009).

Results

Descriptive statistics

Descriptive statistics for the final sample are in Table 2.1 and 2.2. Twin pairs were excluded if they did not have available data on negative emotionality for at least one twin at either 5 or 18 months. Demographic characteristics for the final sample were comparable to the original sample and did not differ in terms of age of mother at birth, weeks of gestation at birth, days premature, or weight at birth.

Model Fitting Analyses For Each Time Point

5 months. The magnitude of genetic and environmental effects was similar for boys and girls at 5 months. Models constraining the correlations among variables to be equal across sex fit equally well as unconstrained models, suggesting no significant sex difference. The ACE models without sex differences and with contrast effects exhibited indices of acceptable fit (Table 2.3).

At 5 months, boys and girls had fairly comparable variability estimates in negative emotionality attributed to genetic, shared environmental, and unique environmental influences. Both sexes demonstrated substantial shared environmental influence and genetic influence.

18 months. The likelihood ratio test showed that the fit was slightly improved in models with sex constraints, but other fit indices (AIC and BIC) indicated that models without sex limitation had better fit. Hence it was more parsimonious to accept the model without sex differences as the final 18-month model. A contrast effect improved the fit of the model (Table

2.3). At 18 months, twins demonstrated similarly substantial shared environmental influence and genetic influence as they had at 5 months.

Longitudinal Model-Fitting Analyses

Longitudinal data were analyzed with a biometric correlational model ($N = 1276$) to evaluate the stability and innovation of gene-environment's influence on negative emotionality. A saturated model was used as a baseline to which the constrained models were compared. Likelihood ratio test indicated a somewhat adequate fit for the model with sex constraints, but other indices (AIC, BIC) indicated that the model without sex limitation had a better fit. The sex-limited model showed interesting preliminary results: girls' innovation effects were attributed to genetic (29.9%) and shared environment (42.2%) factors, whereas innovation effects for boys were genetically mediated (76.3%). However, we lacked the power required to detect sex effects. Again, in the interest of parsimony, the model without sex limitation was accepted as the final model (Table 2.3).

Sibling interaction parameters were estimated for the multivariate model, and set to be equal between males and females as well as between MZ and DZ pairs because the fit was not improved by specifying different values. The path diagram for the resulting model is illustrated in Figure 2.1, and the path estimates are detailed in Table 2.4. Genetic and environmental contributions to each time point as well as their stability and change were computed (as described in above methods) and illustrated in Figure 2.2.

At both 5 and 18 months, estimates of the genetic and environmental contributions to negative emotionality were almost identical. Heritability accounted for approximately 34% of the variance in negative emotionality, showing substantial genetic transmission. Shared environmental influences explained 54% of the variance in this trait.

The extent to which the trait presented interindividual stability over time was due to both genetic (37.8%) and shared environment (57.8%) factors. Innovation effects were similarly mediated, with the proportions of innovation effects attributed to genetic and shared environmental factors at 35.8% and 51.4%, respectively.

Discussion

The current research used a large sample of twin participants followed from birth in order to examine the stability and innovation in the genetic and environmental sources of variance of temperamental negative emotionality in the first 2 years of life. Importantly, the longitudinal approach allowed for an evaluation of the gene-environment underpinnings of the phenotypic stability in this trait. We found that the ACE model with sibling interaction (suggesting significant reciprocal twin interaction) fit our data the best. Within each time point, we found substantial heritable (approximately 34%) and shared environmental (54%) contributions to negative emotionality. As well, both genetic and shared environmental influences mediated the stability in the trait and presented a dynamic influence pattern. Notably, we saw evidence of both genetic and environmental innovative effects coming online at 18 months. These results suggest that in infancy, genetic and environmental influences support the stability of temperamental negative emotionality and that those influences are dynamic.

The magnitude of heritability is substantial and commensurate with the low end of previous research on temperament (between 40% and 48%). While some similar studies have found a negligible contribution of the shared environment and found an AE model as best fit (Goldsmith et al., 1997, 1999; Silberg et al., 2005), the present study evidenced a substantial shared

environment influence. The final multivariate ACE model with a sibling interaction, but invariant with respect to sex, was consistent with the two similar longitudinal twin studies (Rhee et al., 2012; Saudino & Cherny, 2001).

However, contrary to previous research (Rhee et al., 2012; Saudino & Cherny, 2001; Saudino & Wang, 2012; Wilson & Matheny, 1986), the contributions of genetic and environmental factors did not change between 5 and 18 months. The contrast in these findings could be the result of different methodology engaging different processes and measuring different constructs of temperament or behaviour (Saudino & Wang, 2012). The longitudinal consistency of proportions attributed to genetic and environmental factors result indicates some stability in these influences in early infancy.

Furthermore, our results indicated that genetic and shared environmental factors account for why young children stay on course with respect to negative emotionality in the first two years. The finding that genetic effects show stability over time is consistent with the longitudinal twin study literature on negative emotionality (Rhee et al., 2012). The stability of the shared environment effect over time is a more novel result and suggests that the environment plays a larger role in the development of temperament in early infancy than previously estimated.

Similarly, the innovation effect on negative emotionality was mediated by both genetic and shared environmental influences. These substantial genetic innovations are somewhat dissimilar to Rhee et al.'s (Rhee et al., 2012) observation that age-specific influences were mostly limited to non-shared environmental influences. However, there is evidence that new genetic effects begin to shape childhood outcomes of negative emotionality such as affect and adaptability (Saudino & Wang, 2012; Saudino, 2005), as well as physical (Lacourse et al., 2014) and reactive aggression (Paquin, Lacourse, & Ouellet-Morin, 2014). For example, the innovation effect we observed in

negative emotionality at 18 months could represent genetically-driven maturation or age-related increases in socio-cognitive development, such as the emergence of social communication (Saudino & Wang, 2012). As well, the timing and influence of new genetic factors coming online during development could be programmed, in part, by environmentally-driven epigenetic mechanisms (Booij et al., 2013). Thus, we observed that negative emotionality was developmentally dynamic, and that these new genetic contributions may also be accompanied by increasing susceptibility to shared environmental factors (Lewis & Plomin, 2015). In this way, heritable and environmental factors are responsible for stability, and present a dynamic influence pattern.

Despite limited power, our preliminary results suggest possible sex differences (i.e., temperament was increasingly associated with genetic factors for boys, while the shared environment continued to have a significant effect on girls' temperament) that need to be confirmed with larger samples.

This research has limitations that may affect its generalizability. First, we employed single informant measures of temperament of twin infants. As suggested by Silberg et al. (Silberg et al., 2005), a mother could systematically rate her twins differently comparing one to another, which could have led to a rater bias. We minimized such effects by having each member of the pair rated at a different moment in time (approximately two weeks apart). Second, our results were based on a sample recruited in a specific cultural context, and thus may not generalize to all cultural contexts. Regardless, the present research was carried out with a large, relatively diverse sample that was representative of the population of the region (Montreal, Canada).

Further examination of how the environment moderates development of temperament in young children and the developmental timing of key periods for this process to occur are

important next steps for research in this area. For example, Lemery-Chalfant and colleagues (Lemery-chalfant et al., 2013) found that heritability of negative affectivity increased under crowded or unsafe home conditions, exemplifying the complex and influential interaction between genetic and environmental factors on temperament.

To conclude, the current research showed that in the first two years of life, both genetic and shared environmental factors contribute substantially to negative emotionality. The trait's stability across time appears to be both genetically- and environmentally- mediated. Furthermore, evidence of innovative effects indicated that negative emotionality is developmentally dynamic and affected by new genetic and environmental factors at 18 months. The present study's large sample size, longitudinal design, and measures in infancy offer more precise estimates of genetic and environmental contributions, to trait negative emotionality. The results of the present study stand to further improve prevention and intervention programs aimed to reduce mental health problems in high-risk populations. For instance, it has widely been demonstrated that difficult temperament is a predictor of mental health problems such as depression, anxiety, and aggression in childhood (Côté et al., 2009; Tremblay et al., 2004) or adulthood (Lonigan et al., 2003; Sayal et al., 2014; Watson et al., 1988). The substantial environmental influences in the first two years of life support the need for early interventions at a very early stage; i.e. during pregnancy or in the first years of life (Tremblay, 2010).

Table 2.1: Descriptive statistics

Demographic Characteristic	Mean (SD)/ percentage
Age of mother at twins' birth	30 years, 5 months (4 years, 9 months)
Weeks of gestation at birth	36 weeks (2.5)
Days premature	27 days (18)
Weight at birth	2.48 kg (0.5)
Mother has obtained secondary school diploma	76.3%
<u>Race/Ethnicity of mother</u>	
White/Caucasian	81.2%
Non-White/non-Caucasian	18.8%
<u>Family Status</u>	
Twins living with both biological parents (at 5 months)	86.5%
Twins living with both biological parents (at 18 months)	84.3%

Table 2.2: Ratings of twins at 5 and 18 months separated by sex and zygosity

Trait	Twin pairs used in model	N of Males/ Females	MZ m/m	MZ f/f	DZ m/m	DZ f/f	DZ f/m
Negative emotionality at 5 months	598	590/606	111	118	94	95	180
Negative emotionality at 18 months	558	543/573	105	120	87	87	159
Total sample	638	634/642	119	125	101	99	194
Mean rating (SD)			MZ m/m	MZ f/f	DZ m/m	DZ f/f	DZ m/f
Negative emotionality at 5 months			3.12 (0.90)	3.03 (.98)	3.16 (1.10)	3.10 (1.20)	2.98 (1.10)
Negative emotionality at 18 months			3.41 (0.89)	3.34 (0.86)	3.32 (1.00)	3.30 (1.00)	3.30 (1.10)
Intraclass correlation (based on ANOVA)			MZ m/m	MZ f/f	DZ m/m	DZ f/f	DZ f/m
Negative emotionality at 5 months			.28	.42	-.11	-.05	-.10
Negative emotionality at 18 months			.54	.31	-.18	.14	-.12

Note: MZ = monozygotic twins, DZ = dizygotic twins, f/f = female/female twins, m/m = male/male twins, f/m = female/male twins, SD = standard deviation

Table 2.3: Comparison of fit for univariate and multivariate models

Model	Log likelihood	χ^2 (df)	p	AIC	BIC
<i>5 months</i> ($N=1196$)					
1. Saturated	-1711.73			3457.46	3532.15
2. ACE sex-limited	-1715.37	7.27 (8)	.51	3448.74	3488.28
3. ACE no sex limitation	-1716.08	8.70 (11)	.65	3444.16	3470.52
<i>18 months</i> ($N=1116$)					
1. Saturated	-1520.94			3075.87	3149.38
2. ACE sex-limited	-1524.40	6.94 (8)	.54	3066.81	3105.73
<i>Multivariate</i>					
1. Saturated	-3152.84			6397.69	6602.77
2. ACE sex-limited	-3163.50	21.33 (26)	.72	6367.01	6456.18
3. ACE no sex-limitation	-3168.48	31.27 (32)	.50	6364.95	6427.37

Caption: AIC = Akaike information criterion, BIC = Bayesian information criterion

Table 2.4: Longitudinal unstandardized parameter estimates and confidence intervals

Parameter estimates
$a_{11} = .68 (0.21 - 0.89)$
$a_{12} = .28 (0.05 - 0.48)$
$a_{22} = .60 (0.19 - 0.78)$
$c_{11} = .88 (0.45 - 1.4)$
$c_{12} = .40 (0.13 - 0.62)$
$c_{22} = .71 (0.24 - 1.2)$
$e_{11} = .41 (0.13 - 0.57)$
$e_{12} = .06 (-0.00 - 0.13)$
$e_{22} = .36 (0.11 - 0.49)$
Correlations
$r_a = .42$
$r_c = .49$
$r_e = .15$
Sibling effect
$s_1 = s_2 = -.46$

Figure 2.1 Theoretical path diagram: biometric Cholesky decomposition

Caption: Stability effects are denoted by the subscript 12; innovation effects are denoted by the subscript 22

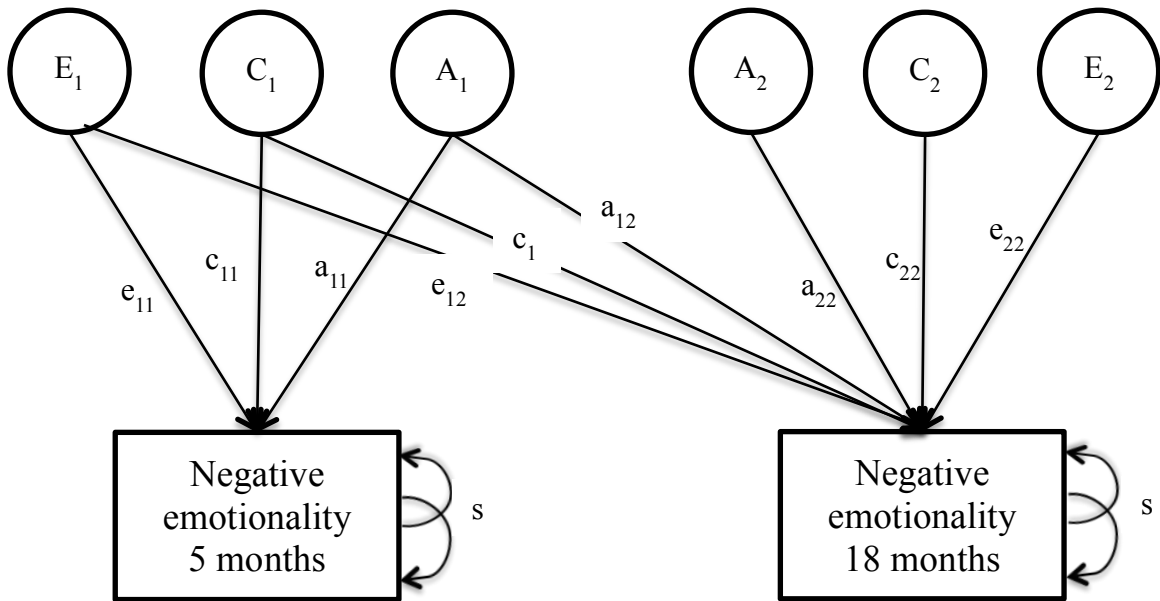
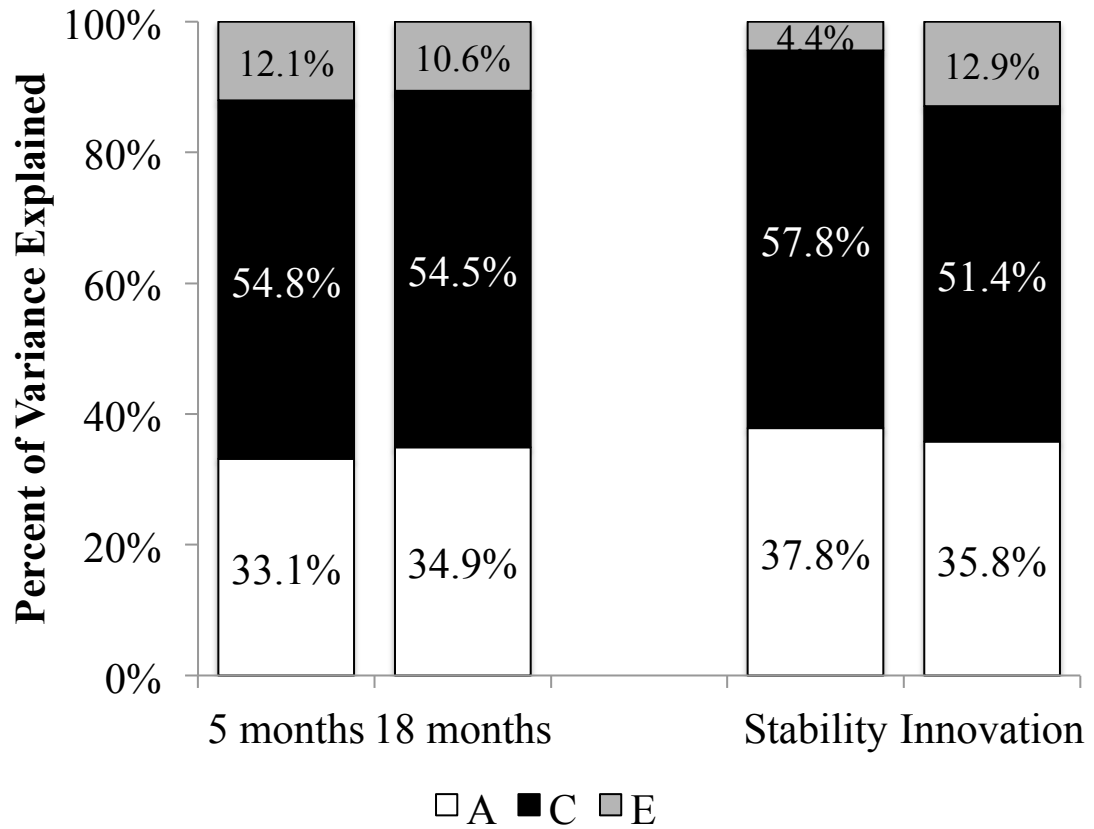


Figure 2.2 Variance attributed to ACE by time and in terms of stability and innovation for negative emotionality



Chapter 3

Childhood psychosocial adversity, psychological abuse, and high internalizing trajectory are associated with peripheral SLC6A4 DNA methylation: A 21-year longitudinal study

Abstract

Adverse exposures happening early in life have often been linked to a variety of mental health problems in adulthood, including major depression and anxiety disorders. Among the many factors that may play a role, one important physiological mechanism driving this process is DNA methylation in genes regulating the neurotransmitter serotonin. An emerging hypothesis is that site-specific serotonin transporter gene (SLC6A4) methylation would alter stress regulation systems and have downstream consequences, including increased internalizing psychopathology. The aim of the present study was to study the association between peripheral SLC6A4 methylation and different types of early life adversity and risk for internalizing trajectories in a large longitudinal sample of adults followed since childhood. Peripheral DNA (whole blood), measures of childhood psychosocial adversity, and experiences of abuse from 300 adult men and women were collected from a community sample of adults (born between 1988 and 1990) followed since kindergarten. Patterns of trajectories of internalizing behaviours were created from 7 time-points from childhood to late adolescence. Linear regression analyses were performed. Psychosocial adversity was associated with peripheral SLC6A4 methylation in males but not in females. Psychological abuse but not physical and sexual abuse was associated with peripheral methylation in both men and women. Notably, long-term patterns of internalizing behaviors were associated with lower peripheral SLC6A4 methylation, irrespective of sex and psychosocial adversity. Our prospective measures of childhood psychosocial adversity and a model that accounts for various forms of adversity, as well as the use of internalizing trajectories rather than single time point measures, provides support for a meaningful link between peripheral DNA methylation and risk for longstanding internalizing problems in a gene-environment context.

Introduction

Exposure to early life adversity such as abuse and neglect in childhood as well as poor socioeconomic conditions like growing up in poverty are associated with increased risk for anxiety and depression later in life (Kendler, Kuhn, & Prescott, 2004; Shonkoff et al., 2012). Although the specific mechanisms through which this occurs are not yet well known, a widely postulated overarching hypothesis is that disruptions in physiological systems that are sensitive to environmental exposures (so called biological embedding) adversely affect developmental trajectories and health outcomes (Shonkoff et al., 2012).

Recent models propose DNA methylation of key neuro-regulatory genes as an important molecular mechanism driving this process (Booij, Tremblay, et al., 2015; Booij et al., 2013; Levesque et al., 2016). Specifically, DNA methylation is considered to be a stable and proximal epigenetic modification, which makes it an excellent candidate for a biomarker of changes in genomic function (Booij et al., 2013). High levels of DNA methylation could silence a gene, resulting in a loss of function (Booij et al., 2013).

Among the many neurobiological systems that are jeopardized by adverse environmental conditions, the serotonin system (5-HT) is of particular interest, given its crucial role in early brain development. Indeed, early disruption of the 5-HT system in animals has been shown to adversely affect brain structure and function as well as emotion processing and stress regulation later in life (Booij, Tremblay, et al., 2015; Esaki et al., 2005; Gaspar et al., 2003). As such, altered 5-HT neurotransmission, acquired through environmental stress, may represent an important biological risk factor for internalizing problems such as major depressive disorder (MDD; Booij, Tremblay, et al., 2015; Jans et al., 2007), and anxiety disorders (Richardson-Jones et al., 2011).

Researchers have explored the possibility of DNA methylation of specific promoter regions as the mechanism that could alter 5-HT system functioning (Glover, O'Connor, & O'Donnell, 2010). Since DNA methylation cannot be accessed directly in the brain (central methylation), peripheral methylation has been used as a viable alternative to measure associated brain function. Specifically, peripheral serotonin transporter gene (SLC6A4) promoter methylation was found to be associated with lower 5-HT synthesis (Dongsha Wang et al., 2012), and decreased hippocampal volume and neural processing of negative emotions (Booij, Szyf, et al., 2015; Frodl et al., 2015). Moreover, alterations in DNA methylation may have downstream consequences for risk for internalizing problems (Booij, Tremblay, et al., 2015; Frodl et al., 2015). The goal of the present study was to investigate the association between an epigenetic measure (peripheral DNA methylation of the serotonin transporter gene, SLC6A4) and different types of early environmental adversity as well as risk for internalizing problems in a large longitudinal community sample followed for 21 years.

Investigations into the association between risk for internalizing problems and/or disorders and SLC6A4 methylation are limited (especially for anxiety), most often have small sample sizes, and have yielded mixed results (for review, see Januar, Saffery, & Ryan, 2015). While some studies have found positive trends or positive associations between peripheral SLC6A4 promoter methylation and MDD symptoms (Kang et al., 2013; Philibert et al., 2008; Zhao et al., 2013) and MDD severity (Okada et al., 2014) and functional impairment and stress sensitivity (Kang et al., 2013), others have found no association between overall SLC6A4 methylation and presence of MDD diagnosis (Okada et al., 2014; Booij et al., 2015). One longitudinal study found only a tendency for association between peripheral SLC6A4 methylation and internalizing disorder diagnoses at 3-year follow-up (after accounting for baseline symptom

scores) in a sample of 939 adolescents (van der Knaap, Van Oort, Verhulst, Oldehinkel, & Riese, 2015). Thus, the gene's association with internalizing problems is not clear, as studies differ in terms of age and specific CpG region(s) tested and possible moderation by other factors such as early life adversity (Booij, Tremblay, et al., 2015; Booij et al., 2013; Januar et al., 2015; van der Knaap, Riese, et al., 2015).

Although the general association between early life adversity and alterations in DNA methylation at key sites has been previously demonstrated (Beach et al., 2010, 2011; Borghol et al., 2012; Devlin et al., 2010; Essex et al., 2013), how these environmental factors relate to risk for internalizing problems is understudied and may depend on how adversity is defined (Levesque, Szyf, & Booij, 2016). Adversity in childhood has been operationalized with different types of stressors, including socioeconomic adversity, experience of abuse/maltreatment (including physical, sexual, and psychological abuse), maternal depression, and maternal separation in animals. Notably, psychosocial adversity factors such as extreme poverty are also risk factors for “toxic stress,” or frequent or prolonged exposure to stress-response systems that has physiological consequences (Shonkoff et al., 2012), including higher risk of aggression and hyperactivity later in life (Mazza et al., 2016). These alterations in stress systems are believed to be facilitated by epigenetic mechanisms (Shonkoff et al., 2012). Indeed, one epigenome wide study in 40 adult males with prospective measures of social economic adversity showed that adult DNA methylation patterns were more associated with disadvantaged social economic status in childhood than in adulthood (Borghol et al., 2012). Links with DNA methylation in specific serotonin genes and in particular the SLC6A4 gene are not known, although variability in 5-HT functioning (as reflected in the endocrine response to a fenfluramine challenge) has been linked to socioeconomic status, irrespective of serotonin transporter genotype (Manuck et al., 2005).

We know from animal research that not all stressors have similar effects and outcomes: chronic unpredictable stressors are more likely to be linked to depressive symptoms, whereas predictable stressors may be associated with resilience to anxious and depressive symptoms (Gobinath, Mahmoud, & Galea, 2015; Suo et al., 2013). In humans, it is hypothesized that youth who are subject to unpredictable interpersonal conflict at home may grow hypervigilant to changes in parental mood or behavior and perceived threat, leading to a bias toward negative emotional content, and increasing risk of internalizing problems (Schleider & Weisz, 2016). The operationalization of psychosocial adversity and abuse varies in terms of chronicity and predictability, making it difficult to compare observed effects of these factors across studies.

To date, previous studies have not investigated separate adversity factors (psychosocial adversity, sexual, physical, and psychological abuse), internalizing problems, and sex within the same individuals to determine their associations with peripheral DNA methylation over and above each other, and thus relative importance. Clarity is needed regarding sex differences in SLC6A4 methylation, as studies have reported both higher (Booij, Szyf, et al., 2015) and lower methylation (Philibert et al., 2008) in males. Additionally, internalizing problems are more often than not measured at one single point in time, rather than longitudinally to establish longstanding patterns (or trajectories) of such problems.

The current project aims to investigate the effects of different types of adversity that take into account both abuse and psychosocial adversity, as well as longitudinal patterns (trajectories) of internalizing symptoms in a longitudinal sample of men and women followed for more than 20 years since childhood. We hypothesize that psychosocial adversity and abuse, as well as internalizing trajectories will be positively associated with peripheral sites-specific peripheral SLC6A4 DNA methylation. We also examine sex differences. We focus on DNA methylation in

a region of the SLC6A4 promoter and associated a-priori chosen specific CpG sites that previously were shown to be most strongly associated with measures of 5-HT synthesis as assessed in vivo (Wang et al., 2012) and with transcriptional activity as assessed in vitro (Wang et al., 2012).

Method

Participants

Three hundred participants were recruited from the Quebec Longitudinal Study of Kindergarten Children (QLSKC), which has followed females and males regularly since their kindergarten year in French-speaking schools in the 1980s. The cohort was followed yearly from age 6-12, at mid-adolescence (mean age = 15), in young adulthood (mean age = 21) and in adulthood (mean age = 27). At the age 27 follow-up assessment, 1255 participants provided a blood or saliva sample for DNA sampling. DNA from a subset of these participants ($N = 300$) was selected for DNA methylation analyses as part of a larger research project on early stress, behavior, DNA methylation and brain processes as part of a team grant led by Dr. Booij. The selection of the 300 samples for SLC6A4 methylation analyses was based on the availability of (a) sufficient DNA concentration, (b) whole blood DNA (c) adversity data as collected at various time points in childhood and adolescence (d) French-Canadian descent. Table 3.1 outlines participant levels of childhood psychosocial adversity, abuse, internalizing behaviours and DNA SLC6A4 methylation.

Measures

Adversity. Adversity was measured using two factors: Childhood psychosocial adversity, and abuse.

Childhood psychosocial adversity. We used the psychosocial adversity index, a measure previously created, validated and used in other studies conducted in this cohort. Seven socioeconomic indices, prospectively collected in childhood at age 6 and again at age 10-16, were included in this index: maternal and paternal years of schooling, mean maternal and paternal occupational prestige, age at birth of their first child, and familial composition. These indices are strongly associated with social economic status, which has previously been associated with DNA methylation in adulthood (Borghol et al., 2012) as well as 5-HT functioning (Manuck et al., 2005; Roy, 2002). A score of 1 was given for familial composition if the participant was not living with his two biological parents in childhood before age 6. All other indices were given a score of 1 if they were below or at the 30th percentile in the present sample and a score of 0 if they were above the 30th percentile, as in our previous studies (Arseneault, Tremblay, & Boulerice, 2002; Booij et al., 2012). Predictive validity of the index in relation to behavioural and cognitive outcome and test-retest reliability throughout childhood ($r = 0.85$; Haapasalo & Tremblay, 1994) has been demonstrated in 3 different large cohort samples, (Arseneault et al., 2002; Séguin, Haapasalo & Tremblay, 1994; Pihl, Harden, Tremblay, & Boulerice, 1995; Tremblay et al., 1991). The finding that this index predicts similar behaviours over time in different cohorts supports the external validity. Only the age 6 measure was analyzed in the present study given our specific interest in adversity in early development and it thus represented the earliest index of the socioeconomic conditions in which the cohort grew up. This childhood psychosocial adversity index in families

at age 6 has found to be highly correlated with age 12 family adversity scores, indicating its static nature (Arseneault et al., 2002; Haapasalo & Tremblay, 1994).

Abuse. Measures of physical, sexual, and psychological abuse were assessed using the Parent-Child Conflict tactics Scale and the Adverse Childhood Experiences Study Questionnaire (ACEQ) measured in early adulthood (Brezo et al., 2010; Felitti et al., 1998; Ouellet-Morin et al., 2016). The ACEQ items assessed the frequent or very frequent physical abuse (push, grab, hit, shove or slap, hit so hard marks were left/injuries), sexual abuse (sexual touch/fondle, have you touch their body in a sexual way, attempt/have oral/anal/vaginal intercourse with you), and psychological abuse (swear at, insult or put you down, act in a way that made you afraid that you would be physically hurt) experienced by participants during their first 18 years of life. Score on the Physical abuse scale was associated with specific monoaminergic genotypic variation (Brezo et al, 2010) and future aggressive and antisocial behaviors in this cohort. (Isabelle Ouellet-Morin et al., 2016; Pagani et al., 2004). The sexual abuse scale has been shown to predict sexual problems in women in this cohort (Lacelle, Hébert, Lavoie, Vitaro, & Tremblay, 2012). Test-retest k coefficient of the original physical abuse scale was $k = .55$ (95% CI, .47-.63); for sexual abuse, $k = .69$ (95% CI, .61-.77) and for psychological abuse, $k = .66$ (95% CI, .55-.76; Dube, Williamson, Thompson, Felitti, & Anda, 2004).

Internalizing problems. A measure of childhood trajectories of internalizing problems was created using data from the Social Behaviour Questionnaire from 7 time points over the course of 7 years (Cronbach α : 0.73, range: 0.71-0.74). Mothers rated their children at home on the following six items: fearful or afraid of things or new situations; is worried, worries about

many things; cries easily; has a tendency to work alone; looks sad, unhappy, tearful; easily distracted. Prior to the present analyses, trajectories were created in the entire initial cohort using semi-parametric group based modeling (SGM; type of growth mixture modeling) that assumes the population is composed of groups following distinct developmental trajectories (Nagin & Tremblay, 1999). The score of internalizing problems is created from the above-mentioned 6 items, where, within each participant, the missing items are imputed by the mean of the non-missing items. The score was defined only if there are at least 3 non-missing items.

The Bayesian Information Criterion (BIC) was used as a basis for selecting the optimal model. For any given model BIC is calculated as: $-2 \log(L) + \log(n) * (k)$, where L is the model's maximized likelihood, n is the sample size, and k is the number of parameters in the model (Nagin & Tremblay, 1999). The BIC indicated that the models with best fit were a 3-group and a 4-group model (i.e. had the highest BIC). Bayesian Information Criterion (BIC) was -46878.67 for the 3-group model and BIC was -46650.28 for the 4-group model. Although the BIC was slightly higher (less negative) for the 4-group model than for the 3-group model (indicating better fit), the 3-group model was chosen for the present study in order to have sufficient sample size in each group. Three groups that were formed from data obtained in the entire cohort had low (35.5%), medium (51.5%), and high (13%) rates of internalizing problems that were highly stable over time throughout childhood and adolescence. The posterior probabilities of group membership for the trajectories also suggested that the fit was good. These probabilities provide a quantitative basis for assigning individuals to the trajectory group that best describes their behaviour (Mazza et al., 2016; Nagin & Tremblay, 1999). Mean assignment probabilities (\pm SD) were 0.91(.15) for the low anxiety group, 0.88 (.15) for the medium anxiety group, and 0.91 (.13) for the high anxiety group. These assignment probabilities also indicated good fit for each group

in each trajectory measure (Mazza et al., 2016; Nagin & Tremblay, 1999). Trajectories were used in order to model the pattern of behaviour, which, though stable, provides more information about the nature and longitudinal course of the behaviours and/or symptoms than a single time point.

DNA methylation protocol. Peripheral blood samples were collected from participants and stored in EDTA coated tubes at 4°C before extraction. Extracted DNA was stored in a -80°C freezer until pyrosequencing. DNA methylation was assessed following our previously-validated pyrosequencing assay procedure (Dongsha Wang et al., 2012) and targeted the same 10 CpG sites as in our previous studies in our laboratory (Booij, Szyf, et al., 2015; Frodl et al., 2015). Greater DNA methylation in these 10 CpG sites has been associated with lower in vivo Positron Emission Tomography measures of brain 5-HT synthesis, reduced hippocampal volume and greater frontal- limbic responses to negative stimuli (Booij, Szyf, et al., 2015; Frodl et al., 2015; Schumann et al., 2015), and reduced in vitro transcriptional activity (Dongsha Wang et al., 2012). Site-specific methylation analyses were performed by pyrosequencing using PyroMark Q24 (Qiagen, Venlo, Limburg, Netherlands) at CFI Imaging and Molecular Biology Platform at McGill University in the Department of Pharmacology and Therapeutics in the lab of Dr. Moshe Szyf. Each CpG site was analyzed in triplicates. For each CpG site, a mean score was computed across the 3 CpG sites. If a sample did not pass quality control, the mean for a CpG site was calculated from the 2 replicates. Overall mean from the 10 CpG sites was calculated from the averages of the 10 CpG sites (CpG 5-15), and used as outcome measure for the present study.

Data Analysis

Data were analyzed using univariate General Linear Models (GLM) and linear regression in Statistical Package for the Social Sciences (SPSS) version 22. Linear regression analyses were used to test our primary hypotheses. Three primary analyses were conducted to study (1) the association between peripheral SLC6A4 methylation and childhood psychosocial adversity; (2) the association between peripheral SLC6A4 methylation and abuse. We included sex as an independent variable in both sets of analyses based on our previous study showing sex differences in SLC6A4 methylation (Booij et al., 2015). In both analyses, mean percentage in peripheral SLC6A4 methylation across all 10 investigated CpG sites was the dependent variable. Childhood psychosocial adversity as assessed at age 6 and sex were the predictors in the first set of analyses. In the second set of linear regression analyses, abuse and sex were the predictors. For each of the two sets of analyses, a second block of variables represented the interactions between the primary independent variables (childhood psychosocial adversity and sex in the first regression and abuse and sex in the second analyses). The childhood psychosocial adversity and abuse indices were centered and the sex variable was dummy coded (male =1) prior to the regression analyses.

The third set of regression analyses was to examine the association between peripheral SLC6A4 methylation and internalizing problems. A two-way Factorial ANOVA for General Linear Models was conducted, in which peripheral SLC6A4 methylation was the independent variable and sex and internalizing trajectory (low, medium, and high, as obtained in the trajectory analyses above) were the predictor variables.

Lastly, we also investigated the SLC6A4 methylation's association with early adversity and internalizing trajectories in one model. Regression analyses were conducted, in which the internalizing trajectory sex, childhood psychosocial adversity, and their interaction were the predictors. The internalizing group trajectories categories were dummy coded, in which the

moderate and high anxiety was included in the analyses, and low anxiety was used as a reference category, and then collapsed into two categories (low vs. medium/high), since the ANOVA analyses demonstrated that this comparison between low and medium/high was the meaningful contrast. A similar model was run for the abuse variables except that the predictors were now sex, abuse type, and internalizing behaviors.

Statistical Assumptions. Univariate normality and homoscedacity were both determined to be acceptable via visual inspection and plotted residuals, respectively. Levene's test indicated acceptable equality of variance. Outliers were examined using Cook's D, standardized residuals and Mahalanobis distances. In case of outliers, the specific analysis was run with and without the outlier and results were compared. Multicollinearity was examined using Tolerance Statistics.

Results

Data screening

Eighteen participants were missing the psychosocial adversity index measure at age 6 that was specifically used in this study. Abuse data were available for 194 participants. For 4 participants, DNA was analyzed for DNA methylation but the obtained methylation data did not pass quality control for most of the first 5 CpG sites (CpG 5-10), in spite of multiple attempts. DNA of these participants was therefore omitted from analyses.

The association between SLC6A4 methylation and social adversity

In our first regression model, sex and social adversity was included in the first block and the sex*social adversity interaction in the second block. The model was only significant when the interaction between early psychosocial adversity and sex was entered into the second block of variables ($F(3, 274) = 3.88, p = .01$), with an R of .202 and R^2 of .041 (overall model; Table 3.2). The standardized beta of the interaction term was .208 ($p = .004$). Adversity was significantly positively associated with DNA methylation in males only (Figure 3.1). Exclusion of the 4 outliers who had a z-score larger than 3 gave similar results.

The association between SLC6A4 methylation and abuse

Regression analyses were run with sex and each type of abuse separately (physical, sexual, and psychological abuse) in order to avoid problems of multicollinearity that occurred when all abuse types were entered into one model (reflected in low Tolerance values). Each model also had the interaction between abuse and sex entered in a second block of variables. While the initial model with psychological abuse and sex and their interaction as predictors was not significant, a rerun of the analyses with the removal of 2 outliers ($z > 3$), the model with psychological abuse and sex was significant ($F(2, 189) = 3.22, p = .042$), with R of .207 and an R^2 of .043 (Table 3.3). In block one (no interaction term), psychological abuse ($\beta = .176, p = .015$) was significantly associated with peripheral SLC6A4 DNA methylation levels. Higher psychological abuse score was associated with greater level of peripheral SLC6A4 methylation. Once the interaction term was added to the model in the second block, psychological abuse was no longer a significant predictor, although the overall model was significant ($F(3, 188) = 2.80, p$

= .041). Sex was not a significant predictor in either block. Physical and sexual abuse models were not significant.

The association between SLC6A4 methylation and internalizing trajectories

GLM ANOVA analyses showed that there was a significant association between internalizing trajectory and peripheral SLC6A4 DNA methylation ($F(2, 296) = 5.09, p = .007, \eta^2 = 0.03$). A Tukey post-hoc test revealed that SLC6A4 DNA methylation was statistically lower in the high internalizing group ($3.58 \pm 1.10, p = .01$) and medium internalizing group ($3.73 \pm 1.37, p = .02$) compared to the low internalizing group (4.23 ± 1.39). There were no statistically significant differences between the medium and high internalizing group ($p = .75$). Similar results were obtained after removal of 3 outliers ($z > 3$).

SLC6A4 methylation and early psychosocial adversity and internalizing problems

The regression model was significant ($F(4, 273) = 6.05, p < .001$), with R of .285 and an R^2 of .08 (Table 3.4). Sex ($\beta = .140, p = .018$), the adversity X sex interaction ($\beta = .192, p = .007$), and internalizing trajectory (low vs. medium/high; $\beta = -.210, p = .001$) were all significantly associated with peripheral SLC6A4 DNA methylation levels. Male sex, male sex with higher early psychosocial adversity, and low internalizing trajectory were associated with greater level of peripheral SLC6A4 methylation. Further analyses including higher order interaction terms into the model yielded no other significant results.

SLC6A4 methylation and psychological abuse and internalizing problems

The final regression model was significant ($F(3, 189) = 5.50, p = .001$), with R of .283 and an R^2 of .08 (Table 3.5). Psychological abuse ($\beta = .153, p = .031$), and internalizing trajectory (low vs. medium/high; $\beta = -.231, p = .001$) were both significantly associated with peripheral SLC6A4 DNA methylation levels. Sex was not a significant predictor in the model. Psychological abuse and low internalizing trajectory were associated with greater level of peripheral SLC6A4 methylation. Interaction terms did not yield significant results.

Discussion

The present study's longitudinal design allowed for a unique investigation into specific differences among types of abuse, internalizing trajectories, and their association with peripheral SLC6A4 methylation. Three relevant findings emerged.

First, childhood psychosocial adversity was associated with peripheral SLC6A4 methylation in males but not in females. Our results provides evidence to support Shonkoff et al.'s (2012) theory of biological embedding of psychosocial (socio-economic) adversities during sensitive developmental periods. The results are also line with Manuck's (2005) and Borghol's (2012) findings that link socioeconomic status with 5-HT functioning and adult DNA methylation patterns, respectively. Interestingly, a number of studies have linked socioeconomic adversity to mental health problems in which serotonin plays a major role, including persisting depression (Lorant et al., 2003; Mazza et al., 2016). The present study is in line with a very recent study showing a link between greater SLC6A4 methylation and early socio-economic adversity (Swartz, Hariri, & Williamson, 2016).

Yet a significant interaction with sex indicated that adversity's association with the proposed SLC6A4 methylation mechanism may operate differently in males and females. Although this result is somewhat in line with the literature finding sex differences in methylation, it differs in that Booij et al. (2015) found that males had higher levels of peripheral SLC6A4 methylation independent of childhood abuse in a sample of depressed adults.

Second, we observed psychological abuse was associated with peripheral methylation in both males and females. Notably, we did not find associations between methylation and other abuse types (physical and sexual). It is possible that the unique nature of psychological abuse makes it a more influential factor in how environment affects further development. Although far less studied than physical or sexual abuse, Yates' (2008) review of the socioemotional consequences of psychological abuse asserts that it disrupts early neurodevelopment of stress response systems and that the resulting alterations are significant and long-lasting. Indeed, researchers have found that psychological abuse predicted depression, anxiety, post-traumatic stress (Spertus, Yehuda, Wong, Halligan, & Seremetis, 2003), chronic emotional inhibition in adulthood (Krause, Mendelson, & Lynch, 2003), and other psychopathology (Kent, Waller, & Dagnan, 1999) even when controlling for other forms of abuse and lifetime abuse exposure.

Some evidence now suggests that psychological/emotional abuse is one of the most damaging forms of abuse and is the central defining feature to any abuse history (Kent et al., 1999; Yates, 2008). Specifically, psychological abuse is the least prevalent type of abuse (Dube et al., 2004; Felitti et al., 1998; U.S. Department of Health and Human Services, 2013), but has been shown to disrupt development across social, emotional, cognitive and biological domains, thereby altering the neurophysiological stress response system and heightening the vulnerability for internalizing disorders (Yates, 2008). Indeed, the overlap and influence of different types of abuse are difficult

to disentangle in human participants. Although a trend, our results suggest that psychological abuse may have particular relevance for SLC6A4 epigenetic processes, thereby helping to explain the literature on this specific form of abuse and its importance.

Nevertheless, these first two findings may help elucidate why the link between peripheral SLC6A4 methylation and adversity is inconsistent in the literature. It is possible that operationalization of adversity, abuse or SLC6A4 methylation is crucial to discovering different associations. Our findings are in line with studies that used interview measures that included more in depth assessment of psychological distress associated with the adversity/abuse (I. Ouellet-Morin et al., 2013; Van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach, & Philibert, 2010). Previous research has failed to find this link when employing short form self-report measures of abuse (Okada et al., 2014). Furthermore, it has been suggested that different genomic regions (CpG units) of the SLC6A4 may have opposite associations with early adversity (Okada et al., 2014). We used specific CpG sites of SLC6A4 methylation and found a positive association with psychosocial adversity (in males) and abuse, whereas previous research failed to find a link between overall SLC6A4 methylation and abuse (Okada et al., 2014).

Third, our finding that long-term trajectories of internalizing problems were negatively associated with peripheral SLC6A4 methylation, or in other words, that trajectories of high levels of internalizing problems were linked to lower levels of SLC6A4 methylation is somewhat unexpected. Some of our own and others' previous research suggests that higher levels of SLC6A4 methylation at these key sites are linked to reduced 5-HT synthesis, smaller hippocampal volume, and increased threat-related amygdala activity; alterations traditionally associated with an increased risk of internalizing problems (Booij, Tremblay, et al., 2015; Booij et al., 2013; Nikolova et al., 2014). However, the strength and directionality of peripheral

SLC6A4 DNA methylation's link with internalizing disorders in the literature are not clear (Januar et al., 2015; van der Knaap, Van Oort, et al., 2015). A number of factors could account for these differences, including sample tissue heterogeneity, definition of cases, low statistical power, age of participants, and degree of early adversity included in the sample (Frodl et al., 2015; Januar et al., 2015). While our study involved a community sample of approximately equal groups of males and females, other studies with dissimilar findings used samples of clinically depressed populations and/or assessments at a single time point (Frodl et al., 2015; Kang et al., 2013; van der Knaap, Van Oort, et al., 2015). The direction of the effect appears to rely on a complex interplay of factors and may differ across region of the SLC6A4 gene (Booij, Szyf, et al., 2015). Nevertheless, our methodology, specifically using a large longitudinal sample with community males and females who experienced various forms of adversity and with assessments of the longitudinal course of internalizing symptoms provides evidence for a meaningful link between peripheral SLC6A4 methylation and risk for longstanding internalizing problems in a gene-environment context.

Our study has several strengths. First and foremost, the study benefited from the genetic homogeneity of our predominantly French Canadian Caucasian sample. Second, participants were all recruited and tested at the same age, thereby minimizing possible age effects on SLC6A4 methylation (Booij, Tremblay, et al., 2015). Third, many of the measures were assessed prospectively rather than retrospectively than in most studies. Fourth, internalizing symptoms were assessed by multiple ratings taking place over many years, rather than at a single point in time as is the case in most other studies. Finally, the investigated SLC6A4 region and CpG sites were chosen a-priori, based on previous work and validation in vivo and in vitro in an independent sample.

Our research has some limitations. The first is that our DNA methylation was analyzed from peripheral tissues, and there is some evidence that development of 5-HT receptors and transporters is brain-region-specific (Booij, Tremblay, et al., 2015). Despite this, Wang et al. (2012) found high correlation between peripheral methylation of the SLC6A4 gene and in vivo measures of 5-HT synthesis in the brain, providing indications for a functional role for methylation in brain serotonergic regulation (discussed in more detail in Booij, Tremblay, et al., 2015). Secondly, our study did not investigate the possible role of the DNA sequence (genotype) in SLC6A4 methylation levels, which may or may not be a relevant factor. Although the DNA sequence may have a bearing on methylation levels, the research is not consistent (Booij, Szyf, et al., 2015; Philibert et al., 2007; Wang et al., 2012; Wankerl et al., 2014). Third, our study did not include information about current anxiety and depression. However, the long-term trajectories of internalizing behaviors were very stable over time throughout the period in which the behaviors were assessed. In fact, long-term trajectories of internalizing symptoms have been shown to be predictive of future depressive or anxiety disorders (Rueter, Scaramella, Wallace, & Conger, 1999). Fourth, as psychosocial adversity correlated highly with abuse, we were not able to reliably investigate the additional contribution. Similarly, our regression results on different types of abuse suffered from an issue of multicollinearity and possible restricted range of variance. We present these findings as an interesting trend for these reasons, and attempted to reconcile this by three separate models for each abuse variable. An ideal design to further test the impact of specific types of adversity and abuse on the phenotype would be to recruit individuals with one type of abuse (e.g., only psychosocial adversity vs. specific physical abuse vs. specific psychological abuse) and compare the impact on DNA methylation and the phenotype. However,

recruitment of such individuals would be extremely challenging at if feasible at all, and may not be generalizable to real world populations.

To conclude, the current research showed a relationship between peripheral SLC6A4 methylation and both childhood psychosocial adversity in males and psychological abuse in both sexes. We also found an association between peripheral SLC6A4 methylation and internalizing trajectory. The longitudinal design including internalizing patterns over 21 years and inclusion of different types of adversity offer critically important and nuanced information about how these factors are associated with DNA methylation and associated biological systems. Our finding suggesting that psychosocial adversity and psychological abuse in childhood/adolescence are relevant factors in the prediction of peripheral SLC6A4 methylation is an important advancement in understanding adversity's role in epigenetic alterations in GXE models of risk for internalizing psychopathology.

Table 3.1: Descriptive statistics for $N = 300$

Demographic Characteristic	Mean (SD)/percentage	Count	Range
Female	182 / 60.7%		
Abuse			
Physical abuse	4.39 (3.86)		0-20
Sexual abuse	3.62 (2.98)		0-8
Psychological abuse	4.58 (4.04)		0-17
Psychosocial Adversity	.267 (.268)		0-1
Internalizing trajectory			
Low = 1		84 (28.0%)	
Medium = 2		158 (52.7%)	
High = 3		58 (19.3%)	
Peripheral SLC6A4 DNA methylation	3.84 (1.35)		0.15- 8.87

Table 3.2: Parameters and collinearity of the childhood psychosocial adversity model

Model	Standardized beta	<i>t</i>	<i>p</i> value	Tolerance
Childhood psychosocial adversity model				
Childhood psychosocial adversity	-.115	-1.61	.109	.688
Sex (male)	.110	1.86	.064	.996
Sex X childhood psychosocial adversity interaction	.208	2.92	.004	.688

Caption: Peripheral SLC6A4 DNA methylation is the outcome variable.

Table 3.3: Parameters and collinearity of the abuse models

Model	Standardized beta	<i>t</i>	<i>p</i> value	Tolerance
Psychological abuse model*				
Psychological abuse	.176	2.45	.015	.993
Sex (male)	.062	.868	.385	.993
Sexual abuse model				
Sexual abuse	.060	.830	.407	.987
Sex (male)	.060	.827	.409	.987
Physical abuse model				
Physical abuse	.067	.928	.354	.996
Sex (male)	.057	.791	.430	.996

Caption: Peripheral SLC6A4 DNA methylation is the outcome variable.

**When the interaction term, Psychological abuse X sex was entered into the psychological abuse model, psychological abuse was no longer significant, but the overall model was significant ($F(3, 188) = 2.80, p = .041$).*

Table 3.4: Parameters and collinearity of the internalizing trajectory and childhood psychosocial adversity model

Model	Standardized beta	<i>t</i>	<i>p</i> value	Tolerance
Internalizing trajectory and childhood psychosocial adversity model				
Childhood psychosocial adversity	-.054	-.749	.454	.648
Sex (male)	.140	2.38	.018	.976
adversity X sex	.192	2.74	.007	.685
Internalizing trajectory (low vs. medium/high)	-.210	-3.48	.001	.919

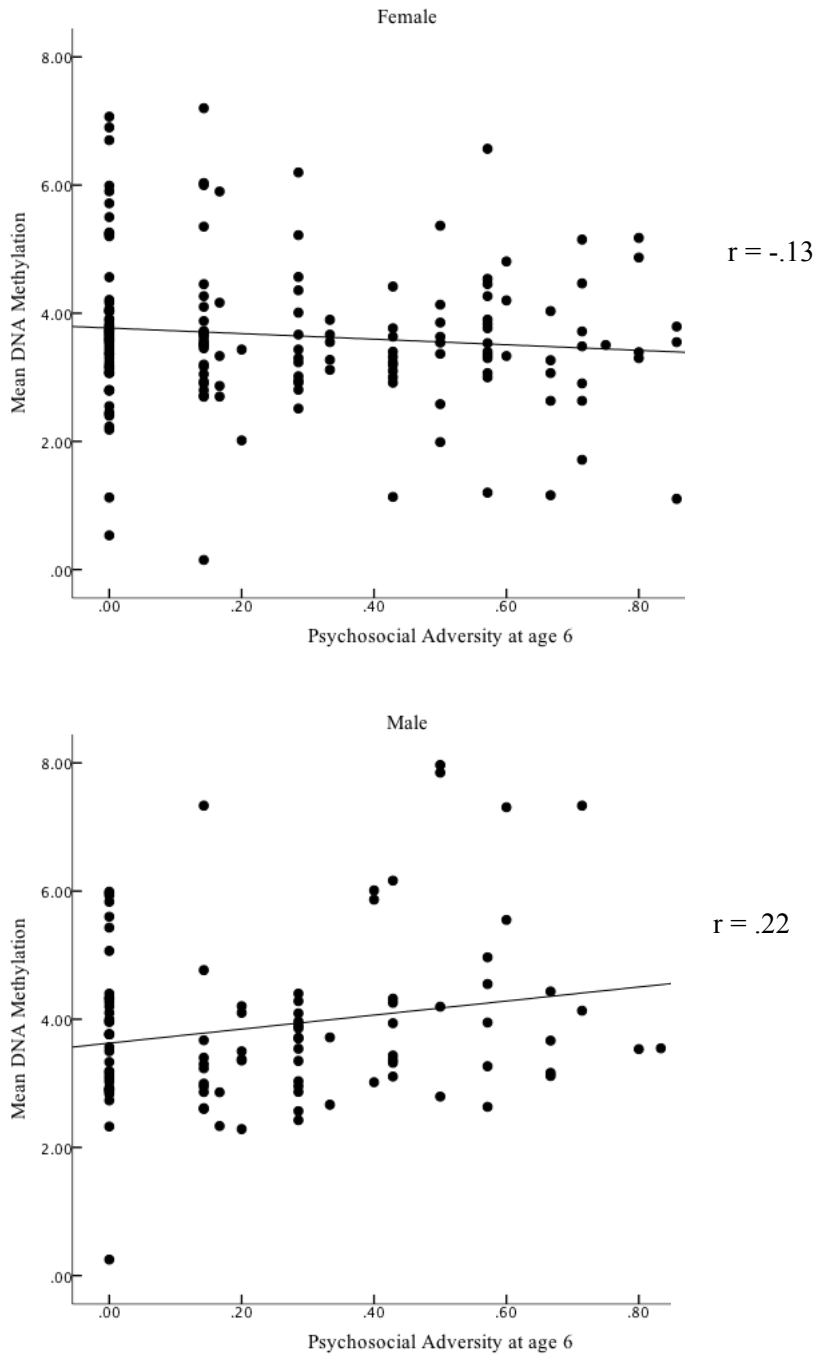
Caption: Peripheral SLC6A4 DNA methylation is the outcome variable.

Table 3.5: Parameters and collinearity of the psychological abuse and internalizing trajectory model

Model	Standardized beta	<i>t</i>	<i>p</i> value	Tolerance
Psychological abuse and internalizing trajectory model				
Psychological abuse	.153	2.18	.031	.992
Sex (male)	.111	1.57	.119	.978
Internalizing trajectory (low vs. medium/high)	-.231	-3.28	.001	.986

Caption: Peripheral SLC6A4 DNA methylation is the outcome variable.

Figure 3.1: Peripheral SLC6A4 methylation associated with childhood psychosocial adversity in males only



Chapter 4

Enhanced control and efficiency in emotional processing in depressed adolescents after mindfulness treatment and associations with SLC6A4 methylation: A pilot study

Abstract

We applied a cognitive-emotional fMRI task in depressed adolescents to investigate the effect of Mindfulness-Based Cognitive Therapy, including task related modulations of brain connectivity during an emotional attention set-shifting task and SLC6A4 DNA methylation changes. Depressed adolescents (N = 13; mean age 16 years, 3 months; 10 females) underwent a functional Magnetic Resonance Imaging scan while completing an emotional attention shifting task before and after an outpatient MBCT group treatment. Average percentage of methylation in specific CpG sites of the SLC6A4 gene was also determined from participant saliva pre- and post- treatment. Seed-to-voxel, ROI-to-ROI and graph theoretical analyses suggested that participant's control for cognitive and emotional tasks, reflected in increased activity in the frontal operculum anterior cingulate (BA32) dorsolateral-prefrontal cortex (DLPFC) network after 6 weeks of MBCT treatment, while activity in brain regions involved in self-reference and focus on emotional state (paracingulate and insula) decreased for the cognitive condition. Increased communication between anterior cingulate cortex (ACC) and frontal cortex post-treatment was reflected by increased functional connectivity within the ACC BA 32 and BA 24, DLPFC/frontal operculum, and precuneus (PC) network. The enhanced cognitive control post treatment was also reflected in global efficiency analyses within these networks. Peripheral DNA methylation and depression symptoms decreased over treatment and were associated with fronto- limbic activation changes. Though preliminary, the results are consistent with cognitive theory and indicate that MBCT treatment may work by suppressing activity in certain brain regions

involved in self-referential thought, while enhancing functional connectivity between brain regions implicated in controlling emotion (i.e., fronto-limbic/temporal structures). In essence, improvement after MBCT in depressed adolescents was associated with enhanced functional connectivity and coupling in key brain regions implicated in emotional-cognitive regulation and contributing to variation in depression symptoms.

Introduction

Mindfulness-Based Cognitive Therapy (MBCT) is a treatment derived from a model of cognitive vulnerability to depressive relapse. The therapy works to retrain awareness and cognitive reactivity to increase conscious awareness of thoughts and emotions and cognitive control of attention (Ma & Teasdale, 2004; Teasdale, Segal, & Williams, 1995). Documented improvements in emotional reactivity, attentional control, anxiety, and distress tolerance (Britton, Shahar, Szepsenwol, & Jacobs, 2012; Farb, Anderson, & Segal, 2012; Ives-Deliperi, Howells, Stein, Meintjes, & Horn, 2013; Strawn et al., 2016; Tang, Hölzel, & Posner, 2015), suggest that MBCT can be used successfully to target the cognitive-emotional vulnerabilities underlying depression and other disorders characterized by emotion processing disturbances (Marchand, 2012; Strawn et al., 2016). Although the efficacy of MBCT has been quite well demonstrated, our understanding of how MBCT may affect brain processes, including the biological mechanisms of depression in the context of psychotherapy, is limited.

Recent investigations of functional connectivity (a measure of how brain regions communicate) of the amygdala, anterior cingulate cortex (ACC), and prefrontal cortex (PFC) in individuals with MDD (Carballedo et al., 2011; Ho et al., 2014; Klumpp, Keutmann, Fitzgerald, Shankman, & Phan, 2014) supports the theory that decreased prefrontal control of subcortical regions results in the prolonged activation of limbic structures and is associated with increased negative cognitive processes such as rumination and over-identification (De Raedt & Koster, 2010). FMRI studies assessing neural activity while practicing mindfulness in the scanner suggest that MBCT enhances deidentification /disidentification from emotional experience by enhancing regulation and connectivity of the brain networks involved in cognitive control of emotion, namely among the ACC, prefrontal cortex (PFC), precuneus (PC), and insula (Farb et al., 2012;

Ives-Deliperi, Solms, & Meintjes, 2011). A number of studies have investigated the effects of MBCT treatment on neural processing. Although the majority of the studies were done in healthy samples, these studies provided evidence that MBCT treatment can lead to increased engagement of regions involved in emotion control and regulation after MBCT intervention (Gotink et al., 2016). However, very few imaging studies have studied the impact of MBCT treatment on neural processing of emotions in clinical populations, and even less so in youth populations with mental health problems. Recently a pilot study in 9 youth with anxiety disorder who were at risk for Bipolar disorder showed an increased neural response to negative emotional stimuli in the ACC, insula and DLPFC, after MBCT relative to pre-treatment (Strawn et al., 2016). While these studies identified key regions that exhibit altered functioning during emotional processing after MBCT treatment, none of these studies included investigations of functional connectivity within the frontal-limbic network. In other words, whether MBCT treatment alters the functional relationship between limbic activity elicited by emotional stimuli and brain regions during cognitive regulation of emotional information is unknown.

In the present study, we use a well-established visual emotional attention task (Frodl et al., 2015; Lisiecka et al., 2013) to compare functional connectivity within the frontal-limbic network during cognitive regulation of an emotional response, before and after 6 weeks of MBCT treatment, in a sample of depressed adolescents. We specifically focus on the ACC, insula and PFC given their role in processing of emotional information, attention, introspection and visual awareness, and previous reports on greater activation in these regions following mindfulness meditation practices (Chiesa and Serretti 2010, review). We hypothesized that during emotional regulation, we would observe increased functional connectivity in the frontal limbic

circuitry (in particular ACC, insula and the PFC) in the post-MBCT treatment condition relative to the pre-MBCT treatment condition.

Our secondary aim to explore the link between functional connectivity in emotion processing and epigenetic changes is based on recent observations in depressed adults showing that frontal limbic brain activation in response to emotional stimuli correlated with DNA methylation in the serotonin transporter (SLC6A4) gene. This gene codes for the functioning of the serotonin transporter and is highly involved in brain development and risk for depression (Booij, Tremblay, et al., 2015; Booij & Van der Does, 2007). We previously investigated the DNA methylation of SLC6A4 and functional activation in key brain areas in adult with major depressive disorder (MDD) patients and healthy controls. Peripheral (of the body, excluding brain) SLC6A4 DNA methylation state was significantly associated with fMRI responses in various frontal-limbic brain regions responsible for emotional processing and cognitive control (Frodl et al., 2015). The present study extends this line of research to depressed adolescents and MBCT treatment.

We hypothesized that during emotional processing, we would observe increased functional connectivity in the frontal limbic circuitry (in particular ACC, insula and the PFC) in the post-MBCT treatment condition relative to the pre-MBCT treatment condition. We also expected that the peripheral SLC6A4 DNA methylation state was significantly associated with fMRI responses in various frontal-limbic brain regions responsible for emotional processing and cognitive control.

Methods

Participants

Fifteen adolescents were recruited from 5 MBCT treatment groups from 2013 to 2014 as part of outpatient child and adolescent psychiatry services (as referred from their psychiatrist) and participated in the post-treatment measures. Each participant and a parent gave informed assent and consent, respectively, to participate. Exclusion factors were active suicidality, active psychosis, current alcohol or drug abuse, and meeting criteria for mental retardation. Presence or absence of current and/or past Diagnostic and Statistical Manual-IV (DSM-IV) Axis I diagnoses was confirmed using the Schedule of Affective Disorders and Schizophrenia (Kaufman et al., 1997). Those participating in the group intervention were stabilized on medication by the time the MBCT started, having been on medication for 4-6 weeks at minimum (4 weeks for selective serotonin reuptake inhibitors, or SSRIs, 6 weeks for other medication¹). Thirteen (3 males and 10 females) out of 15 participants' pre and post scans (6 weeks apart) passed quality control parameters and were included in the final sample. The average age was 16.25 years ($SD = 9$ months). All procedures were performed in accordance with the Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans and approved by the ethics committee of Queen's University, Kingston Ontario.

Measures

MBCT Protocol. The protocol was based on the cognitive behavioural model and combined elements of distress tolerance and mindfulness (Segal et al., 2012), and was tailored to

¹ Two were medication free; two were taking antidepressant medication only; two were taking a different class of medication (stimulant) only; and seven were taking more than one psychoactive medication (in all but one case, this included an antidepressant).

a depressed adolescent population. Participants completed the depression scale of the Beck Youth Inventory (Dolle et al., 2012) on both days of the fMRI session.

Neuroimaging acquisition. All participants were scanned twice (pre and post MBCT; before and after 6 weeks of MBCT treatment) using a 3T whole-body MRI system (Siemens Magnetom Trio; Siemens, Erlangen, Germany), with a 12-channel head coil, at the Centre for Neuroscience Studies at Queen's University in Kingston, Canada. The scan consisted of a high-resolution structural scan (TR = 1760ms, TE = 2.2 ms, FOV = 256 X 256 mm, voxel size = 1 mm³), followed by a functional scan (TR/TE = 2000ms/30 ms, voxel size = 3.3mm³, 550 dynamic scans). During the functional scan, participants were asked to complete a well-established emotional attention-shifting task, as previously used in adult patients with Major Depressive Disorder (Frodl et al., 2015; Lisiecka et al., 2013). Briefly, participants were presented a picture from the International Affective Pictures System database for two seconds, and were asked about its emotional content (emotional condition: positive, negative or neutral) or its orientation (cognitive condition: horizontal, vertical). Participants had thus either to focus on the emotion of the picture (emotional condition) or had to shift their attention away from the emotion and answer a question about the shape of the picture (cognitive condition). The conditions were pseudorandomly distributed in the experiment. Participants did not know before the start of each trial and during the picture viewing which of the two types of questions would be asked.

Participants had two seconds to give their answer using a response box. There were 180 trials; 90 belonging to the emotional condition and 90 to the cognitive condition. The trials were alternated with a picture of a fixation cross (2 seconds). Participants were trained in the task outside the scanner prior to the scanning.

DNA methylation

Saliva samples (3-6 ml) were collected to determine DNA methylation, using Oragene genotek kits (Lévesque et al., 2014). DNA methylation in the SLC6A4 gene was assessed using pyrosequencing methods according to previously used procedures, in the same 10 CpG sites as in our studies in adult depressed patients (Booij, Szyf, et al., 2015; Frodl et al., 2015). Means were calculated across the 10 CpG sites (Cpg 5-15), as in previous work (Booij, Szyf, et al., 2015; Frodl et al., 2015).

Functional connectivity neuroimaging analyses

Functional data were pre-processed using the Diffeomorphic Anatomical Registration Through Exponentiated Lie Algorithm (DARTEL) Statistical Parametric Mapping Program (SPM8) (The Wellcome Department of Cognitive Neurology, London, United Kingdom, <http://www.fil.ion.ucl.ac.uk/spm/software/spm8>). All functional images were realigned to the first volume using a six-parameter rigid body transformation. The mean images generated were spatially normalized into standard stereotactic space, using the Montreal Neurological Institute (MNI) echo planar imaging (EPI) template and then coregistered to the structural T1 images. Computed transformation parameters were applied to all functional images, interpolated to isotropic voxels of 2 mm³ and the resulting images were smoothed using an 8-mm full-width half-maximum (FWHM), isotropic Gaussian kernel. Using the SPM8 segmentation tool, structural images for each participant were segmented into grey matter (GM), white matter (WM), and cerebro-spinal fluid (CSF) masks. The five principal components of the blood-oxygen-level dependent (BOLD) signals from WM and CSF were regressed out (Whitfield-Gabrieli & Nieto-Castanon, 2012), and a temporal band-pass filter of 0.009–0.08 Hz was applied

to the time series (Chai, Ofen, Gabrieli, & Whitfield-Gabrieli, 2014) to remove possible confounding noise.

Functional connectivity analyses were performed using the CONN-fMRI Functional Connectivity toolbox v14 (<http://www.nitrc.org/projects/conn>), which uses the General Linear Model to estimate correlation connectivity. The five principal components of the BOLD signals from WM and CSF were regressed out (Whitfield-Gabrieli & Nieto-Castanon, 2012), and a temporal band-pass filter of 0.009–0.08 Hz was applied to the time series (Chai et al., 2014) to remove possible confounding noise.

The ACC was chosen as a seed region, given the extensive evidence of studies showing that the ACC (dorsal and ventral) is a key structure highly involved in the etiology of MDD (Pessoa, 2008; Wu et al., 2016). Moreover it is considered an important connectivity hub in the integration of cognitive and emotional information (Pessoa, 2008; Wu et al., 2016). Regions-of-interest (ROI) were based on neurocognitive theories of depression (see De Raedt & Koster, 2010): the ACC corresponding to the affective division: Brodmann area (BA) 25, 33 and rostral area 24, and the cognitive division: caudal areas 24 and 32, the insula, in addition to the executive region of the frontal pole, i.e., the dorsolateral prefrontal cortex/frontal operculum (DLPFC BA9 and BA10; see review in Pessoa 2010; Wu et al. 2016). We performed bivariate-regression analyses (first level) – estimating the correlation coefficient between the seed signal over time and all other brain voxels – for each participant. The emotional condition consisted of negative positive and negative stimuli. To limit the number of contrasts given sample size in order to keep Type I error under control, we collapsed the positive and negative stimuli in one analysis. Nevertheless, further analyses showed that results for the positive and negative stimuli were very similar (data available on request). Hence, the contrast presented here was emotional condition

vs. cognitive condition. In the second-level analysis, the whole-brain connectivity pattern of each condition was generated for the group. The magnitude and extent of temporal connectivity were thresholded using a false discovery rate (FDR) correction of $p < 0.01$ for the whole brain volume with a minimum cluster extent of five contiguous voxels.

We performed seed-to-voxel, ROI-to-ROI analysis and graph theoretical analysis. From the Conn ROI-to-ROI toolbox we computed the correlation coefficients between/from the six selected ROIs (BA 25, 33, 24, 32, insula and DLPFC/frontal operculum) for each condition. The 2nd level beta values ≥ 0.30 represented Fisher-transformed correlation coefficient values. The r -values were acquired by using inverse Fisher transformation. The threshold for significance was set to $p = 0.01$ whole brain cluster level FDR corrected $p = 0.001$, uncorrected at the voxel level. Subsequently, we generated a graph theory, which is a framework for the mathematical representation of complex networks, where brain regions are represented by nodes and the functional links between regions by edges (Dosenbach et al., 2007; He & Evans, 2010). For each node (ROI), we computed the global efficiency, to get a measure of the node's efficiency of information transfer within the network (Achard & Bullmore, 2007; Latora & Marchiori, 2001). We thresholded global efficiency based on correlation scores indices at p -FDR < 0.01 , corrected for multiple comparisons with a network edge of 0.15. CONN-fMRI computes the mean BOLD time series across all voxels within each ROI. For all connectivity analyses (i.e., seed-to-voxel analyses and ROI-to-ROI analyses) we set the bidirectional explorations of connectivity (i.e. positive and negative associations) at a peak voxel threshold of $p \leq 0.001$ and a cluster extent threshold of $p \leq 0.01$.

Regression analysis was conducted to investigate the association between depressive symptoms and functional connectivity. Symptoms were entered as a regressor to investigate links

to activation before and after MBCT. A similar approach was used for the association between DNA methylation and functional connectivity pre and post MBCT. T-values were calculated for these associations ($p \leq 0.01$ p -FDR corrected at cluster and $t \geq 4.0$).

Results

Depression

Average depression score (Beck Youth Inventory, or BYI-II, depression subscale) decreased from within the clinical range ($T = 70$) to the subclinical level ($T = 66$), suggesting our participants experienced meaningful symptom improvement. The within subjects t-test was not significant ($p = .091$) but yielded a moderate effect size of Cohen's $d = .51$.

ACC-related networks

Seed-to-voxel and ROI-to-ROI analyses showed in the post-treatment condition, relative to pre-MBCT treatment, functional connectivity changes between our brain seed region (ACC) and brain regions hypothesized to be involved in emotional and cognitive processing in relation to our task. Specifically, in the emotional condition, we observed significant increased connectivity between the cognitive and affective divisions of the ACC, BA32 and 24 (Post beta = 0.62, $F = 44.22$, p -FDR $\leq .01$). Additionally, while in the pre-treatment condition there was relatively strong connectivity between the ACC and the frontal operculum region (Pre beta = 0.24, $F = 39.61$, p -FDR $\leq .01$) and between the ACC and insula (Pre beta = 0.25, $F = 39.61$, p -FDR $\leq .01$), connectivity in these networks were normalized post-MBCT treatment. (Figure 4.1)

In the cognitive condition, we observed enhanced connectivity after 6 weeks of MBCT treatment in brain regions representing parts of the cognitive network, such as the cognitive division of the ACC BA 24 and 32 and the executive region of the frontal pole, i.e., the dorsolateral prefrontal cortex/frontal operculum (DLPFC BA9 and BA10) (post beta 0.64, $F = 52.72$, $p\text{-FDR} \leq 0.01$) (Figure 5). The DLPFC was not involved before MBCT. Notably, while before MBCT treatment, the insula was highly connected to the frontal operculum region during the cognitive condition, we noticed a decrease in connectivity within the ACC-frontal-insula network (Pre beta=0.24, $F = 60.05$, $p\text{-FDR} \leq .01$), with no insula involvement post MBCT (Figure 4.1).

Graph-Theory Global Efficiency Analysis

For the emotional condition, nodes corresponding to the DLPFC, ventral ACC (BA24 affective and dorsal ACC (BA32) cognitive parts) all exhibited increases in global efficiency index post MBCT indicating closer connections to other nodes of the network (post beta = 1.25, $F = 1836.63$, $df = 11$, $p\text{-FDR} \leq .001$). Interestingly, we observed a less connected emotional processing network pre MBCT as depicted by the low involvement of the frontal and the high involvement of the insula (pre beta = 0.44, $F = 324.25$, $df = 11$, $p\text{-FDR} \leq 0.001$) (Figure 4.2).

For the cognitive condition, we note that in the pre-treatment condition, graph theory depicted an enhanced connectivity between the self referential regions of the insula and the frontal, specifically between the insular cortices, the paracingulate, the affective region of the ACC and the frontal operculum bilaterally (pre beta = 0.43, $F = 175.92$, $df = 11$, $p\text{-FDR} \leq .001$).

In the post-treatment condition, there was an increased level of global connectivity of the network involved in cognitive control (post beta = 1.14, $F = 714.78$, $df = 11$, $p\text{-FDR} \leq .001$), specifically in the ACC, frontal operculum and the DLPFC as shown in Figure 4.2.

DNA Methylation

Average peripheral DNA methylation level in the SLC6A4 gene significantly decreased after MBCT (post – pre; $t(12) = -3.60$), $p = .004$). Cohen’s d was .92. Seed-to-Voxel functional connectivity in regions corresponding to the insula and ACC were associated with peripheral SLC6A4 methylation before (Emotional: ACC = 1598 voxels, insula = 143 voxels, $F = 21.86$; $p = .001$; Cognitive: ACC = 1293 voxels, insula = 120 voxels, $F = 22.47$; $p = .001$) and after MBCT treatment (Emotional: ACC = 1699 voxels, insula = 58 voxels, $F = 22.18$; $p = .001$; Cognitive: ACC = 1576 voxels, insula = 64 voxels, $F = 22.47$; $p = .001$). Hence, relative to before MBCT, the intensity of the association (as measured by the number of voxels) in both conditions diminished after MBCT treatment for the insula, but increased slightly for the ACC (Figure 4.3).

Discussion

Studying fMRI task modulations of brain connectivity is critical to understanding brain functional connections that support cognitive and affective processes. This study investigated the relationships among brain networks involved in emotional and cognitive processing and how they respond to MBCT in depressed adolescents. We also explored how changes in peripheral SLC6A4 DNA methylation were associated with brain activation in these networks before and after 6 weeks of MBCT treatment.

Firstly, activation patterns in the DLPFC/frontal operculum and ACC indicated improved cognitive processing after 6 weeks of MBCT relative to pre-MBCT, and decreased focus on emotional state and self-reference. The observed changes in patterns of regional activation and connectivity related to emotion processing post-treatment are in accordance with both the cognitive and fMRI literature on depression. According to Beck's theory of depression, improvement in cognitive bias to negative emotional information and decreased self-focus should be seen after successful cognitive treatment. The increased activations we saw in the DLPFC following MBCT appear to indicate enhanced prefrontal control of subcortical regions, and could underlie the improvements in deidentification and cognitive control documented after MBCT (De Raedt & Koster, 2010; Williams, Teasdale, Segal, & Soulsby, 2000). Furthermore, researchers have found activation changes similar to our results after cognitive therapy in adolescents in the left amygdala, left hippocampus and bilateral subgenual ACC (sgACC), and linked these changes to successful psychotherapy and pharmacological treatment for depression (Straub et al., 2015). Our study provides further detailed support for critical changes in brain activation that underlie observed improvements in cognitive and emotional processing, which helps integrate the neurobiological and cognitive theories of depression treatment.

Secondly, global efficiency within the ACC-frontal network improved after 6 weeks of MBCT treatment. Key areas involved in cognitive regulation (ACC, frontal pole, frontal operculum and paracingulate) became more highly integrated in the network, with the ACC serving as an information-integrating hub (Pessoa, 2008, 2010). The improvements in functional connectivity between the ACC and DLPFC/frontal operculum post MBCT treatment suggests that MBCT treatment in depressed adolescents may work to optimize the connectivity of brain networks involved in cognitive control of emotion (Farb et al., 2012; Ives-Deliperi et al., 2011;

Perlman et al., 2012; Xue, Tang, & Posner, 2011). We can conclude that the constant challenge of cognitive/emotional thoughts during MBCT sessions may have played an important role in brain plasticity expressed in enhanced connectivity strength of brain regions implicated in self-regulation and auto-control (i.e., frontal regions) due to co-activation.

Thirdly, and similarly to a previous study conducted in our laboratory (Frodl et al. 2015), we saw that degree of peripheral SLC6A4 methylation of depressed adolescents was positively associated with activation in response to emotional content, in particular the insula. Furthermore, DNA methylation decreased over 6 weeks of MBCT treatment and we observed a change in the association between insula activation in response to emotional stimuli and SLC6A4 methylation. As MBCT treatment alleviates depressive symptoms, it could be speculated that MBCT may also work to alter the relationship between SLC6A4 methylation and the limbic activation elicited by negative emotional stimuli in depressed participants observed by Frodl et al. (2015). Our preliminary results require further investigation to elucidate this relationship and the degree to which it reflects a mechanism for depression remission.

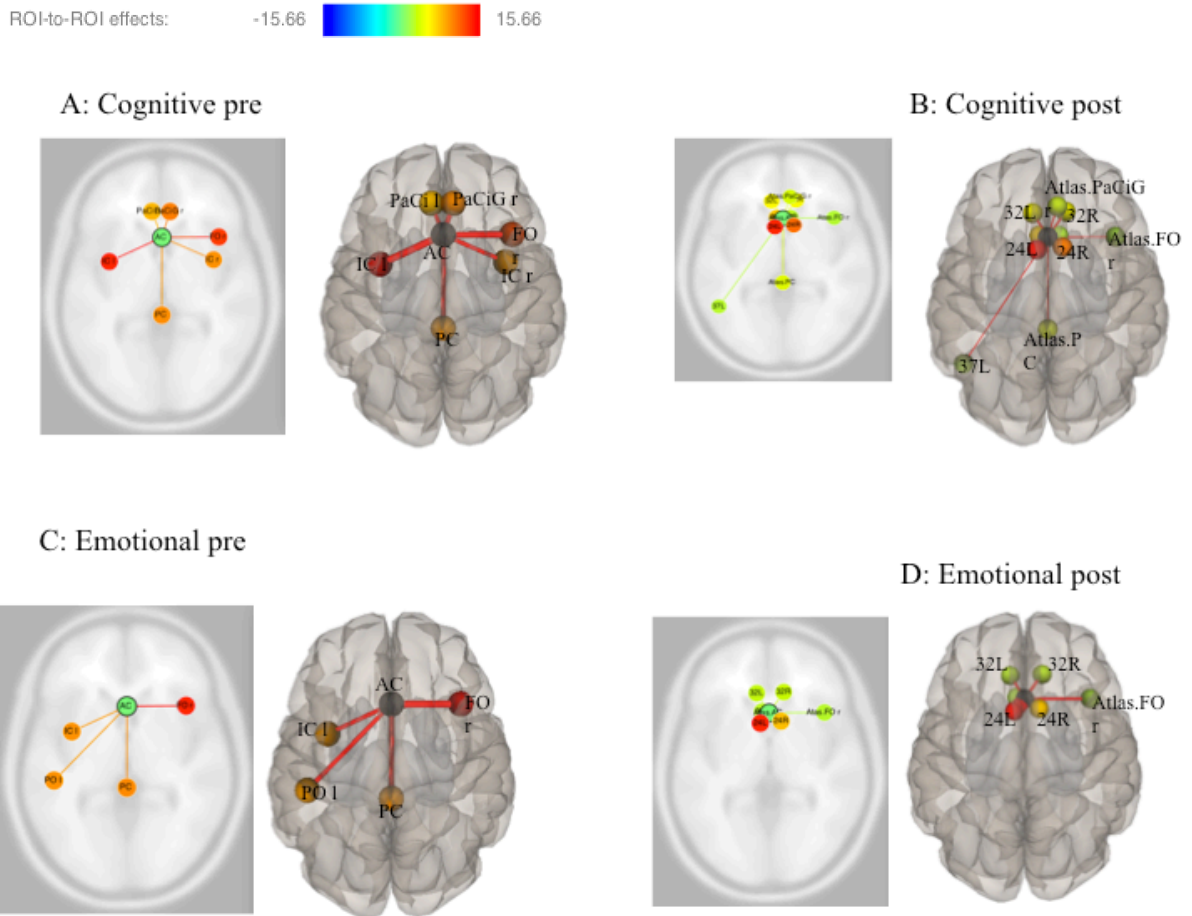
Some limitations should be taken into account when interpreting the results. First, as in the few other published MBCT –imaging studies in clinical samples (Goldin & Gross, 2010; Strawn et al., 2016), this was a naturalistic study and we did not use another treatment in patients or included a healthy control sample to compare results. However, we did observe that depression remittance was positively correlated with altered left hippocampus – left orbitofrontal connectivity. Changes in neural activity in these regions have previously been linked to psychotherapy and antidepressant medication responses in adolescents (Straub et al., 2015). Moreover, although the sample size is comparable with other imaging studies on the effects on

MBCT on brain processes in clinical youth samples, the sample size must be considered small. Consequently, the change in depression symptoms was not statistically significant. However effect sizes were moderate, and comparable as what has been observed in trials on behavioural efficacy of MBCT (Burke, 2009; Ives-Deliperi et al., 2013). Nevertheless, replication in a larger sample is needed.

Some previous research has raised the possibility that SLC6A4 methylation and, in particular, changes in SLC6A4 methylation throughout treatment, could be informative for prediction of successful psychotherapeutic treatment. Specifically, one previous study in children with an anxiety disorder showed that the direction of change in percentage of SLC6A methylation during CBT treatment was significantly different between treatment responders and non-responders (Roberts et al., 2014). Longitudinal follow up studies with a large number of participants are needed to study the relationships among activation, network efficiency, DNA methylation, and depression remission and relapse.

Though preliminary, these data may elucidate how functional connectivity in cognitive and emotion processing networks, along with DNA methylation, may change in the context of MBCT psychotherapy in depressed adolescents. In particular, our study illustrates how DNA methylation and fMRI measures can be employed in order to get a fuller picture of how remittance and relapse occurs in the context of neurophysiological systems and networks and other developmental changes throughout adolescence. This result may help us better understand the neurophysiological mechanism that allows MBCT to effectively prevent relapse, including the importance of the ACC in these processes.

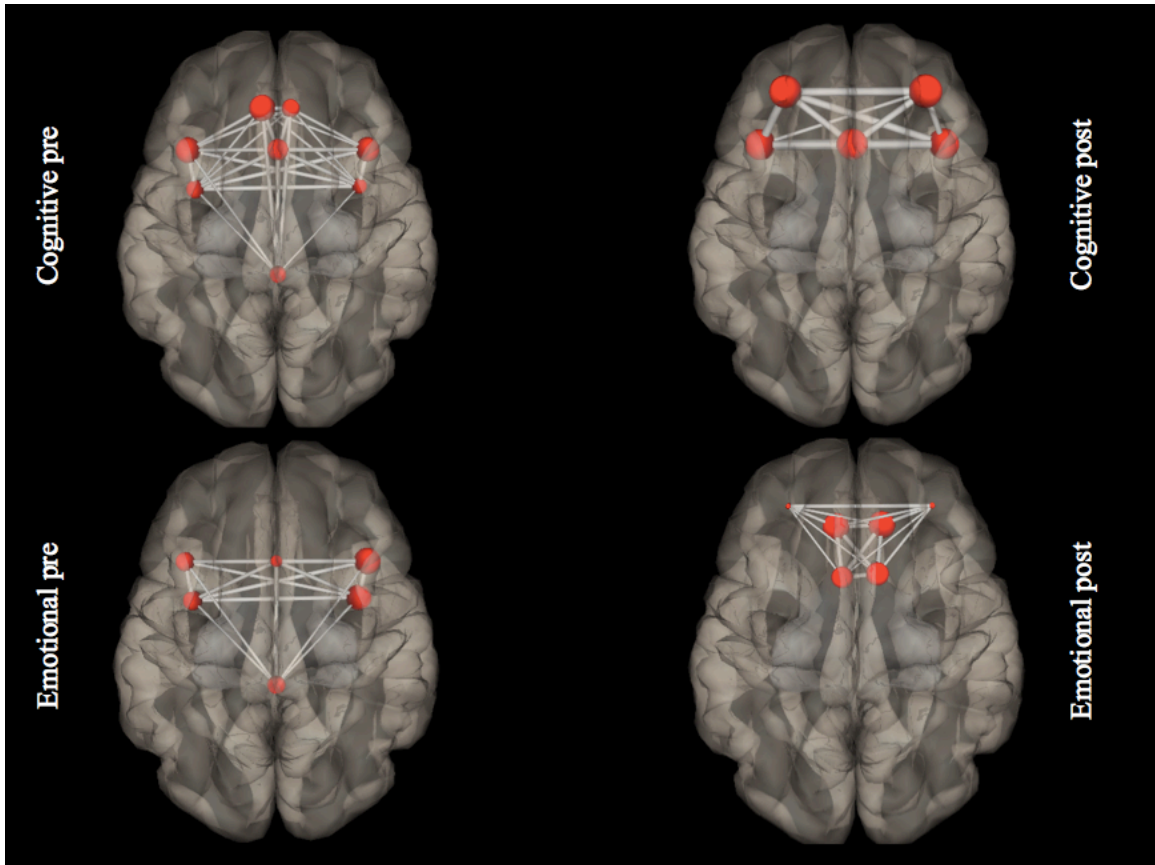
Figure 4.1: ROI-to-ROI functional connectivity with ACC seed area



Caption: Greater connectivity increases before (A and C) and after (B and D) MBCT treatment: the anterior cingulate seed region (AC) is represented by the brown circle. (A). represents the ROI-to-ROI network related to the cognitive condition, where pre MBCT FO = frontal pole, PaCiG = paracingulate gyrus, IC l = left insula, IC r = right insula, and the PC = precuneus. (B). represents the post MBCT network for the cognitive condition, where 32L, 32R and 24L, 24R = represent Brodmann areas of respectively the left and right cognitive subdivisions of the anterior cingulate cortex, and 37L = left angular gyrus. (C). represents the

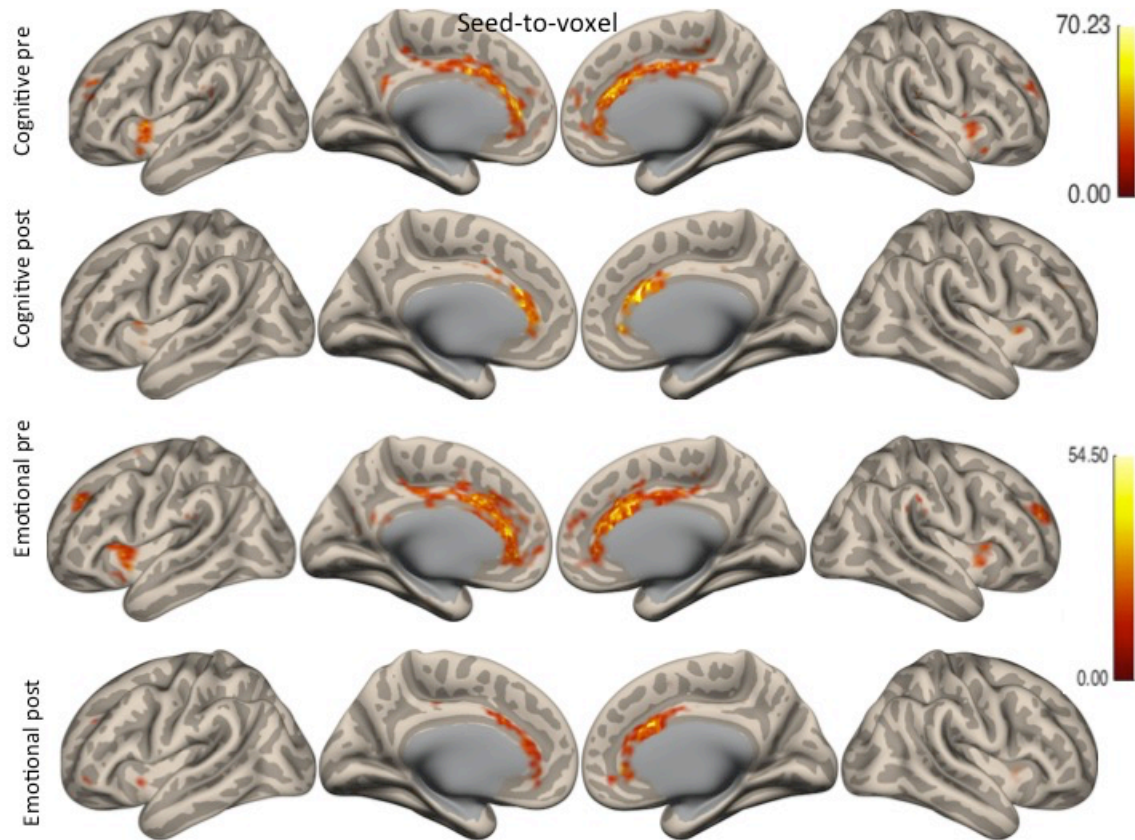
network involved in processing the emotional condition pre MBCT, where Pol =the left parietal pole. (D). represents the Post MBCT processing of the emotional condition. The thicker the line the stronger the connection. Note the strong connections between the ACC and frontal operculum, and ACC and insular cortices pre MBCT and the normalized connections post. Also notice the lack of the insular cortex in the post measure, which is involved in self-referential cognitive-emotional processing. Instead there is the implication of a stronger connection between BA 32 and 24, which is involved in cognitive control.

Figure 4.2: ROI-level analysis of global efficiency



Caption: Global efficiency of each ROI (a measure of ROI centrality, and shown proportional to circle sizes in the left display) in the network defined by associated ROIs (ROIs defined from Talairach atlas Brodmann areas). Small world properties (highly connected hubs and modularity; Bullmore & Sporns, 2009) are observed at the chosen cost threshold level ($K=0.15$). Note that lines and circle sizes are proportional to functional connectivity network efficiency. Additionally, we observed increased network global efficiency within the prefrontal cortex (implicated in auto-control and self-regulation) post MBCT, and less involvement of the insular cortex, which is implicated in self-referential emotional processes.

Figure 4.3: Seed-to-voxel analyses for the association between ACC related network and SLC6A4 methylation during the cognitive and emotional condition



Caption: Seed-to-Voxel positive correlations with the peripheral SLC6A4 methylation during the cognitive and emotional task condition before and after MBCT treatment. Colored bar depicts T values. Note that the intensity of the correlation in both conditions diminished after MBCT treatment in particular for the insula.

General Discussion

This thesis investigated the influence of the early environment on vulnerability to internalizing disorders, specifically depression, within a developmental framework, including exploration of DNA methylation as an environmental programming mechanism in the 5-HT system. Together, our three related studies contribute richer, more developmentally-informed information to the current models of depression pathogenesis. Additionally, these studies provide ideas for future avenues of research that could substantially contribute to common goals of better early prediction and intervention for individuals with chronic depression trajectories.

Our investigation began in infancy with study one, which examined the contributions of G X E in an important early temperamental vulnerability factor for internalizing disorders known as negative emotionality. We found that both genetic and shared environmental factors contribute substantially to negative emotionality in the first two years of life. The trait's stability across time appeared to be both genetically- and environmentally- mediated. We also saw evidence of both genetic and environmental innovative effects coming online at 18 months. We concluded that heritable and environmental factors present a dynamic influence pattern from infancy for this temperamental vulnerability.

Following our exploration of very early G X E interactions in vulnerability for internalizing disorders, we continued to investigate the association between life-long patterns of these disorders and early environmental adversity in study two. We were interested in a potential epigenetic mechanism (SLC6A4 methylation) for the G X E interaction involved in vulnerability for internalizing behavioural trajectories, as well as the role of the different characteristics of

stressors (abuse type, demographic disadvantage, predictability of stressor). We found relationships between peripheral SLC6A4 methylation and indices of childhood psychosocial / socio-economical adversity in males. We also found associations between peripheral SLC6A4 methylation and psychological abuse and internalizing behavioural trajectory in males and females. These findings underscore Shonkoff et al.'s (2012) theory of biological embedding of early adversity through alterations in development of stress regulation systems, and its long-term health impacts. Our finding not only provides support for the idea that DNA methylation of the SLC6A4 gene is a potential epigenetic mechanism for the G X E interaction involved in vulnerability for internalizing patterns over time, but also helps to clarify and underscore the relative importance and damaging consequences of specific types of adversity.

With a deeper understanding of how vulnerability to internalizing disorders develops from infancy to adulthood and the roles of the early environment as well as a DNA methylation mechanism, study three addressed how brain function and DNA methylation in depressed adolescents may change in the context of 6 weeks of Mindfulness-Based Cognitive Therapy (MBCT). Our fMRI measures before and after 6 weeks of MBCT demonstrated alterations in connectivity between the ACC and DLPFC/frontal operculum post treatment relative to pre-treatment suggesting that MBCT may work to optimize the connectivity of brain networks involved in cognitive control of emotion. Changes in depressive symptoms and SLC6A4 methylation also indicated that as MBCT treatment works to alleviate depressive symptoms, it may also normalize the relationship between SLC6A4 methylation and activation patterns in frontal-limbic circuitry in depressed adolescents.

These findings help fill out and enrich common neurodevelopmental frameworks of psychopathology, such as the one the described in (Booij, Tremblay, et al., 2015) and the one

described in the introduction of this dissertation. This framework integrates early G X E interactions that lead to disruption of the 5-HT system driving subtle structural and functional alterations in brain development that establish vulnerability for psychopathology (Figure 1.1). However, very little is known about the mechanism of recurrent depression (Willner et al., 2012). Our study provides more detailed and nuanced information about how early developmental changes, environmental factors, functional alterations, and a proposed G X E mechanism could contribute to enduring and multilevel diatheses for depression that illuminate a neurodevelopmental model of vulnerability for chronic depressive patterns.

First, even in infancy, we observed genetic and environment contributions establishing personality-based vulnerabilities to internalizing disorders, like negative emotionality, that are developmentally dynamic over the first two years of life at least. The first environmental stresses are thought to influence development in utero (e.g., exposure to drugs of abuse, maternal depression) interacting with genetic factors that are already directing construction of the embryo and foetus, including the 5-HT system (Booij, Tremblay, et al., 2015). After birth, early environmental stresses may trigger changes in 5-HT functioning that then have long-term effects on temperament, brain morphology, and 5-HT neurotransmission (Booij, Tremblay, et al., 2015; Willner et al., 2012). Indeed, differences in temperament in infancy have been linked to cerebral cortex architecture differences (Schwartz et al., 2010). We speculate here that this dynamic G X E interplay is driven in part by epigenetic mechanisms that program the timing and influence of new genetic factors coming online during development.

Future research endeavours that involve longitudinal collection of DNA methylation levels and 5-HT synthesis beginning in infancy could help elucidate the nature and timing of environmental programming and subtle changes in 5-HT homeostasis that may be taking place at

key developmental stages. Although some studies have found links between exposure to stress and DNA methylation in the umbilical cord or at birth (Devlin et al., 2010; Essex et al., 2013; Koestler, Avissar-Whiting, Houseman, Karagas, & Marsit, 2013), only recently have researchers begun to investigate levels in post-birth infancy and/or attempted longitudinal sampling in the context of environmental adversity (Montirosso et al., 2016; Deli Wang et al., 2012). In order to further these lines of inquiry, we suggest that future longitudinal investigation of infant SLC6A4 methylation in the context of abuse includes epigenetic, neuroimaging and behavioural measures.

During childhood and adolescence, our results highlighted links with peripheral SLC6A4 methylation that supported epigenetic programming by the early environment. Importantly, we found that the chronicity of MDD may be correlated with this potential physiological mechanism, since adult levels of SLC6A4 methylation were associated with life-long patterns of internalizing behaviors.

Moreover, we found a link between peripheral SLC6A4 methylation with psychological abuse, an unpredictable and trauma-based stressor. This finding is consistent with the theories that unpredictability and the emotionally damaging nature of childhood trauma elicits distress, chronic threat response arousal, hypervigilance, and a sense of being unsafe, leading to a neurobiological sensitivity to stress (Foa & Hearst-Ikeda, 1996; Gobinath et al., 2015; Heim, Shugart, Craighead, & Nemeroff, 2010; Suo et al., 2013). Interestingly, the experimental literature also suggests that both unpredictability and aversive nature of the threat are key factors that need to be present together in order to trigger a potentiated stress response (Grillon, Baas, Lissek, Smith, & Milstein, 2004). This sustained level of anxiety produced by the unpredictable, aversive threat would then lead to down-stream changes in the stress regulatory systems in our neurodevelopmental stress-diathesis model. It would be worthwhile to investigate patterns of

brain development and emotional regulation specific to this type of adversity using longitudinal measures of structure and functional connectivity in children and adolescents.

Interestingly, socio-economic factors such as growing up in poverty may be a risk factor for “toxic stress” (Shonkoff et al., 2012), with putative additive consequences for the 5-HT system. Indeed, in the present dissertation, it was shown that greater peripheral SLC6A4 methylation is associated with lower SES, a finding consistent with another recent study (Swartz et al., 2016). Although our findings differ from Swartz et al. in that the relationship between low SES and SLC6A4 methylation was in our study primarily observed in males, and the specific mechanism for this sex specificity not known, the findings are in line with research showing that low socio-economic conditions is a particular risk factor for 5-HT associated behaviors that generally are more common in boys than in girls, like aggression and hyperactivity (Mazza et al., 2016).

Thus, it could be speculated that chronic depressive patterns may develop from enduring infant vulnerabilities interacting with unpredictable childhood stress factors that establish physiologically and cognitive based diatheses. Fitting these findings into our neurodevelopmental model of recurrent MDD, we theorize that individuals with early vulnerabilities such as high degree of negative emotionality and early stresses such as psychosocial adversity and unpredictable psychological abuse experience sustained anxiety resulting in an early disruption of 5-HT homeostasis, leading to epigenetic changes that program subtle alterations in brain development and heighten risk for internalizing disorders like depression and anxiety.

Additionally, the risk for depression intensifies after a major depressive episode. The recurrent nature of MDD is also related to the hypothesis of “kindling”: due to morphological, functional, neurochemical, and cognitive alterations after a depressive episode, successive episodes of depression are prompted by weaker stresses (Willner et al., 2012). In this way, a

previous depressive episode becomes a diathesis for a future episode. Although our pilot project results highlighted changes in functional connectivity in depressed adolescents after treatment, it would be interesting to monitor the depressed sample over time and observe the connectivity changes throughout eventual recovery or depression remittance. Nevertheless, our results provided preliminary evidence that functional connectivity between prefrontal and subcortical regions in depressed adolescents is associated with epigenetic changes, which could hint at a role for DNA methylation in the remediation and kindling processes.

Such a role for epigenetic “memory-like” mechanisms in kindling or long-lasting increases in vulnerability to recurrence was recently suggested by Post (2015). The depressive episode itself, and its related symptoms, including poor diet and lack of physical activity, hopelessness, and social isolation are aversive experiences that could trigger methylation alterations that accumulate and increase vulnerability to successive episodes through long lasting changes in various neurophysiological systems. Importantly, alterations in 5-HT neurotransmission and prolonged activation of limbic structures may then underlie more enduring cognitive phenomena, such as negative cognitive response style, attentional biases, and rumination (Carballedo et al., 2011; De Raedt & Koster, 2010; Disner et al., 2011).

In fact, we observed a “decoupling” effect after MBCT treatment in the association between brain activation and SLC6A4 methylation that could be interpreted as this treatment’s ability to weaken, or douse the vulnerability to recurrence. This result in the context of the kindling theory along with further inquiry may help us better understand the neurophysiological mechanism that allows MBCT to effectively prevent relapse, including the importance of the ACC in these processes.

Thus, the idea that epigenetic alterations could accumulate after a major depressive episode and heighten risk for successive episodes highlights the importance of investigating DNA methylation changes in the context of treatment. If treatments for MDD can target dismantling the epigenetic processes by which kindling occurs and the associated altered patterns of functional connectivity of circuits involved in emotion regulation, the heightened sensitivity to further episodes of depression could possibly be attenuated, preventing recurrence of MDD and its deleterious consequences.

Whether psychotherapy or pharmacological or combination interventions would be most effective at targeting dismantling epigenetic processes in this way is an exciting topic for future research. It would also be of interest to explore if treatments that can attenuate the effects of kindling are developmental-stage dependent, and whether such processes are more effective in adolescence when the serotonergic system and cerebral cortex are not yet fully developed, compared to adulthood. It should be stressed that recurrence of depressive symptoms and episodes should be measured in multiple ways: with longer-term follow-up interview measures, collateral interviews, and DNA methylation and fMRI measures in order to get a fuller picture of how remittance and relapse occurs in the context of neurophysiological systems and networks and other developmental changes.

Our research has several overall limitations. First, there is a high degree of complexity in models of depression and internalizing disorders in general, involving many more neurotransmitters and hormones than serotonin. Although our investigation does not include many other key factors (e.g., glucocorticoid receptor, norepinephrine, dopamine, brain-derived neurotrophic factor), we chose to focus on 5-HT due to the substantial literature detailing its pivotal role in neurotypical development and psychopathology. We aimed to gain a better

understanding of the model as it relates to one neurotransmitter in the hope that this work can inform future endeavours involving one or more other neurotransmitters.

Second, we used measures of peripheral 5-HT methylation as a proxy for central 5-HT methylation when analyzing DNA, as this is the only option. While there is some evidence that development of 5-HT receptors is brain-region-specific, there is also evidence of a high correlation between peripheral methylation of the SLC6A4 gene and in vivo methylation, as well as with 5-HT synthesis in the human brain (Booij, Tremblay, et al., 2015; Dongsha Wang et al., 2012). Additionally, peripheral SLC6A4 promoter methylation has been linked to limbic activity (Nikolova et al., 2014) and functional connectivity of emotional regulation networks in the brain (Muehlhan et al., 2015). More generally, early life adversity in rhesus macaques was associated with genome-wide methylation in both T-cell and prefrontal cortex cells, indicating that epigenetic response to adversity is system-wide (Provençal et al., 2012).

On a related note, our study did not investigate the possible role of the DNA sequence (genotype) in SLC6A4 methylation levels, which may or may not be a relevant factor. Although the DNA sequence may have a bearing on methylation levels, the research is not consistent (Booij, Szyf, et al., 2015; Philibert et al., 2007; Dongsha Wang et al., 2012; Wankerl et al., 2014). Moreover, our preliminary findings of a change in DNA methylation throughout MBCT treatment, as well as the recent observation of changes in DNA methylation in anxiety disorder patients after Cognitive Therapy (Roberts et al., 2014), support the notion of relevant DNA methylation changes occurring irrespective of DNA sequence effects.

Third, our sample sizes were sufficient in some cases, but in others were small (N=13 in pilot project, study 3). In the future, longitudinal follow up studies with more participants could extend this line of research in order to study the relationships among depressive remission and

relapse, activation, and physiological mechanisms. However, our investigations in studies 2 and 3 used sample sizes that are somewhat consistent with, if not larger than, recent studies of a similar nature (Borghol et al., 2012; Devlin et al., 2010; Essex et al., 2013; Goldin & Gross, 2010; Strawn et al., 2016).

Fourth, the diagnosis of MDD is a heterogeneous one, and even more confounds are added when comorbid conditions are introduced. We were often in a position to group individuals together with different symptom manifestations of internalizing disorders, comorbidities, pharmaceutical regimes, and previous depression history including previous treatment in order to evaluate relationships among factors. Nevertheless, a mixed sample of patients is often the reality in clinical research and practice. Every possible effort was made to exclude participants who did not meet criteria in order to balance both clinical and research concerns in a way that was ethical and feasible.

Despite these limitations, our studies boast many strengths. In two studies, we employed large longitudinal samples where possible that enabled us to observe key changes over months and years in development of personality and internalizing problems. We also integrated behavioural data, adversity factors, (f)MRI measures, and epigenetic information in order to synthesize a model that attempts to clarify a highly complex disorder on multiple levels.

Although some future directions for research have been discussed above, an overarching goal would be to improve prediction of individuals at risk for recurrent depression and prevention and interventions that target and reduce first and future depressive episodes. The key to achieving these goals is longitudinal data collection that begins in infancy in order to better elucidate the sequence of events and changes and thus causal relationships between vulnerability factors, DNA methylation, adversity, and symptoms. Objective, multi-informant measures of adversity/abuse,

including neglect, may also help determine vulnerable groups. We also need to redefine the remittance of depression in our research endeavours if we hope to truly remediate chronic trajectories. With our expanded knowledge of depression as a life-long struggle and the kindling of physiological systems, assessing depressive relapse after a treatment evaluation with a single self-report measure feels like an afterthought. Remittance and relapse should be examined with longer-term follow-up assessments that include interview, fMRI, and epigenetic measures.

Models of depression have made substantial gains and stand to improve significantly from continued research into environmental factors and the biological mechanisms for environmental programming related to vulnerability for MDD throughout development and the lifespan. This thesis investigated how environmental influences during development interacted with genetic factors in order to alter neurotransmission of 5-HT related to changes in neurophysiological systems and vulnerability to depression, and a possible epigenetic mechanism. Our results supported the theory that environmental influences are instrumental in establishing early vulnerability factors, driving epigenetic processes, and altering brain processes as an individual undergoes treatment, or experiences remittance and/or relapse. The results and conclusions drawn from the current studies stand to help further develop models of depression vulnerability and recurrence and highlight critical interactions among environmental factors, symptoms, personality factors, functional connectivity of emotional processing networks, and SLC6A4 methylation. Improving our knowledge of who is likely to become depressed, how we can prevent or inoculate these individuals, and how best to intervene to break the pattern of chronic depressive episodes stands to improve outcomes for those afflicted as well as the wider society that bears the burden of this public health problem.

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