Elucidating the Link Between the Environment, the Brain and Depression: Genetic and Epigenetic Mechanisms

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ABSTRACT

Elucidating the Link Between the Environment, the Brain and Depression: Genetic and Epigenetic Mechanisms

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The development and maintenance of depression is influenced by a combination of genetic and environmental factors, as well as their interactions. In recent decades, a number of candidate genes involved in stress-related systems have been implicated in major depressive disorder, shedding light on the genetic contributions to depression. Further, the study of epigenetic mechanisms has provided a potential mechanism by which the expression of genes implicated in depression are shaped by the environment, offering a bridge between environmental (e.g., early life adversity) and genetic factors. However, relative to adulthood, our knowledge of the genetic and epigenetic mechanisms of depression in childhood and adolescence remains limited. The current thesis aimed to address several gaps in our understanding of the genetic and epigenetic basis of depression in childhood and adolescence, while simultaneously exploring novel epigenetic methodology, which, in turn, could inform our understanding of depression across the entire lifespan. The projects that are described in this thesis used a variety of measures, including longitudinal ACE modelling of twin cohort data, (functional) magnetic resonance imaging, peripheral assessments of DNA methylation and *in vivo* assessment of epigenetic processes using positron emission tomography.

In study 1, we showed in a longitudinal cohort of 1344 twins that the association between externalizing symptoms at the preschool age and internalizing symptoms in early adolescence

was essentially accounted for by genetic factors, suggesting a shared genetic vulnerability to externalizing and internalizing symptoms that is stable throughout childhood. In study two, we examined the association between DNA methylation at key genes within the serotonin and HPA axis systems (i.e., *SLC6A4* and *FKBP5* respectively) in 25 adolescents with major depressive disorder and 20 healthy controls. We found associations between DNA methylation, diagnostic status and frontolimbic structure/function that both converged with and diverged from findings in adults. In study three, we examined the link between childhood adversity, epigenetics and depression vulnerability while simultaneously addressing a common limitation in the epigenetic literature also present in study 2, the reliance on peripheral epigenetic measures. Using the novel [^{11}C]Martinostat tracer in combination with positron emission tomography, we assessed levels of histone deacetylase (HDAC) *in vivo*. We found, in a sample of 14 healthy adult males, that HDAC density was associated with depressive symptoms and neuroticism in key frontolimbic regions. We found no association between early life adversity and HDAC density.

The results from the projects described in this dissertation may offer novel contributions to our understanding of the epigenetic and genetic basis of depression across childhood and adolescence, and validate the use of the novel [^{11}C]Martinostat in the study of depressive symptoms and personality traits relevant to depression.

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CONTRIBUTION OF AUTHORS

Manuscript 1

Manuscript one was conducted using previously collected data from the Quebec Newborn Twin Study (QNTS) cohort. In collaboration with my supervisor Dr. Linda Booij and co-leader of this project, Dr. Michel Boivin, I was involved in the determination of the research question and the selection of data variables to be extracted from the QNTS database. I assisted statistician, Bei Feng, in the analysis of the data. Under the guidance of co-authors Dr. Booij, Dr. Boivin, Bei Feng and Dr. Mara Brendgen, and with the input of other co-authors, I interpreted the analyses and wrote the manuscript.

Manuscript 2

The second manuscript made use of data that were partly collected while I was a graduate student in Clinical Psychology at Queen's University (also supervised by Dr. Linda Booij at that time). It draws on data collected by myself, previous graduate student Dr. Lyndall Schumann and research assistants. The study is an expansion of a treatment study designed by Dr. Linda Booij and Dr. Lyndall Schumann, who selected constructs and measures and established study protocols. For the current study, a control group was added in order to make baseline comparisons between depressed adolescents and controls pre-treatment. I was involved in the design of the "baseline" study alongside Dr. Linda Booij, using the previously established measures, constructs and protocols. With the guidance of Dr. Florence Pomares, a past post-doc in our laboratory at Concordia University, and Dr. Linda Booij, I analysed and interpreted the regional structural and functional imaging and behavioural data and wrote the manuscript.

Manuscript 3

The study on which the third manuscript is based was designed by and supervised by Dr. Linda Booij alongside Dr. Chawki Benkelfat. I recruited participants, collected and interpretated data and drafted the research manuscript. Jocelyn Fotso Soh, Naomi Azar and Dr. Mari Sild aided with recruitment and data collection. Dr. Kevin Casey and I analyzed the data. Drs. Gassan Massarweh, Jean-Paul Soucy and Jacob Hooker were integral in the determination of study protocols as they relate to radiochemistry. Dr. Leyton provided input on the analysis. Drs. Frank Vitaro and Richard Tremblay were responsible for the development and continuation of the QLSKC cohort.

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1. CHAPTER 1: GENERAL INTRODUCTION

1.1 Depression: Symptomatology, epidemiology and risk factors

Major depressive disorder (MDD) is a debilitating mood disorder and is the leading cause of disability worldwide (World Health Organization, 2017). Furthermore, MDD is common, with a 12 month and lifetime prevalence of 10.4% and 20.6%, respectively in 2018, and more than 300 million people affected worldwide (Hasin et al., 2018; World Health Organization, 2017). The prevalence of depression has risen more than 18% since 2005 in the United States and suicide rates have increased more than 30% in half of US states since 1999 (CDC, 2018; World Health Organization, 2017). More recently, since the onset of the COVID-19 pandemic, there has been an estimated 27.6% increase in MDD globally (Santomauro et al., 2021). Moreover, the costs of depression to society are large, accounting for approximately 400 million disability days per year in the United States pre-pandemic (Greenberg et al., 2015; WHO). In 2010, the incremental economic burden of depression was estimated at $210.5 billion, with $77.5 billion in direct costs (i.e., medical services and prescription costs; Greenberg et al., 2015). In 2016 it was estimated that MDD costs Canada 32.3 billion dollars per year in lost productivity (Sutherland & Stonebridge, Carole, 2016). Since the pandemic, the number of years of healthy life lost to mortality and disability linked to depression is thought to have risen from 38.7 to 49.4 million as of 2020 (Santomauro et al., 2021).

In order to receive a diagnosis of MDD according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), individuals must endorse 5 or more out of 8 possible symptoms over a 2-week period. At least one of these symptoms must include either a period of depressed mood and/or the loss of interest or pleasure in nearly all activities lasting at least two weeks (American Psychiatric Association, 2013). Other possible symptoms include a change in

appetite or sleep, psychomotor agitation or retardation, feelings of worthlessness or guilt, difficulty concentrating, a loss of energy and thoughts of death and/or suicide.

Depression is associated with a number of biological and environmental risk factors. Biological factors include genetic makeup and hereditable neural markers, as well as temperamental factors. Environmental factors include both early life predisposing factors such as childhood maltreatment, as well as precipitating factors such as stressful live events (e.g., job loss, divorce; American Psychiatric Association, 2013). Depression tends to co-occur with a variety of other mental disorders including anxiety, substance use, personality and eating disorders (American Psychiatric Association, 2013).

In addition to the costs to society, depression has a profound impact on the individual affecting not only worker productivity, but also relationships, academic performance, educational attainment, physical health and general quality of life (Angermeyer et al., 2002; Burke, 2003; Fletcher, 2008; Ibrahim et al., 2013). Given the high cost of depression to both society and the individual, it is imperative that we further our understanding of both the etiology and treatment of this burdensome disorder.

1.2 Depression and development

While depression across the lifespan could have profound implications, adolescent depression is particularly problematic given its association with a wide number of physical and health problems and a chronic course of illness (American Psychiatric Association, 2013; Noyes et al., 2022). One study suggests that of those adolescents who develop depression, 10-40% will continue to experience clinically significant symptoms for longer than a year, and of those who remit, 50-70% will experience another depressive episode within 5 years (Thapar et al., 2012). Moreover, adolescent depression in particular is predictive of a number of negative outcomes

such as unemployment, early parenthood, lower educational achievement and increased rates of substance abuse (Fergusson & Woodward, 2002). Between 2005 and 2014, the 12-month prevalence of major depressive episodes has increased from 8.7% to 11.3% in adolescents and 8.8% to 9.6% in young adults (Mojtabai et al., 2016). With the onset of the global pandemic, a recent Canadian meta-analysis suggested that estimates of youth depression have doubled, and it is now believed that 1 in 4 youth are experiencing clinically elevated depressive symptoms in Canada (Racine et al., 2021). Such increases in prevalence are alarming given the particularly negative outcomes associated with adolescent depression. Indeed, even subthreshold levels of depression have serious deleterious effects on quality of life in adolescents and predict later development of MDD (Bertha & Balázs, 2013)

Interestingly, while the prevalence of depression is similar across genders in childhood and rises rapidly during puberty for both boys and girls, rises are greater for girls with the rate of depression in females growing to be twice that of males by the end of puberty and into early adulthood (Thapar et al., 2012). Further, the symptoms endorsed by males and females in adolescents vary, with, for example, adolescent girls being more likely to experience feelings of worthlessness and guilt as well as disturbances in weight and appetite and boys experiencing greater levels of anhedonia, irritability, depressed morning mood and morning fatigue (Bennett et al., 2005; Khesht-Masjedi et al., 2017; Lewinsohn, 1998). Understanding the development of such sex differences in adolescents may provide clues to the etiology of this disorder.

1.3 Models of depression

1.3.1 Biopsychosocial model

In order to provide a framework for understanding the etiology and progression of depression, a number of different models have been proposed. The biopsychosocial model of depression stresses the importance of considering biological, psychological and social factors in

the etiology of the disorder (Schotte et al., 2006). It was first proposed by physician George Engel as a challenge to the reductionist biomedical model of disease prominent in medicine, and has since greatly influenced how psychiatric disorders, and illness more broadly, are conceptualized (Engel, 1977; Fava & Sonino, 2007). Potential biological factors which contribute to the etiology and maintenance of depression include alterations in brain structure, function and neurochemistry, as well genetic and epigenetic vulnerabilities. Psychological factors include, personality, coping skills, biases in information processing and maladaptive behaviours which may maintain depression. Social factors include, for example, education, socioeconomic status, immediate social support as well as cultural context.

The biopsychosocial model also acknowledges that in order to understand depression, one must consider all of such factors, as well as their interactions (see Figure 1.). For example, prolonged social stress leads to lasting neurobiological (e.g., epigenetic, brain structure/function) and psychological (e.g., influencing attentional biases) changes which predispose individuals to further depressive episodes in a way which reinforces these neurobiological and psychological effects. Further, negative cognitions about the self, future and the world and underlying neurobiological changes may worsen the social environment by, for example, leading to social withdrawal or the pursuit of maladaptive relationship patterns characterized by social rejection. Thus, the biopsychosocial model provides a framework for understanding such interactions over time.

In addition, the biopsychosocial model has important implications for the treatment of depression, suggesting the importance of a multipronged approach in which social, psychological and biological variables are viable intervention targets.

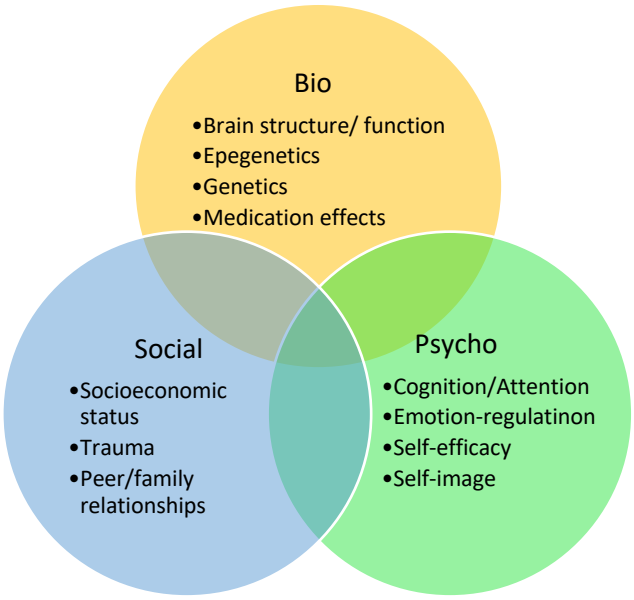

Figure 1. A graphical depiction of the biopsychosocial model along with examples of biological, social, and psychological determinants of depression

1.3.2 Diathesis-stress and differential susceptibility

According to the diathesis stress model of depression, stressful life events can activate a vulnerability to depression, leading those individuals with a predisposition to develop the disorder in the face of such life stressors (Monroe & Simons, 1991). While this model was initially used to explain the etiology of schizophrenia in the 1960s, it was applied to depression in the 1980s and has since remained one of the dominant models of depression and has received a great deal of support in the literature (Colodro-Conde et al., 2018). Indeed, one seminal study showed that in 50 to 80 percent of individuals with depression, the onset of the disorder is preceded by a major stressful life event (SLE; Monroe & Simons, 1991). Later research has supported this initial estimate, suggesting that a severe stressful life event precedes approximately 70% of first episodes of depression and 40% of recurrences (Monroe & Harkness, 2005).

Since Monroe and colleague's (Monroe & Simons, 1991) seminal study, much research has been conducted in order to understand the relationship between stressful life events and depression. Current thinking understands that the relationship between depression vulnerability, SLEs and major depressive episodes (MDEs) is complex and mediated through multiple pathways. More specifically, depression vulnerability can influence risk for MDD through both 'within the skin' and 'outside the skin' pathways. The 'within the skin' pathway involves neurobiological, cognitive and temperamental factors which directly increase the risk for a major depressive episode (MDE). The 'outside the skin' path involves the selection or creation of high-risk environments that increase the change of experiencing SLEs (Kendler & Gardner, 2016). The relationship between SLEs, depression vulnerability and MDD is complex and evolves over time. SLEs are more predictive of depressive episodes early in life and the association between SLEs and depression are greater in those experiencing their first episode of depression compared to previous episodes (Kendler et al., 2001; Kendler & Gardner, 2016; Post, 1992). Moreover, the effect of stress on depression may be dose-dependent; greater stress predicts greater symptom severity and duration, as well as increased risk of relapse (Chapman et al., 2004; Hammen, 2005; Mazure, 1998). This diathesis-stress model is particularly relevant to genetic research studying gene by environment interactions (GxE). In this context, the diathesis component can be understood to represent an underlying genetic vulnerability, while the stress component accounts for environmental stressors present both early in life (e.g., childhood maltreatment) as well as those which precipitate the disorder such as job loss, death or divorce. In a seminal study by Caspi and colleagues (Caspi et al., 2003), individuals who possessed at least one copy of the short allele at the serotonin transporter gene promoter were at greater risk of developing depression in the face of stressful life events compared to those homozygous for the long allele.

Thus, it was suggested that the long allele was protective against life stress while the s allele put individuals at risk for a MDE in the face of stressful life events. This model has inspired a great deal of research into GxE interactions and research using advanced statistical techniques in genetics (i.e., polygenic risk scores) continue to provide support for the diathesis-stress model (Colodro-Conde et al., 2018).

While the diathesis-stress model has fueled decades of productive research, some have criticized this model, arguing that, at least in regards to certain candidate genes, individuals who are more susceptible to adverse effects of a stressful early environment are also more likely to experience the beneficial effects of a supportive environment (Belsky & Pluess, 2009). Indeed, there is an accumulation of evidence suggesting that individuals who are vulnerable to the negative effects of adversity may also be more susceptible to the positive effects of supportive and enriching environments. This has led to the development of the differential susceptibility model, in which certain genes act more like "plasticity factors" than "vulnerability factors," making individuals more susceptible to both positive and negative environmental influences (Belsky & Pluess, 2009; Uher & McGuffin, 2008). For example, in a study of young adults homozygous for the risk allele at the serotonin transporter gene promoter, individuals displayed greater depressive symptomatology compared to those with other allele combinations if they had been exposed to early adversity, but fewer symptoms if they were raised in a supportive early environment or had recently had positive experiences (Belsky & Pluess, 2009). Similar findings have been reported for genes related to dopaminergic functioning (Bakermans-Kranenburg & van Ijzendoorn, 2011). Nevertheless, in the context of psychopathology and stressful life events, the diathesis-stress model continues provides a meaningful framework for understanding GxE interactions.

1.4 Depression: Neurobiology

Since the discovery in the 1950s that specific psychoactive compounds could have an antidepressant effect, suggesting that there might be a biological basis to depression, our understanding of the neurochemical and neurobiological basis of depression has grown immensely (Booij, Tremblay, et al., 2015). Some of the most well studied neural systems involved in depression, the serotonergic and glucocorticoid systems, will be reviewed below.

1.4.1 Neurotransmitter and hormonal underpinnings

1.4.1.1 Serotonergic system

One of the earliest theories of the neurobiology of depression is the monoamine hypothesis. This theory was developed in the 1950s in light of findings that chronic administration of Reserpine, an anti-adrenergic drug, induced depression, and later that antidepressants which acted on monoamine systems (e.g., serotonin and norepinephrine) were effective in treating depression (Booij, Tremblay, et al., 2015; Freis, 1954; Goldberg et al., 2014; Hirschfeld, 2000). Although we have since come to understand that the monoamine hypothesis of depression does provide a complete understanding of the neurobiology of depression, it has nonetheless prompted fruitful research into the involvement of monoamine systems in depression. One of such monoamines which has received considerable attention is serotonin (5-Hydroxytryptamine, 5-HT).

Models developed in the 1970s and 1980s hypothesized that low serotonin in particular was the cause of depression given the effectiveness of serotonin enhancing drugs in treating depression as well as the depressogenic effect of serotonin depleting agents such as reserpine.(Albert et al., 2012; Cowen & Browning, 2015). However, limitations with this model rapidly became clear. Firstly, not all individuals with depression showed alterations in 5-HT functioning nor did all individuals respond to serotonergic medications (Booij, Tremblay, et al.,

2015). Further, medications which act on other neurotransmitter systems such as noradrenergic or dopaminergic medications are also effective in treating depression and the effect of certain SSRIs are at least partly mediated by indirect changes in noradrenergic activity (van Praag et al., 1988). In addition, serotonergic medications were found to be effective for other disorders such as obsessive compulsive disorder (van Praag et al., 1988). Perhaps more conclusive evidence came from studies suggesting that while depressed individuals do display altered serotonergic neurotransmission, so do healthy first-degree relatives who may share a predisposition but not the depressive phenotype. For example, first-degree relatives are also more sensitive to the low mood inducing effects of tryptophan depletion, a procedure in which serotonergic levels are experimentally lowered (Benkelfat et al., 1994; Klaassen et al., 1999). Further, tryptophan depletion has been shown to trigger a relapse of depression in recovered individuals (Booij, 2002; Leyton et al., 2000). Such evidence has led researchers to propose a model which postulates that altered serotonergic neurotransmission acts as a vulnerability factor which predisposes individuals to depression in the face of stressful life events. In line with the diathesis-stress model, it is thought that genetic factors and early maladaptive environmental conditions may alter serotonin homeostasis in a way which makes it more likely that individuals will become depressed in the face of life stressors (Booij, Tremblay, et al., 2015). See Figure 2 below for a graphic depiction of this model.

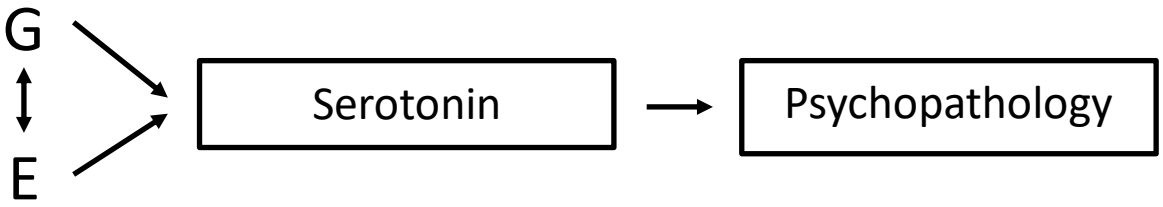

Figure 2. Serotonin diathesis stress model adapted from Booij and colleagues (2015). This model postulates that genetic and environmental factors influence the serotonergic system in such a way that predisposes individuals to psychopathology. G = gene; E = environment.

However, as noted by Booij and colleagues (2015), there are at least 5 key findings which the vulnerability model is unable to explain. First, while many serotonergic genes have been investigated in relation to psychopathology, none has produced consistently replicable results. Second, risk alleles for 5-HT genes are not consistently associated with 5-HT expression levels. Third, the vulnerability model is overly deterministic and does not take into account resilience and non-stress induced relapses. Fourth, SSRIs administered early in life in animal models increases rather than decreases anxiety. Finally, tryptophan hydroxlase-2 (a key enzyme required to produce 5-HT) knockout mice survive without any clear changes in brain morphology or depressive and anxious behaviours. Overall, the preceding suggests that the 5-HT vulnerability model is too simplistic to explain depression vulnerability.

Adding further to the complexity, the serotonergic system is implicated not just in depression and anxiety, but also externalizing symptoms such as aggression and anger, a link which was recognized early on (Coccaro et al., 2015; Olivier, 2004; van Praag et al., 1988). More specifically, childhood aggression and depression have both been found to be associated with changes in serotonin synthesis (Booij et al., 2010; Rosa-Neto et al., 2004; Tuvblad et al., 2019). Further, certain SSRIs and SNRIs, while most often used to treat anxiety and depression,

have some effectiveness in treating attention deficit hyperactivity disorder (ADHD) and aggressive behaviours (Bond, 2005; Ghanizadeh et al., 2013). Finally, there is evidence that juvenile onset depression in particular is linked to externalizing symptoms such as suicide attempts, self-harm, hyperactivity and alcohol abuse (Chanen et al., 2016; Hill et al., 2004). Indeed, anger is elevated in depression and associated with greater severity and poorer response to treatment (Cassiello-Robbins & Barlow, 2016). Irritability as well is common in depression and associated with greater severity of depressive symptoms, anxiety comorbidity and suicidality (Perlis et al., 2009; Verhoeven et al., 2011).

It is perhaps not surprising that the link between depression and serotonergic functioning is not simple given the immense complexity of the serotonin system. There are currently 14 known receptors divided into 7 families, many of which act through different mechanisms and have opposing effects on synaptic firing and, complicating matters father, are located both post-synaptically and pre-synaptically as autoreceptors (Köhler et al., 2016; Morrissette & Stahl, 2014). Further, the major serotonergic projections relevant to MDD arise from the dorsal and medial raphe nuclei and project to a wide variety cortical and subcortical regions relevant to depression including the prefrontal cortex, hippocampus, amygdala and striatum. Such widespread projections also influence almost all other neurotransmitter systems, accounting for its role in regulating a wide variety of psychological and behaviour processes including not only mood, but also sleep, appetite and aggression (Morrissette & Stahl, 2014).

In an attempt to reconcile limitations of the diathesis stress model outlined above with recent advances in neurobiology, a *neurodevelopmental* diathesis-stress model has been proposed which suggests alterations in serotonergic neurotransmission, induced either by genetic or environmental stressors, at key developmental periods, may lead to permanent changes in

brain development which underlie the predisposition to a number of different psychopathologies, including depression (Booij, Tremblay, et al., 2015). Indeed, the serotonergic system undergoes several major changes in utero and early life and some 5-HT receptors do not stabilize until adulthood (see Booij et al., 2015 for a review). See Figure 3 below for a visual depiction of the neurodevelopmental diathesis-stress model.

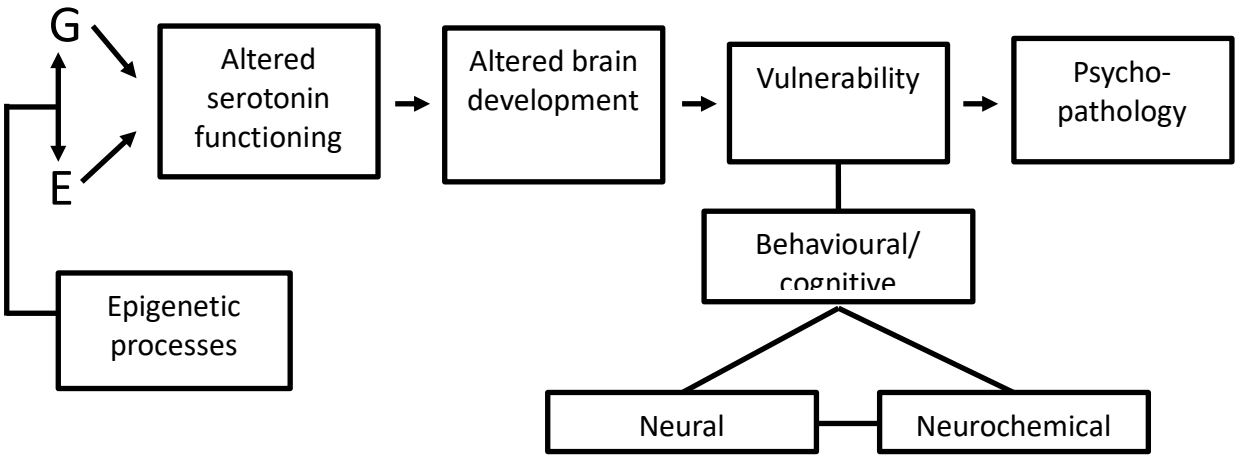

Figure 3. Neurodevelopmental diathesis stress model adapted from Booij and colleagues (2015). E= environment, G = gene.

In addition to serotonin receptors, another important serotonergic protein implicated in depression is the serotonin transporter (5-HTT). Selective Serotonin Reuptake Inhibitors (SSRIs) act by inhibiting 5-HTT, which is thought to lead to an increase in serotonergic neurotransmission accounting for its effects (Morrissette & Stahl, 2014). However, the exact mechanisms of action of antidepressants and the role of 5-HTT in depression remain to be completely elucidated.

1.4.1.2 HPA Axis

Beyond the serotonergic system, another biological system which has been strongly implicated in depression is the hypothalamic-pituitary-adrenal (HPA) axis (Pariante & Lightman,

2008; Parker et al., 2003). The HPA axis is activated in times of perceived stress and leads to the initiation of the fight-or-flight response. When a threat is perceived, the amygdala responds, leading to stimulation of the autonomic sympathetic axis and the HPA axis. While the former is involved in the rapid response to stressors by directly stimulating the secretion of epinephrine by the adrenal glands, the latter is activated minutes later and involves a more complex cascade of hormones (Juruena et al., 2018). More specifically, corticotropin-releasing factor (CRF) is released from the paraventricular nucleus of the hypothalamus which, in turn, stimulates the release of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. ACTH stimulates the release of glucocorticoids such as cortisol from the adrenal cortex which bind to glucocorticoid (GR) and mineralocorticoid (MR) receptors in order to regulate functions such as metabolism, immunity and cardiovascular output (Juruena et al., 2018; Pariante & Lightman, 2008). Corticosteroid binding at GRs and MRs at various sites in the brain, including the hypothalamus and pituitary, mediates a negative feedback mechanism, decreasing the release of CRH and ACTH (Keller-Wood, 2015).

Depression is associated with dysregulated basal cortisol functioning (e.g., diurnal or cortisol awakening response; Juruena et al., 2018; Rothe et al., 2020). Twenty to eighty percent of depressed individuals display hypersecretion of cortisol, greater number of episodes of cortisol secretions and greater cortisol release upon secretion (Guerry & Hastings, 2011; Stetler & Miller, 2011). In addition, individuals with depression also display an altered cortisol response to psychosocial stressors (e.g., preparing and giving a speech to and/or performing mental arithmetic in from of a panel of judges). More specifically, adults, adolescents and school-aged children with depression have been found to show an altered cortisol response to psychosocial stressors compared to controls (Foley & Kirschbaum, 2010; Guerry & Hastings,

2011; Lopez-Duran et al., 2009; U. Rao et al., 2008; Rothe et al., 2020; Zorn et al., 2017). Finally, depressed adults, adolescents and children also display a greater cortisol response (non-suppression) in response to administration of dexamethasone (DEX) or simultaneous DEX/CRH, compounds which are administered in an attempt to initiate the negative feedback loop within the HPA axis (Guerry & Hastings, 2011; Ising et al., 2005; Rothe et al., 2020; Sher, 2006). Such results suggest dysfunction of this negative feedback mechanism (Guerry & Hastings, 2011; Ising et al., 2005; Lopez-Duran et al., 2009; Mokhtari et al., 2013; Sher, 2006; Stetler & Miller, 2011).

Interestingly, there is evidence from a number of studies, including prospective longitudinal studies, that altered HPA axis functioning, as indicated by elevated daytime cortisol levels or cortisol awakening response (CAR) levels, in adolescents may represent a vulnerability marker for the later development affective disorders (Adam et al., 2010; Ellenbogen et al., 2011; Goodyer et al., 2003; Halligan et al., 2007; Vrshek-Schallhorn et al., 2013). For example, Halligan and colleagues (2007) found that an elevated morning and evening cortisol levels in 13-year-old adolescents predicted depressive symptomatology at 16 years of age. In another study, elevated cortisol awakening response in adolescents aged between 16 and 18 years was associated with greater risk for MDD at 1 year and 2.5 years later (Adam et al., 2010; Vrshek-Schallhorn et al., 2013). Thus, altered HPA axis functioning may serve as an important early marker in youth at risk for depression with the potential of guiding intervention programs. Further, the preceding findings are in line with research that suggests that prolonged cortisol release may induce alterations in brain regions key for emotion regulation, such as the hippocampus and amygdala (Lupien et al., 1998; Pagliaccio et al., 2014).

1.4.2 Genetics

It is well-established that there is a relatively strong genetic component to depression; heritability estimates from twin studies range from 35-40% (Shih et al., 2004; Sullivan et al., 2000). Two genes which have been most commonly associated with depression in the context of life stress are reviewed below; the *SLC6A4* gene and the *FKBP5* gene.

1.4.2.1 Serotonin Transporter Gene (*SLC6A4*)

Since its discovery as the target of antidepressant medications, the serotonin transporter (5-HTT) has received much attention in the context of depression, as has the serotonin transporter gene (*SLC6A4*). The serotonin transporter is a protein involved in the reuptake of serotonin from the synaptic cleft. Both selective serotonin reuptake inhibitors (SSRIs) and serotonin norepinephrine reuptake inhibitors (SNRIs) block the activity of this protein, resulting in increased levels of serotonin in the synaptic cleft. 5-HTT, along with other serotonergic proteins, plays a role in brain development and is expressed in a number of frontolimbic regions such as the cingulate cortex, amygdala, hippocampus and insular cortex (Booij, Tremblay, et al., 2015; Kish et al., 2005; Varnäs et al., 2004). Like most genes investigated in depression, the association between depression and *SLC6A4* genotype appears to be moderated by the presence of life stress (Caspi et al., 2003; Gatt et al., 2009; Nugent et al., 2011; Vaske et al., 2009). One of the earliest studies to show this association was conducted by Caspi and colleagues (2003) who studied a single nucleotide polymorphism (SNP) in the promoter regions of the serotonin transporter gene. They showed that individuals who had at least one copy of the risk allele were at greater risk of developing depression in the face of stressful life events. However, it should be noted that later findings have been mixed, with one meta-analysis failing to find an association and another finding that the strength of the association may vary by type of environmental stressor (Karg et al., 2011; Risch et al., 2009). It may also be important to consider a

developmental model during which stress at critical periods of development interact with *SLC6A4* genotype to confer a vulnerability to depression. Such a model is consistent with the neurodevelopmental diathesis-stress model outlined previously which is in agreement with findings that the serotonergic system undergoes key periods of development and that some aspects of the serotonergic system do not mature until adulthood (Booij, Tremblay, et al., 2015). Further, inconsistencies in the link between *SLC6A4* genotype, stressful life events and depression may be suggestive of the interaction with other genes, or the existence of other biological mechanisms which must be included in our models, such as epigenetic mechanisms, which will be reviewed later.

1.4.2.2 FK506 binding protein 5 gene (*FKBP5*)

Another gene which has been implicated in depression is the FK506 binding protein 5 (*FKBP5*) gene (Rao et al., 2016). Located on chromosome 6p21.31 (GRCh38), this gene codes for the FK506 binding protein 51 (FKBP5; also known as FKBP51 or FKBP54), a protein involved in the regulation of glucocorticoid receptor sensitivity (Fries et al., 2017). When FKBP5 is bound to the GR complex, cortisol binds to the GR complex with decreased affinity, decreasing GR translocation to the nucleus and thus decreasing the negative-feedback loop within the HPA axis. In this way, FKBP5 is thought to be an amplifier of the stress response (Matosin et al., 2018).

Transcription of the *FKBP5* gene is itself regulated, in part, by glucocorticoids. When glucocorticoids bind to GR, GR translocates to the nucleus and acts as a transcription factor, binding to DNA called glucocorticoid response elements which act as enhancers or suppressors (Matosin et al., 2018). Induction of *FKBP5* mRNA by GCs is moderated by a haplotype at the *FKBP5* gene which includes rs380033, rs9296158, rs1360780 and rs9479989, the functional

variant of which is the SNP rs1360780 (Matosin et al., 2018). Rs1360780 is associated with altered stress response in both healthy and clinical populations, disruption of the HPA-axis negative feedback and prolonged cortisol response after psychosocial stress (Matosin et al., 2018). Further, variation in *FKBP5* genotype is associated with differences in brain structure and function of limbic regions (Matosin et al., 2018; Tozzi et al., 2018). Clinically, carriers of an *FKBP5* risk allele are more likely to experience PTSD in the face of trauma compared to carriers of the protective allele, and depressed individuals carrying the risk allele experience a greater number of depressive episodes, better response to antidepressant and greater susceptibility to early life stress (Binder et al., 2004; Keijser et al., 2021; Klengel et al., 2013; Q. Wang et al., 2018). Further, FKBP5 expression has been shown to predict response to antidepressant medication in depression (Ising et al., 2019).

1.4.3 Brain structure and function

1.4.3.1 Etiological Models

Current neurobiological models broadly posit that depression results from increased bottom-up reactivity to emotional stimuli in limbic regions in combination with reduced capacity for top-down cognitive control over these limbic regions (Disner et al., 2011; Mayberg et al., 1999; M. Phillips et al., 2008; Pizzagalli & Roberts, 2022). Regions involved in the bottom-up production of the emotional response include the amygdala, hippocampus, subgenual cingulate and ventral and rostral anterior cingulate cortex. Regions involved in cognitive control include the dorsolateral prefrontal cortex, ventrolateral prefrontal cortex and dorsal anterior cingulate cortex (Disner et al., 2011). Results from structural, functional, and resting-state connectivity studies provide support for this model and are outlined below.

1.4.3.2 Brain structure

Structural brain imaging results across studies are often inconsistent, likely due to variation in sample size, demographic and clinical characteristics as well as imaging protocols (Wang et al., 2016). Thus, meta-analyses have shown to be particularly important to uncover enduring effects seen across studies.

Meta-analyses suggest that depression is characterized by both increases and decreases in gray matter density in a number of brain regions, many of which are part of the frontolimbic system (Bora et al., 2012; W. Wang et al., 2017; Wise et al., 2017; Zheng et al., 2021). In particular, depression appears to be most consistently associated with decreased gray matter in the right dorsolateral prefrontal cortex, right inferior temporal gyrus and right supplementary motor area as well as increased gray matter in the right insula, right putamen, left temporal pole, left lateral orbitofrontal cortex and bilateral hypothalamus. Although alterations in the amygdala, hippocampus and anterior cingulate are some of the region's most often discussed in relation to depression, reports of decline in these regions show some inconsistency (Bora et al., 2012; Frodl et al., 2002, 2008; Gray et al., 2020; Salvadore et al., 2011; W. Wang et al., 2017; Wise et al., 2017; Zheng et al., 2021). However, there is research to suggest that gray matter declines in these regions occurs over the course of the illness, perhaps suggesting that may be associated with the disease state itself and may occur as a reaction to chronic stress (Espinoza Oyarce et al., 2020; Frodl et al., 2008; Stratmann et al., 2014). Thus, selection criteria across meta-analyses (e.g., using first episode sample vs. multiple episodes) may explain these discrepancies. In addition, there is evidence that reduced amygdala volume is associated not with depression per se, but with the presence of a comorbid anxiety disorder (Bora et al., 2012; Espinoza Oyarce et al., 2020). Anxiety disorders and depression are highly comorbid and anxiety disorders, especially social anxiety or separation anxiety disorder, frequently precede

depression in adolescence and childhood (Cummings, Caporino & Kendall, 2014; Giedd, Keshavan & Paus, 2008). Further, individuals with generalized anxiety disorder likely share a similar genetic and/or environmental predisposition, making it difficult to separate these disorders conceptually, perhaps making the distinction between the two less useful (Cummings et al., 2014).

1.4.3.3 Brain function

In addition to differences in brain structure, individuals with depression display altered blood oxygen level dependant (BOLD) response during a variety of tasks, most notably those involving emotion and reward processing. However, there is great inconsistency across studies, with one meta-analysis of cognitive and emotion processing tasks failing to find any difference between depressed individuals and controls, which the authors attribute to a lack of spatial convergence across findings (Müller et al., 2017). Controlling for medication status, comorbidities and developmental stage (i.e., geriatric vs. adult depression) had no effect on results, although using only studies with corrected statistics revealed convergence in the left thalamus and hippocampus in response to negative stimuli. They suggest that heterogeneity in experimental design and procedures (including choices of analytic techniques) are a likely contributor to lack of spatial convergence (Müller et al., 2017).

Other meta-analyses have found consistencies across studies. Evidence from one meta-analysis of first-episode, drug-naïve individuals provided evidence for functional alterations which both converge and diverge with structural alterations (Wang et al., 2017). Co-joint alterations were found in the left lateral orbitofrontal cortex and right supplementary area, with dissociated alterations in a number of frontolimbic regions including the hippocampus and right dorsolateral prefrontal cortex. Another meta-analysis reported consistent findings of altered

activation in frontolimbic regions including increased amygdala and hippocampal reactivity to negative stimuli, decreased basal activity in response to positive stimuli, increased activity to positive stimuli in the ganglia/thalamus and increased ventro-rostral anterior cingulate cortex to emotional stimuli more generally (Jaworska et al., 2015). However, as with Müller and colleagues (2017), they report a lack of consistencies in prefrontal regions, with the exception of medial prefrontal cortex hyperactivity. Findings from a meta-analysis of resting-state fMRI data in medication naïve individuals with a first episode of depression by Ma and colleagues (2019) supports the involvement of the hippocampus and amygdala in depression. More recently, Gray and colleagues conducted a combined meta-analysis of structural and functional resting-state studies and found convergent findings of the involvement of the subgenual cingulate cortex, hippocampus, amygdala, and putamen in depression (2020).

Emotional valence seems to be an important factor in moderating the neural response to emotional stimuli in depression with one meta-analysis finding that individuals with depression displayed activity in the opposite direction (hyper *vs*. hypoactivity) in frontolimbic regions depending on whether they were viewing positive or negative stimuli (Groenewold et al., 2013). More specifically, they displayed hypoactivity in response to positive and hyperactivity in response to negative stimuli in a number of regions such as the amygdala, striatum and anterior cingulate cortex (with the exception of reduced activity in the left dorsolateral prefrontal cortex for negative stimuli and increased activity in the orbitofrontal cortex for positive stimuli). Also of note, response in the anterior cingulate cortex was modulated by facial *vs*. non-facial stimuli, with individuals with depression showing increased activation relative to controls during facial processing and decreased activation during non-facial processing, regardless of stimulus valence (Groenewold et al., 2013).

In summary, meta-analyses have shown alterations in frontolimbic functioning in depression. In particular, the anterior cingulate cortex (including the subgenual cingulate cortex), hippocampus and amygdala emerge as core regions involved in emotion processing alterations observed in depression. Further, other prefrontal and limbic areas emerge such as the orbitofrontal cortex, dorsolateral prefrontal cortex and striatum.

1.4.3.4 Brain connectivity

Advances in our understanding of the coordinated functioning of multiple brain regions has led to a wealth of research examining not only the isolated functioning of specific regions, but also the coordinated activity of intrinsic brain networks. Resting-state functional connectivity is a method which allows for the study of core intrinsic brain networks active while individuals are at rest (Greicius et al., 2003). More specifically, resting-state functional connectivity analysis allows researchers to examine correlations in spontaneous activity across brain regions and over time when no information is presented externally, offering the opportunity to characterize intrinsic networks which are spatially distinct and are thought to underlie specific brain functions active in the absence of a tasks (He, Snyder, Zemple, Smyth, & Raichle, 2008). Such intrinsic brain networks are thought to underlie complex cognitive and emotional functions and are present as early as childhood, with the core nodes of these networks remaining consistent into adulthood (Jolles et al., 2011; Thomason et al., 2011).

A meta-analyses by Kaiser and colleagues (2015) suggested that depression is characterized by a number of alterations within and between such brain networks. Firstly, individuals with depression display *hyper*connectivity between the medial prefrontal cortex and other seeds within the default mode network, a network thought to support self-referential thought and internally focused attention and which has been implicated in depressive rumination

(Andrews-Hanna, 2012; Hamilton et al., 2011; Kaiser et al., 2015; H.-X. Zhou et al., 2020). The anterior portion of the default mode network, which is centered on the medial prefrontal cortex, appears to be more involved in self-referential processing and emotion regulation through its connection with the amygdala while the posterior region, consisting of the posterior cingulate cortex and precuneus is more involved in memory processing in concert with the hippocampus (Andrews-Hanna, 2012; Raichle, 2015; Vincent et al., 2006). Further, individuals with MDD also display *hypo*connectivity within the frontoparietal network, which is involved in the top-down regulation of attention and emotion, perhaps suggesting a disruption in the coordination of cognitive control systems. A mega-analysis focusing solely on the default mode network found a reduction in functional connectivity in the default mode network only in individuals with recurrent MDD (Yan et al., 2019). Further, they found that, in those with recurrent MDD, greater functional connectivity was associated with greater symptom severity.

In addition to within-network connectivity, studies have also investigated between-network connectivity with the aim to understand how networks involved in externally and internally directed attention and emotional responses interact with those involved in emotion regulation. Depression appears to be associated with altered connectivity between the frontoparietal network and networks involved in externally (dorsal attention network) or internally (default mode network) directed attention. In particular, the dorsolateral prefrontal cortex within the frontoparietal network, an area important for top-down control of cognitive functions, was hyperconnected with internally-directed attention systems (default mode network) and hypo-connected with externally directed attention systems (dorsal attention network), which may support the tendency for depressive rumination. This hyperconnectivity may be specific to anterior default mode network regions as one review found hypoconnectivity between the

frontoparietal network and posterior default mode network regions (Mulders et al., 2015). Further, depressed individuals show hypoconnectivity between salience network regions involved in emotion processing and medial prefrontal regions involved in emotion regulation. In particular, hypoconnectivity was associated with blunted positive communication (medial prefrontal cortex-anterior cingulate cortex) and excessive negative communication (medial prefrontal cortex-amygdala) between limbic regions involved in mediating affective response (Mulders et al., 2015). MDD is also associated with hypoconnectivity between various salience network seeds and the posterior default mode network, but increased connectivity between the salience network and the anterior default mode network (Kaiser et al., 2015; Mulders et al., 2015). Further, there is also evidence for altered connectivity between the salience network and frontoparietal network, perhaps suggesting altered ability to recruit cognitive resources in response to salient events. However, both *hypo* and *hyper* connectivity was observed, which is perhaps reflective of the importance of considering the valence of stimuli (Kaiser et al., 2015) given that depressed individuals show an increased attention towards negative stimuli and decreased attention towards positive stimuli compared to controls (Peckham et al., 2010).

Thus, findings from resting-state functional connectivity studies support the model of depression as arising from an imbalance between top-down emotion regulation and bottom-up emotion production processes. Further, they also expand this model to suggest that depression is not only associated with difficulties regulating affect , but also altered communication between top-down control and networks important for internally vs. externally directed attention (Kaiser et al., 2015). Therefore, they provide an account of both affective and cognitive characteristics of depression. More specifically, resting-state functional connectivity may account for a number of known phenomena in depression including poor emotion regulation (frontoparietal network-

default mode network and frontoparietal network hypoconnectivity), excessive rumination/internally directed attention (default mode network hyperconnectivity, frontoparietal network-default mode network hyperconnectivity) in combination with decreased externally directed attention (frontoparietal network-dorsal attention network hypoconnectivity) as well as attentional biases towards potentially negative stimuli (altered frontoparietal network-salience network connectivity).

1.4.3.5 Brain imaging and depression: Conclusions

Though inconsistencies across studies exist, a number of meta-analyses investigating structural and functional findings point to involvement of key frontolimbic regions in depression, namely the amygdala, hippocampus, orbitofrontal cortex, ventromedial prefrontal cortex, anterior cingulate cortex and striatum. Some inconsistencies in the literature may be explained by distinguishing brain alterations associated with depression itself from comorbid anxiety, as well as brain alterations which are associated with early depression from those that are caused by the disease state itself. In particular, there is evidence that altered function and structure of the amygdala may be more strongly associated with anxiety, and that reduced hippocampal volume may be a result of prolonged depressive state.

Studies investigating functional connectivity suggest that depressive symptoms may be more closely associated with the coordinated activity of multiple brain regions in the form of networks as opposed to activity in any one region. Indeed, there is now strong evidence associating specific depressive tendencies with the function of specific brain networks and their interaction (i.e., rumination and the default mode network). Ultimately, a combination of network-based analyses and analyses investigating specific regions will be necessary to have a more complete understanding of MDD.

1.4.3.6 Depression, neurobiology and typical vs. atypical development

In addition to evidence that the brains of individuals with depression differ from healthy controls, there is also evidence that their neural development differs as well. In typical development, cortical gray matter volume in the frontal and parietal lobes increases until puberty after which it gradually declines, with the rate of decline varying across regions (Fuhrmann et al., 2015; Paus et al., 2008). White matter volume, on the other hand, increases linearly throughout childhood and adolescence until as late as the 30s (Fuhrmann et al., 2015; Paus et al., 2008). Further, there are also a number of typical developmental changes in neurotransmitter systems that occur throughout the lifespan. For example, animal models suggest that the serotonergic system continues to develop until adulthood and undergoes important changes during adolescence that depends on the region being investigated (Booij, Tremblay, et al., 2015). Notably, 5-HTT density increases until adulthood in the forebrain (Moll et al., 2000). In the dorsal raphe, 5-HTT levels decline until they reach a stable level during adolescence, which persists into adulthood (Sidor et al., 2010). $5HT_{1A}$ levels appear to increase rapidly in the raphe and hippocampus (Sidor et al., 2010). There is ample evidence to suggest that alterations in the serotonergic system can lead to structural and functional changes in key frontolimbic regions such as the amygdala, anterior cingulate cortex and medial prefrontal cortex (Booij, Tremblay, et al., 2015; Holmes, 2008)

There is evidence that compared to individuals who do not develop depression, individuals who go on to develop depression display developmental patterns which diverge from typical development . For example, Pagliaccio and colleagues (2020) found that children of parents with a history of depression displayed smaller right putamen volume compared to healthy children. Further, a longitudinal study found that individuals who would go on to

develop depression displayed attenuated hippocampal growth as well as attenuated putamen reduction as well as altered amygdala development (Whittle et al., 2014). Interestingly, the nature of altered amygdala development was mediated by gender, with females displaying exaggerated growth and males attenuated growth. Further, in females, but not males, decreases in nucleus accumbens volume were associated with depression (Whittle et al., 2014). Another longitudinal study of adolescents scanned twice between ages 11 and 15 found that depressive symptomatology was associated with blunted development of reward related ventral striatum activity (Hanson et al., 2015). In a sample of adolescent females, blunted reward positivity, an event-related potential indicating monetary gain (*vs*. loss), predicted greater depression scores 18 months later. (Nelson et al., 2016). Similar findings of altered structural and functional development in adolescents who go on to develop depression have been found in prefrontal regions such as the medial prefrontal cortex and lateral orbitofrontal cortex (Bos et al., 2018; Vilgis et al., 2018). Some differences are seen as early as childhood, with children at high risk for depression showing greater activation in the ventrolateral and ventromedial prefrontal cortex when processing positive self-referential words (P. Liu et al., 2020).

1.4.3.7 Brain structure, function and connectivity in youth *vs*. adults

Although depressed youth have received less attention than their adult counterparts in the neuroimaging literature, there is now emerging evidence suggesting that adolescence is associated with neurobiological alterations which are both consistent and inconsistent with findings in adults.

Various findings from the functional imaging literature in adolescents appear to align well with those found in adults. One review of emotion processing studies in younger (13-18) and older (19-25) youth adolescent found that results generally aligned with those in adult

studies, and suggested that adolescents display a hyperactive "extended medial network" consisting of the anterior cingulate cortex (specifically the pregenual and subgenual anterior cingulate cortex), ventromedial prefrontal cortex, orbitofrontal cortex, striatum and amygdala (Kerestes et al., 2014). They reported that this was observed across five domains of fMRI tasks: emotion processing, cognitive control, affective cognition, reward processing and resting-state functional connectivity. They did point out, however that there were some differences between adolescents and adults in neural activity during tasks of cognitive control and affective cognition. In particular, during studies of emotional distraction, only the amygdala was consistently hyperactive across the youth and adult literature. For cognitive reappraisal studies, they report a lack of significant findings during cognitive reappraisal of negative emotional stimuli in youth compared to adults, which they propose may be due to that cognitive impairments tend to become more severe with recurrent MDD. They also suggest that later maturation of prefrontal regions (which are vital for cognitive control) compared to subcortical regions may explain some of these differences. More recently, a meta-analytic review of electroencephalogram (EEG) and functional magnetic resonance imaging (fMRI) studies related to reward processing in depression found reduced striatal activation in anticipation of reward in depression, with this effect being larger in adolescence (Keren et al., 2018).

Another meta-analysis showed that, consistent with reports by Kerestes and colleauges (2014), studies comparing depressed adolescents to healthy controls implicate many similar regions to adult studies (Miller et al., 2015). In their meta-analysis, they found that youth MDD was associated with overactive in central hubs of the default mode network including the subgenual anterior cingulate cortex and thalamus. They also suggest evidence for difficulty in shifting from the default mode network to the task-positive network (a network which, as its

name suggests is active when individuals are completing tasks) during cognitively demanding tasks, as well as anhedonia resulting from cuneus and posterior insula hypoactivity during reward processing. However, in adolescents, they found over-activity in emotion regulation regions associated with ineffective regulation during affective processing, which appears to be inconsistent with the adult literature. They suggest that this may represent a compensatory shift that occurs over development or the course of illness.

A more recent meta-analysis investigated neural response to emotion processing tasks in studies involving adults with MDD *vs*. those involving youth with MDD (X. Li & Wang, 2021). They found that great degree of overlap in affected structures in participants with MDD compared to controls in both adult and youth studies, particularly in the anterior cingulate cortex, insula, superior and middle temporal gyrus, and occipital cortex. However, they found that hyperactivity in the striatum was only observed youth, while adults showed more pronounced increases in response to emotional stimuli in the insula, middle frontal gyrus and hippocampus, as well as less altered activity in the middle temporal gyrus, middle occipital gyrus, lingual gyrus and striatum. The authors interpreted these findings as suggesting that adults may be more impaired in emotional appraisal and reactivity, while youth may be more impaired in emotional perception (X. Li & Wang, 2021).

Another recent meta-analysis found increased bilateral amygdala activation in youth with depressive and other internalizing disorders, with findings most robust for anxiety disorders (Ashworth et al., 2021). Interestingly, there is some evidence that while the amygdala may play an important role in both adolescent and adult depression, amygdala resting-state functional connectivity dysfunction may occur in different brain networks in adults and adolescents. More precisely, results from a recent meta-analysis suggest that adults may experience more amygdala

resting-state functional connectivity dysfunction in the affective network, reflecting emotional dysregulation, whereas adolescents may experience more amygdala resting-state functional connectivity dysregulation in networks related to cognitive control, perhaps reflecting deficits in affective cognition (Tang et al., 2018).

In terms of structural data, an international study of 2148 individuals with MDD and 7957 controls scanned at 20 different centres suggests that structural alterations in adolescents vary from those in adults, with adults displaying cortical thinning in the orbitofrontal cortex, anterior cingulate cortex, PCC, insula and temporal lobes, and adolescents displaying decreased surface in the medial orbitofrontal cortex and SFG, as well as primary and higher-order visual, somatosensory and motor areas (Schmaal et al., 2017). However, more studies (including reviews and meta-analyses) will be necessary to confirm these findings.

Together, these results suggest both overlap and differences between brain processes in adolescents and adults. Understanding these differences further, as well as biological factors which may account for these differences, is an important topic for future research as they may suggest alternate courses of treatment and inform early intervention programs.

1.5 Early life adversity

1.5.1 Early life adversity and depression

While the heritability of depression appears to be substantial, a substantial majority of the variability in MDD (~60%) is accounted for environmental factors (American Psychiatric Association, 2013). Therefore, equally important as characterizing neurobiological characteristics of depression is understanding how these characteristics develop in response to, or are shaped by, environmental factors. One of the strongest environmental predictors of depression is early life adversity (Chapman et al., 2004; Zare et al., 2018). Childhood maltreatment alone is associated not only with risk of depression, but also unfavorable illness

course in the form of recurrent and persistent episodes and lack of response or remission during treatment (Dahl et al., 2017). In addition to childhood maltreatment, other forms of early life adversity such as the presence of parental illness, incarceration, death, disability or and psychiatric diagnosis, disruption of the family and out-of-home care are also associated with depression (Nanni et al., 2012).

The link between early life adversity and mental health is not specific to depression (Green et al., 2010). One longitudinal study investigating the link between early childhood stress and later psychopathology found that early stress in the family was associated equally with both internalizing and external disorders, but had no disorder specific effects (Conway et al., 2018). In a survey of over 50 000 adults across 21 countries conducted by the WHO, early life adversity was associated with 29.8% of the 20 mental disorders investigated, with little specific effect on any given disorder (Kessler et al., 2010). Further, adverse experiences during childhood associated with poor family functioning (e.g., parental mental illness, abuse and neglect) were the strongest predictors of the development mental health disorders. Interestingly, early life adversity is associated with not only mental, but also physical health: the presence of six or more adverse childhood experiences associated with a 20-year shorter lifespan (Brown et al., 2009).

While the link between early life adversity and depression is non-specific, there is significant overlap between the effects of adversity on the brain and those of depression, suggesting that adversity may lead to changes in brain development which predispose individuals to developing depression in the face of stressors later in life. One line of thinking suggests that early life adversity may interact with specific genetic and neurobiological vulnerabilities characteristic of depression to produce depressive symptoms, as opposed to symptoms characteristic of other psychiatric disorders associated with childhood adversity. Indeed, there is

now substantial evidence that early life adversity interacts with key genes, such as the 5-HTT, *FKBP5* and *BDNF* genes, to lead to depressive symptoms and underlie morphological changes associated with depression (Aguilera et al., 2009; Keijser et al., 2021; Y.-K. Kim et al., 2018; Tozzi et al., 2016; Uher & McGuffin, 2010; Q. Wang et al., 2018).

1.5.2 Early life adversity and neurobiology

Early life adversity is associated with neurobiological alterations which bear great resemblance to those seen in depression. The effect of childhood adversity on the brain is thought to be largely mediated through its effects on HPA axis functioning, with repeated and excessive HPA axis activation leading to sustained overabundance of glucocorticoids in the brain (Booij et al., 2013). Research to date suggests that childhood maltreatment is associated broadly with altered development of the "threat-detection and response circuit," which also forms part of the negative valence system (LeDoux, 1996, 2003; Teicher et al., 2016). Core areas of this circuitry associated with childhood maltreatment include the anterior cingulate cortex, medial prefrontal cortex, orbitofrontal cortex, hippocampus, thalamus and amygdala (Kraaijenvanger et al., 2020; Teicher et al., 2016). Further, many of these regions are also form part of the reward circuitry of the brain.

There is evidence that childhood maltreatment influences depressive symptoms through its effect on connectivity between the prefrontal cortex and ventral striatum in response to reward (Hanson et al., 2015, 2017). More specifically, Hanson and colleagues (2017) found that greater functional connectivity between the left ventral striatum and medial prefrontal cortex during a reward task in individuals who had experienced greater levels of childhood adversity in addition to greater levels of recent life stress. They also found that this pattern of connectivity was associated with increased levels of depression and that it partially mediated the link between

childhood adversity and internalizing symptoms. Interestingly, there is some evidence that different types of childhood adversity (e.g., maternal deprivation vs. emotional deprivation vs. trauma) may differential affect reward processing and frontostriatal white matter tracts (Dennison et al., 2019)

In addition to the limbic system, some have stressed the link between childhood adversity and development of perceptual systems (Teicher et al., 2016). More specifically, Teicher and colleagues suggest that various forms of abuse lead to altered development of sensory areas which correspond the type of abuse experience (e.g., verbal abuse and auditory cortex, interparental violence and visual cortex). Interestingly, one study found that childhood sexual abuse was associated with thinning of the area somatosensory cortex representing the genitals (Heim et al., 2013).

On a network level, childhood maltreatment is associated with reduced centrality, a measure of the importance of a given node within a network, in the left anterior cingulate cortex, temporal pole and middle frontal gyrus, as well as increased centrality in the right anterior insula and precuneus (Teicher et al., 2014). There is now an accumulating body of evidence that childhood adversity is associated with altered connectivity *within* the default mode network and salience network, as well as reduced connectivity *between* the default mode network and Salience Network (Rakesh et al., 2021; Teicher et al., 2016). Interestingly, increased default mode network activity has been associated with depressive rumination while decreased activity has been associated with depersonalization and derealization (Hamilton et al., 2015; Tursich et al., 2015; H.-X. Zhou et al., 2020).

1.6 Epigenetics: Bridging the gap between genes and the environment

Given the link between early life adversity, psychopathology and brain development, a crucial question research has been tasked with answering is the following: What are the biological mechanisms by which early life experiences can lead to lasting changes in brain development? A fruitful body of research has pointed to epigenetic mechanisms as strong candidate for one of such biological mechanisms (Bale, 2015; Kundakovic & Champagne, 2015; Lin & Tsai, 2019; Meaney, 2010, 2017; Nestler et al., 2016).

1.6.1 Genetic vs. epigenetic mechanisms

While the genetic code is stable throughout the lifespan and identical across different cell types, gene expression is not. Epigenetics refers to heritable and potentially enduring changes to DNA and chromatin that can affect gene expression without affecting the DNA sequence itself (Levenson & Sweatt, 2006; Morris et al., 2010). The term epigenetics encompasses a variety of mechanisms including modifications to histone proteins and to DNA directly (acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation; Jenuwein & Allis, 2001).

Variation in gene expression allows for different cells to express different phenotypes. Epigenetic mechanisms are responsible for the long-term programming of gene expression patterns that permit such variation to occur. For example, epigenetic mechanisms play a critical role in development processes such as X chromosome inactivation and cell differentiation (Feng et al., 2007; Heard, 2004). Two of the most well understood and studied of these mechanisms in the context of mental health are DNA methylation and histone deacetylation.

1.6.2 DNA methylation

DNA methylation is an epigenetic process whereby the nucleotide cytosine is methylated predominately where it is found next to a guanine (at what are referred to as CpG sites) by a class of enzymes known as DNA methyltransferases (Dnmts) in such a way that typically

silences gene transcription (Meaney, 2010). DNA methylation can alter gene expression through two mechanisms. First, methylation allows for the binding of methyl-CpG-binding domain proteins (MBDs) which recruit proteins involved in the compacting of chromatin, reducing access by transcription factors. Second, methylation of CpG islands (regions of DNA with a high density of CpG sites) in or near promoter regions can also cause gene silencing by inhibiting access of transcription factors directly. Both of these mechanisms allow for the stable, yet reversible, programming and re-programming of gene expression (Bestor, 1998; Bird, 2002).

While methylated cytosine was first identified in 1925, the role of DNA methylation in inhibiting gene transcription was not discovered until the 1980s (Mattei et al., 2022). Since then, DNA methylation has been shown to play a critical role in human development. For example, DNA methylation has been implicated in genetic imprinting and X-chromosome inactivation and plays an active role in guiding embryonic, germline and somatic cell development(Greenberg & Bourc'his, 2019). On the other end of the life cycle, DNA methylation has been implicated in aging and age related cognitive decline, as well as cancer (Skvortsova et al., 2019; Xu, 2015). In more recent years, beginning in the 2000's a wealth of research has accumulated implicating DNA methylation in psychiatric disorders including schizophrenia, eating disorders, autism spectrum disorder, post-traumatic stress disorder and major depressive disorder (C. Liu et al., 2018).

1.6.3 Histone (de)acetylation

Another epigenetic mechanism allowing for the regulation of gene expression is the modification of chromatin structure through histone acetylation. The basic unit of chromatin is the nucleosome, a 146 base pair (bp) long section of DNA wrapped around eight histone proteins. Histone proteins possess N-terminal tails which can be acetylated or deacetylated at

lysine residues. Two enzyme families allow for the regulation of histone acetylation, the histone acetyltransferases (HATs) and histone deaceylases (HDACs), which add and remove acetyl groups, respectively. Acetylation of histone protein tails by HATs neutralizes their positive charge, making them more strongly repel negatively charged DNA leading to a more relaxed chromatin state (Haberland et al., 2009). This typically leads to increased gene transcription. HDAC engages the opposite process, deacetylation of histone tails, leading to greater attraction to DNA and, typically, decreased gene transcription. In this way, HDACs, in concert with other histone modifying machinery, regulate the conformation of chromatin across a continuum from an inactive condensed state called heterochromatin to an active and open state called euchromatin (Nestler et al., 2016).

The HDACs are widely distributed throughout the body, including the brain. There are 5 main classes of HDACs: I, IIa, IIb, III, and IV. The class of HDACs thought to be the most pertinent to disease states are the class I HDACs which consist of HDAC 1, 2, 3 and 8 (Haberland et al., 2009). Class I HDACs are found in the cell nucleus and, with the exception of HDAC 8, are widely expressed throughout the brain (Haberland et al., 2009; Morris et al., 2010). There is evidence that HDAC 1 and 2 may act to regulate excitatory synapse maturation early in development (Akhtar et al., 2009). In mature neurons, it appears that histone acetylation is involved in the formation and extinction of conditioned fear responses (Morris et al., 2010). Histone acetylation in the hippocampus seems to be important for the learning of fear conditioned responses and HDAC inhibitors lead to enhanced LTP in the hippocampus and amygdala (Barrett & Wood, 2008; Levenson et al., 2004; Vecsey et al., 2007; Yeh, 2004).

Thus, basic research points to a potential role of HDACs in regulating learning and memory related processes which may be relevant to psychological disorders. Indeed, evidence

for the involvement of HDACs in psychopathology is growing (Misztak et al., 2018; H.-S. Park et al., 2021; Sild & Booij, 2019). Despite the evidence for the role of HDACs in regulating brain processing relevant to psychopathology, most research to date investigating epigenetic mechanisms in humans in relation to mental health has focused on DNA methylation. However, it is imperative that the role of HDACs in psychiatric disease be further elucidated in order to have a complete picture of the epigenetic basis of mental illness.

1.6.4 Epigenetics, early life adversity and depression

A seminal study by Weaver and colleagues (Weaver et al., 2004) showed that early life experience can influence HPA axis functioning through epigenetic mechanisms in such a way that alters stress related behaviours. These researchers studied rats whose mothers displayed high or low levels of maternal licking and grooming behaviour (LG-ABN) behaviour. Offspring of high LG-ABN mothers had previously been shown to be less fearful and to display more modest HPA axis response to stress compared to offspring of low LG-ABN. In this study, they showed that the reduced HPA-axis response to stress in offspring of high LG-ABN mothers was mediated by DNA methylation at the glucocorticoid receptor gene promoter, as well as co-occurring changes in histone acetylation. These results were taken as evidence that early life experience can affect the epigenome in a way which has lasting changes on both physiology and behaviour. Research in humans has since supported a link between early life experience, epigenetic mechanism and stress related behaviours and pathology. This includes DNA methylation at specific genes as well as broader histone modifications.

Research in humans has shown a link between *SLC6A4* hypermethylation and childhood trauma (Provenzi et al., 2016b). A recent study suggested that greater proximal family risk factors and childhood maltreatment may be associated with increased *SLC6A4* methylation act in

a dose dependent manner (F. Craig et al., 2021). A systematic review concluded that most studies investigating the link between *SLC6A4* methylation and depression have found that depression is associated with *SLC6A4* hypermethylation (M. Li et al., 2019). Further, they concluded that studies rated as more methodologically and statistically rigorous were more likely to find such an association. Their review supports the hypothesis that *SLC6A4* hypermethylation and, in turn, decreased *SLC6A4* expression and 5-HT reuptake may increase susceptibility to developing depression at critical stages of development (M. Li et al., 2019). Interestingly, *SLC6A4* methylation levels are also predictive of antidepressant response, with one study showing that *SLC6A4* hypomethylation was predictive of reduced response to antidepressant treatment (Schiele et al., 2021; Webb et al., 2020).

Similarly, *FKBP5* hypomethylation has been associated with both childhood adversity and depression, as well as other forms of psychopathology such as PTSD (Grasso et al., 2020; Klinger-König et al., 2019a; Matosin et al., 2018). There is evidence that alternations in methylation can be transmitted to offspring (Bierer et al., 2020; Grasso et al., 2020). Using *FKBP5* as a model, Matson, Halldorsdottir and Binder (2018) have proposed how epigenetic mechanisms may interact with pre-existing genetic differences in the context of psychopathology. More specifically, they suggest that a genetic predisposition towards increased *FKBP5* expression after activation of GR leads to delayed HPA-axis negative feedback, resulting in a prolonged cortisol response to stress or trauma which leads to demethylation at GREs in *FKBP5*, thus leading to even higher *FKBP5* expression over time. They hypothesize that might lead to lasting changes in limbic circuitry, thus predisposing individuals to psychopathology.

In addition to DNA methylation, histone modifications have also been investigated in the context of early adversity and depression (H.-S. Park et al., 2021). HDAC2 is the most investigated HDAC in the context of preclinical and clinical studies of depression, although HDAC5 has also received significant attention (Misztak et al., 2018). In rodents, chronic stress increases histone acetylation in limbic regions along with levels of HDAC2 (Uchida et al., 2018). Systemic administration of HDAC inhibitors or more specific injection of selective inhibitors into limbic regions has been shown to reduce depression-like symptoms in mice exposed to the chronic social defeat paradigm as well as maternal separation (Nestler et al., 2016). Given these findings, it has been hypothesized that increases in HDAC levels related to early stress and depression may lead to decreased transcription of genes important to neuronal plasticity (e.g., brain derived neurotrophic factor and glial cell-derived neurotrophic factor; Uchida et al., 2018). Indeed, greater levels of HDAC2 (Hobara et al., 2010; Otsuki et al., 2008; Takebayashi, Hisaoka, Nishida, Tsuchioka, Miyoshi, Kozuru, Hikasa, Okamoto, Shinno, Morinobu, et al., 2006), HDAC4 and HDAC 5 (Hobara et al., 2010; Iga et al., 2007) have been observed in peripheral tissues of depressed individuals (Uchida et al., 2018). Further, as with DNA methylation, changes in histone acetylation via HDACs and HATs appears to be a vital part of the response to antidepressant therapy (Lin & Tsai, 2019).

1.6.5 Peripheral DNA methylation as a marker for methylation in the brain: methodological issues and possible solutions

Research investigating epigenetic mechanisms in living humans relies on sampling peripheral tissues such as blood, saliva and buccal cells in order to study DNA methylation. This poses a problem as researchers are most concerned with DNA methylation in the brain, and not in the periphery. However, as noted by others, it is possible that peripheral measures of epigenetic regulation may reflect brain regulation (Nestler et al., 2016). Indeed, there is

evidence from animal studies that DNA methylation changes induced by glucocorticoids in the brain and peripheral blood cells are correlated; however further research needs to be done to break down associations by cell type, gene locus and brain region (Ewald et al., 2014; Husby, 2020; R. S. Lee et al., 2010; Matosin et al., 2018).

Further evidence for the validity of peripheral methylation measures comes from research in humans. Peripheral levels *SLC6A4* methylation have been found to be associated with in-vivo measures of brain serotonin synthesis (D. Wang et al., 2012). Further, both *SLC6A4* and *FKBP5* methylation measured in the periphery appear to be associated with brain structure, function and functional connectivity in depressed individuals and healthy controls, although the brain regions or networks implicated appears affected by the type of peripheral tissue analysed (Di Sante et al., 2018; Ismaylova et al., 2017; Ismaylova, Lévesque, et al., 2018; Tozzi et al., 2018). In addition, one study investigating methylation differences in rhesus macaque monkeys who experienced maternal vs surrogate–peer rearing showed that, while there were extensive differences in methylation between t-cells and prefrontal cortex cells, there were also similarities (Provencal et al., 2012). Perhaps most notably, a region upstream of the NR3C1 analog, A2D681, was more methylated in surrogate-peer reared monkeys in both cell types.

In an attempt to address the issue inherent in measuring epigenetic mechanisms in the periphery, Wang and colleagues (2014) have developed a radioactive tracer which can image HDAC density directly in the brain using Positron Emission Tomography (PET) imaging. The tracer developed ([^{11}C]Martinostat) binds selectively to HDAC 1, 2 and 3 (Class I) and 6 (Class IIb; C. Wang et al., 2014b) offering the opportunity to study HDAC subtypes implicated in depression and other psychiatric disorders *in vivo*. Validation studies of this tracer in both animal and human studies suggest that the tracer possesses biological processes which will allow

it to be used further in human studies investigating the epigenetic basis on psychopathology (C. Wang et al., 2014; Wey et al., 2015). Further human studies are now underway, with the first clinical study using this tracer finding that individuals with schizophrenia or schizoaffective disorder were found to have lower HDAC expression levels in the dorsolateral and dorsomedial and orbitofrontal regions of the prefrontal cortex (Gilbert et al., 2019). More recently, the same group found reduced HDAC density in the in the bilateral thalamus, orbitofrontal cortex, right hippocampus and right amygdala in individuals with bipolar disorder compared to controls (Tseng et al., 2020). To date, no study has yet investigated the link between [^{11}C]Martinostat density and early life adversity or depressive symptoms.

1.7 Summary and Goals

In summary, major depressive disorder is a common and pervasive mental health disorder which has a profound impact on both the individual and society. To date, research has been successful in taking the steps necessary to elucidate the genetic, epigenetic and neurobiological mechanisms underlying depression. However, despite these important advancements in our understanding of major depressive disorder, our knowledge of the genetics and epigenetics of depression in childhood and adolescence lags behind our understanding of adults. Moreover, our understanding of the epigenetics of depression in humans relies heavily on peripheral epigenetic measures. The present dissertation aimed to address these important limitations and expand our understanding of the genetics and epigenetics of depression across the lifespan.

First, while the genetic and environmental basis of the co-occurrence of externalizing and internalizing behaviours have been studied, the genetic and environmental contributions to their relationship across development have not. Study one sought to identify the genetic and environmental contributions to the longitudinal association between externalizing symptoms in

preschool and internalizing symptoms in early adolescence. Given the shared neurobiological underpinnings between externalizing and internalizing symptoms (e.g., serotonin system), we hypothesized that genetic factors would account for the shared variability between externalizing and internalizing symptoms over time.

Second, although both *SLC6A4* and *FKBP5* methylation have been studied in the context of major depressive disorder, research to date has for the most part focused on adulthood, with comparatively less attention paid to adolescence, a crucial time period in the development of depressive disorders. Specifically, the link between *SLC6A4* methylation and brain structure and function in adolescence has received little attention, while the link between *FKBP5* methylation and resting-state connectivity in adolescents had yet to be studied before our investigation. To address these gaps in the literature, in study two, we investigated the link between *SLC6A4/FKBP5* methylation and brain structure/function in adolescents with major depressive disorder and healthy controls.

Finally, due to ethical constraints associated with studying epigenetic mechanisms *in vivo*, research investigating the role of epigenetic mechanisms in mental health have focused either on peripheral epigenetic markers or post-mortem samples. In study three, we applied the novel [^{11}C]Martinostat tracer in combination with positron emission tomography imaging to study the association between histone deacetylase levels *in vivo*, depression vulnerability and early life adversity in a sample of healthy adults.

2. CHAPTER 2: A longitudinal examination of the genetic and environmental contributions to preschool externalizing symptoms and internalizing symptoms in early adolescence

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2.1 Abstract

While the genetic and environmental contributions to the co-occurrence of internalizing and externalizing symptoms have been investigated, the contributions to their association across time have not. This study examined the genetic and environmental contributions to the association between externalizing behaviours in preschool (60 months) and internalizing symptoms in adolescence (12 years) in a longitudinal sample of 1344 twins (50% girls; 85.6% White). The association between preschool externalizing and adolescent internalizing symptoms was accounted for by a strong genetic (r= .55, p = .02), but not environmental, correlation. The results suggest that genetic factors account for a temporally stable vulnerability to internalizing and externalizing symptoms and highlight the importance of incorporating elements of internalizing symptom prevention in interventions targeting externalizing symptoms.

2.2 Introduction

Internalizing disorders present a large burden to society, with major depressive disorder (MDD) alone being the leading cause of disability world-wide (World Health Organization, 2017). Developmentally, internalizing symptoms arise as early as childhood and the prevalence of internalizing disorders increases at the onset of puberty (Leigh & Clark, 2018; Thapar et al., 2012). Compared to externalizing symptoms, internalizing symptoms in childhood and adolescence are typically viewed as less problematic by parents, teachers and caregivers due to their more inward manifestation (de Lijster et al., 2017; Luby, 2010; Tandon et al., 2009) However, research suggests that childhood and adolescent internalizing symptomatology is associated with a number of serious negative psychological and functional outcomes into adulthood (Luby, 2010; Tandon et al., 2009; Thapar et al., 2012). It is therefore important to be able to identify those at risk for developing internalizing disorders in childhood and adolescence so that early prevention programs can be targeted appropriately.

Both genetic and environmental factors play a role in the etiology of internalizing disorders. Heritability estimates for internalizing disorders range from 11% to 72% during childhood and adolescence, stabilizing at approximately 30-40% in adulthood (Thompson et al., 2017). Environmental risk factors which may contribute to internalizing disorders include stressful life events, childhood abuse and neglect, and low social support(American Psychiatric Association, 2013; Tandon et al., 2009).

One well-established risk factor for internalizing disorders that can be reliably assessed as early as preschool is externalizing symptomatology (Luby, 2010). Indeed, childhood externalizing behaviours are predictive of internalizing symptoms in adolescence and adulthood (Loth et al., 2014; Mesman et al., 2001). This is unsurprising given the overlap in their

symptomatology – for example, anger and irritability are core symptoms of both internalizing (e.g., generalized anxiety disorder and MDD) and externalizing (e.g., oppositional defiance disorder) disorders. Importantly, previous twin studies suggest that the co-occurrence of internalizing and externalizing difficulties in childhood and adolescence is most consistently accounted for by a shared genetic factor (Cosgrove et al., 2011; Lahey et al., 2011; Mikolajewski et al., 2013). In contrast, certain unique environmental factors seem to be particularly important in shaping the expression of the aforementioned vulnerability throughout development. More specifically, whereas the stability of internalizing symptoms over time (the homotypic pathway) seems to be mediated by peer rejection, the transition of externalizing to internalizing symptoms (the heterotypic pathway) is mediated by academic difficulties and asociality (i.e., the absence of social interaction; Jobs et al., 2019; Masten et al., 2005; Mesman et al., 2001; Patterson & Stoolmiller, 1991).

Together, the preceding findings suggests that there exists a shared genetic vulnerability to externalizing and internalizing symptoms whose expression is shaped by the unique environment. However, to our knowledge, all studies have been cross-sectional and no study has yet examined the extent to which genetic or environmental factors might account for this shared vulnerability *over time*. Two particularly important time points worthy of investigation are preschool and early adolescence. Oppositional, aggressive and hyperactive behaviours are identifiable as early as preschool and have been reported to be consistent across childhood, with this stability accounted for largely by genetic factors (Lacourse et al., 2014; Petitclerc et al., 2011; Silberg et al., 1996). Early adolescence, on the other hand, represents a time where internalizing symptoms rise sharply and mental disorders such as MDD and anxiety disorders tend to first emerge (Leigh & Clark, 2018; Thapar et al., 2012).

The aim of the current study was to investigate the extent to which the longitudinal association between externalizing behaviours in early childhood and internalizing symptoms in adolescence is driven by genetic compared to environmental factors. To this end, we studied the predictive association between externalizing symptoms in preschool and later internalizing symptoms in early adolescence using a longitudinal twin design. Specifically, we employed common pathway modelling in a twin sample to assess genetic and environmental correlations across these timepoints. We expected that a genetic factor would underlie the shared variability among externalizing and internalizing symptoms across time. Given our specific hypothesis and the use of an adequately powered twin design, our analyses can be considered confirmatory in nature.

2.3 Method

2.3.1 Participants

Participants were recruited as part of the Quebec Newborn Twin Study (Boivin et al., 2019). All participants were born between April 1995 and December 1998 and were recruited from the Greater Montreal Area. In total, 989 families were contacted and 672 agreed to participate in the cohort and were included in the present study. Of the 672 twin pairs, 251 were MZ twins and 421 DZ. Of the MZ twin pairs, 123 were male-male pairs and 128 were female-female pairs. Of the DZ twin pairs, 109 were male-male pairs, 105 were female-female pairs and 207 were male-female pairs. Measures of externalizing symptoms (aggression, hyperactivity and opposition) were collected at 60 months while measures of internalizing symptoms (anxiety and depression) were collected at age 12. Measures of externalizing symptoms at 60 months were available for 190 monozygotic and 254 dizygotic twin pairs. Measures of internalizing symptoms at age 12 were available for 156 monozygotic and 232 dizygotic pairs. 85.6% of participants were White, 4.3% were Black, 0.3% were Indigenous, 1.7% were Southwest Asian

or North African, 2.4 % were Asian and 5.7% were of mixed ethnicity or another ethnicity. Household income and maternal education were assessed when children were 5 months old. Data regarding household income was missing for 10.1% of participants. Of participants who responded, 18.3% of participants had a household income below $20 000, 37.0 % had an income between $20 000 and $50 000, 31.4 % had an income between $50 000 and $80 000 and 13.2 % had an income over $80 000. 22.2% of mothers opted not to disclose their highest level of educational achievement and data was missing for 6.6%. Of participants whose response was obtained, 30.4 % had a bachelor's degree, 8.0 % had a graduate degree, 21.1% had a community college degree, 14.8% had a vocational degree. Further details regarding the characteristics of the cohort have been reported previously (e.g., Boivin et al., 2019).

2.3.2 Measures

In order to assess physical aggression, hyperactivity and opposition, mothers were administered a variation of the Children's Social Behaviour Questionnaire used in a number of previous studies working with data from the QNTS cohort (Lacourse et al., 2014; Petitclerc et al., 2011). Children were approximately 60 months old when the questionnaire was administered. Responses were rated on a three-point scale (never, sometimes and often). Physical aggression was assessed using three items which asked how often the child hits, bites/kicks, and fights/attacks other children. Hyperactivity was assessed using five items (e.g., is impulsive/acts without thinking, can't sit still). Opposition was assessed using three items (e.g., does not have remorse after misbehaving, was rebellious/refused to behave). All externalizing items ranged from 0–2. A total score was obtained by calculating the average across all items within each scale. Ordinal alpha ratings were calculated and were $\alpha = .84$, $\alpha = .85$ and $\alpha = .78$ for physical aggression, hyperactivity and opposition respectively (Zumbo et al., 2007).

Anxiety was assessed using a variation of the Revised Children's Manifest Anxiety Scale (RCMAS; Reynolds & Richmond, 1985) entitled the "My Emotions" questionnaire, which was administered directly to the children at age 12. The "My Emotions" questionnaire is a seven item self-report questionnaire focusing on oversensitivity and physical anxiety (e.g., being afraid of a number of things, waking up afraid, having difficult falling asleep at night). Content and strength of factor loadings in the standardization samples were used previously to select seven items (Reynolds & Richmond, 1985; Serra Poirier et al., 2017). Items were rated on a four-point scale with responses ranging from never occurring (1) to occurring very often (4). A total anxiety score was obtained by summing the seven item scores for each participant (Ordinal alpha = .87).

A brief version of the Children's Depression Inventory was also administered to children at age 12 to assess depressive symptoms(Kovacs, 1983). Participants were asked to rate the frequency with which they experienced seven items (e.g., feeling alone, being disagreeable, crying) during the previous two weeks. Answers ranged from rarely (1) to often (3). A total depression symptoms score was obtained by summing the seven item scores for each participant (Ordinal alpha = .83).

Means and standard deviations for all measures are listed below in Table 1.

Table 1. *Means and standard deviations for externalizing and internalizing measures.*

	Mean	Standard Deviation	Scale range	Skewness	Kurtosis
60 Months					
Aggression	0.64	0.48	0-2	.29	-.60
Hyperactivity	0.87	0.46	0-4	.15	-.38
Opposition	0.81	0.48	0-2	.33	-.41
12 Years					
Depression	8.71	1.90	7-21	1.54	2.27
Anxiety	11.47	3.81	7-28	1.37	2.22

2.3.3 Statistics

Descriptive statistics were analysed with SPSS statistic 27. Univariate genetic analyses and common pathway modelling were performed in Mplus 8.1(Muthén & Muthén, 1998). Missing data percentage for all variables ranged from 10.6% to 38.5%. Missing data was estimated using full information maximum likelihood (FIML) estimation. Depression scores were winsorized with the 98th percentile due to excess skewness. Bootstrapping N =2000 times was performed for common factor models. Model specific specifications are indicated in the results section.

2.4 Results

2.4.1 Univariate Genetic Analyses

Univariate genetic modelling (more specifically ACE modelling) was run separately for aggression, hyperactivity and opposition at 60 months as well as depression and anxiety at age 12 in order to determine estimates of genetic and environmental influences. ACE modelling is predicated on the assumption that MZ twins share 100% of their DNA, while DZ twins share 50%. Therefore, any measurable differences between MZ twins must be a result of environmental factors, while any excess in similarity between MZ twins above and beyond similarity between DZ twins can be expected to be a result of genetic factors. Building off of this basic logic, one can estimate what proportion of variation in a given phenotype is a result of additive genetic (A), common or shared environmental (C) and unique environmental factors (E).

Upon visual inspection of intraclass correlation (ICC) values (Table *2*) it was noted that ICC (DZ) values for hyperactivity at 60 months and anxiety at age 12 were unusually small relative to ICC (MZ) values. These unexpected values were addressed differently for each measure. For hyperactivity, the low DZ values suggest a contrast effect, a well-documented phenomenon whereby mothers tend to contrast their DZ co-twins against each other when rating,

leading to overestimated differences among those twins (Hartman et al., 2007). To address this, we used a statistical indicator calculated from two equal regression paths stemming from one twin to their co-twin. We found that accounting for a contrast effect produced a better fitting model as assessed by comparing AIC and BIC values as fit indices (See Table 3). Smaller AIC and BIC values generally indicate a better model fit.

Table 2**.** *Intraclass Correlation Coefficient (ICC) values*

Variable	Time point	ICC (MZ)	N (MZ)	ICC (DZ)	N (DZ)
Aggression	60 months	0.670	186	0.506	250
Hyperactivity	60 months	0.622	186	0.053	249
Opposition	60 months	0.437	186	0.288	249
Depression	Age 12	0.337	152	0.159	223
Anxiety	Age 12	0.433	155	0.067	224

ICC = intraclass correlation coefficient, MZ = monozygotic, DZ = dizygotic

To address the large discrepancy in ICC (MZ) and ICC (DZ) values for anxiety in grade six, a different method was used since anxiety symptoms were assessed through self-report and thus cannot be influenced by a rater bias leading to a contrast effect. First, we tested for correlation differences for same sex compared to opposite sex dizygotic pairs, as this could account for the observed discrepancy. We did this by comparing the fit of a univariate ACE model in which the correlation for opposite sex dizygotic and same sex dizygotic twins are constrained to be equal, to the fit of a saturated model. The fits of the models were compared using a chi-square difference test. We identified a significant correlation difference ($\chi 2$ = 8.806, df = 2, p = .012) between mixed-sex and same-sex DZ twins for anxiety that could account for the discrepancy in ICC (MZ) and ICC (DZ). This could be resolved by excluding mixed-sex

twins from the analyses. However, removing mixed-sex twins from the analysis would significantly reduce power. Further, visual inspection of the ICC values suggested that a sex effect accounted for the discrepancy in ICC values; therefore, we included sex as a regressor in our model. This resulted in a better model fit as assessed by comparing Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values (see Table 3).

In addition to the full ACE model, nested models were run in order to assess the relative fit of AE, CE and E models compare to the full ACE model. The best fitting models for aggression, hyperactivity and opposition were ACE, AE and AE models respectively. The ACE model was the best fitting models for anxiety and depression. Results are displayed in Table 3 below.

Table 3. *Model fit parameters*

	Model	AIC	BIC	PA	PC	PE
60 Months						
Aggression	**ACE**	**1037.63**	**1054.02**	**0.38**	**0.3**	**0.32**
	AE	1044.46	1056.75	0.704	-	0.296
	CE	1047.88	1060.17	-	0.57	0.43
	E	1217.71	1225.9	-	-	1
Opposition	ACE	1137.53	1153.92	0.35	0.11	0.55
	AE	**1036.26**	**1148.55**	**0.47**	**-**	**0.53**
	CE	1140.30	1152.59	-	0.35	0.65
	E	1194.66	1202.85	-	-	1
Hyperactivity (with contrast)	ACE	1063.04	1083.51	0.78	0	0.22
	AE	**1061.04**	**1077.42**	**0.78**	**-**	**0.22**
	CE	1117.03	1133.41	-	0.2	0.8
	E	1115.03	1127.32	-	-	1
Hyperactivity (without contrast)	ACE	1080.86	1097.25	0.57	0	0.43
	AE	**1078.86**	**1091.15**	**0.57**	**-**	**0.43**
	CE	1115.03	1127.32	-	0.28	0.72
	E	1148.97	1157.16	-	-	1
Age 12						
Anxiety (without sex control)	ACE	4199.14	4218.96	0.38	0	0.62
	AE	**4197.14**	**4213.00**	**0.38**	**-**	**0.62**
	CE	4207.96	4223.80	-	0.22	0.78
	E	4223.33	4235.221	-	-	1
Anxiety (with sex control)	**ACE**	**4185.13**	**4204.95**	**0.38**	**0**	**0.62**
	AE	4187.13	4210.91	0.38	-	0.62
	CE	4196.27	4216.08	-	0.23	0.78
	E	4212.67	4228.53	-	-	1
Depression	**ACE**	**3093.58**	**3109.42**	**0.33**	**0**	**0.67**
	AE	3127.50	3139.38	0.34	-	0.66
	CE	3132.77	3144.66	-	0.21	0.79
	E	3147.50	3155.42	-	-	1

Note: Bold lettering indicates the best fitting model. AIC = Akaike Information Criterion, BIC = Bayesian Information Criterion, PA = Proportion of variance accounted for by additive genetics, PC = proportion of variance accounted for by shared environment, PE = proportion of variance accounted for by unique environment.

2.4.2 Univariate Qualitative and Quantitative Sex Differences

In order to investigate sex differences, we tested for quantitative and qualitative sex effects using a univariate sex-limited model following methodology previously outlined by Karcher and colleagues (2014). To test for quantitative sex differences, a model in which sex differences in means and variances for opposite sex dizygotic twin pains (DZO) were permitted (full model) was compared to a more restrictive model in which values for A, C and E were constrained to be equal across sexes. Fit values were compared using a chi-square difference test. To test for qualitative sex differences, we compared a model in which the genetic correlation (rA) between the latent genetic factors underlying the observed variable in DZO pairs were either allowed to vary or were fixed to .05.

Using a cut-off of $p = .01$ to correct for multiple comparisons across five variables, we identified quantitative sex differences for depression only ($p < .001$). We observed no qualitative sex effects. Modelling these sex differences within a univariate model, we observed that genetic contributions were larger for girls (54.4% vs 15.7%), while unique environmental contributions were larger for boys (84.3% vs 45.6%).

Because we identified a quantitative sex difference for depression, we opted to run secondary bivariate sex-limited analyses to examine the association between depression and the individual externalizing symptoms. These analyses and the rationale for these analyses are reported in more detail in the section labelled “Sex Limited Scalar Correlational Model”.

2.4.3 One-Factor Common Pathway Model

Common pathway models were applied to assess the genetic and environmental contributions to both externalizing symptoms at 60 months and internalizing symptoms at age 12. We used a common-pathway model analogous to the one used previously by Lemelin and colleagues (2007) with the addition of covariates controlling for the contrast effect for

hyperactivity and sex effect for anxiety in order to account for the aforementioned discrepancies in ICC (MZ) and ICC (DZ) values for these variables. The first common-pathway model derived a common factor of externalizing symptoms resulting from shared variance between physical aggression, hyperactivity, and opposition at 60 months (Figure 4). The second common-pathway model derived a common factor of internalizing symptoms resulting from shared variance between anxious and depressive symptoms (Figure 5). Within both models, the variance of the latent factors (i.e., externalizing or internalizing) and the residual specific contributions of each externalizing/internalizing symptom is decomposed into its genetic, shared environmental and non-shared environment components. Additive genetic (A), shared environmental (C), and non-shared environmental (E) general contributions to the general latent factor are shown, as well as specific additive genetic (a), shared environmental (c), and non-shared environmental (e) parameters accounting for each specific internalizing or externalizing behaviour score on the general latent factor, independent of the score's loading on specific symptoms. 95% bootstrap confidence intervals are reported in brackets below estimates.

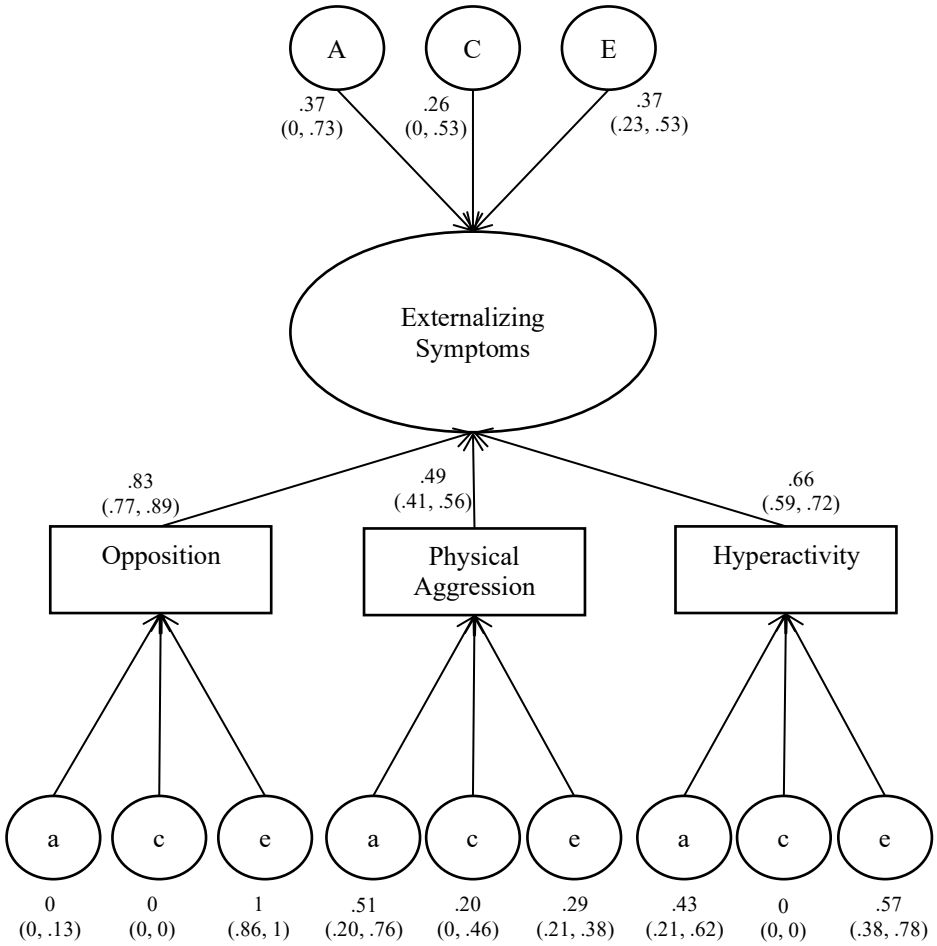

Figure 4. One-Factor Common pathway model applied to externalizing symptoms.

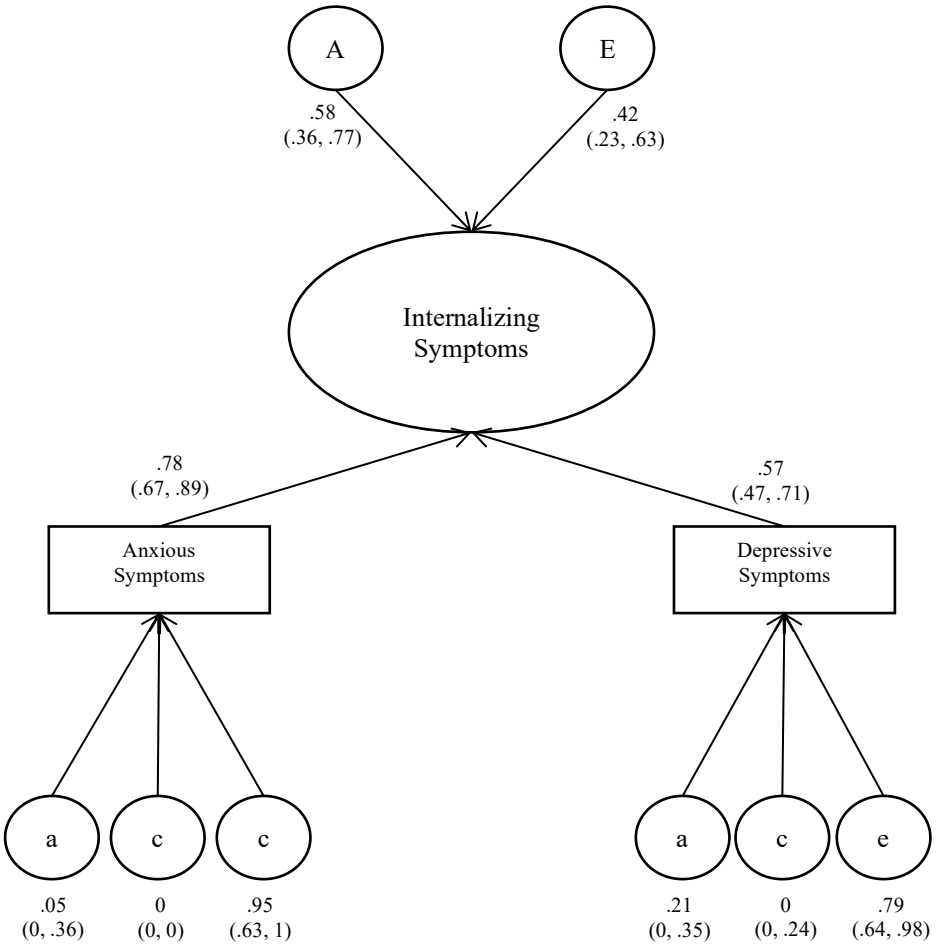

Figure 5. One-Factor Common pathway model applied to internalizing symptoms.

Externalizing symptoms at 60 months were accounted for by additive genetic (A), shared-environment (C) and non-shared environment (E) components. A and E components accounted for an equal proportion of the variability in externalizing symptoms (37%), while the C component accounted for slightly less (27%). Internalizing symptoms at age 12 were entirely accounted for by additive genetic and non-shared environment components, with A accounting for 58% of the variance in internalizing symptoms, C accounting for 0% of the variance and E accounting for 42% of the variance.

2.4.4 Two-Factor Common Pathway Model

To examine the ACE underpinnings of the longitudinal association between externalizing symptoms at 60 months and later internalizing symptoms, we used a two-factor common pathway model (as used in Lemelin et al., 2007). Firstly, we assessed the correlation between the externalizing factor at 60 months and internalizing factor at age 12. We found a significant correlation between externalizing and internalizing factors ($r = 0.27$, $p < .001$). We then fit a two-factor common pathway model in order to examine the association between two general-latent factors of externalizing symptoms at 60 months (derived from covariance between physical aggression, hyperactivity and opposition) and internalizing symptoms (derived from covariance between anxiety and depressive symptoms) at age 12. The variance of the latent externalizing and internalizing factors and the residual specific contributions of each specific externalizing symptom was decomposed into genetic, shared environmental and non-shared environment contributions. Genetic (A), shared environmental (C), and nonshared environmental (E) contributions to externalizing symptoms are shown, as well as genetic (a), shared environmental (c), and nonshared environmental (e) parameters accounting for each specific externalizing/internalizing behaviour score, independent of the score's loading on externalizing symptoms. Finally, we estimated the genetic correlation (R_G) and the non-shared environmental correlation (R_E) between the two general-latent factors. The correlation between C components (R_C) was fixed to zero within the model as there was no C component loading on internalizing symptoms. Results are displayed in Figure 6 and revealed a significant genetic correlation between the two general latent factors $R_G = .55$ ($p = .02$). R_E was non-significant. In other words, the association between externalizing symptoms at 60 months and internalizing symptoms at age 12 was essentially accounted for by common genetic, but not shared or unique environmental,

factors. 95% bootstrap confidence intervals are reported in brackets below estimates. Asterisk indicates statistical significance at $p < .05$ level.

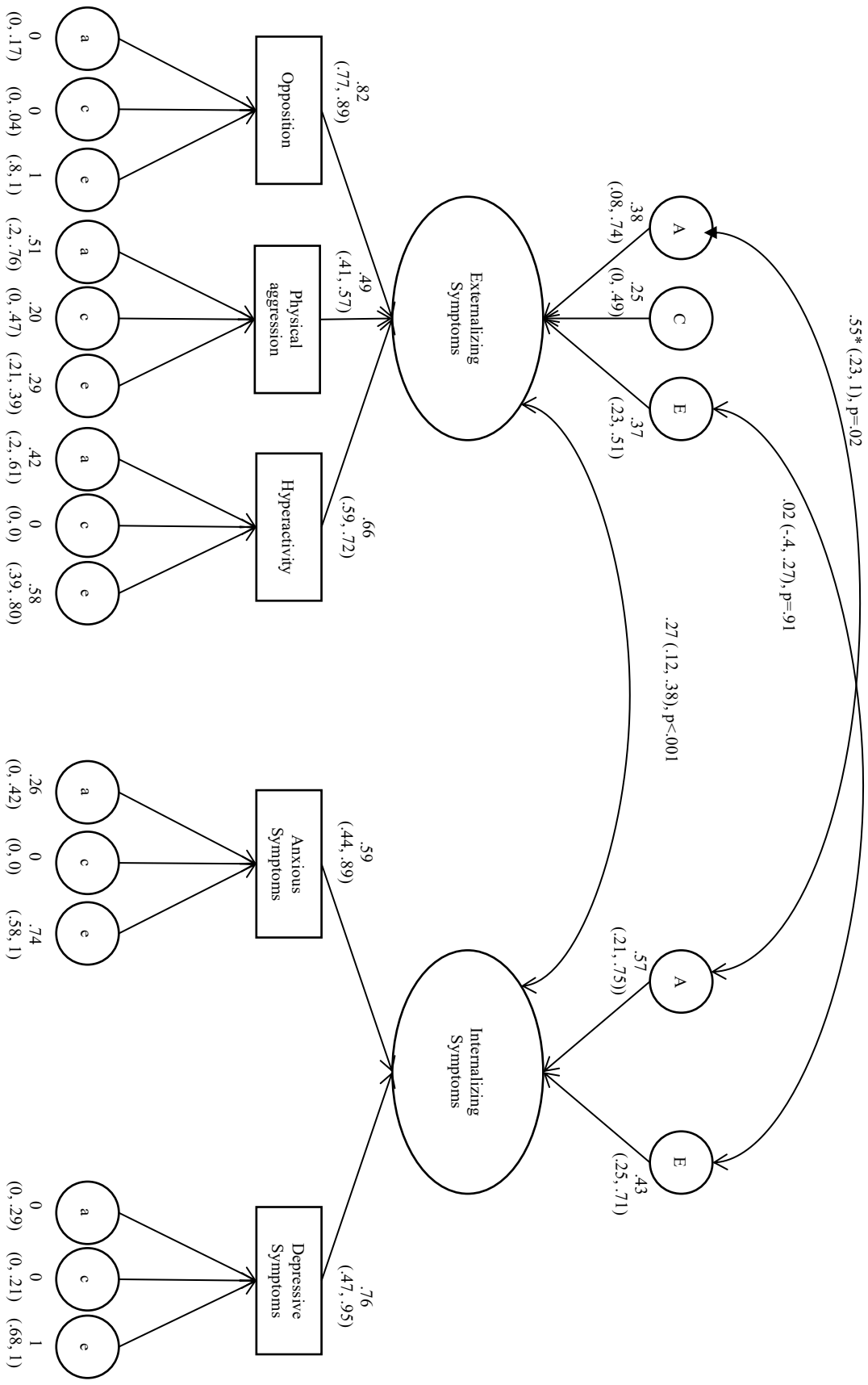

Figure 6. Two-Factor common pathway model applied to externalizing symptoms at 60 months and internalizing symptoms in grade 6.

2.4.5 Sex Limited Scalar Correlational Model

As previously noted, we observed qualitative sex differences for depressive symptoms. To examine the sex effect on the association between depressive symptoms at 60 months and the individual externalizing symptoms at age 12, we opted to run secondary bivariate sex-limited analyses to complement our primary analyses. Specifically, we used the scalar correlational model to assess the genetic and environmental correlations between the following pairs of variables over time: depression-aggression, depression-hyperactivity, and depression-opposition. The scalar correlational model allows for quantitative sex-differences within the model as well as differences in variances between sexes (Haworth et al., 2008; Neale et al., 2006). It provides in its output a single set of genetic (rA), common environment (rC) and unique environment (rE) correlations which is the same for each gender. In the current analysis, as in the two-factor common pathway model, the common environment correlation (rC) was constrained to 0. See Neale colleagues (2006) for a detailed explanation of the scalar correlation model and its comparison to other sex-limited approaches.

The results of the scalar correlational model were largely consistent with findings from the two-factor common pathway model (See Table 4 below). Specifically, as would be expected based on the results from the two-factor common pathway model, we observed significant genetic correlations for hyperactivity-depression and opposition-depression, and no significant unique environmental correlations. Interestingly, the genetic correlation for aggression-depression was not significant at the $\alpha = .05$ level.

Table 4. *Sex limited analyses for depression (scalar correlational model)*

	rA (p-value)	rE (p-value)
Aggression-Depression	.20 (p = .10)	.033 (p = .61)
Hyperactivity-Depression	.27 (p = .019)*	.016 (p = .84)
Opposition-Depression	.39 (p = .026)*	.078 (p = .29)

Ra = genetic correlation, Re = unique environmental correlation
** Indicates significance at p <.05 level*

2.5 Discussion

In the current study, we sought to better understand the longitudinal genetic and environmental associations between externalizing symptoms in preschool and internalizing symptoms in early adolescence. To this end, we assessed the genetic and environmental contributions to externalizing symptoms at 60 months, internalizing symptoms at age 12, and their longitudinal associations. We found that externalizing symptoms at 60 months were accounted for by genetic, shared environment and unique environment components, while internalizing symptoms at age 12 were accounted for solely by genetic and unique environment components. Further, we found that there was a significant moderate correlation between externalizing symptoms at 60 months and internalizing symptoms at age 12. This association was essentially accounted for by common genetic factors. There was no association between unique or shared environmental components of externalizing and internalizing symptoms across time.

As we observed quantitative sex effects specifically for depressive symptoms, we conducted secondary sex-limited analyses in depression only. Consistent with our primary analyses, we found evidence for a genetic, but not environmental, longitudinal association for

depression-hyperactivity and depression-opposition. Interestingly, we did not find a genetic correlation for depression-aggression.

Consistent with previous research, externalizing symptoms at 60 months were moderately correlated with later internalizing symptoms (Loth et al., 2014; Mesman et al., 2001; Reef et al., 2011). Also consistent with the literature, one-factor common pathway analyses revealed significant genetic and unique environmental components explaining variation in both externalizing and internalizing symptoms (Lubke et al., 2018; Thompson et al., 2017; Tuvblad et al., 2019). We also observed a significant contribution of shared environmental factors to externalizing, but not internalizing, symptoms. Indeed, shared environmental contributions to internalizing and externalizing symptoms tend to decrease with age and would be expected to be more prominent in a preschool population (Burt, 2009). Further, while internalizing symptoms were rated by the children themselves, externalizing symptoms were rated by mothers who tend to rate their children as more similar, leading to higher shared environmental estimates.

Longitudinally, a main finding of the present study was that the predictive association between externalizing symptoms at 60 months and internalizing symptoms at age 12 was essentially accounted for by common genetic contributions. Unique or shared environmental factors did not significantly contribute to this predictive association. Our findings extend previous findings of genetic factors accounting for the co-occurrence of internalizing and externalizing symptoms by suggesting that a stable genetic factor also accounts for the overlap between externalizing and internalizing symptoms *over time* (Cosgrove et al., 2011; Lahey et al., 2011; Mikolajewski et al., 2013). This observation is consistent with the overlap in the neurobiology of externalizing symptoms with internalizing symptoms. For example,

the catechol-O-methyltransferase (COMT) gene, a gene coding for a protein involved the breakdown of catecholamines such as dopamine and norepinephrine, has been implicated in depression, anxiety, aggression and conduct difficulties, as well as more generally in neural emotion processing networks (Antypa et al., 2013; L. O. Lee & Prescott, 2014; Tuvblad et al., 2019). Further, medications that act on the serotonin system are effective in treating both internalizing and externalizing disorders (Coccaro et al., 2015; Ghanizadeh et al., 2013).

It is of interest that shared or unique environmental factors did not contribute to the longitudinal association observed given the known role of certain environmental factors (e.g., peer rejection and victimization) in shaping both internalizing and externalizing symptoms (Attar-Schwartz et al., 2019; van Lier et al., 2012). However, this is not entirely unexpected as the impact of shared environmental effects tends to wane with age. Thus, any stability in a shared vulnerability would not necessarily be expected to be accounted for by shared environmental factors. Further, while genetics remain stable across time, the unique environmental landscape in adolescence would be expected to vary widely from, and thus be less correlated to, the unique environmental landscape in preschool.

While secondary sex-limited analyses support the primary analyses by showing a genetic, but not environmental correlation between depression-hyperactivity and depression-opposition, it is of interest that we did not observe a significant genetic correlation between depression-aggression. Indeed, both depression and aggression are associated with alterations in brain serotonin synthesis and depression shares a number of risk genes with aggression (Coccaro et al., 2015; Rosa-Neto et al., 2004; Tuvblad et al., 2019). These results would appear to suggest that while internalizing and externalizing symptoms share genetic a vulnerability across time from preschool to early adolescence, this effect may be driven more strongly by oppositional and

hyperactive behaviours. However, it may be the case that the genetic component underlying aggression is more strongly associated with internalizing symptoms more broadly, as opposed to specifically with depressive symptoms. Indeed, there is evidence that genetically influenced neurobiological systems tend to correlate more strongly with brain processes associated with higher-order constructs such as internalizing and externalizing disorders, than individual syndromes (Antypa et al., 2013; Wolf et al., 2018).

It is important to consider the preceding findings in light of two factors which may affect the generalizability of the findings. Firstly, because this study investigated normal variation in internalizing and externalizing symptoms and did not focus on those children with a psychiatric diagnosis, the extent to which the findings of the present study can be generalized to clinical populations is not known. However, there is a strong argument to be made for studying these variables with a dimensional approach as strictly studying psychiatric groups can hamper our understanding of underlying developmental processes (Haberstick et al., 2005; Loeber & Burke, 2011). In addition, it is important to note that our sample was primarily white. There is evidence that environmental factors such as parental education and socioeconomic status, which act as protective factors for majority groups, do not necessarily act as protective factors for ethnic minority groups, suggesting perhaps larger environmental (i.e., systemic) factors at play (Assari & Islam, 2020). Thus, the relative contribution of genetics and environmental factors may not generalize to other ethnicities.

Our results suggest that it is important to consider the risk for internalizing disorders when staging interventions aimed at early externalizing symptoms. Indeed, previous research has shown that while 50.7% of children follow a homotypic pathway to internalizing symptoms, 44.6% follow a heterotypic pathway (Jobs et al., 2019). A number of effective intervention

studies aimed at addressing early externalizing difficulties have been conducted (e.g., Castellanos-Ryan et al., 2013; Vitaro et al., 2012). Such studies have focused on social skills training and psychoeducation for parents and teachers and have been shown to improve school performance and high school graduation rates as well as reduce criminality and substance use. The findings of the present study suggest that interventions for children with or at risk for early externalizing difficulties should also incorporate elements of depression and anxiety prevention, and to monitor the development of internalizing symptoms at follow-up. Addressing genetically influenced biological systems which directly overlap between externalizing and internalizing symptoms through psychological interventions may be a beneficial adjunctive treatment. Psychological interventions aimed at addressing negative emotionality (the tendency to experience negative aversive emotions broadly) may be particularly worthy of investigation in light of evidence that negative emotionality in-part accounts for the overlap in genetic factors between adolescent internalizing and externalizing symptoms (Mikolajewski et al., 2013). Mindfulness-based interventions provide an evidence-based, transdiagnostic approach by targeting dysregulation of negative emotions broadly and have been shown to be particularly effective in preventing the development of internalizing disorders in at-risk individuals (Bögels et al., 2008). Indeed, there is some evidence that mindfulness-based interventions in preschool may reduce emotionality lability/negativity and improve social competence and social-emotional development (Flook et al., 2014; E. Kim et al., 2020).

In conclusion, our findings extend previous research investigating the co-occurrence of externalizing and internalizing symptoms by providing evidence that longitudinal associations between preschool externalizing symptoms and early adolescent internalizing symptoms are accounted for essentially by shared genetic factors. This has important implications for

understanding the etiology of internalizing difficulties and can help guide early interventions aimed at mitigating their development. Further research will be important to better understand how shared genetic vulnerability and unique environmental pathways interact up until adulthood to characterize the relation between early externalizing and later internalizing symptoms.

2.6 Funding

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3. CHAPTER 3: DNA methylation differences in stress-related genes, functional connectivity and gray matter volume in depressed and healthy adolescents

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3.1 Abstract

Background: Studies in adult depressed patients have indicated that altered DNA methylation patterns at genes related to serotonin and HPA axis functioning (e.g., *SLC6A4, FKBP5*) are associated with changes in frontolimbic functional connectivity and structure. Here, we examined whether these associations can be generalized to adolescents.

Methods: 25 adolescents with depression (Mean age = 15.72 ± .94 SD; 20 girls) and 20 healthy controls (Mean age = 16.05 ± 1.5 SD; 16 girls) underwent a functional and structural magnetic resonance imaging protocol, which included a resting-state assessment and measures of brain morphometry. DNA was obtained from saliva. Levels of *SLC6A4* and *FKBP5* methylation were determined using pyrosequencing.

Results: *SLC6A4* methylation was linked to amygdala-frontal operculum resting-state functional connectivity (rs-FC), regardless of diagnosis, and was differentially associated with inferior orbitofrontal gyrus (IFOG) gray matter (GM) volume in adolescents with depression and controls. Replicating and extending previous findings in adults, *FKBP5* methylation was associated with IFOG GM volume in depressed and healthy adolescents, as well as orbitofrontal cortex (OFC)-rostral prefrontal cortex (RPFC) connectivity in healthy adolescents only.

Limitations: Effects of medication use or genotype cannot be ruled out. Further, the relatively small sample size and predominately female sample may limit generalizability.

Conclusions: These findings suggest that previously observed associations between *SLC6A4* and *FKBP5* methylation and frontolimbic processes in adult depressed patients can be in part generalized to adolescent patients. Further, findings suggest that measuring peripheral methylation at these genes deserves further attention as potential markers of typical and atypical development.

3.2 Introduction

Major depressive disorder (MDD) is a highly prevalent disorder affecting more than 300 million individuals, or 4.4% of the population, worldwide (World Health Organization, 2017). Its symptoms often appear early in life, with the likelihood of developing depression increasing greatly at the onset of puberty (American Psychiatric Association, 2013). Adolescent depression is particularly costly due to its chronicity (50-70% of adolescents experience at least one other depressive episode within 5 years) and its association with a number of negative social and health outcomes including difficulties obtaining and maintaining employment, substance abuse, lower educational achievement and early parenthood (Hauenstein, 2003).

The diathesis-stress model postulates that the presence of genetic risk alleles of stress related genes (e.g., the glucocorticoid receptor, or its chaperone FKBP5) and/or key regulators of neurodevelopment (e.g., the serotonin transporter), in combination with early environmental stressors (e.g. childhood trauma, neglect), confers a biological vulnerability to developing depression in the face of further stressors (Colodro-Conde et al., 2018; Monroe & Simons, 1991). This vulnerability may be reflected in neural changes in frontolimbic brain regions - key regions involved in the regulation of mood and anxiety. Indeed, numerous imaging studies have found differences in structure and function between MDD patients and controls in frontolimbic regions such as the dorsolateral prefrontal cortex, the insula, the lateral orbitofrontal cortex, the anterior cingulate, the amygdala and the hippocampus (Bora et al., 2012; Frodl et al., 2002, 2008; Salvadore et al., 2011; W. Wang et al., 2017; M. Zhou et al., 2017) .

Recent work emphasizes alterations in the functional organization of the brain as a possible risk-specific marker in youth with, and at familial risk for, depression (Singh et al., 2018). One measure used to study core functional networks is resting-state functional

connectivity (rs-FC), which indexes the correlation of spontaneous activity in various brain regions over time in the absence of external information (He, Snyder, Zemple, Smyth, & Raichle, 2008). Several studies have shown that youth with depression display altered rs-FC at key nodes within the default mode network (DMN) as well as altered rs-FC within the limbic, frontal-parietal and salience networks (e.g. amygdala, anterior cingulate cortex, ventromedial prefrontal cortex, dorsomedial prefrontal cortices; Connolly et al., 2017; Kerestes et al., 2014).

Although specific mechanisms by which early-life stressors might translate into a neurobiological vulnerability to depression remain to be elucidated, epigenetic mechanisms have been recognized as a potential mechanism of gene-environment interactions (Booij et al., 2013). Epigenetic changes refer to alterations in gene expression that do not involve changes to the genetic sequence itself (Levesque et al., 2016). The most widely studied epigenetic mechanism, DNA methylation, is a process in which a methyl group is added to the nucleotide base pair cytosine where it is found next to a guanine, a region of the gene referred to as a CpG site (Meaney, 2010; Meaney & Szyf, 2005). Several studies have linked altered DNA methylation patterns at hypothalamic pituitary adrenal (HPA) axis and serotonin system related genes to environmental factors such as early-life adversity (Booij et al., 2013; Booij, Szyf, et al., 2015). Two genes that have received particular attention in the context of MDD are the serotonin transporter (*SLC6A4*) gene and the FK506 binding protein (*FKBP5*) gene.

The serotonin transporter is involved in the uptake of serotonin from the synaptic cleft and is widely expressed in frontolimbic regions (Booij, Szyf, et al., 2015; Kish et al., 2005; Nordquist & Oreland, 2010; Varnäs et al., 2004). Furthermore, the serotonin transporter plays a critical role in brain development throughout childhood and adolescence and into early adulthood (Booij, Tremblay, et al., 2015). Using functional magnetic resonance imaging (fMRI), we

showed that greater peripheral *SLC6A4* promoter methylation levels were associated with hippocampal volume and increased frontal-limbic response to negative stimuli in adults with and without MDD (Booij, Szyf, et al., 2015; Frodl et al., 2015). Furthermore, we and others have shown that *SLC6A4* methylation is associated with rs-FC within the DMN and salience network (SN) in healthy volunteers (Ismaylova et al., 2017; Muehlhan et al., 2015). However, it remains unknown whether the relationship between *SLC6A4* methylation and rs-FC in adolescents is altered in depression.

The few studies examining associations between brain processes in depressed patients and DNA methylation levels of HPA axis genes have focused on the *FKBP5* gene (Chen, Meng, Pei, Zheng, & Leng, 2017) coding for the FK506 binding protein 51, a protein which downregulates the binding affinity of glucocorticoid receptors to cortisol through its interaction with the hsp90 protein. Recently, we showed that *FKBP5* intron 7 methylation is associated with brain structure and function in depressed adults and healthy controls. Most notably, an increase in methylation of these CG positions in *FKBP5* intron 7 was associated with elevated gray matter (GM) concentration of the inferior frontal orbital gyrus (IFOG) in patients and controls, and increased activation in patients (Tozzi et al., 2018). In a sample of healthy adults, we did not find an association between *FKBP5* intron 7 methylation and rs-FC (Di Sante et al., 2018). Yet, the link between *FKBP5* methylation and rs-FC remains to be studied in adolescents. The adolescent time period is of much interest given that *FKBP5* has been implicated in early brain development and plasticity, and therefore merits unique consideration (C. Wang et al., 2018).

The aim of the current study is to examine the association between *SLC6A4* and *FKBP5* methylation, rs-FC and brain structure in healthy adolescents and those diagnosed with depression. We expect that DNA methylation will be associated with altered rs-FC between

frontolimbic regions of the default mode, salience, frontoparietal and limbic networks. We further hypothesize that DNA methylation level of the *SLC6A4* and *FKBP5* gene regions will be associated with frontolimbic GM volume. We explore whether adolescents with and without depression differ in the strength of the methylation-brain associations, and whether *SLC6A4* and *FKBP5* methylation levels are associated with overlapping or distinct frontolimbic regions.

3.3 Methods

3.3.1 Participants

Twenty-eight adolescents with a depressive disorder between the ages of 13 and 18 were recruited from Hotel Dieu Hospital Site, Kingston Health Sciences Centre in Kingston, Ontario. Exclusion criteria included a history of a psychotic disorder, substance use disorder or current suicidality. If patients were using antidepressant medications, they were required to be stabilized on medication for at least 6 weeks. Twenty-three age and sex-matched controls were recruited from the local community. Controls were excluded if they had any previous psychiatric diagnoses (DSM-IV Axis I or Axis II) or had ever taken any psychotropic medications. In addition to group specific exclusion criteria, participants were not eligible for the study if they did not meet the MRI safety criteria, which included having any non-removable metal in their body (e.g., electronic implant, prosthesis, pacemaker, aneurysm clip), as well as being pregnant. Three participants in the control group were excluded due to the presence of a current psychiatric disorder as determined by the Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS; Kaufman et al., 1997). One participant in the depression group was excluded from the final analyses due to the presence of artifacts in the structural data, and two others were excluded as the participants wished to stop the scan prematurely.

The study was approved by the Health Sciences Research Ethics Board of Queen's University in Kingston, Ontario. Written informed consent was obtained from the adolescent or a

parent or guardian if the participant was under 16 years of age. If under the age of 16, assent was obtained from the participant.

3.3.2 Interviews and questionnaires

Diagnosis. In order to confirm the presence or absence of a psychiatric diagnosis, the K-SADS (Kaufman et al., 1997) was administered by one of two clinical psychology graduate students (JC, LS) under supervision of a licensed clinical psychologist (LB).

Depressive symptomatology. The Beck Depression Inventory for Youth (BDI-Y), second edition, was employed to assess depression severity (Beck et al., 2001).

3.3.3 DNA methylation Analysis

Participants provided 2 ml saliva samples for DNA methylation analysis by passive drooling using the Oregene Genotek kit. DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega). DNA methylation in *SLC6A4* and *FKBP5* gene regions was assessed as reported previously (Tozzi et al., 2018; D. Wang et al., 2012). At the *SLC6A4* promoter region, methylation was assessed at CpG sites 5-7, and CpG sites 10-14, given findings of our previous study showing an association between methylation at these sites and brain serotonin synthesis (D. Wang et al., 2012). At the *FKBP5* gene, methylation was assessed at CpG sites 6 and 7 of the intron 7 region (as numbered by Resmini et al., 2016 and Di Sante et al., 2018; please note that CpG 6 was named as Bin3, CG1 by Klengel et al., (2013). The PyroMark Q96 (Qiagen) was used to assess site specific methylation levels at the CFI-Imaging and Molecular Biology Platform in the Department of Pharmacology and Therapeutics, McGill University. Triplicates were obtained for each CpG site and data were checked for quality using the PyroMark CpG 1.0.11 software (Qiagen). Mean methylation across sites 6 and 7 of the *FKBP5* gene, and sites 5-14 of the *SLC6A4* gene (excluding sites 8 and 9) were computed across all replicates as in previous studies.

3.3.4 Resting-state MRI acquisition and rs-FC analysis

MRI scans were obtained using a Siemens Trio 3 T MRI scanner with a 12-channel head coil. A localizer sequence was acquired to position subsequent scans, followed by a 7.5 minute-long high resolution 3 dimensional T_1-weighted structural scan (TR = 1760 ms; TE = 2.2 ms; FOV anterior to posterior = 256 mm; matrix size = 256x256; slice thickness = 1.0 mm; voxel size = 1.0 mm x 1.0 mm x 1.0 mm). Participants then underwent a 6 minute resting-state functional scan with eyes closed (spin-echo echo-planar imaging sequence; TR = 2000 ms; TE = 30 ms; FOV anterior to posterior = 211 mm; matrix size = 64x64; slice thickness = 3.3 mm; 33 slices; voxel size = 3.3 mm x 3.3 mm x 3.3 mm, 180 volumes).

Images were pre-processed according to the default preprocessing and denoising pipeline in the CONN functional connectivity toolbox v17 (Whitfield-Gabrieli & Ford, 2012) as implemented in MATLAB R2010a (Mathworks, Sherborne, MA). Regions of interest (ROIs) were identified using the Harvard-Oxford atlas and the CONN network atlas, and ROI-to-ROI functional connectivity was assessed. Source ROIs included frontolimbic regions which comprise major nodes within the DMN (posterior cingulate gyrus, medial prefrontal cortex) as well as ROIs within the salience, fronto-parietal and limbic networks (bilateral insula, bilateral orbitofrontal cortex, anterior cingulate gyrus, amygdala, hippocampus). Target ROIs included all 173 default ROIs in the CONN toolbox. All possible comparisons between each source ROI and target ROIs were done. ROI-ROI connectivity was regressed onto variables of interest, controlling for sex and age.

ROI analyses were conducted at both the analysis- and seed-level using a false discovery rate (FDR) correction ($p_{FDR}<.05$; Benjamini, 2010). The analysis-level corrects for all 11 source ROIs as well as all 173 target ROIs. The seed-level analysis applies a more liberal correction

which corrects for the number of target ROIs, but not the number of seed ROIs. For significant interactions, ROI-ROI connectivity values were extracted and analyzed in SPSS Version 25 in order to examine the nature of the interactions.

Independent Component Analysis (ICA) was conducted using the CONN toolbox in order to study the DMN specifically in addition to specific frontolimbic regions. First, independent components of the DMN, a network reliably identified in rs-FC analyses which is known to be affected by depression, were identified with a match to template method and visual confirmation. Second, connectivity between independent DMN components and the rest of the brain was assessed using a voxel-wise comparison in order to identify clusters significantly associated with DMN connectivity. Connectivity between DMN components and significant clusters was regressed onto variables of interest, controlling for age and sex. A false discovery rate (FDR) correction at the cluster level was applied ($p_{FDR} < .05$).

The main effect of *SLC6A4* and *FKBP5* methylation on brain rs-FC (both ROI-ROI and ICA), as well as methylation by diagnosis interactions, were assessed. For significant interactions, connectivity values were extracted and analyzed in SPSS Version 25 in order to examine the nature of the interactions.

3.3.5 Voxel Based Morphometry

Pre-processing of structural images and extraction of GM volumes were done using the default pipeline within the CAT12 toolbox within SPM12 (Wellcome Department of Cognitive Neurology, London UK). This included segmentation of gray and white matter, normalization, quality check and smoothing. Individual GM values were extracted at each of the following ROIs derived from the Neuromorphometrics Atlas (2012; http://neuromorphometrics.com) based on findings from our previous work in adults (Tozzi et al., 2018) and the involvement of

these brain regions in depression (Drevets et al., 2008; M. L. Phillips et al., 2003): amygdala, anterior cingulate cortex (ACC), anterior and posterior insula (aIns, pIns), anterior orbital gyrus (AOG), frontal operculum (FO), hippocampus, inferior frontal gyrus (IFG), inferior frontal orbital gyrus (IFOG), lateral orbital gyrus (LOG), middle frontal gyrus (MFG) and superior frontal gyrus (SFG).

GM concentration was analyzed using generalized estimating equations as implemented in SPSS. GM volume was set as the dependent variable. Side was included as a within-subjects factor. Between-subjects variables were diagnosis, age, sex, total intracranial volume and methylation. Models included main effects of all the independent variables and the diagnosis*methylation interaction. The model was run for *SLC6A4* and *FKBP5* methylation separately.

Predictors were evaluated using Wald tests. An FDR correction was applied for multiple comparisons. Results are reported at the $p_{FDR} < .05$ level. Yet, because of the decreased power of morphological analysis with smaller sample sizes, we also discuss results at the uncorrected level of $p < .001$ in ROI analyses examining the link between methylation and GM volume (Ismaylova, Di Sante, et al., 2018). In case of significant interactions, post-hoc analyses were run within each factor level.

3.4 Results

3.4.1 Participants

Twenty-five adolescents with depression and 20 controls were included in the analyses. All participants in the depression group were either currently in a major depressive episode (n=13), in partial remission (n=10) or diagnosed with Depressive Disorder Not Otherwise specified (n=2). (Table 5).

Table 5. *Sample characteristics and patient clinical information.*

	HC	MDD	Test
N	20	25	
Age	16.05 ± 1.5	15.72 ± .9	$t = .84, p = .41$
Sex (M:F)	4:16	5:20	Chi-square = 0, $p > 0.99$
SLC6A4 methylation (%)	3.02 ± .9	3.21 ± .8	$t = -.70, p = .49$
FKBP5 methylation (%)	53.96 ± .4.8	49.79 ± 5.8	$t = 2.58, p = .013$
BDI (*t*-score)	51.29 ± 8.4	69.84 ± 11.1	$t = -5.83, p < .001$
MDD history			
Age of onset of first episode	N/A	13.32 ± 1.4	
Number of episodes	N/A	1.84 ± .1	
Cumulative duration of MDEs (months)	N/A	22.04 ± 19.6	
Depression Status (n)			
Definite	N/A	9	
Probable	N/A	4	
Partial Remission	N/A	10	
Depressive Disorder Not Otherwise Specified	N/A	2	
Psychoactive-medication (n)*	0	23	

Data are means ± SD unless stated otherwise. Note. HC = Healthy Control; MDD = Major Depressive Disorder; N/A = Not Applicable; BDI = Beck Depression Inventory; *antidepressant (*n*=16), adjunctive atypical antipsychotic medication (*n*=7), anxiolytic (*n*=2), stimulant (*n*=6).

3.4.2 DNA methylation and depressive symptomatology

A multivariate regression analysis was run in order to test for significant associations between participant variables and *FKBP5* and *SLC6A4* methylation. Sex, diagnosis and the sex*diagnosis interaction were included as predictor variables while *FKBP5* and *SLC6A4* methylation were included as dependent variables. Results indicated that *FKBP5* methylation was significantly associated with sex ($F(1,41)=13.86$, $p< .001$) and diagnosis ($F(1,41)=5.82$,$p= .02$). Patients ($M=49.79$, $SD =5.8$) had lower levels of *FKBP5* methylation than controls ($M=53.96$, $SD=4.8$), and boys ($M=46.31$, $SD=4.6$) had lower levels of *FKBP5* methylation than

girls (*M*=52.98, *SD*=5.2). There were no diagnosis*sex interactions. No significant associations were found for *SLC6A4* methylation.

Multivariate regression analyses did not show any associations between *SLC6A4* and *FKBP5* and depression severity. There were no differences in *SLC6A4* and *FKBP5* methylation levels between those patients who used stimulants and those who were not on stimulants.

3.4.3 DNA methylation level and resting-state rs-FC: ROI-ROI analysis

There was a significant main effect of *SLC6A4* promoter methylation on right amygdala – left frontal operculum connectivity such that greater methylation was associated with increased connectivity, T(39)=4.04, $p_{\text{-FDR}}$=.041, β=.524 (FDR corrected at seed-level; See Figure 7). Results were not significant when FDR corrected at the (more stringent) FDR correction at the analysis level.

No main effect of *FKBP5* intron 7 methylation was observed. However, there was a significant diagnosis**FKBP5* methylation interaction (T(39)=5.04, *p*=.002; FDR corrected at analysis-level; see Figure 8). Follow up analysis indicated that lower *FKBP5* intron 7 methylation was associated with increased connectivity between the left orbitofrontal cortex (OFC) and left rostral prefrontal cortex (RPFC) in controls (β=-.728, *p*=.002) but that there was no association in the MDD group (β=.340, *p*=.129).

There were no differences in ROI-ROI rs-FC between those individuals who were taking stimulants and those who were taking other medications or no medication.

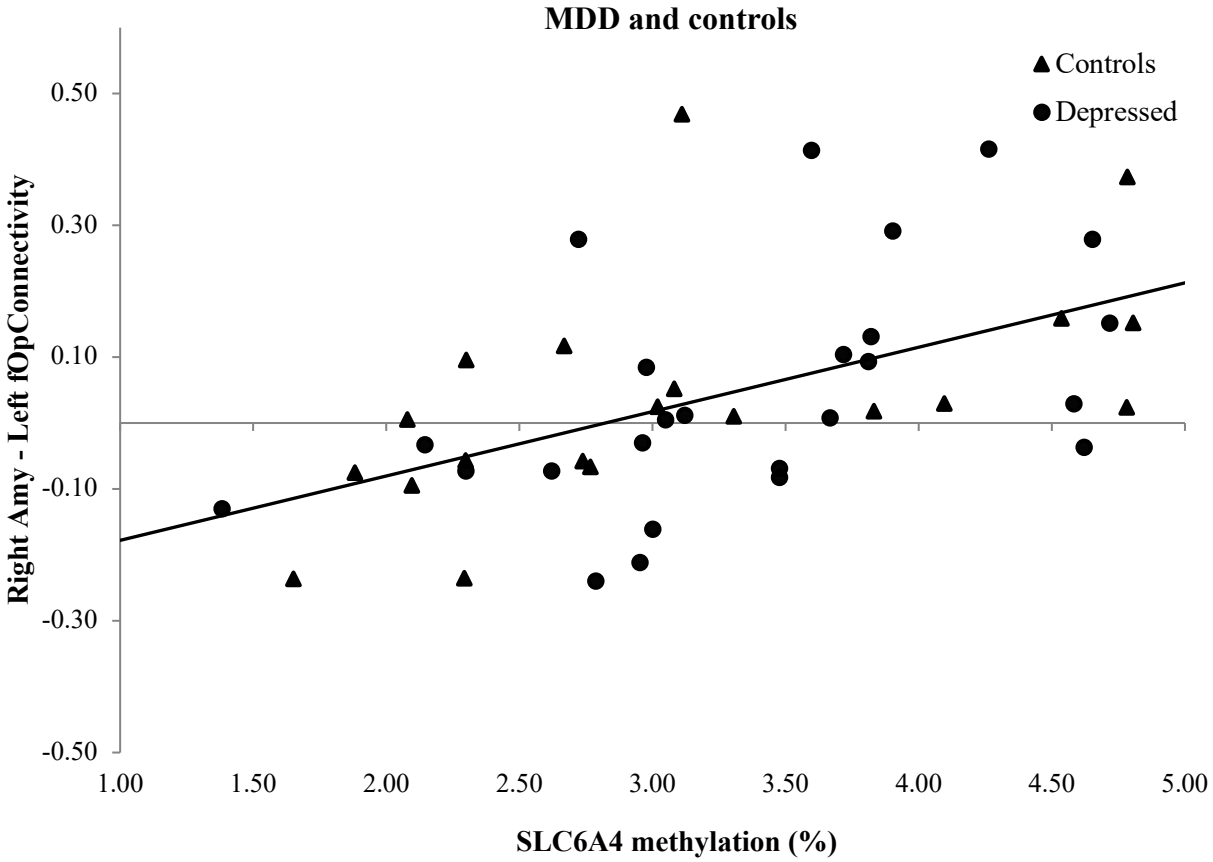

Figure 7. Greater *SLC6A4* promoter methylation was associated with increased right amygdala – left frontal operculum resting-state connectivity, regardless of diagnosis.

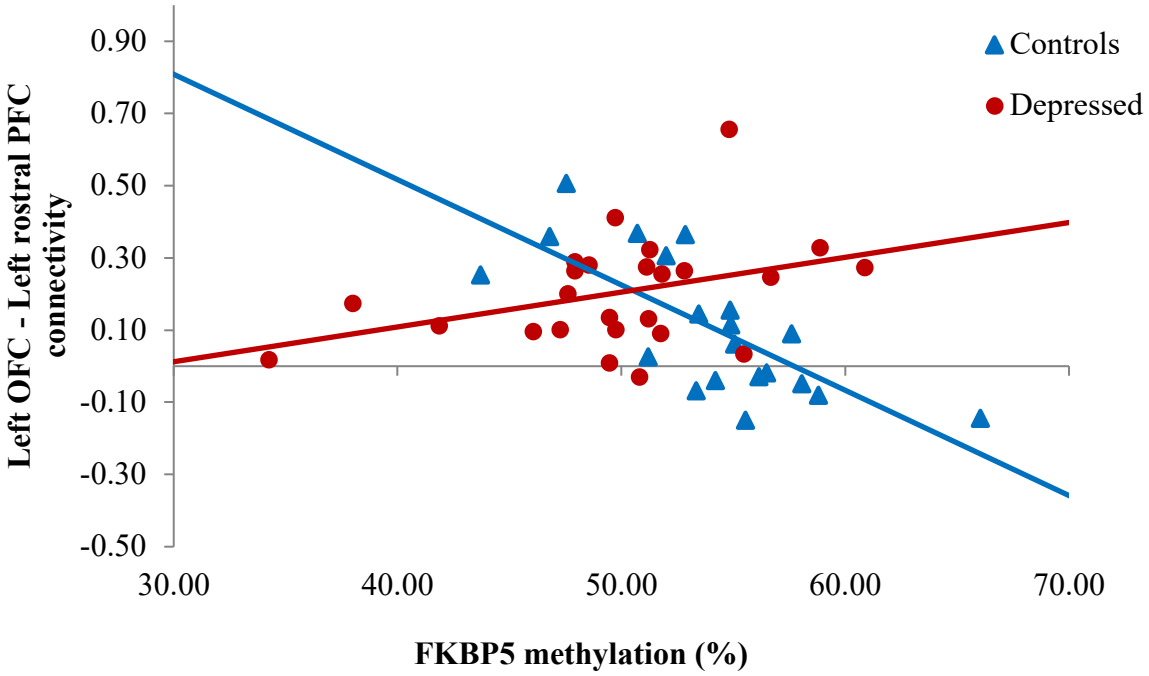

Figure 8. Greater *FKBP5* intron 7 methylation was associated with decreased resting-state connectivity between the left orbitofrontal cortex and right rostral prefrontal cortex in controls only.

3.4.4 DNA methylation level and rs-FC: ICA

Two independent spatial components were identified as distinct components within the DMN. Regions which loaded onto each component were identified using the default anatomical labels within CONN. Regions which loaded most strongly on the first component (ICA 2) included the precuneus, bilateral middle frontal and superior frontal gyri, bilateral lateral occipital cortex, frontal medial cortex, right middle temporal gyrus and parahippocampal gyri. Regions which loaded most strongly on the second component (ICA 17) included the posterior cingulate, the precuneus and the bilateral lateral occipital cortex.

For ICA 17, there was a significant Diagnosis**SLC6A4* methylation interaction ($t(39)$= 3.31, k=256, p_{FDR}< .001) in a cluster containing the right pre and postcentral gyrus. This

interaction was followed up with correlations within the significant cluster in each group (Figure 9). Greater *SLC6A4* promoter methylation was associated with decreased connectivity between IC 17 and the cluster within adolescents with depression (r=-.687, p<. 001) but increased connectivity within controls (r =.566, p=.009).

Similarly, for IC 17, there was significant Diagnosis**FKBP5* methylation interaction within a cluster containing the left postcentral gyrus (t(39)=3.31, k=121, pFDR=.0016). Specifically, lower *FKBP5* intron 7 methylation was associated with increased connectivity between the DMN (ICA 17) and the postcentral gyrus in adolescents with depression (r = -.637, p =.001) but decreased connectivity in controls (r = .788, p <.001) (Figure 10).

For ICA 2, there was also a significant Diagnosis**FKBP5* Methylation interaction in two separate clusters. The first cluster contained voxels within the left inferior frontal gyrus and the left middle frontal gyrus (t(39)=3.31, k=118, pFDR=.002). Lower *FKBP5* intron 7 methylation was associated with increased connectivity between ICA 2 and the cluster in adolescents with depression (r=-.56, p=.003) but decreased connectivity in controls (r=.722, p<.001; see supplemental Figure 12). The second cluster contained voxels within the right frontal pole (t(39) = 3.31, k=118, pFDR=.014). Lower *FKBP5* intron 7 methylation was associated with increased connectivity between ICA 2 and the right frontal pole in adolescents with depression (r=-.526, p=.007) but decreased connectivity in controls (r=.616, p=.004; see supplemental Figure 13).

There were no differences in ICA 2 or ICA 17 functional connectivity between those individuals who were taking stimulants and those who were taking other medications or no medication.

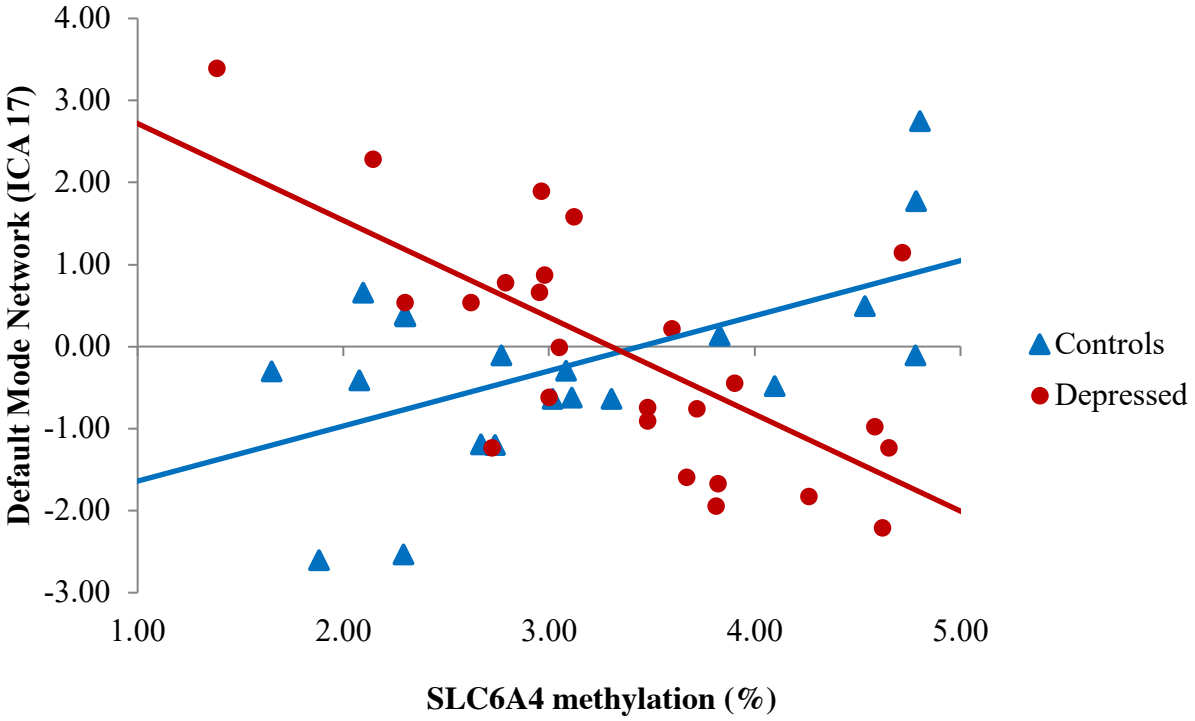

Figure 9. Greater *SLC6A4* promoter methylation was associated with decreased DMN connectivity in adolescents with depression, but with increased connectivity in healthy adolescents.

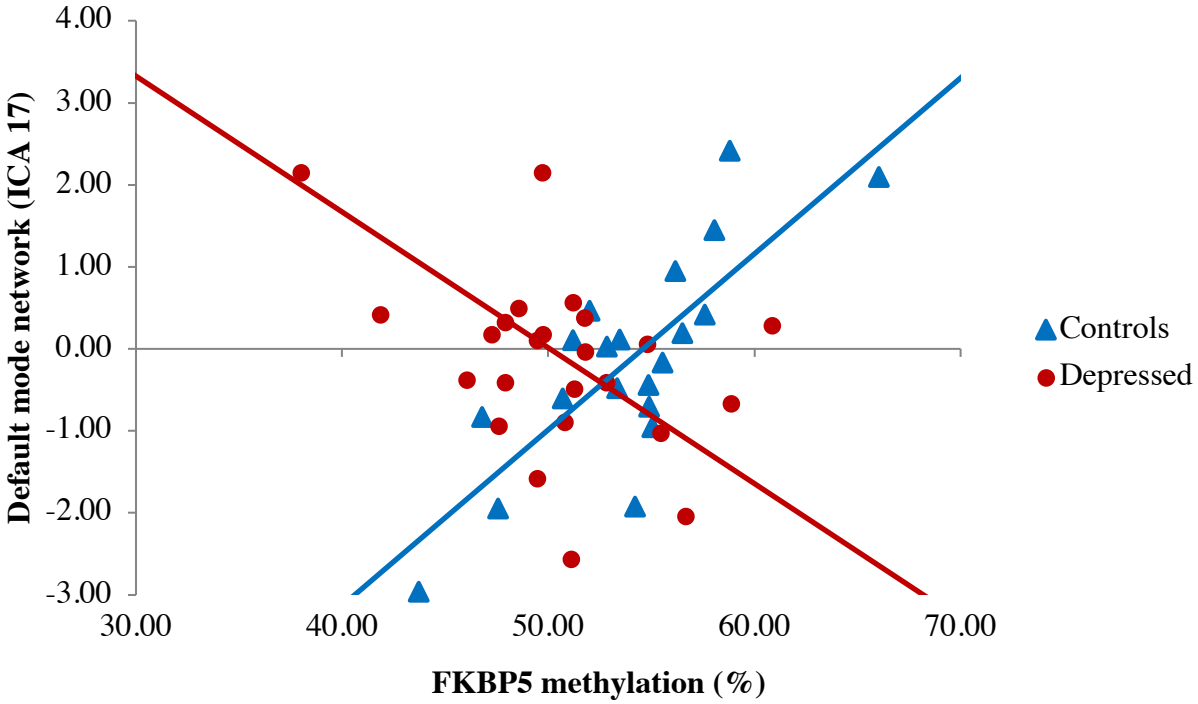

Figure 10. Greater *FKBP5* intron 7 methylation was associated with decreased DMN connectivity within adolescents with depression but increased connectivity within controls.

3.4.5 DNA methylation and GM volume

There was a diagnosis**SLC6A4* promoter methylation interaction (Wald chi-square= 6.59, p_{FDR}=.036) in the inferior frontal orbital gyrus. Follow up analysis indicated that greater *SLC6A4* methylation was associated with increased volume in adolescents with depression (Wald chi-square=3.98, p=.046) and a trend towards decreased volume in controls (Wald chi-square= 2.93, p= .087).

Lower *FKBP5* intron 7 methylation was associated with decreased GM volume in the anterior cingulate (Wald chi-square=11.00, p_{FDR}= .004), frontal operculum (Wald chi-square= 12.30, p_{FDR}< .001) and inferior frontal orbital gyrus (Wald chi-square=9.79, p_{FDR}=.007), regardless of diagnosis (see supplemental Table 6.). At the uncorrected level, there was a

diagnosis**FKBP5* methylation interaction (Wald chi-square=5.65, *p*= .017) in the hippocampus (Figure 11) such that lower *FKBP5* intron 7 methylation was significantly associated with increased hippocampal volume in adolescents with depression (Wald chi-square=11.05, *p*= .001), but not controls (Wald chi-square=.457, *p*= .499). A rerun of the analyses with stimulant use as covariate did not change the results.

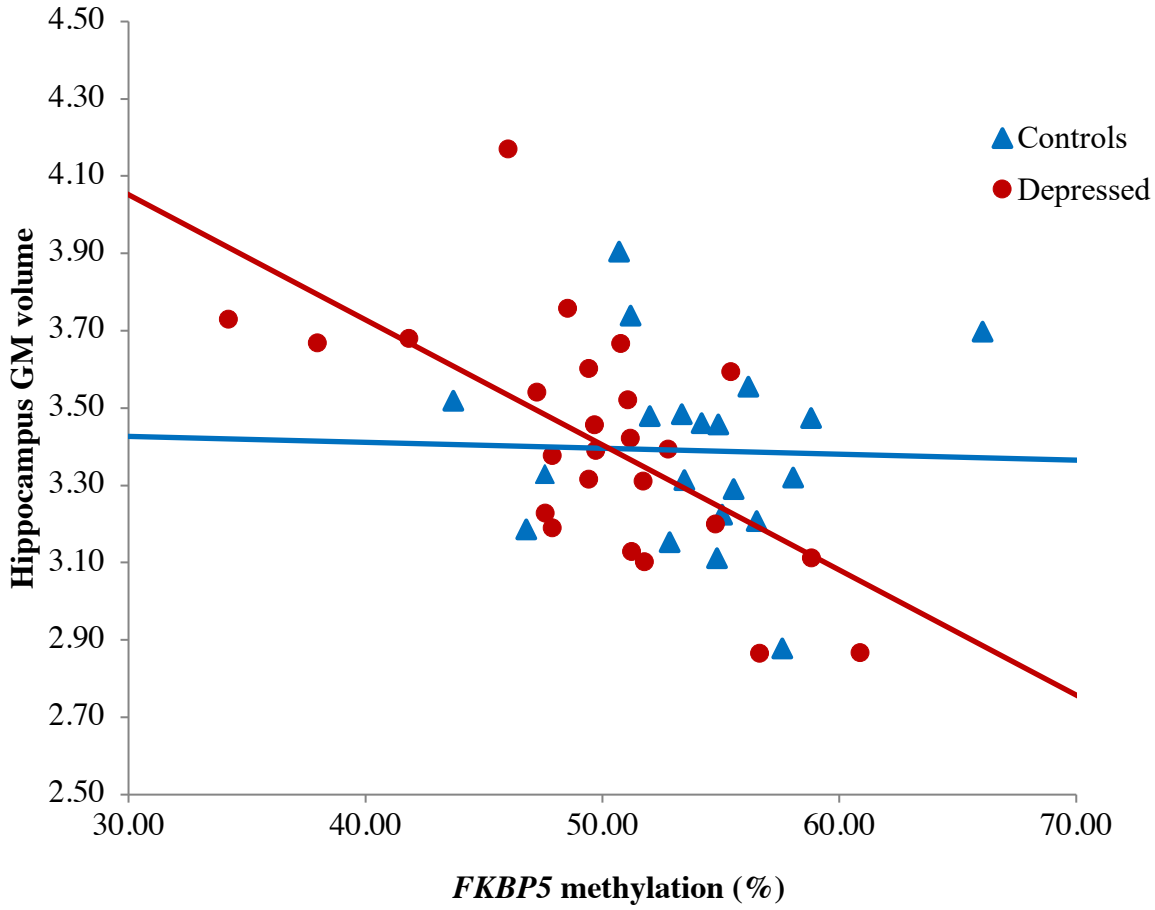

Figure 11. Greater *FKBP5* intron 7 methylation was associated with decreased hippocampal volume in adolescents with depression, but not in healthy controls.

3.5 Discussion

Following up on our previous work in adults, the current study aimed to investigate the relationship between DNA methylation levels of the *SLC6A4* and *FKBP5* genes as assessed in saliva, rs-FC and frontolimbic brain structure in adolescents with depression and healthy controls. Consistent with findings in adults, we find an association between greater *SLC6A4* methylation and increased amygdala-FO connectivity, and identify a novel negative association between *FKBP5* methylation and OFC-rostral PFC connectivity in healthy adolescents. Further, we showed that diagnosis modulated the association between DNA methylation and DMN rsFC. Finally, we extend findings of an association between lower *FKBP5* methylation and smaller IFOG GM volume in adults to an adolescent population.

3.5.1 Peripheral DNA methylation and rs-FC

Healthy controls had higher *FKBP5* methylation levels than patients. Notably, in the control group, lower *FKBP5* intron 7 methylation was associated with greater connectivity between the left orbitofrontal cortex (OFC) and left rostral prefrontal cortex (RPFC) in controls. Such as association was not observed in the patient group.

The observation of lower *FKBP5* methylation in patients is in line with a recent study showing an association between low *FKBP5* methylation and depressive symptoms (Klinger-König et al., 2019b). Furthermore, *FKBP5* methylation has also been associated with awakening and bedtime cortisol reactivity (R. S. Lee et al., 2018). Likewise, increased *FKBP5* expression has been associated with lower cognitive flexibility (Sabbagh et al., 2014). At the neural level, both the OFC and RPFC have been implicated in the cognitive control of emotions. Specifically, the OFC has been implicated in emotion-regulation, motivation, behavioural (dis)inhibition, and in the ability to predict and evaluate behavioural consequences of potential actions (Rudebeck & Murray, 2014). Although much less is known about the specific function of the RPFC, unlike

other prefrontal regions, the RPFC is principally interconnected with the supramodal cortex (Dumontheil et al., 2008; Ramnani & Owen, 2004), and seems to play an important integrative role in cognitive regulation by offsetting the contribution of internal and external attentional sources (Henseler et al., 2011). Hyperconnectivity between the OFC and RPFC may reflect an increased ability to cognitively regulate emotional states and cognitively evaluate behavioural outcomes. Although speculative, the greater OFC-RPFC connectivity in individuals with low methylation but with no lifetime psychopathology may reflect a neural substrate of a resilience mechanism of a more optimal and/or more efficient use of attentional control strategies when regulating emotions. Follow-up studies in healthy individuals, with repeated methylation and neural assessments over time, are needed to further test such hypothesis.

This is also the first study, to our knowledge, to show a link between *FKBP5* intron 7 methylation and frontolimbic rs-FC. A previous study of ours which examined seed-to-voxel rs-FC at four DMN seeds (posterior cingulate cortex, medial prefrontal cortex, right/left lateral parietal areas) did not find an association between saliva *FKBP5* intron 7 methylation and rs-FC in a sample of 51 healthy adults (Di Sante et al., 2018). However, this previous study examined only DMN seeds, while our study also included frontolimbic regions which are part of other large-scale brain networks. Significant regions in the current study were not included in the previous study, and regions included in both studies (i.e., mPFC and PCC) were not associated with *FKBP5* methylation in either study. While the current study found an association between DMN rsFC and *FKBP5* methylation using the IC analysis, Di Sante and colleagues (2018) used a seed-to-voxel analysis. Thus, the IC analysis, which examines activity in the DMN as a whole as opposed to particular nodes, may be more sensitive to the relationship between rsFC and *FKBP5* methylation.

Greater saliva *SLC6A4* promoter methylation was associated with increased connectivity between the right amygdala and left frontal operculum regardless of diagnosis. These regions are involved in emotional arousal and emotional awareness of the self and others, respectively (A. D. Craig, 2009; Frodl et al., 2015; Jabbi et al., 2007; Singer et al., 2009; Wicker et al., 2003; Zald, 2003). Consistent with this finding, Muehlhan et al. (2015) found a positive association between *SLC6A4* methylation and rs-FC between the amygdala and a cluster within the salience network which contained the operculum in healthy adults. We have shown an association between peripheral *SLC6A4* promoter methylation and activity in both the amygdala and frontal operculum during emotion processing in depressed and healthy adults (Frodl et al., 2015). In addition, Dannlowkski et al. (2014) showed a link between *SLC6A4* methylation and amygdala volume in healthy individuals. The results of the current study are consistent with such findings, and suggest that the association between peripheral *SLC6A4* methylation, the amygdala and the frontal operculum extends to healthy and adolescents with depression. Interestingly, Singh and colleagues (Singh et al., 2018) found that both depressed and healthy youth at familial risk for depression displayed altered amygdala rs-FC compared to healthy low-risk controls, suggesting that amygdala rs-FC may represent a risk marker for depression. Given the association between *SLC6A4* promoter methylation and amygdala rs-FC in our study, it is possible that peripheral S*LC6A4* methylation at specific loci may serve as a useful proxy for one or more neurobiological risk factors for depression in adolescence.

Both *FKBP5* and *SLC6A4* methylation were also associated with the connectivity between the DMN and various brain regions outside the DMN in healthy controls and with decreased connectivity in adolescents with depression. Notably, greater *FKBP5* methylation was associated with decreased connectivity between the DMN and frontal pole in adolescents with

depression but with increased connectivity in controls. Interestingly, increased methylation at both the *SLC6A4* and *NR3C1* (glucocorticoid receptor) genes, have been linked to increased connectivity between the DMN and frontal pole in healthy adults in previous studies by our research group (Di Sante et al., 2018). Furthermore, this finding is of direct relevance to MDD as DMN dysregulation is thought to be a key factor underlying depressive rumination in depressed individuals (Hamilton et al., 2015).

3.5.2 DNA methylation and brain structure

Lower *FKBP5* intron 7 methylation was associated with smaller volume of the IFOG, ACC and FO, independent of diagnosis. The link between lower *FKBP5* methylation and smaller IFOG is consistent with previous research by our group in adult MDD patients and healthy controls (Tozzi et al., 2018), as well as research showing that the minor (risk) *FKBP5* allele of the rs9470080 single nucleotide polymorphism (SNP) is associated with increased inferior orbitofrontal volume in healthy controls (Hirakawa et al., 2016). Further, Fujii and colleagues (2014) found that healthy adult carriers of the minor rs9470080 allele had smaller dorsal ACC GM volume. The present study is a first reporting an association between *FKBP5* methylation, ACC and FO volume. In the current study, *SLC6A4* promoter methylation was also associated with greater volume of the IFOG gyrus in adolescents with depression while a trend towards decreased volume was observed in controls. We previously found that greater blood *SCL6A4* promoter methylation was associated with decreased serotonin synthesis in the lateral OFC in healthy adults (D. Wang et al., 2012). Moreover, in healthy adolescent monozygotic twin pairs, twins with greater *SLC6A4* promoter methylation relative to their co-twins displayed greater left orbitofrontal cortex activation and greater left amygdala-right OFC connectivity in response to sad faces (Ismaylova, Lévesque, et al., 2018). Thus, structural, functional and

neurochemical findings converge on the link between peripheral *SLC6A4* promoter methylation and the orbitofrontal cortex in adolescents and adults.

Unlike a previous study investigating the link between *SLC6A4* promoter methylation in adult patients and healthy controls at the same CpG sites as the current study (Booij, Szyf, et al., 2015), we did not find an association between *SLC6A4* promoter methylation and hippocampal volume. This may suggest the importance of developmental considerations in understanding the relationship between DNA methylation and brain structure and function. However, we did observe that greater *FKBP5* intron 7 methylation was associated with decreased hippocampal GM volume in depressed individuals, albeit at the uncorrected $p < .001$ level. Although this finding should be interpreted with caution, it is of importance given the high density of glucocorticoid receptors in the hippocampus and the consistent link between MDD and reduced hippocampal volume (Bremner et al., 2000; Campbell et al., 2004; D. Wang et al., 2012). Interestingly, another study using whole blood found that lower *FKBP5* methylation levels were associated with increased volume of the right hippocampal head in females who had experienced childhood adversity and healthy controls (Klengel et al., 2013). However, in a non-clinical sample, we did not find an association between *FKBP5* intron 7 methylation and hippocampal volume (Di Sante et al., 2018). Together, these results suggest that peripheral *FKBP5* methylation may be associated with hippocampal development.

3.5.3 Limitations and future directions

One limitation is that we assessed only one gene region at each candidate gene. Secondly, we did not obtain participants' genotype at common polymorphisms of the *FKBP5* or *SLC6A4* genes. On the other hand, because of the small sample size of the current study, it is unlikely that a reliable genotype effect (or lack of) could be detected. Furthermore, results may be confounded

by medication use. However, it is also important to recognize that excluding individuals based on medication use may lead to an unrepresentative sample. Additionally, we did not exclude participants based on comorbid psychopathology (with the exception of substance abuse, suicidality and psychosis), yet this also makes our results more generalizable to the population. Further, our sample was mostly female. However, this gender bias is representative of the gender difference in the clinic from which are sample was recruited, and is likely a result of the greater prevalence of depression among adolescent females than males, as well as the greater willingness of females to seek services (Addis & Mahalik, 2003; Call & Shafer, 2018). Thus results are likely generalizable to the treatment seeking population. Finally, as brain-DNA methylation associations of the *SLC6A4* and *FKBP5* gene appear to be, in part, tissue-specific (Di Sante et al., 2018; Ismaylova et al., 2017), our results cannot be necessarily generalized to other peripheral tissues and the observed peripheral methylation patterns would not necessarily reflect the state of methylation of the same gene regions in the brain (Booij, Szyf, et al., 2015; Nikolova et al., 2014).

In conclusion, the current study showed that both salivary *FKBP5* and *SLC6A4* methylation levels are associated with brain connectivity and structure in regions relevant to adolescent depression. Further, we found associations between peripheral DNA methylation and brain structure and connectivity which mirror and extend findings from past research in both adults and adolescents. We replicated our previous finding in adults suggesting a link between *FKBP5* intron 7 methylation and IFOG GM volume in depressed adults and healthy controls, and extend these findings to show a link between *FKBP5* intron 7 methylation and OFC-RPFC rs-FC in healthy adolescents. Future studies using a longitudinal design with repeated measures from childhood to adulthood in individuals with and without (risk for) depressive disorder are needed

to better understand how the relationship between DNA methylation at stress-related genes and brain processes changes throughout the lifespan.

3.6 Declaration of Funding

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3.7 Acknowledgements

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3.8 Supplemental tables and figures

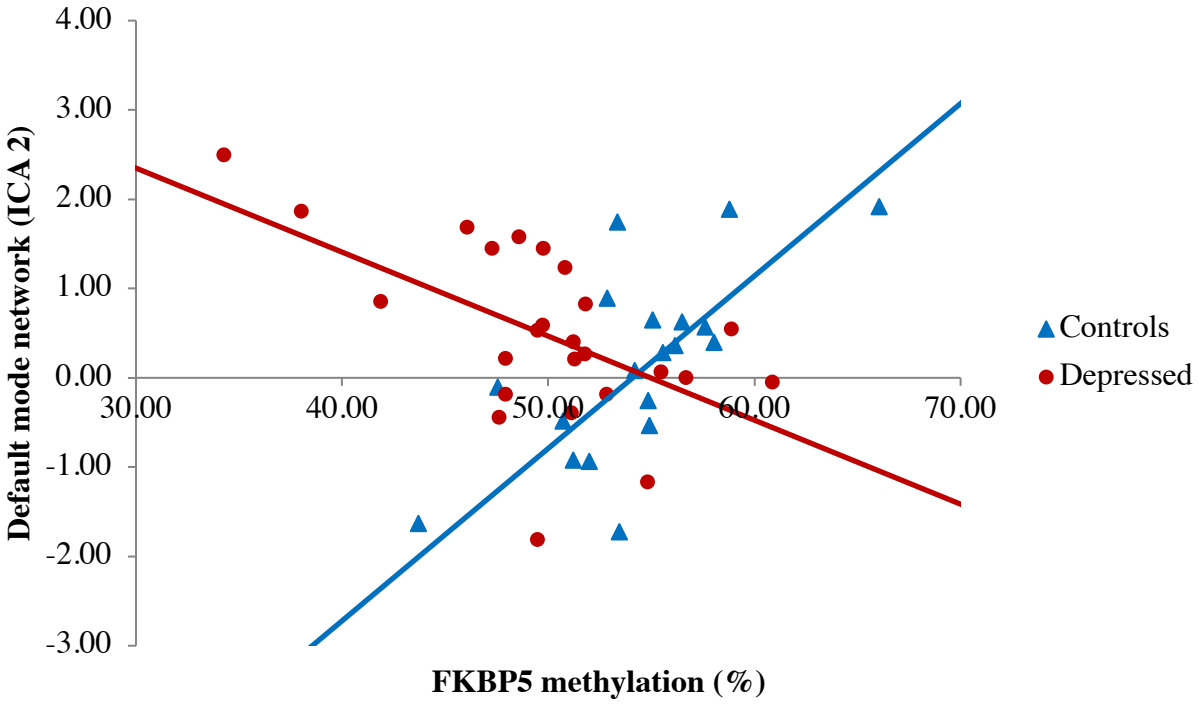

Figure 12. Association between *FKBP5* methylation and ICA 2 of the default mode network. The cluster of the ICA 2 displayed above contained voxels within the left inferior frontal gyrus and the left middle frontal gyrus.

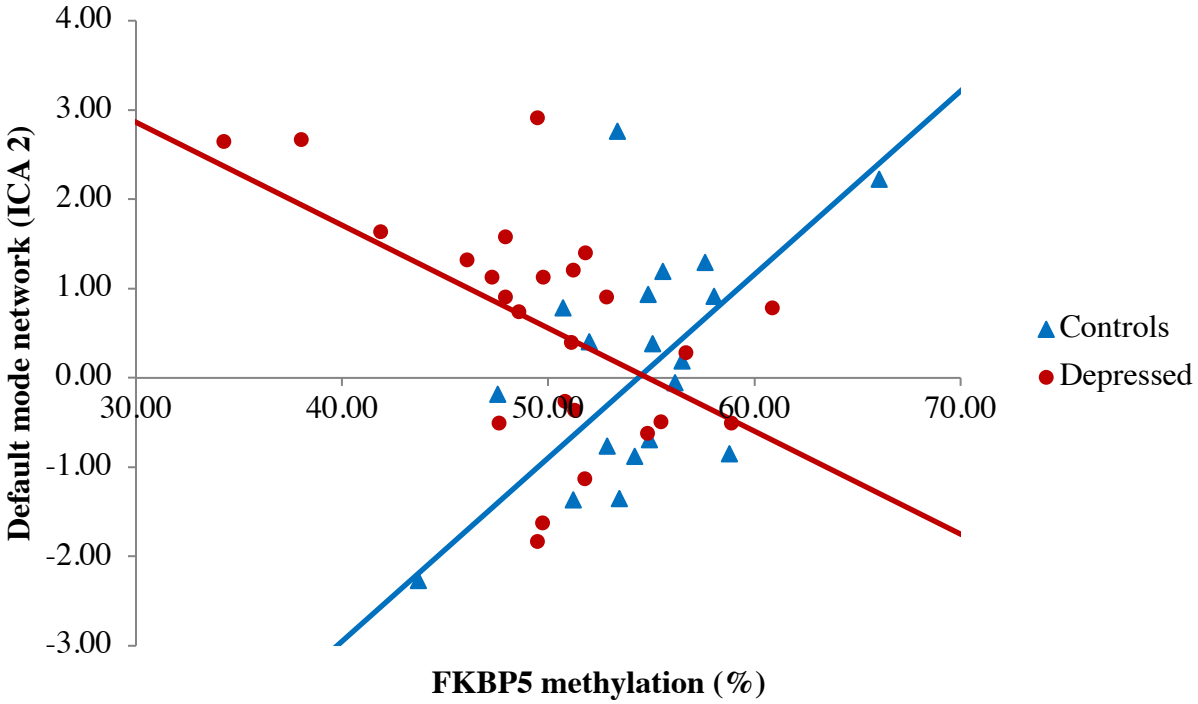

Figure 13. Association between *FKBP*5 methylation and ICA 2 of the default mode network. The cluster of the ICA 2 displayed above contained voxels within the right frontal pole.

Table 6. *Association between FKBP5 methylation and gray matter volume.*

	Wald chi-square	p	pFDR	Direction of Association
Anterior cingulate	11.00	.001	.004	+
Frontal operculum	12.30	<.001	<.001	+
Inferior frontal orbital gyrus	9.79	.002	.007	+
Hippocampus X Diagnosis	5.65	.017	.056	Interaction
*Controls	.457	.499	N/A	None
*Depressed adolescents	11.05	.001	N/A	-

Association between *FKBP5* Methylation and gray matter volume in depressed adolescents and healthy controls. * Indicates follow-up analysis. X indicates interaction.

4. CHAPTER 4: The association between early life adversity, depression vulnerability and *in vivo* measures of histone deacetylases in healthy adults: A positron emission tomography study

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(In preparation for submission to European Neuropsychopharmacology)

4.1 Abstract

Early life adversity is associated with lasting effects on the brain and mental health. Epigenetic processes (e.g., DNA methylation, histone acetylation) have received attention as a group of biological mechanisms through which the early life environment is thought to affect brain development. Due to limitations inherent in measuring epigenetic processes *in-vivo*, human research has focused on proxy measures of epigenetic processes in peripheral tissues or on post-mortem samples. In the current study, we used the [^{11}C]Martinostat tracer in 14 healthy adult males aged between 35 and 40 years old, to study the association between early life adversity, risk for depression and *in-vivo* brain HDAC density. We found no associations between HDAC density and early life adversity. Decreased HDAC density in the orbital frontal cortex was associated with higher levels of depressive symptoms, while greater HDAC density in the hippocampus was associated with higher levels of neuroticism. This is the first study to investigate the link between epigenetic processes in the living brain, early life adversity and risk for depression. These findings suggest that early life adversity in healthy adults may not be associated with changes in HDAC expression that can be identified using the [^{11}C]Martinostat tracer. However, results suggest the [^{11}C]Martinostat tracer may be sensitive to subtle brain changes underlying risk for depression, even in a healthy population. Future research investigating early life adversity in the clinical populations may provide a better understanding the role of HDACs in early life adversity.

4.2 Introduction

It is now well established that adversity early in life is associated with lasting adverse effects on emotion, behaviour and their underlying neurocircuitry (Callaghan & Tottenham, 2016; Krugers et al., 2017; Maccari et al., 2014). Adversity occurring *in utero* (e.g., exposure to teratogens, malnutrition), in the home (e.g., maladaptive parenting practices, low socioeconomic

status) and outside the home (e.g., peer rejection, lack of social support) are associated with increased risk for a number of different psychopathologies including major depressive disorder, the leading cause of disability worldwide (Conway et al., 2018; Fergusson & Woodward, 2002; Green et al., 2010; Lovallo, 2013; World Health Organization, 2017). Over the past two decades, the role of epigenetic processes has been studied extensively as a potential biological mechanism by which the early environment impacts brain development and associated behavioural, emotional and cognitive outcomes.

While DNA methylation is the epigenetic process which has received the most attention to date, there is clear evidence that histone acetylation also plays an important role in epigenetic regulation of emotion and behaviour in the context of mental health (Borbély et al., 2022; D. Chen et al., 2017; Cui et al., 2013; Frieling et al., 2010; Hobara et al., 2010; Iga et al., 2007; Nestler et al., 2016; Otsuki et al., 2008; Takebayashi, Hisaoka, Nishida, Tsuchioka, Miyoshi, Kozuru, Hikasa, Okamoto, Shinno, & Morinobu, 2006; Uchida et al., 2018; Weaver et al., 2004). More specifically, substantial body of literature has emerged focusing specifically on the role of HDACs in early life adversity and depression. Animal models have shown that: (1) adversity (e.g., maternal separation, chronic social defeat) early in life can lead to lasting changes in histone acetylation and associated changes in HDAC expression in limbic regions such as the hippocampus and nucleus accumbens; (2) early life adversity is associated with concurrent increases in anxious-depressive behaviours and; (3) anxious-depressive behaviours associated with early life adversity can be alleviated by administration of HDAC inhibitors (W.Y. Chen et al., 2019; Nestler et al., 2016; Qian et al., 2021; Uchida et al., 2018; Weaver et al., 2004). Further, several human studies have emerged providing evidence for altered levels of HDACS in peripheral tissues of depressed individuals (Hobara et al., 2010; Iga et al., 2007; Misztak et al.,

2018).

While the aforementioned findings have been insightful, a limitation of research conducted to date is that, due to the technical and ethical constraints inherent in measuring epigenetic processes in the brain *in-vivo*, studies have focused either on preclinical animal models, post-mortem samples, or peripheral measures of HDAC RNA expression. To circumvent this limitation, the tracer [^{11}C]Martinostat has been developed which allows for *in vivo* imaging of specific HDAC proteins (C. Wang et al., 2014). This tracer binds with specificity to class I and IIb HDACS, namely HDAC1, HDAC2, HDAC3 and HDAC 6, and has been shown to possess biological and kinetic properties which make it appropriate for use in PET brain imaging studies (Wey et al., 2015). Class I and IIb HDACs are of particular relevance given a growing body of research implicating them in in psychiatric disease and early adversity (Haberland et al., 2009; Misztak et al., 2018).

Results from early studies investigating psychiatric populations using [^{11}C]Martinostat are promising and provide support for the implication of HDACs in the development of psychopathology. Gilbert and colleagues (2019) showed that individuals with schizophrenia or schizoaffective disorder displayed decreased HDAC density in a number of prefrontal regions, including the dorsolateral prefrontal cortex (DLPFC; Gilbert et al., 2019). In a more recent study by the same group, individuals with bipolar disorder showed reduced HDAC density in the bilateral thalamus, orbitofrontal cortex, right hippocampus and right amygdala compared to controls (Tseng et al., 2020). However, to our knowledge, no study to date has used [^{11}C]Martinostat to study early life adversity or depressive symptoms.

In addition to studying psychiatric populations, it is important to study the neurobiology of subclinical symptomatology and risk factors in healthy populations in order to better

understand the basic neurocircuitry affected in psychiatric disorders. One possible area for understanding the epigenetic basis of risk for depression and early life adversity is the study of personality. Personality traits are thought to relate to a number of basic brain systems which can become dysregulated in psychopathology (Cai et al., 2020; DeYoung et al., 2010; Hsu et al., 2018; Markett et al., 2018). Neuroticism is perhaps the most relevant personality trait to depression. Neuroticism is linked to negative affectivity and the functioning of the behavioural inhibition system, a neural system thought to be responsible for controlling responses to conditioned stimuli signaling punishment (DeNeve, 1999; Gore & Widiger, 2018; Jorm et al., 1998). Indeed, depression has been hypothesized to be characterized by an overactive BIS (Jylhä & Isometsä, 2006; Kasch et al., 2002).

The aim of the current study is to investigate the association between HDAC density in the living brain, early life adversity and depression vulnerability. To this end, we will assess levels of HDAC density as assessed by the [^{11}C]Martinostat tracer, early life adversity, depressive symptoms and neuroticism in a sample of healthy adult males. We expect that early life adversity, depressive symptoms and neuroticism will be associated with HDAC density in key frontolimbic regions. Given inconsistencies in the direction of findings in human and animal research, we do not hypothesize a direction of this effect. We will also perform exploratory analyses investigating associations between the other Big Five personality traits (extraversion, openness to experience, conscientiousness and agreeableness).

4.3 Experimental Procedures

4.3.1 Participants

Participants were recruited from the Quebec Longitudinal Study of Kindergarten Children (N=3185). A representative sample of 2000 boys and girls was randomly selected from this larger cohort and was followed regularly from age 6 to 14 with measures focused on child

and family related characteristics, and again from age 18 to mid 30s in order to assess psychosocial functioning, neurobiology and behaviour. Fourteen healthy male participants a range of early life adversity sores were recruited. All participants were between 35 and 40 years old.

Participants underwent one PET and one MRI scan at the Montreal Neurological Institute (MNI). In addition to PET and MRI scans, all participants underwent the Structured Clinical Interview for DSM-5 (SCID-5) in order to assess for past and current psychopathology. Participants were excluded if they endorsed any current or past psychiatric diagnoses. Further, participants completed a number of questionnaires outlined below.

4.3.2 Interviews and questionnaires

Screening. In order to confirm the absence of a current psychiatric diagnosis, the SCID-5 was administered by a senior graduate student with significant training and experience under the supervision of a licensed clinical psychologist (LB).

Early life Adversity: An adversity index used in previous studies in this cohort was administered which combines seven socioeconomic indices including parental occupational prestige, parental age at birth of their first child, parental education level, and familial status (Tremblay et al., 1991). Information on these indices was collected at ages: 6, then 10-16 and averaged across these timepoints, with scores ranging between 0 and 1. Research by our group has previously used this index to investigate links between early adversity and serotonin synthesis as well as physical aggression (Arseneault et al., 2002; Booij et al., 2012; Haapasalo & Tremblay, 1994). Further, this index has previously been associated with childhood externalizing disorders in a sample of 3000 children (Vitaro et al., 1992).

Personality. Personality characteristics were assessed using the Revised NEO Personality Inventory (NEO PI-R). The NEO-PI-R is a commonly used personality measure consisting of 240 items. Participants are presented with descriptions of behaviours and asked to rate the which extent to which they agree that these descriptions apply to them on a 5-point Likert scale ranging from strongly disagree to strongly agree. The scale measures five domains of personality functioning: Neuroticism, extraversion, openness to experience, conscientiousness, and agreeableness. Internal consistency of individual domain scores is high with domain scores ranging from .88 to .92 (Costa et al., 1991) and neuroticism showing the greatest internal consistency.

4.3.3 MRI Imaging

MRI scans were obtained using a Siemens Trio 3 T MRI scanner for anatomical co-registration (TR = 2300 ms; TE = 3.42 ms; field of view = 256 mm; matrix size = 256x256; slice thickness = 1.0 mm; resolution = 1.0 mm isotropic voxel).

4.3.4 PET Imaging

[^{11}C]Martinostat Radiosynthesis

The method used to synthesize [^{11}C] Martinostat is described in detail in a previous paper (C. Wang et al., 2014).

Image Acquisition

A licensed nuclear medicine technologist injected a minimum of 7 mCi (range = (259 MBq - 370 MBq) of [^{11}C]Martinostat as manual bolus through an intravenous (IV) catheter placed in the antecubital vein. Participants were asked to remain still during the scan. PET images were acquired using a high-resolution research tomograph (HRRT) scanner. Acquisition began upon injection of the radioactive tracer and 33 frames of increasingly longer duration were

obtained in order to image identical amounts of radiation in each frame. The last 6 frames, corresponding to the last half hour of the scan (60 – 90 minutes) were used in the analysis.

Preprocessing

In a sample of healthy volunteers, Wey et al., (2016) previously showed that [^{11}C]Martinostat can reliably be assessed without using arterial blood sampling, and using instead regional standard uptake values from 60 to 90 minutes post [^{11}C]Martinostat injection ($SUV_{60\text{-}90min}$), an image based measure of radiotracer binding (SUV= mean radioactivity/injected dose/weight). In order to avoid the invasive procedure of arterial blood sampling required to calculate V_T values, we used the standardized uptake value ratio (SUVR), calculated as the ratio of $SUV_{60\text{-}90min}$ values between each ROI compared to whole brain.

Preprocessing was conducted using the MINC toolbox (version 1.9.18). First, all frames except those from 60-90 minutes were discarded, and values were averaged across this time period. Averaged PET images for each individual were then co-registered to their T_1-weighted structural image. If their T_1-weighted image was not available, which was the case for two participants as they did not meet the MRI safety requirements, PET images were co-registered to the PET image of another participant; the participant whose image resulted in the best co-registration, as determined by visual inspection, was used. After images were visually inspected for successful registration, they were intensity normalized to whole brain to obtain $SUVR_{60\text{-}90min}$ values, and then smoothed using a 6mm kernel. Finally, $SUVR_{60\text{-}90min}$ values were extracted for further analysis from the participants' native space at the following ROIs, as identified using the Automated Anatomical Labeling Atlas: Orbitofrontal Cortex (OFC), Cingulate, Hippocampus, Amygdala, Caudate, Putamen and Thalamus (Tzourio-Mazoyer et al., 2002).

Analyses

In order to assess the association between early life adversity and HDAC density, bivariate Pearson correlation analyses between SUVR $_{60\text{-}90min}$ values at each ROI and the total score on the adversity index were conducted. Bivariate Pearson correlation analyses were also conducted between SUVR $_{60\text{-}90min}$ values at each ROI and total score on the CES-D in order to determine the relationship between depressive symptoms and HDAC density. Finally, in order to test the association between HDAC density and personality, bivariate Pearson correlation analyses were conducted between SUVR $_{60\text{-}90min}$ values at each ROI and each of the Big 5 personality traits (neuroticism, extraversion, openness to experience, conscientiousness, and agreeableness). False discovery rate (FDR) correction for multiple comparisons was applied across regions for each measure individually using the Benjamini-Hochberg procedure. However, given the small sample size and exploratory nature of the study, uncorrected results at $\alpha = .05$ are also reported. Results significant at the FDR corrected level are indicated by an asterisk.

4.4 Results

4.4.1 Participants

PET imaging data was collected for all 14 individuals included in the study. MRI data was collected for all but 2 participants due to the participants not meeting the MRI safety requirements. All other measures (i.e., adversity index, CESD, NEO-PI-R) were obtained for all 14 participants.

4.4.2 SUVR Data

Signal intensity was greater in all gray matter regions examined compared to background and there was moderate regional and inter-individual variability (Figure 14). There was adequate variability among participants within ROIs.

SUVR values were highest in the putamen (*M* =1.23 , *SD* = 0.07), thalamus (*M* = 1.16, *SD* = 0.086) and cingulate (*M* = 1.13, *SD* = 0.09), followed by the caudate (*M* = 1.0, *SD* = 0.11) and OFC (*M* = 1.0, *SD* = 0.033), and then the amygdala (*M* = 0.88, *SD* = 0.061) and hippocampus (*M* = 0.83, *SD* = 0.56).

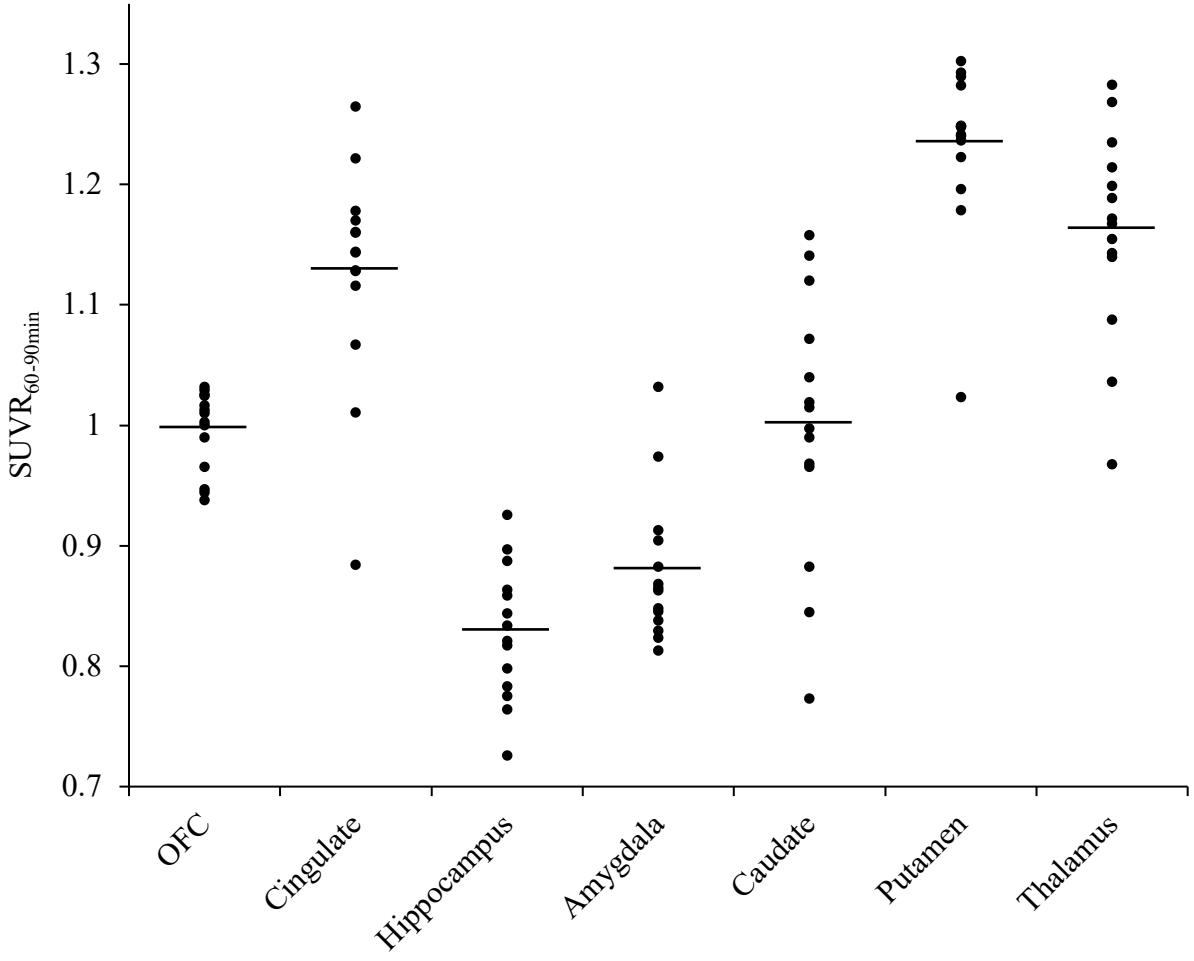

Figure 14. Scatter plot indicating $SUVR_{60\text{-}90min}$ across the 7 ROIs investigated. Dots indicate individual participants. The mean SUVR60-90min value within each ROI is indicated by a horizontal bar.

4.4.3 Early life Adversity and HDAC density

No significant associations were found between the early life adversity index and SUVR $_{60\text{-}90min}$ values at any of the ROIs investigated ($p > .22$, $-.36 < r < .29$)

4.4.4 Depressive Symptoms and HDAC density

Simple bivariate correlation analysis between CESD scores and SUVR $_{60\text{-}90min}$ values at each ROI revealed a significant association between depressive symptoms and HDAC density in the OFC ($r = -.60$, $p = .023$), such that greater depressive symptoms was associated with decreased HDAC density (Figure 15).

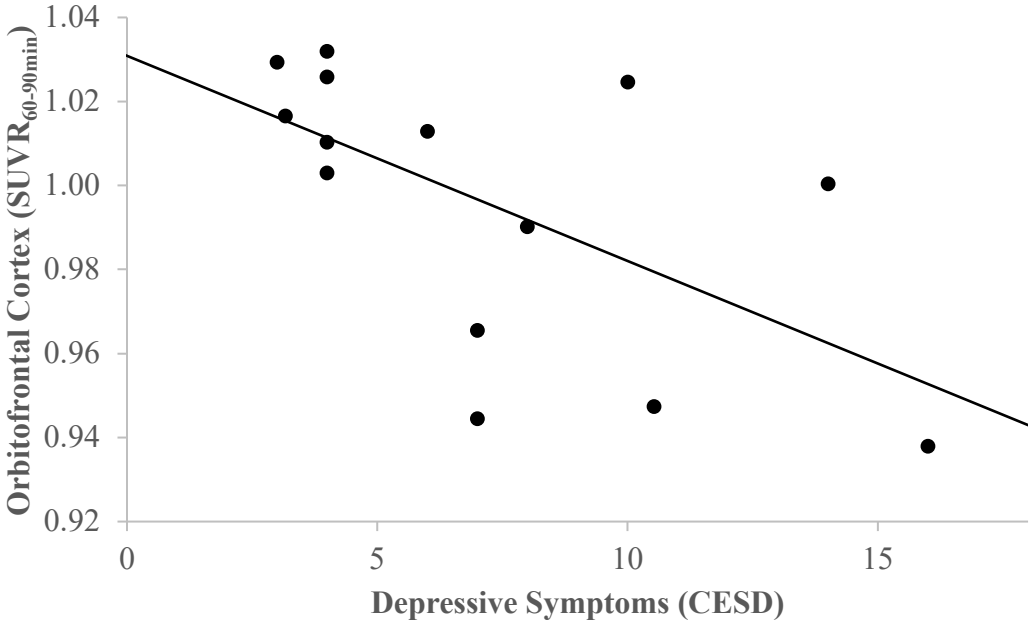

Figure 15. Greater depressive symptoms were associated with decreased SUVR$_{60\text{-}90min}$ in the orbitofrontal cortex.

4.4.5 Neuroticism and HDAC density

There was a significant association between neuroticism and HDAC density such that greater neuroticism was associated with increased SUVR $_{60\text{-}90min}$ in the hippocampus (r = .626, p = .017; Figure 16).

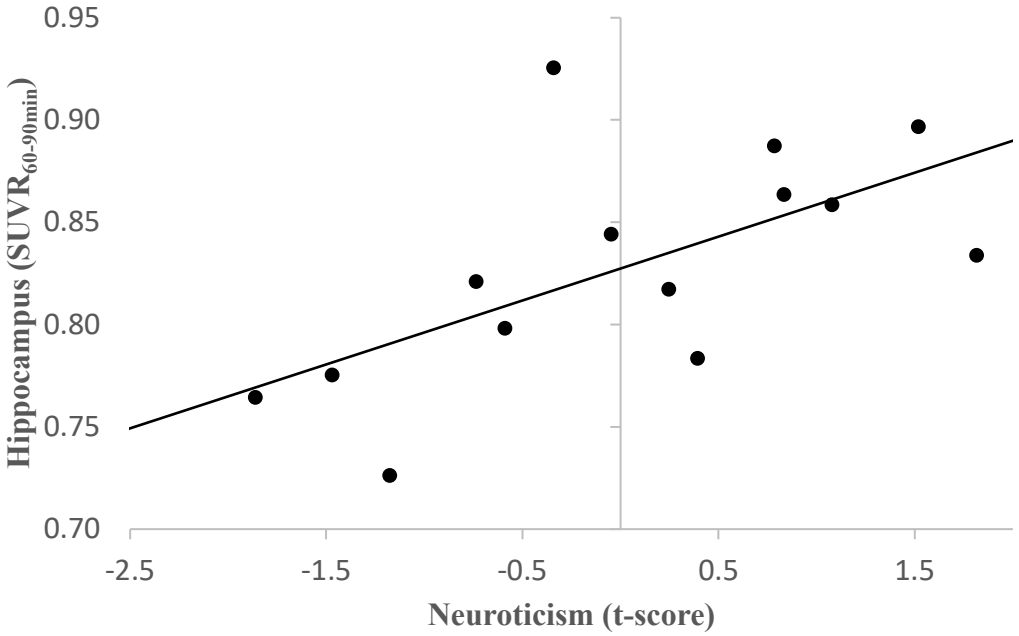

Figure 16. Greater neuroticism was associated with increased SUVR$_{60\text{-}90min}$ in the hippocampus.

4.4.6 Other personality traits and HDAC density

There was a significant association between extraversion and HDAC density such that greater extraversion was associated with decreased SUVR $_{60\text{-}90min}$ in the amygdala (r = -.60, p = .024; supplemental figure 1). In addition, greater openness to experience was associated with decreased SUVR $_{60\text{-}90min}$ in the amygdala (r = -.69, p = .007*; supplemental figure 2), and increased SUVR $_{60\text{-}90min}$ in the caudate (r = .71, p = .004*; supplemental figure 3). There was no significant association between conscientiousness or agreeableness and HDAC density.

4.5 Discussion

In this study examining a sample of healthy adult males, we did not find a significant association between early life adversity and HDAC density. Further, we found that greater levels of depressive symptoms were associated with decreased HDAC density in a brain region previously implicated in depression, the orbitofrontal cortex (OFC). In addition, greater HDAC density in the hippocampus was associated with greater levels of neuroticism. Secondary analyses showed further associations with HDAC density and personality variables; increased extraversion was associated with decreased amygdala HDAC density; increased openness to experience was associated with decreased HDAC density in the amygdala and increased density in the caudate.

Our finding that greater levels of depressive symptoms were associated with decreased HDAC density in the OFC are consistent with findings from Tseng and colleagues who, using the [^{11}C]Martinostat, found that individuals with bipolar disorder, a disorder distinct but related to depression, displayed decreased HDAC density in the orbitofrontal cortex compared to controls (Tseng et al., 2020). It is interesting that we find comparable results, even in a healthy population. This suggests that the [^{11}C]Martinostat tracer may be sensitive not only to neurological changes in associated with disease state, but also more subtle changes underlying variation in subclinical symptomatology that may reflect risk for depression. The association between increased depressive symptoms and decreased OFC HDAC density is of interest given that the OFC is implicated in a number of cognitive and emotional processes relevant to sadness and depression including emotion regulation and motivation (Rudebeck & Murray, 2014). Indeed, the orbitofrontal cortex and the adjacent and cytoarchitecturally related part of the inferior frontal gyrus (IFG) have been implicated in depressive symptoms and depression

(Chiarella et al., 2020; Ismaylova, Lévesque, et al., 2018; Rolls et al., 2020; Tozzi et al., 2018; C. Wang et al., 2014; Xie et al., 2021).

In the present study, we also found a link between HDAC density in the hippocampus and neuroticism, but not depressive symptoms. Research in clinical samples of individuals with major depressive disorder (MDD) have repeatedly found reduced hippocampal volume in patients with MDD (Frodl et al., 2010; Frodl et al., 2008; Treadway et al., 2015). A number of neuroplastic changes, including changes in neurogenesis, are thought to underlie these findings, with evidence that antidepressant may work in part by reversing such neuroplastic changes (N. D. Hanson et al., 2011). While results have tended to suggest that changes in hippocampal volume are, at least in part, a consequence of the disease state itself, there is also evidence that visible changes in hippocampal structure and function are associated with neuroticism and vulnerability to depression more broadly (DeYoung et al., 2010; Joffe et al., 2009; Servaas et al., 2013; Tzschoppe et al., 2014). Further, genes implicated in depression and hippocampal function, namely brain derived neurotrophic factor (*BDNF*) and the serotonin transporter gene (*SLC6A4*) have been implicated in neuroticism in addition to depression (Arias et al., 2020; Booij, Szyf, et al., 2015; Frustaci et al., 2008; Yu & Chen, 2011). The *BDNF* gene is particularly relevant to hippocampal neuroplasticity and has been shown to be associated with HDAC activity (S. W. Park et al., 2018; Shirata et al., 2018; Zhao & Liu, 2020). Together, our results suggest that HDAC density in the hippocampus may be more sensitive to variability in neuroticism than to depressive symptoms themselves, at least in a healthy population. Indeed, in animal models, HDACs have been linked not uniquely to depression, but to both anxious and depressive symptoms, the two traits most highly associated with neuroticism.(Bartlett et al., 2017; W.-Y. Chen et al., 2019; Weaver et al., 2006).

In addition to a link between HDAC density and neuroticism, secondary analyses showed that greater levels of extraversion were associated with decreased HDAC density in the amygdala. This finding is consistent with previous studies showing an association between the amygdala and extraversion (Clauss, 2019; Cremers et al., 2011; Pang et al., 2016). This finding is of interest given that extraversion is associated with positive affectivity and the functioning of the behavioural activation system, a neural system implicated in depressive symptoms (Jylhä & Isometsä, 2006; Rasmussen et al., 2012). We also found that greater levels of openness were associated with decreased HDAC density in the amygdala and increased HDAC density in the caudate. Although findings of a link between depression and personality have been stronger with regard to neuroticism, associations between a link depression and openness has been observed previously (Khoo & Simms, 2018). It is interesting that we find an association between openness and the reward system as openness to experience has been hypothesized to relate to the dopaminergic system and associated "cognitive exploration" (Käckenmester et al., 2019).

Despite evidence of the involvement of HDACs in the effects of early life stress, we did not find an association between early life adversity and HDAC density in any of the ROIs investigated (Bonomi et al., 2022). In rodents, chronic stress increases histone acetylation in limbic regions along with levels of HDAC2 (Uchida et al., 2018). Systemic administration of HDAC inhibitors or more specific injection of selective inhibitors into limbic regions has been shown to reduce depression-like symptoms in mice exposed to the chronic social defeat paradigm as well as maternal separation (Nestler et al., 2016). Further, it is known that in mature neurons, histone acetylation is involved in the formation and extinction of conditioned fear responses (Morris et al., 2010) and that histone acetylation in the hippocampus seems to be important for the learning of fear conditioned responses and HDAC inhibitors lead to enhanced

LTP in the hippocampus and amygdala (Barrett & Wood, 2008; Levenson et al., 2004; Vecsey et al., 2007; Yeh, 2004). Thus, it is unexpected that we did not find an association between early life adversity and HDAC density in the hippocampus, amygdala or other limbic regions. However, it should be noted that our sample consisted of healthy individuals. Indeed, none of our participants developed stress related psychopathology in response to early life adversity experienced. It may be that our group was a resilient one, and that HDAC changes may be associated with phenotypic changes resulting from early adversity that our group did not display. Indeed, adverse effects to traumatic experience and their effects on the brain can be buffered by a number of important resilience factors ranging genetic, to familial to sociocultural factors (Holz et al., 2020). Research investigating HDACs in *vivo* in individuals who developed stress related psychopathology in response to early life adversity could provide more context for the effect of trauma on HDACs in *vivo.*

It is important to consider these novel findings in light of the study's limitations. Firstly, the study sample is not a clinical sample, but rather a sample of healthy adults. As such, caution should be taken in generalizing to clinical populations. In addition, the homogenous nature of the sample (all males aged 35-40) limits generalizability, but also increased the power of our study by eliminating the necessity of including covariates for age and gender in the analyses. Further, while sample size was limited, we were able to detect meaningful effects within a small sample size, which may speak to the sensitivity of the [^{11}C]Martinostat tracer and, in conjunction with other [^{11}C]Martinostat studies to date, provides promise for using this tracer to understand the biological basis of psychopathology and epigenetic mechanisms more generally. Finally, the sample size of the current study is limited to 14 participants. However, small sample sizes are not uncommon in PET imaging due to the cost prohibitions associated with this imaging modality

and the invasive nature of the procedure. Further, our sample size is comparable to that of Tseng and colleagues (Tseng et al., 2020) who also found similar brain associations as our study and other PET imaging studies investigating links with personality traits have detected significant associations using comparable sample sizes (Baik et al., 2012; S. H. Kim et al., 2008).

The finding that we observed associations that overlap with Tseng and colleagues in a small sample, further attests to the potentially sensitive nature of the [^{11}C]Martinostat tracer.

4.6 Author Disclosures

4.6.1 Role of the Funding Source

Funding for this study was provided by the NARSAD independent investigator award from the Brain & Behavior Research Foundation, as well as the Quebec Network on Suicide, Mood Disorders and Related Disorders. LB was supported by a career award from the Fonds de Recherche du Quebec Santé (FRQS; *chercheur-boursier Senior*). Julian Chiarella was supported by the Frederick Banting and Charles Best Canada Graduate Scholarship.

The above funding agencies were not involved in the study design; collection, analysis and interpretation of data; manuscript writing; or in the decision to submit the paper for publication.

4.6.2 Contributors

Linda Booij and Chawki Benkelfat designed the study. Linda Booij, Julian Chiarella, Jocelyn Fotso Soh, Naomi Azar and Mari Sild collected the data. Kevin Casey, Julian Chiarella, Marco Leyton and Linda Booij performed or provided input on the statistical analyses. Julian Chiarella and Linda Booij wrote the manuscript. Gassan Massarweh, Jean-Paul Soucy and Jacob Hooker developed and implemented the PET 11C Martinostat ligand. Frank Vitaro and Richard Tremblay were responsible for the development and continuation of the QLSKC cohort. Linda Booij supervised the study. Authors critically reviewed and approved the manuscript. Dr. Chawki Benkelfat passed away during preparation of this manuscript for publication.

4.6.3 Conflict of Interest

All authors declare that they have no conflicts of interest.

4.6.4 Acknowledgements

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5. CHAPTER 5: INTEGRATED DISCUSSION

5.1 Introduction

The present chapter will discuss and synthesize findings from the three studies that make up this dissertation. Below is a summary of the studies followed by an integrated discussion of the results, strengths and limitations, disciplinary implications, and overarching conclusions.

5.2 Summary and main findings

The purpose of this doctoral thesis was to advance our understanding of the etiology and development of depression and to elucidate the biological mechanisms (genetic and epigenetic) underlying its development throughout the lifespan. In study one, using a longitudinal twin design, we aimed to understand the genetic and environmental contributions to the longitudinal association between preschool externalizing (aggressive, hyperactive and oppositional) symptoms and early adolescent internalizing (anxious and depressive) symptoms. Using common pathway modelling in a longitudinal sample of 1344 twins (671 boys, 673 females), we found that externalizing symptoms at 60 months of age were associated with internalizing symptoms at 12 years old. Further, we observed that this association was accounted for by a genetic, but not environmental, correlation.

In study two, we sought to better understand the link between DNA methylation and brain structure and function in adolescent depression. Studying a sample of 25 adolescents with depression and 20 healthy controls, we found that *SLC6A4* methylation was linked to amygdala-frontal operculum resting-state functional connectivity across all participants, and differentially associated with inferior orbitofrontal gyrus gray matter volume in adolescents with depression and healthy adolescent controls. Further, we found that *FKBP5* methylation was associated with inferior orbitofrontal gyrus gray matter volume in both depressed and healthy adolescents, as well as orbitofrontal cortex-rostral prefrontal cortex connectivity in healthy adolescents.

In study three, we aimed to investigate the link between early life adversity, depression vulnerability and HDAC while simultaneously addressing an important limitation in the literature also present in study two, the reliance on peripheral measures of DNA methylation. To this end we employed, by means of a pilot project, PET imaging along with the use of a novel tracer [^{11}C]Martinostat to image histone deacetylase (HDAC) proteins *in vivo* in a sample of 14 healthy adults with a range of early life adversity and depressive symptoms. While we hypothesized a link between early life adversity and in vivo HDAC density, we found no such association in any of the regions examined. We did, however, find that greater levels of depression were associated with decreased HDAC density in the orbitofrontal cortex and greater levels of trait neuroticism were associated with increased HDAC density in the hippocampus.

In the following sections, I will integrate findings across the three studies included in this dissertation with the broader literature and elaborate on the overarching implications for the fields of behavioural genetics, epigenetics, neuroimaging, and mental health.

5.3 Overarching discussion points

5.3.1 Implications for neurobiology of depression

A key finding of study one was that of a significant moderate correlation between externalizing symptoms at 60 months and internalizing symptoms at age 12 which was accounted for solely by common genetic factors. This suggests the presence of a stable genetic vulnerability that accounts, in part, for the overlap between externalizing and internalizing symptoms across time from preschool to early adolescence. This novel finding is consistent with what is known about the neurobiological overlap between internalizing and externalizing symptoms. For example, the Monoamine Oxidase A (*MAOA*) and Serotonin Transporter (*SLC6A4*) genes, which code for proteins involved in the breakdown and neurotransmission of

serotonin, have been extensively associated with depression and aggression (Coccaro et al., 2015; Nutt, 2008; Tuvblad et al., 2019). Further, the catechol-O-methyltransferase (*COMT*) gene, which codes for a protein responsible for breaking down catecholamines such as dopamine and norepinephrine, has been linked to depression, aggression, conduct difficulties in ADHD and the functioning of neural emotion processing networks (Antypa et al., 2013; Tuvblad et al., 2019). Further, selective serotonin reuptake inhibitors (SSRIs) as well as serotonin and norepinephrine reuptake inhibitors (SNRIs), which act on the serotonin and norepinephrine systems, show efficacy in the treatment of both internalizing (e.g., anxiety, depression) and externalizing (e.g., aggression, ADHD) difficulties (Bond, 2005; Coccaro et al., 2015; Ghanizadeh et al., 2013). Moreover, dopamine agonists are used to treat not only hyperactive behaviours, but also depressive behaviours (Corp et al., 2014; Faraone & Buitelaar, 2010).

Interestingly, this link between internalizing and externalizing symptoms may be especially present in adolescence. On a phenotypic level, irritability and/or anger are core symptoms not only of externalizing disorders, but also MDD and generalized anxiety disorder in adolescence (American Psychiatric Association, 2013). Further, there is evidence that juvenile onset depression, in particular, is linked to a host of externalizing behaviours including suicide attempts, self-harm, hyperactivity and alcohol abuse (Hill et al., 2004). In addition, MDD in adolescence is particularly linked to borderline personality disorder, a disorder characterised often by impulsivity and difficulty with emotion regulation (Chanen et al., 2016). Therefore, extending study one to understand the genetic relation between internalizing and externalizing symptoms past adolescence and into adulthood may be important to obtain a more fine-tuned picture of how these symptoms develop together. Indeed, prefrontal regions involved in regulating aggressive and impulsive behaviours do not fully develop until adulthood, which may

change how this shared vulnerability is expressed over time, as well as the overall genetic contribution to these symptoms (Teffer & Semendeferi, 2012).

5.3.2 Brain regions of interest: Recurrent findings

In the present dissertation, we observed several associations between behavioural/epigenetic measures and brain regions which appear to reflect recurrent findings in the literature. Perhaps most interestingly, two regions were consistently found to be associated with epigenetic measure across methodology: the hippocampus and the orbitofrontal cortex.

5.3.2.1 Orbitofrontal cortex

We observed that orbitofrontal cortex structure and functional connectivity was associated with *FKBP5* methylation in study two, and that depressive symptomatology was associated with orbitofrontal HDAC density in study three. The orbitofrontal cortex is a region involved in functions relevant to emotion processing and stress-related disorders such as encoding of value, inhibiting responses and emotional regulation/appraisal (Fettes et al., 2017). Moreover, the orbitofrontal cortex has consistently been linked to depression and the serotonin system, and its structure/function has been shown to correlate with epigenetic measures (Fettes et al., 2017; Rudebeck & Murray, 2014). Further, it is sensitive to stress and glucocorticoids, and thus amenable to epigenetic changes induced by stress (Gulyaeva, 2019; Sequeira & Gourley, 2021). Findings pertaining to the orbitofrontal cortex have appeared consistently in studies conducted by our group. For example, our lab had previously found that greater blood *SCL6A4* promoter methylation was associated with decreased serotonin synthesis in the lateral orbitofrontal cortex in healthy adults (Wang et al., 2012). Another study in our lab observed that MZ twins with greater levels of *SLC6A4* methylation compared to their co-twin displayed greater left orbitofrontal cortex activation and left amygdala- right orbitofrontal cortex connectivity

when viewing sad faces (Ismaylova et al., 2018b). It appears that a number of findings, both functional and neurochemical, suggest a link between DNA methylation at stress-related genes and orbitofrontal cortex structure and function.

5.3.2.2 Hippocampus

Like the orbitofrontal cortex, the hippocampus, a structure involved in a variety of processes ranging from memory consolidation and spatial navigation to representation of the self, has been consistently linked to depression, the serotonin system and epigenetic measures (Raichle, 2015; Rolls, 2019). Further like the orbitofrontal cortex, the hippocampus is also sensitive to the effects of glucocorticoids. (Gulyaeva, 2019; Sequeira & Gourley, 2021). In both study two and three, we observed an epigenetic correlation with the hippocampus. In study two, greater *FKBP5* methylation was associated with decreased hippocampal gray matter volume in depressed individuals. In study three, greater levels of neuroticism was associated with greater HDAC density in the hippocampus. It is of interest that in study three, hippocampal HDAC density was associated with neuroticism, and not depressive symptoms, but that in study two, hippocampal *FKBP5* methylation was associated with depressive symptoms more specifically. There is a large body of evidence implicating the hippocampus in depression and stress related psychopathology, possibly through its involvement in neurogenesis (Baptista & Andrade, 2018). This raises the question of the precise nature of the relationship between *FKBP5* methylation, HDAC density, the hippocampus and depression. Indeed, DNA methylation and histone acetylation/deacetylation processes are intricately linked, and the hippocampus appears to be a key region in understanding the brains reaction to stress and glucocorticoid exposure, a processes which is thought to be in part mediated by *FKBP5* methylation (Matosin et al., 2018; Pagliaccio et al., 2014; Weaver et al., 2004). It should be noted that the link between *FKBP5* methylation

and the hippocampus was found in depressed adolescents, while that between hippocampal HDAC density and neuroticism was found in healthy adults. Thus, this discrepancy may be explained by developmental factors. It will be important for future research to clarify the complex interaction between *FKBP5* methylation, HDAC density, hippocampal structure and function, and depression over time.

5.3.3 Moving away from diagnosis?

Beginning in 2008, there has been a push initiated by the National Institutes of Mental Health (NIMH) to encourage the movement away from understanding mental health in terms of diagnostic categories and towards understanding psychopathology on a continuing from normal to abnormal. This push has culminated in the Research Domain Criteria (RDoC) framework, a framework which emphasizes the study of six categories of basic brain systems (Cuthbert & Insel, 2013; Insel et al., 2010). These categories were derived in a "bottom-up" fashion from research data, as opposed to the more top-down approach of guiding research based on pre-existing mental health categories. This framework is relevant to the current dissertation as there are important ways in which each of the three studies point towards the utility of a RDoC approach to mental health.

In study one, we identified a genetic vulnerability shared by both internalizing and externalizing symptoms across time, and thus not specific to any given diagnosis. Previous research investigating the co-occurrence of internalizing and externalizing symptoms in childhood has found that a significant degree of co-occurrence is explained by negative affect, a concept which encompassed by basic RDoC category of the negative valence system (Savage et al., 2017). It is possible that the genetic vulnerability underlying both internalizing and externalizing symptoms over time in study one might also be accounted for, at least in part, by

negative affect. Testing this hypothesis could shed further light on the developmental behavioural genetics of the negative valence system.

In addition, in study two, we observed a number of transdiagnostic associations between DNA methylation and brain structure and function. More specifically, we observed that, regardless of diagnosis, *SLC6A4* methylation was associated with amygdala-frontal operculum resting-state functional connectivity and *FKBKP5* methylation was associated with inferior orbitofrontal gyrus gray matter volume. If further research continues to replicate such links between individual brain regions or circuits and methylation at specific genes, it could suggest that epigenetics could plays role in regulating broad neurocircuitry associated with the continuous categories outlined by RDoC. Indeed, a number of researchers have argued that neurotransmitter systems are more reliably associated with broader neural processes of the sort contained in the RDoC framework than specific disorders (Canli & Lesch, 2007; Wolf et al., 2018).

Finally, in study three, we observed that HDAC density was associated with both neuroticism and trait extraversion. Indeed, the negative valence system outlined by RDOC is closely correlated with trait neuroticism, while the positive valence system is correlated with extraversion (Cai et al., 2020; DeYoung et al., 2010; Gore & Widiger, 2018; Hsu et al., 2018; Markett et al., 2018). Trait neuroticism is a personality trait which is characterised by the tendency to experience negative affect broadly, including both affects typically associated with externalizing symptoms such as anger and irritability, as well as those associated with internalizing symptoms such as anxiety and depression. This observed link between HDAC and broad personality constructs has important implication for future [^{11}C]Martinostat studies, which to date have focused on specific syndromes. Our findings of a link between HDAC and

continuous personality constructs suggest that it may be fruitful for further research using the [^{11}C]Martinostat tracer to incorporate an RDOC approach.

Of note, some researchers have argued that the RDOC approach has not paid enough attention to the specific neurobiological mechanisms underlying homotypic and heterotypic pathways of development (Beauchaine & Hinshaw, 2020). We echo this concern and suggest that, in light of our findings discussed above, studying the role of epigenetic mechanisms in homotypic and heterotypic pathways could be a fruitful avenue of research to complement our findings of a common genetic factor in study one. Both methylation and *in-vivo* HDAC studies could be used to elucidate such mechanisms in the context of a MZ twin design. More specifically, given the assumed 100% genetic similarity between MZ twins, changes in externalizing and internalizing symptoms, as well as their relationship, over time could be correlated with changes in epigenetic processes.

5.3.4 Epigenetics, depression, and adolescent development: Comparison with adult findings and the broader literature

While the genetic and biological basis of youth depression is less well-studied compared to adult depression, youth represents a particularly sensitive period in the development of depression and a time at which the rate of depression increases rapidly (Leigh & Clark, 2018; Thapar et al., 2012). Study two presents a number of findings which are consistent with the broader literature and are deserving of further discussion, particularly findings pertaining the link between both *FKBP5* and *SLC6A4* methylation and brain function and structure.

5.3.4.1 FKBP5

In regards to findings surrounding *FKBP5* methylation, we observed a positive association between *FKBP5* methylation and inferior orbitofrontal gyrus gray matter volume, as well as an association between *FKBP5* methylation and hippocampal volume in adolescents with

MDD only. Using comparable methodology in a sample of adults (identical pre-processing/analysis pipeline and the same *FKBP5* CpG sites), our group previously observed a comparable, positive association between *FKBP5* inferior orbitofrontal gyrus gray matter volume in a sample of adults with MDD and healthy controls. This suggests that a relationship between *FKBP5* methylation and inferior orbitofrontal gyrus gray matter volume may be present as early as adolescence and persist into adulthood.

It is also of interest that we observe a link between *FKBP5* methylation and hippocampal volume in adolescents with MDD, given the debate as to whether hippocampal changes in response to chronic stress are a cause of depression, or are a consequence or cause of depression. While our findings cannot directly answer this question, if hippocampal degeneration does indeed result from depression, they suggest that this link may be observed as early as adolescence and provide a possible mechanism underlying such an association.

In study two, we observed an association between *FKBP5* methylation and brain processes as early as adolescence in both depressed adolescents and controls. This is of interest given findings from a number of studies that altered HPA axis function (elevated daytime cortisol and/or cortisol awakening response) may serve as a vulnerability marker for the development of affective disorders (Adam et al., 2010; Ellenbogen et al., 2011; Goodyer et al., 2003; Halligan et al., 2007; Vrshek-Schallhorn et al., 2013). Thus, altered HPA axis functioning as assessed by *FKBP5* could eventually serve useful as an early biomarker in youth at risk for depression, with the potential of guiding interventions. In depressed individuals, *FKBP5* as a proxy measure for brain functioning and risk for depression could even serve to guide psychological interventions. That is, as the link between *FKBP5* methylation and stress and depression related neurocircuitry becomes more clear, measuring peripheral *FKBP5* methylation

levels could guide interventions aimed at targeting that specific neurocircuitry. However, methylation levels will likely need to be understood in a larger context of genetic, psychological and environmental factors in order to have a more complete picture. This point is discussed further in section entitled "Clinical implications".

5.3.4.2 SLC6A4

In a recent systematic review, *SLC6A4* methylation was found to be associated with amygdala resting-state functional connectivity or reactivity across the lifespan (Wheater et al., 2020). This is consistent with our finding of an association between *SLC6A4* methylation and amygdala-frontal operculum functional connectivity in adolescents.

In study 2, *SLC6A4* methylation was differentially associated with the structure of the inferior orbitofrontal gyrus in adolescents with depression and controls. A previous study done by our group observed that *SLC6A4* promoter methylation assessed in saliva within monozygotic adolescent twin pairs was associated with greater orbitofrontal cortex activity and left amygdala-anterior cingulate cortex and left amygdala-right OFC connectivity, as well as increased anterior cingulate cortex-left amygdala connectivity in when viewing images of fearful stimuli (Ismaylova, Lévesque, et al., 2018). Thus, similar to *FKBP5*, there is convergent evidence of an association with *SLC6A4* methylation and the orbitofrontal region that appears as early as adolescence.

5.3.5 Early life adversity

Contrary to our hypothesis, early life adversity was not associated with HDAC density in study three. Animal research provides evidence that the early environment does impact behaviour and neurobiology through both DNA methylation/demethylation and HDAC acetylation/deacetylation (e.g., Weaver et al., 2004). Further, human studies have also found that childhood adversity is associated with *SLC6A4* hypermethylation and *FKBP5* hypomethylation

(Matosin et al., 2018; Provenzi et al., 2016a). Our lack of finding of a link between childhood adversity and DNA methylation or *in vivo* HDAC density may be due to methodological reasons. More specifically, in study three, we excluded individuals with a history of psychopathology. Thus, we would only expect to find an association between early life adversity and HDAC density if HDAC density were not necessarily associated with stress related phenotypes, but rather an underlying vulnerability. It may be the case that HDAC density is more associated with psychopathological phenotypes related to trauma (e.g., PTSD, depression) as opposed to the effects of trauma itself. Indeed, research has already shown that that HDAC density in specific regions in linked to both schizophrenia and bipolar disorder (Gilbert et al., 2019; Tseng et al., 2020). It should also be noted that [^{11}C]Martinostat tracer used binds selectively HDAC 1, 2 and 3 (Class I) and 6 (Class IIb). However, there exist 18 classes of HDAC, and while type I HDACs are the most investigated in the context of psychopathology and early adversity, research has implicated other HDACs such as HDAC 4 and 5 (Hobara et al., 2010; Iga et al., 2007; Uchida et al., 2018). The complex relationship between the different classes of HDAC and childhood adversity will be difficult, but perhaps necessary, to disentangle in order to fully understand the link between HDACs and childhood adversity. Finally, in regards to study three, the limitation of sample size must be acknowledged. Due to the relatively invasiveness of the procedure and for feasibility reasons, PET studies combined with a radioligand are usually not done on a large-scale basis. For our pilot study, we used a modest sample size of 14 participants. Gilbert and colleagues (2019) detected a difference between individuals with depression and controls using 14 participants with MDD and 17 control participants, while Tseng and colleagues (2020) detected a difference between 11 participants with MDD and 11 controls. In contrast, in

the current study, we recruited 14 participants using a correlational methodology. It may be that our study lacked the power necessary to identify an effect.

5.3.6 Methodological considerations

One strength of the current thesis is its use of a range of methodologies including those derived from behavioural genetics, epigenetics and three different types of brain imaging (MRI, fMRI, PET). Study one aimed to investigate the underlying genetic and environmental contributions to youth anxious and depressive symptoms using a powerful research design available to answer such questions, the twin design. Such a design is unique in allowing for the precise estimation of environmental and genetic contributions to a given trait and association among traits (Chiarella et al., 2015). Study two combined epigenetic measures with neuroimaging measures of resting-state and brain structure. Study three utilized PET imaging combined with a novel radioligand which allows for the in-vivo imaging of a specific component of the epigenetic machinery. Together these studies offer a broad range of methodology for investigating depressive symptoms over the life span. Methodological implications and future directions are elaborated on below

5.3.6.1 Bridging animal and human research

Study three provides an important bridge from animal to human research in HDAC. Research in animals has provided clear rational for studying HDACs in human. Studying HDACs *in vivo* in humans will allow us to bridge better understand how a larger body of research investigating a broad range of epigenetic mechanisms in animal models applies to humans. Study three offers insights into the validity and applicability of this methodology, showing that HDACs are associated with in psychological processes related to depression in humans. Further, and perhaps more importantly, study three raises questions worthy of

investigation (e.g., why are HDACs associated with early life stress in animal models but not early life adversity in humans in the current study).

5.3.6.2 Understanding the link between peripheral and brain epigenetic measures.

In light of findings from study three, one important avenue of future research is the study of the direct association between HDAC and peripheral measures of DNA methylation. Methylation and acetylation processes are intricately linked (Weaver et al., 2004). Directly comparing peripheral methylation and *in vivo* HDAC density could allow for a better understanding of the extent to which peripheral measures of methylation reflect epigenetic processes in the brain. Further, demonstrating associations between peripheral and central epigenetic measures will provide further evidence that peripheral measures are a useful proxy. However, understanding the relationship between peripheral methylation and central HDAC density will need to take into account the complexity inherent in comparing DNA methylation at specific sites in the periphery with a somewhat crude epigenetic measure of the density specific subset of HDACs in the brain (HDAC 1, 2, 3 and 6). Studies comparing peripheral proxies of HDAC expression and central HDAC levels could offer a more direct and interpretable comparison. Moving forward, the development of tracers which allow for the imaging of proteins more closely involved in DNA methylation, such as DNA methyltransferases, will allow for further expansion of our understanding of this complex set of epigenetic processes.

Combining methodology of study one and studies two and three using the monozygotic (MZ) twin design would allow for further advancement of our understanding of the environmental contributions to epigenetic processes, and their effects on brain development and various phenotypes. For example, it is unclear the extent to which HDAC signatures observed in study three are the result of genetic or environmental influences. Using a twin design could help

determine the specific proportion of genetic vs. environmental contributions to HDAC levels. Further, using a monozygotic twin design could allow for the evaluation of the associations between HDAC density due to environmental factors (controlling for genetic variation) with various psychological/environmental factors (Chiarella et al., 2015).

5.3.7 Strengths and limitations

In addition to those limitations already discussed, one important limitation to consider is that the epigenetic studies above were conducted in humans, and so are not as amenable to rigorous experimental control as animal studies. As a result, results in the above studies are correlation in nature, making it hard to discern causal epigenetic and genetic mechanisms as they related to depression. On the other hand, this evidently makes results more generalizable to human's and treatment of psychopathology, and also offers a series of bridges between the animal and human literature as it relates to our understanding of the role of epigenetic and neurobiological mechanisms of stress-related disorders and depression more specifically.

In terms of strengths, the current thesis attempts to understand depression from a perspective which incorporates both environmental and biological factors. The strength of the concept of epigenetic mechanisms itself is in the possibility of offering a mechanism by which the environment influences neurobiology. The current thesis attempted to examine the intersection of environmental and genetic factors, using epigenetic mechanisms as a bridge to join these two. In this sense, it, along with the body of research it draws on, offers a language and conceptual framework with which to advance the age-old question as to the role that nature and nurture play in our development, and to break down the somewhat arbitrary distinction between the two. Further, the current thesis examines the link between genes and the environment across the lifespan from childhood to adulthood. Where possible, we have

considered a developmental perspective. Where not possible due to ethical reasons (i.e., in study three), we have attempted to interpret these findings in light a developmental perspective.

5.3.8 Clinical implications

The finding in this dissertation perhaps most directly applicable to clinical practice is that from study one, which showed that the moderate association between preschool externalizing symptoms and adolescent internalizing symptoms is accounted for largely by a stable genetic factor. Indeed, a substantial portion of children who develop an internalizing disorder in adolescents do so through a heterotypic pathway (i.e., from early externalizing to later internalizing symptoms; Jobs et al., 2019). This transition over time from externalizing symptoms to co-occurring internalizing symptoms is thought to be accounted for, at least in part, by the Dual Failure Model. According to this model, externalizing symptoms lead to internalizing symptoms over time through noxious behaviour which disrupts social and academic functioning, leading to academic failure and peer rejection/victimization (Boutin et al., 2020; Capaldi, 1992; Patterson & Stoolmiller, 1991). Boutin and colleagues recently argued for the importance of early screening and intervention for externalizing difficulties and an appreciation of the transition from early externalizing difficulties to later internalizing problems through peer victimization (2020). Our findings add to the importance of such screening as they suggest that there may exist a substantial genetic component which makes individuals vulnerable to this dual failure pathway and the transition from externalizing to internalizing symptoms over time. Further, it suggests that this genetic component is expressed as early as preschool. Better understanding this genetic component and its phenotypic manifestations could be useful in guiding early preventative interventions.

Although effective interventions targeting early externalizing difficulties have been studied (e.g., Castellanos-Ryan et al., 2013; Vitaro et al., 2012), they have tended to focus on social skills training and psychoeducation for parents and teachers. Our findings suggest that it may be fruitful to directly address genetically influenced biological systems influenced by this longitudinal genetic association as early as preschool. While the exact nature of this genetic association would need to be better elucidated, it could be hypothesized that addressing heritable traits such as neuroticism, negative affectivity and emotion regulation could be fruitful targets. Indeed, negative emotionality, the tendency to experience negative aversive emotions broadly, has been shown to at least partially account for the genetic co-occurrence between adolescent internalizing and externalizing symptoms (Mikolajewski et al., 2013). Mindfulness-based therapeutic approaches may be appropriate here as they tend to conceptualize psychopathology in a transdiagnostic framework and aim to teach skills to regulate negative emotions present in both externalizing (e.g., anger) and internalizing (e.g., sadness, anxiety) disorders (Bögels et al., 2008). Indeed, there is evidence that mindfulness-based interventions as early as preschool can reduce negative emotionality and emotional lability, as well as lead to a number of positive functional outcomes such as greater social competence and academic achievement (Flook et al., 2014; E. Kim et al., 2020)

Another implication of the present dissertation for clinical practice is the potential utility of epigenetic measures to guide diagnosis and treatment. In study two, we showed several associations between peripheral methylation at stress related genes and brain functional connectivity and structure. In study three, we showed that *in vivo* measures of epigenetic processes are associated with depressive symptomatology and big 5 personality traits, most notably neuroticism. Though we are still in the early stages of understanding the epigenetic basis

of mental health, these findings suggest that peripheral methylation levels may one day be used to infer disruptions in brain circuitry relevant to both pathology and healthy functioning. Although we might be some ways away from this reality, perhaps in the nearer future, as suggested by Lester and colleagues (Lester et al., 2016), epigenetic measures could be used to guide early identification of those at risk for psychopathology. Further, by understanding which neural circuits are related to methylation at stress related genes, we may gain a clearer picture both of how our both our psychotherapeutic and pharmacological interventions for mental health difficulties work. These findings could even be used to guide psychological interventions aimed at targeting specific neural systems known to be involved in response to stress such as the frontolimbic system or limbic-cortical-striatal-pallidal-thalamic circuit (Beauregard, 2014). The current dissertation suggests a number of specific subcircuits within these systems (i.e., amygdala – frontal operculum and orbitofrontal cortex – rostral prefrontal cortex) that warrant further study in this area. Study three also has important implications along the same lines. While it is not practical to use PET imaging to infer behavioural symptoms which can be measured by a questionnaire, PET imaging will at minimum be useful in so far as it can be utilised to validate less invasive measures. Further, when combined with yet-to-come in-vivo epigenetic imaging techniques, it may be important in improving our diagnostic and treatment abilities.

5.4 Overarching conclusions

In conclusion, the current dissertation provides novel contributions to the fields of behavioural genetics, epigenetics and neurobiology of depression. It showed for the first time the presence of a longitudinally stable genetic factor accounting for the association between externalizing and internalizing symptoms over childhood and into adolescence. Further, it

identified a number of patterns in brain structure and functional connectivity which are associated with peripheral measures of DNA methylation at stress related genes in adolescence. In addition, it makes possible important methodological contributions to the fields of epigenetics and mental health by presenting to first study to investigate the link between epigenetics *in vivo*, early life adversity, depressive symptoms and personality.

On a broader level, these results have implications for our conceptualization of mental health. Firstly, they suggest that a dimensional approach may be helpful in understanding the link between certain epigenetic processes and behaviour. Secondly, they add nuance to the conceptual distinction between internalizing and externalizing symptoms, highlighting the genetic overlap between these broad mental health categories. Further, they help bridge together the age-old debate of nature *vs*. nurture, contributing to our understanding of specific mechanisms by which nature and nurture interact and highlighting the, at times, arbitrary nature of this distinction.

Finally, the current dissertation brings with it certain clinical implications for the diagnosis and treatment of mental health disorders. More specifically, it highlights potential targets for early prevention programs, measures with putative diagnostic utility, and possible mechanisms of psychotherapeutic and pharmacological intervention to be further explored in future research.

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