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Frontal-limbic brain processes in healthy individuals: environmental, epigenetic and behavioral correlates

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Résumé

Des altérations au niveau du cerveau ont été observées dans le circuit fronto-limbique (incluant le cortex préfrontal, le cortex cingulaire antérieur, hippocampe et amygdale), densément innervé avec la sérotonine, chez les individus souffrant de troubles affectifs. La relation entre les processus fronto-limbiques et le bien-être émotionnel peut être influencée par la génétique et l'environnement, ainsi que par leur interaction (GxE). Toutefois, les mécanismes spécifiques sous-tendant cette relation ne sont pas connus à ce jour. Un mécanisme physiologique sous-jacent l'effet GxE sur l'expression des gènes est la méthylation de l'ADN. Le but de cette thèse était, donc, d'étudier les effets environnementaux sur et la pertinence de la méthylation de l'ADN pour les processus fronto-limbiques chez des individus en bonne santé. Dans la première étude, l'association entre l'humeur au quotidien (évaluée à l'aide de la méthode de journal quotidien) et les processus cérébraux a été étudiée. Dans la deuxième étude, l'association entre la méthylation périphérique du gène du transporteur de la sérotonine [*SLC6A4*] (liée au fonctionnement émotionnel), provenant de différents tissus, et les processus cérébraux a été étudiée. Dans la troisième étude, nous avons examiné si l'association entre la méthylation périphérique du gène *SLC6A4* et les processus cérébraux était indépendante de la variation génétique (en utilisant un échantillon de jumeaux homozygotes). Sommairement, l'humeur négative et positive était positivement associée à la connectivité fonctionnelle au repos entre le cortex cingulaire postérieur et antérieur. La méthylation du gène *SLC6A4* était positivement associée au volume cortical préfrontal lorsqu'elle était dérivée du sang, de la salive et des cellules buccales. La méthylation du gène *SLC6A4* dérivée des cellules buccales était également positivement associée au volume cortical antérieur cingulaire et à la connectivité fonctionnelle au repos entre les régions pariétales et le cortex cingulaire antérieur. Aussi, la méthylation périphérique du gène *SLC6A4* était positivement associée à l'activité corticale orbitofrontale ainsi qu'à la connectivité fonctionnelle entre l'amygdale, le cortex orbitofrontal et le cortex cingulaire antérieur en réponse à des stimuli émotionnels négatifs, indépendamment de la séquence d'ADN des individus. Dans l'ensemble, les résultats actuels pourraient indiquer que la fonction et la structure cérébrale dans les régions fronto-limbiques, particulièrement dans le cortex cingulaire antérieur et préfrontal, sont positivement associées à l'humeur dans la vie

quotidienne et à la méthylation périphérique du gène *SLC6A4* chez des individus en bonne santé. En outre, la relation entre ces processus fronto-limbiques et la méthylation périphérique du gène *SLC6A4* serait largement sous influence de l'environnement. Aussi, les résultats actuels suggèrent que les cellules buccales constitueraient un tissu préférable pour étudier la méthylation du gène *SLC6A4* et les processus neuraux apparentés. Des études supplémentaires sont nécessaires pour valider ces résultats auprès de populations cliniques ou chez les individus exposés à des conditions environnementales différentes.

Mots-clés: Cerveau, émotion, gène du transporteur de la sérotonine, méthylation de l'ADN, tissu périphérique, imagerie cérébrale, humeur, santé

Abstract

Neural alterations have been observed in the frontal-limbic circuitry (including the prefrontal cortex, the anterior cingulate cortex, the hippocampus and the amygdala), densely innervated with serotonin, in the individuals with affective disorders. Relationship between the frontal-limbic processes and emotional well-being is affected by the genetics and the environment as well as by their interaction (GxE). Yet, the specific mechanisms are not known to this day. The aim of the present thesis was to study the environmental effects on and the relevance of DNA methylation (a physiological mechanism underpinning the GxE influences on gene expression) for the frontal-limbic brain processes in healthy individuals. In the first study, association between the daily-life mood (assessed using a daily diary method) and the brain processes was studied. In the second study, association between the peripheral DNA methylation in the serotonin transporter [*SLC6A4*] gene (linked to emotional functioning), derived from different tissues, and the brain processes was examined. In the third study, we examined whether the association between the peripheral *SLC6A4* gene methylation and the brain processes was independent of the genetic variation (using a monozygotic twin sample). Briefly, daily-life negative and positive mood was positively associated with the resting-state functional connectivity between posterior and anterior cingulate cortices. The *SLC6A4* gene methylation was positively associated with the prefrontal cortical volume when derived from blood, saliva and buccal cells; buccal-derived *SLC6A4* gene methylation was also found to be positively associated with the anterior cingulate cortical volume and the resting-state functional connectivity between parietal areas and the anterior cingulate cortex. The peripheral *SLC6A4* gene methylation was also positively associated with the orbitofrontal cortical activity as well as with the functional connectivity between the amygdala, the orbitofrontal and the anterior cingulate cortices in response to negative emotional stimuli, regardless of individuals' DNA sequence. Overall, current findings might indicate that brain function and structure in the frontal-limbic regions, particularly in the anterior cingulate and the prefrontal cortices, are positively associated with the daily-life mood and the peripheral *SLC6A4* gene methylation in healthy individuals. Additionally, the relationship between these frontal-limbic processes and peripheral *SLC6A4* gene methylation appear to be largely driven by the environmental influences. Also, current results suggest that buccal cells may be a suitable

peripheral tissue for studying the *SLC6A4* gene methylation and its related neural processes. Future studies are necessary to validate these results in the clinical population as well as in the individuals exposed to differential environmental conditions.

Keywords: Brain, emotion, serotonin transporter gene, DNA methylation, peripheral tissue, brain imaging, mood, healthy

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5-HT: Serotonin

5-HTTLPR: Serotonin-transporter-linked polymorphic region

AAL: Automated Anatomical Labeling

ACC: Anterior cingulate cortex

ART: Artifact detection tools

ATD: Acute Tryptophan Depletion

BDI: Beck Depression Inventory

BDNF: Brain-derived neurotrophic factor

BOLD: Blood Oxygen Level Dependent

CBT: Cognitive-behavioral therapy

CH_3 +: Methyl cation

CpG: Cytosine-phosphate-guanine dinucleotide

CSF: Cerebrospinal fluid

dlPFC: Dorsolateral prefrontal cortex

dmPFC: Dorsomedial prefrontal cortex

DMN: Default-Mode Network

DNA: Deoxyribonucleic acid

DSM: Diagnostic and Statistical Manual of Mental Disorders

DZ: Dizygotic

EPI: Echo-planar imaging

EPQ: Eysenck Personality Questionnaire

FA: Flip angle

fMRI: Functional magnetic resonance imaging

FOV: Field of view

FWE: Family-wise error

FWHM: Full-Width Half-Maximum

GLM: General linear model

GM: Gray matter

K-SADS: Kiddie Schedule for Affective Disorders and Schizophrenia

LLP: Left lateral parietal area

LPFC: Lateral prefrontal cortex

MAO-A: Monoamine oxidase A

MDD: Major depressive disorder

MNI: Montreal Neurological Institute

mPFC: Medial prefrontal cortex

MPFC: Medial prefrontal cortex

MPRAGE: Magnetization-prepared rapid gradient-echo sequence

MRI: Magnetic resonance imaging

MZ: Monozygotic

OFC: Orbitofrontal cortex

OXTR: Oxytocin receptor

PANAS: Positive and Negative Affect Schedule

PET: Positron emission tomography

PFC: Prefrontal cortex

PCC: Posterior cingulate cortex

PTSD: Post-traumatic stress disorder

QLSCD: Québec Longitudinal Study of Child Development

QNTS: Quebec Newborn Twin Study

RLP: Right lateral parietal area

ROI: Region of interest

RSQ: Response Styles Questionnaire

rsFC: Resting-state functional connectivity

rsfMRI: Resting-state functional magnetic resonance imaging

SCID: Structured Clinical Interview for DSM

SD: Standard deviation

SLC6A4: Serotonin transporter

SPM: Statistical Parametric Mapping

TE: Echo time

TPH1: Tryptophan hydroxylase 1

TPH2: Tryptophan hydroxylase 2

TR: Repetition time

VBM: Voxel-based morphometry

vlPFC: Ventrolateral prefrontal cortex

vmPFC: Ventromedial prefrontal cortex

WM: White matter

Liste des abréviations

Min: Minute

À ma maman, qui m'a toujours donnée l'amour, la force et le courage pour réussir

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Introduction

The burden of the mental health disorders measures up to that of the physical health diseases [1]. In fact, mental health disorders, affecting the school engagement, the employment, as well as the peer and personal relationships [2], were ranked as a leading cause of years lived with disability globally [3]. It was estimated that by the time Canadians reach forty years of age, half of them would have personally suffered from a mental health disorder [4]. Affective disorders, in particular depression, were found to be among the most prevalent mental health disorders in the general population [5]. A vast majority of studies on affective disorders has focused on such emotional and cognitive aspects as high negative affect and rumination [6-11]. However, these characteristics were also argued to be relevant for healthy individuals with and without subclinical depressive symptoms [12, 13], allowing a deeper understanding of the mental health spectrum, along which one might observe a variation between positive and negative affect potentially culminating at the psychopathological state of depression.

A meta-analysis of genetic epidemiological studies of major depressive disorder in the monozygotic twins showed that depression heritability is in the range of 31%-42% [14]. Perhaps the most widely studied genetic component in the context of depression is the 5-HTTLPR polymorphism of the serotonin transporter (*SLC6A4*) gene [15-23]. Briefly, the serotonin transporter is one of the fundamental elements of the serotonin system because it generates the protein transporting serotonin from the synaptic cleft to the presynaptic neuron, upholding its availability to the organism [21]. A large number of studies has demonstrated that animal and human carriers of the short allele of the 5-HTTLPR polymorphism were at higher risk of presenting anxiety and depressive symptoms, especially in the presence of early-life adversity such as child abuse and neglect [15, 18-20, 24]. However, there is also a number of results showing a lack of association between the short 5-HTTLPR variant and affective disorders [25-29].

In addition to the genotypic variation, various early-life environmental factors have been associated with the onset and the persistence of affective disorders [30-40]. For instance, such family characteristics as low parental education and early childbearing age have been

associated with an increased risk of belonging to a high depression-anxiety trajectory [30-33]. Early exposure to abusive, hostile and over-controlling parental attitudes/behaviors has been associated with depressive symptoms in late childhood, adolescence and adulthood [36-40]. However, some studies looked for but found no relationship of parental neglect or socioeconomic conditions with the offspring's depressive symptoms later in life [41-43]. That being said, emotional development appears to be quite sensitive to the environmental conditions in early life.

Essentially, environmental signals are assumed to interact with the genetic blueprint to foster developmental trajectories in the central nervous system [44, 45]. In turn, these developmental trajectories were argued to regulate organism's assessment of the environment and its subsequent reactivity to the latter [34]. DNA methylation of the *SLC6A4* gene has been considered by multiple studies and reviews to potentially enact the way in which adverse environmental experiences mesh with the genetic functioning, shaping neural and emotional processes [21, 46-49].

As part of the present thesis, genetic and environmental influences on the relationship between the peripheral *SLC6A4* gene methylation and brain features (e.g., the activity in response to emotional stimuli, the volume and the functional connectivity) are examined. The association between the brain features and the daily emotions is also studied. Here, these important methodological questions are studied in healthy members of the prospective longitudinal cohorts. Doing so allows a thorough consideration of individuals' early- and daily-life environment as well as their history of emotional well-being.

In the following section, the neural bases of emotion processes will be covered. Next, the role of serotonin in brain development will be delineated. Then, the role of early-life environment in brain processes will be described. Finally, DNA methylation of the serotonin transporter gene will be discussed as a potential underlying mechanism linking these elements together.

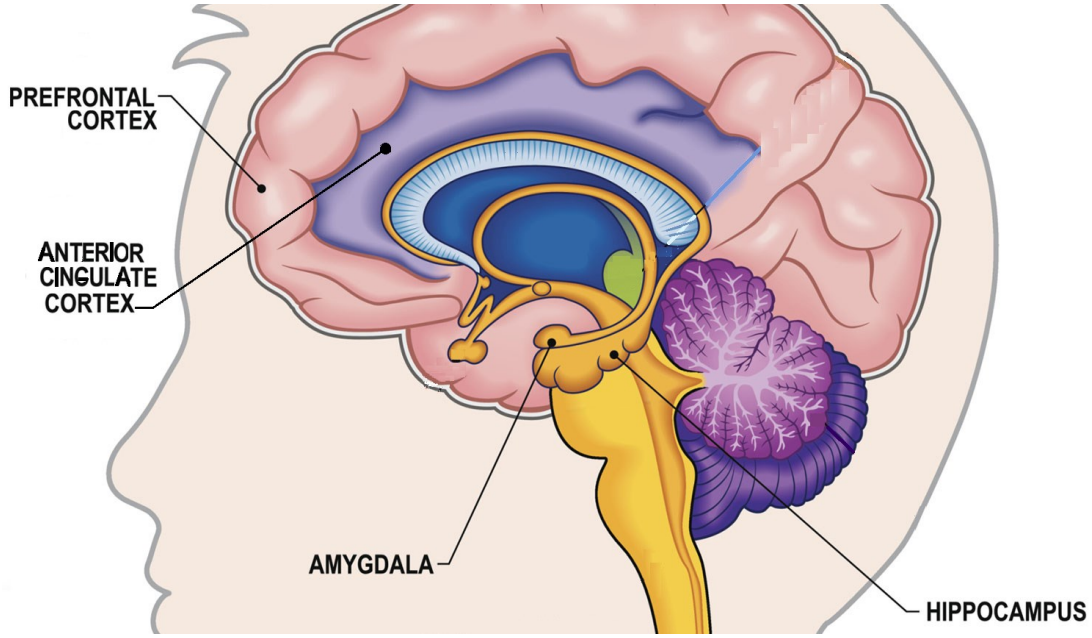

Figure 1. Frontal-limbic neural circuitry. Adapted from https://medium.com/@brainandspace/the-connection-point-brain-computer-interface-the-cerebral-cortex-89a80ebdcb3e

Neural background of emotion processes

At the heart of the neural models of affective (depressive, bipolar) disorders, as argued by Mayberg [50, 51] and Phillips [52], lies the frontal-limbic neurocircuitry (**Figure 1**), which includes various cortical and subcortical brain regions involved in emotional reactivity and emotion regulation. For instance, the posterior cingulate cortex [PCC], parietal cortex as well as prefrontal cortex – subdivided into medial prefrontal cortical regions (e.g., ventromedial prefrontal cortex [vmPFC], dorsomedial prefrontal cortex [dmPFC], orbitofrontal cortex [OFC], anterior cingulate cortex [ACC]) and the lateral prefrontal cortical regions (e.g., ventrolateral prefrontal cortex [vlPFC], dorsolateral prefrontal cortex [dlPFC]) – are considered to be the higher-order cortical regions the functioning of which has been linked to the (dys)regulation of cognitive-emotional processes in depressed individuals, such as rumination [53-56]. Additionally, the frontal-limbic neurocircuitry includes such regions as amygdala, hippocampus, and insula, located within the temporal lobe [50, 51]. These relatively archaic brain regions have been associated with the reactivity to emotional stimuli

and affective behavior. For instance, the amygdala has been associated with assessment, recognition of and responsivity to emotional stimuli [57-62]. The hippocampus and temporal cortex (in particular, medial temporal region) have been linked to encoding, consolidation and retrieval of the emotional memory [63-66]. The insula, PCC and precuneus have been implicated in the awareness of personal emotional and mental states as well as those of others [67-69], while the ACC has been associated with the dampening of emotional responsivity to the threatening stimuli [70-72]. What's more, the medial PFC, PCC as well as parietal and medial temporal areas are also part of the Default-Mode Network [DMN]. The DMN represents a set of brain regions shown to simultaneously activate when the brain is at rest and deactivate when the brain is engaged by a specific task [73-78]. The major nodes of the DMN are the vmPFC, PCC and lateral inferior parietal areas [77, 78]. The DMN is typically more active – hence, presenting greater functional connectivity between its neural components – during rest or while pondering over one's own attitudes, emotions and behaviors in the past, present and future [73, 78, 79].

Neuroimaging studies examining the frontal-limbic regional connections showed that the ventromedial PFC has connections with and receives input from such regions as the lateral prefrontal cortex, the (anterior and posterior) cingulate cortex, the insula, the hippocampus and the amygdala, which together have been associated with such meta-/cognitive processes as self-awareness, decision-making and the adaptive responses to emotional environmental stimuli [65, 80]. One way to examine the neural bases of emotion processes in humans consists in administering an emotion-eliciting task containing stimuli of emotional nature during the functional magnetic resonance imaging [fMRI]. Generally, individuals with a disorder characterized by altered emotion regulation (relative to healthy controls) were found to exhibit altered activity in the (orbital, dorsolateral, superior, inferior) frontal regions, ACC, insula and amygdala as well as in PCC, pre/cuneus and middle temporal regions in response to sad and fearful facial expressions [81-87]. Additionally, relative to healthy controls, affected individuals were found to display altered functional connectivity in response to the negative emotional facial expressions between such frontal-limbic brain regions as the (e.g., orbital, medial, superior) frontal regions, insula, hippocampus and amygdala [88, 89].

While maladaptive affect related to emotional disorders has been associated with altered frontal-limbic processes, transient changes in mood were also found to alter neural functioning in these regions. This has been demonstrated by experimental studies conducted in healthy individuals, combining the fMRI with experimental mood-induction techniques such as emotional music or videos, and autobiographical recall of emotional information. In healthy adults, experimental induction of negative mood has been associated with heightened brain activity in the amygdala, hippocampus, vlPFC, and ACC as well with lowered resting-state functional connectivity between PCC and mPFC, and between ACC and insula [90-93]. On the other hand, induction of positive mood in healthy adults has been associated with greater neural activity in the mPFC, dlPFC, and PCC [91, 93]. While the combination of the fMRI and a mood induction technique permits to temporarily prompt the emotion of interest and examine its neural substrates, the former does not allow examining neural correlates of the mood states occurring naturally in the everyday life. The latter can be achieved by coupling the fMRI technique with a daily diary of mood states. The daily diary, typically in the form of an online questionnaire, would assess the extent to which various emotions and social conflicts are experienced in the everyday life. A growing number of studies employ the combination of the fMRI with the daily diary, the majority of which are primarily focused on clinical or at-risk samples. For instance, Hooker and colleagues (2010; [94]) conducted an fMRI study in healthy young adult couples to examine the link between daily mood following an interpersonal conflict and neural activity in response to emotional stimuli. Results demonstrated that greater daily negative mood and rumination following an interpersonal conflict were associated with lowered vlPFC responses to negative emotional stimuli [94]. Quite similarly, greater daily negative mood following a distressing interpersonal conflict was associated with lowered dlPFC activity in individuals with schizophrenia, relative to their healthy counterparts, during cognitive control of negative emotional stimuli [95]. Additionally, Collip and colleagues (2013; [96]) combined a structural MRI with the daily diary method in order to examine the link between daily emotional reactivity and hippocampal gray matter [GM] volume in adult patients with schizophrenia, their at-risk first-degree relatives and healthy controls. As a result, greater daily emotional reactivity, in particular negative affect, was found to be associated with greater hippocampal volume in healthy controls and with smaller left hippocampal volume in affected and at-risk individuals [96].

Considering that the aforementioned frontal-limbic regions are densely innervated with serotonergic receptors [46], this neurocircuitry and related emotional functioning might be susceptible to any alterations occurring in the serotonin system [46, 97]. In the following section, the association between altered functioning of the serotonin system – in particular, *SLC6A4* – and the frontal-limbic processes will be discussed.

Role of serotonin in neural and emotion processes

Serotonin (5-hydroxytryptamine; 5-HT) is a neurotransmitter mainly synthesized from the amino acid tryptophan in the brain cells with the help of tryptophan hydroxylase (*TPH1* and *TPH2*) enzymes [98, 99]. Serotonin is carried across the brain by the *SLC6A4* and, eventually, degraded by the monoamine oxidase A (*MAO-A*) [98]. While the 5-HT neurons, emerging early during gestation, are scattered throughout the entire brain, the 5-HT population originates and is mostly contained in the raphe neurons, the ascending projections of which wind up in the hypothalamus as well as the PFC, the cingulate cortex and the limbic regions [46, 100, 101].

The implication of the 5-HT in the brain development has been delineated in several reviews [46, 102, 103]. In rodents, first 5-HT-containing raphe neurons are spawned within first two weeks of gestation in rodents, while the mature patterns – in terms of the density and innervation of the 5-HT fibers – are attained by the end of the third postnatal week [46, 102-105]. In humans, first 5-HT neurons are generated within the first two months of pregnancy [46, 103] and continue to proliferate throughout the first five postnatal years upon which they gradually wane until reaching mature levels at 14 years of age [46, 103]. During its expansion in the brain, the 5-HT system is involved in various developmental and lifelong adaptive brain processes including neuronal formation, migration, proliferation and differentiation, synaptic remodelling, axonal myelination as well as the effectiveness of neurotransmission [102, 106, 107].

Alterations in the 5-HT system, in particular during the early-life critical developmental periods, have been associated with the alterations in the brain processes. For instance, pharmacological studies examining the effect of lowered 5-HT levels in rats, by implementing a depletion of the 5-HT concentration or an inhibition of the serotonin

transporter levels during the early postnatal period, reported a long-lasting reduction in the hippocampal dendritic spine density [108] and an increase in the anxious-depressive behaviors in adult rodents [109]. One way to (transiently) lower 5-HT levels in humans is acute tryptophan depletion [ATD] [110]. Since the fMRI connectivity levels depend on the frequencies of the blood oxygen level dependent [BOLD] activation in the brain regions [111], the BOLD signal within the brain regions that are densely innervated with serotonergic receptors might be influenced by the serotonin functioning. Indeed, compared to placebo condition, healthy young adults displayed an ATD-induced lowered resting-state functional connectivity of precuneus and OFC [112]. Moreover, this resting-state functional connectivity, in particular within the middle orbital region, was negatively correlated with depressive mood [112]. In other words, orbital frontal region appears to be involved in serotonin depletion-induced neural and emotional alterations. Furthermore, findings of a systematic review of the fMRI ATD studies conducted in healthy individuals reported, among others, a decreased middle frontal cortical activation as well as an increased dorsomedial and inferior frontal cortical, middle temporal cortical, amygdala and angular activation during detection, processing and recognition of sad and fearful (relative to neutral) emotional stimuli [113]. In addition to altered brain activity, a transient diminution of the 5-HT levels in the brain was associated with a heightened bias towards identification and recognition of negative emotional stimuli [113]. Results of a more recent fMRI ATD study conducted in healthy adults indicated an association between lowered 5-HT neurotransmission in the brain and greater functional connectivity between the amygdala, the insula, the PCC, the precuneus and the superior temporal gyri at rest [114]. Together, these animal and human studies highlight the relationship of the 5-HT functioning with the frontal-limbic processes and the emotional processes.

Considering that the (dys)functions in the 5-HT system occurring early in life have been argued to modulate brain processes [46], the following section will cover the role of the early-life environment in neural processes, particularly in the 5-HT-containing brain regions.

Role of the early-life environment in brain processes

The early-life environment is crucial in calibrating the brain processes since the brain is undergoing significant development during that period of time [115-118]. In other words, the brain is particularly vulnerable to the early environmental context. For instance, the early-life adverse conditions such as childhood maltreatment and poverty has been associated with the short- and long-term alterations in the neural features in such regions as the prefrontal cortex [PFC], the ACC, the hippocampus and the amygdala [119-129]. For instance, pre-/adolescents (age range 8 to 19 years) exposed to childhood domestic violence displayed lower hippocampal volume and responses to threatening faces in a realistic (e.g., school playground) context and greater hippocampal functional connectivity with vlPFC, relative to their non-exposed peers [130]. Greater hippocampus-vlPFC functional connectivity was also associated with a poor context encoding in the presence of a threat [130]. These results may suggest that childhood exposure to violence might be related to a magnified attention to threat at the expense of contextual information [130]. The structural magnetic resonance imaging studies pondered on the association between childhood maltreatment and GM structure. For instance, exposure to childhood maltreatment in healthy individuals (age range 13 to 36 years) was found to be associated with smaller GM volume in the hippocampus and lowered cortical thickness in the medial and lateral PFC and in various temporal regions including the parahippocampal gyri [131, 132]. Moreover, the parahippocampal GM structure was found to mediate the association between childhood maltreatment and the subsequent antisocial behavior [131]. Results of another MRI study showed that the childhood maltreatment was associated with an impaired hippocampal growth later in life (age range 14 to 28 years), even after controlling for the emerging psychiatric symptoms [133]. Taken together, these findings suggest that early-life environment is related to the frontal-limbic brain processes; however, this relationship does not clearly translate into psychopathology.

In fact, not everyone who has been exposed to an early-life stress will develop psychopathology later in life. This is where the concept of resilience comes into play. Resilience can be used to describe the processes and characteristics enabling an individual to adapt well to environmental challenges [134]. For instance, among the individuals who have been exposed to inter-parental violence, those who have developed depressive symptoms were

found to display lower self-confidence and self-control, relative to those who remained healthy [43]. While concept of self-confidence refers to the trust in one's own ability to succeed in various situations, self-control refers specifically to the ability to regulate one's own emotional reactivity [135], which might be reflected in the altered frontal-limbic features. Indeed, as the vulnerability for psychopathology is not likely to be transmitted through one single gene, endophenotypic strategy – referring to the investigation of the characteristics that reflect the actions of (epi)genomes either predisposing the individual to or "buffering" him from the disorder – is typically used to examine the characteristics related to the risk and the resilience to the disorder. Indeed, results of a longitudinal fMRI study, investigating risk and resilience endophenotypes for major depressive disorder in adults indicated a distinct pattern of neural activity during self-regulatory task that included the superior frontal and anterior cingulate cortical regions as part of a brain-based resilience endophenotype [136]. Additionally, greater superior frontal GM volume has been associated with self-regulation in youth who experienced high levels of adversity but displayed positive adaptation [137].

Although the early-life environment is noticeably linked to the frontal-limbic brain processes, the DNA methylation should also be considered to play a role in shaping the neural and the emotional processes.

DNA methylation of the serotonin transporter gene

*DNA sequence and DNA methylation***.** First, it is important to grasp the difference between the genome and the epigenome. Essentially, the genomic (i.e. DNA) sequence is identical across the entire body and throughout the lifespan [138, 139]. The vast majority of the (f)MRI studies of 5-HT genes in the healthy and the depressed individuals outlined the importance of the short (versus long) allele of the 5-HTTLPR polymorphism of the *SLC6A4* gene, suggestive of lowered serotonin transporter function, in the frontal-limbic brain features [22, 140-143]. Altered frontal-limbic features included but were not limited to smaller GM volume in the amygdala, hippocampus and ACC as well as greater amygdala activity and connectivity with dlPFC and ACC in response to emotional stimuli [22, 139-143].

In contrast to the genome, the epigenome varies across body tissues and its workings drive the expression of the genes [21, 138, 144, 145]. The epigenetic processes are involved in calibrating the gene expression in response to environmental stimuli, thereby adding plasticity to the rigid genome [138, 139, 145, 146]. Precisely, epigenetic processes are characterized by a biochemical modification to the DNA functioning without altering the structural composition of the DNA sequence [138].

One of the most widely studied epigenetic processes is the DNA methylation (see reviews [46, 138, 145, 147-149]. Essentially, DNA methylation entails the addition of a methyl group (CH_3+) onto cytosine, one of the fundamental components of the DNA sequence located on a cytosine-phosphate-guanine [CpG] dinucleotide [138, 147, 150, 151]. This "annexation" is catalyzed by a class of enzymes called the DNA methyltransferase, which are crucial for detecting the genome's regulatory (i.e. promoter) site [138, 147, 150, 151]. Once on site, the enzymes covalently bind with the genome, detach the cytosine, plant the methyl group onto the cytosine and breaks away while consolidating the now-methylated cytosine back in its place [138, 147, 150, 151]. DNA methylation is linked to the repression of the gene expression at the promoter site, by hindering the DNA transcription factors' binding onto the altered promoter region of the gene [138, 147, 150, 151]. DNA methylation patterns are, for a large part, formed early during development and are, therefore, susceptible to the influence of the early-life environment [138, 147, 150-153]. Furthermore, DNA methylation patterns have been suggested to confer variation in the emotion processes and personality in humans.

There exists a pattern of mixed evidence that epigenetic modifications confer individual differences in personality in humans. Perhaps the most widely used model of personality traits is the five-factor model, also known as the Big Five [154]. The Big Five model describes the personality based on five dimensions, namely the openness to experience, conscientiousness, extraversion, agreeableness and neuroticism.

The openness to experience, extraversion and agreeableness might be considered as prosocial personality traits. Specifically, extraversion characterizes a tendency to engage the environment and people with vigor and enthusiasm, and agreeableness refers to a tendency to be altruistic and sympathetic to others [154]. The openness to experience reflects the degree of intellectual curiosity and preference for novelty [154]. In light of the implication of the

neurotransmitter oxytocin in the social behaviors, a recent study examined the relationship between the oxytocin receptor [*OXTR*] gene methylation and prosocial personality traits in healthy adults [155]. Results showed a negative association between the openness to experience and the *OXTR* DNA methylation [155]. No other significant association was reported [155].

On the other hand, neuroticism is defined as a tendency to be prone to psychological distress and experience such negative emotions as guilt, embarrassment, fear, or sadness [154]. The dimension of conscientiousness is characterized by a tendency to show self-discipline, act dutifully, and to be generally organized and dependable [154]. It has been hypothesized that the interplay of low conscientiousness, low extraversion and high neuroticism would contribute to the onset of emotional disorders such as depression [156]. In term of the underlying epigenetic processes of the pathogenesis, research evidence appears to point to an etiological link between the development of depression and the functioning of the brain derived neurotrophic factor [*BDNF*], notably based on an increase in the *BDNF* gene expression related to the administration of antidepressants [157, 158]. In light of this evidence, a recent study examined the relationship between the *BDNF* gene methylation and the Big Five personality traits, in particular conscientiousness, extraversion and neuroticism, in healthy young adults [159]. Results showed that individuals with higher neuroticism scores displayed higher levels of the *BDNF* gene methylation [159]. No other personality traits were found to be associated with the *BDNF* DNA methylation [159]. Further, results of a Positron Emission Tomography [PET] study indicated a positive correlation between neuroticism and serotonin transporter availability in the healthy young adult males' brains [160]. Yet another study's findings, mainly focusing on the functioning of the *BDNF*, *OXTR* and *SLC6A4* genes in the elderly women with and without internalizing (i.e. anxiety and depressive) symptoms, showed that greater DNA methylation levels in the *BDNF* and *OXTR* genes (but not *SLC6A4* gene), were observed in the clinical group, relative to healthy controls [161]. The differential findings in the aforementioned studies may be attributed to their methodological differences. Nonetheless, the link between neuroticism, characterized by a tendency to cope poorly with stress and to experience negative emotions [154] and the *SLC6A4* DNA methylation remains of interest.

DNA methylation of SLC6A4 gene. Considering the role of the serotonin transporter in the neural and emotional processes and that both methylation and serotonin patterns are largely shaped early in life, the DNA methylation in the *SLC6A4* gene will be further discussed as a potential underlying mechanism linking early-life environment, brain features and affective behavior. In light of the difficulty in assessing the central DNA methylation in the living human brain, peripheral tissues – such as blood – are essential in psychiatric epigenetics. The peripheral methylation in the serotonin transporter gene has been associated with lower *in vivo* measures of the brain 5-HT synthesis in adults [162], suggesting that the peripheral methylation patterns are informative of the central 5-HT functioning.

To the best of our knowledge, a total of seven (f)MRI studies so far investigated the association between the peripheral DNA methylation in the *SLC6A4* gene and the brain features in depressed as well as in healthy individuals [49, 163-168]. Results of the (f)MRI studies, comparing the peripheral (blood-derived) *SLC6A4* gene methylation between the depressed adults and the age-matched healthy controls, showed that greater blood-derived *SLC6A4* gene methylation was associated with smaller hippocampal GM volume and greater insula activity in response to the negative emotional content, independent of the diagnosis [163, 165]. Interactions between the methylation and diagnostic group have also been reported, whereby greater blood-derived *SLC6A4* gene methylation has been associated with greater temporal/hippocampal activity in response to the negative emotional stimuli in healthy controls [165] and with greater amygdala activity in the clinical group [168]. Additionally, greater blood-derived *SLC6A4* gene methylation has been associated with greater amygdala response to the negative emotional stimuli in a mixed sample of adolescents [49, 167] as well as with greater amygdala, hippocampal and insula GM volumes and greater amygdala resting-state connectivity with the ACC and the insula in the healthy adults [164, 166]. Similar to the blood-derived *SLC6A4* gene methylation, the saliva-derived methylation was found to be positively associated with the amygdala response to the threatening cues in the healthy adolescents [167].

Amidst the seven (f)MRI studies of the peripheral *SLC6A4* gene methylation, two studies looked for and found an association between the childhood maltreatment and the peripheral (blood-derived) *SLC6A4* gene methylation, independently of the individuals'

diagnosis [163, 165]. Additionally, one study reported the association between the recent stressful events and the peripheral (blood-derived) *SLC6A4* gene methylation, irrespective of the individuals' diagnosis [168].

Also among the aforementioned (f)MRI studies, some studies looked for but found no the environment or the genotype effect [163, 167, 168], while one study found an indirect effect of the environment but the no genotype effect [49] on the relationship between peripheral *SLC6A4* gene methylation and brain processes. Yet other studies reported no environment effect and did not test for genotype [165, 166] or vice versa [164]. It is unclear whether these results are underpowered or indicative of a genuine lack of an association.

Several questions arise out of the existing (f)MRI research on the peripheral *SLC6A4* gene methylation. For instance, the environmental influence on the relationship between the *SLC6A4* gene methylation and the brain features – including early-life conditions and daily mood – is open to question. Moreover, the current insight on the peripheral tissue specificity and/or convergence – implying that the relationship between methylation patterns and brain processes is either different or similar across peripheral tissues – is scarce. The genetic influences on the relationship between the *SLC6A4* gene methylation and the brain processes also remain unclear. As a step to address these issues, the peripheral tissue specificity, the role of genetics and that of the daily life are examined. Considering the intricacy of these methodological questions, the latter are studied in the healthy members of the prospective longitudinal cohorts. Doing so allows a careful documentation and investigation of the individuals' early- and daily-life environment as well as their history of emotional well-being.

Objective

First aim of this thesis is to investigate the association between the frontal-limbic features and daily mood. Specifically, in chapter 1, we examine the association between the daily mood and the neural activity in response to the emotional stimuli, the GM volume and the resting-state functional connectivity within the frontal-limbic neural circuitry.

In the second chapter, we test the hypothesis of the peripheral tissue specificity by examining the *SLC6A4* gene methylation derived from which tissue – blood, saliva or buccal cells – corresponds best to the neural activity in response to the emotional stimuli, the GM volume and the resting-state functional connectivity within the frontal-limbic neural circuitry.

In the third chapter, we test the hypothesis that the peripheral *SLC6A4* gene methylation is associated with the frontal-limbic activity and functional connectivity in response to the emotional stimuli, independently of the genotype. The monozygotic [MZ]-twin study design offers a unique means to infer genetic influences because a MZ twin pair originates from a single zygote and, therefore, both twins share identical DNA sequence [169-172]. The neural correlates of the daily mood and he peripheral *SLC6A4* gene methylation are expected to be found in the frontal-limbic neurocircuitry, including the PFC, the ACC, the insula, the hippocampus and the amygdala.

This thesis ends with a discussion of the implications of the results and of current methodological challenges of this type of research as well as the avenues for future research are proposed.

Chapter 1 – Daily-life mood and brain processes

Foreword

In this first chapter, we assess the brain function in response to the emotional stimuli and during the resting state as well as the brain structure in the Quebec Longitudinal Study of Kindergarten Children (QLSKC) cohort at mean age of 33 years. Drs. Richard E. Tremblay and Frank Vitaro are responsible for the QLSKC cohort. Dr. Jean-Philippe Gouin created the online daily stress questionnaire in collaboration with Dr. Linda Booij. Dr. Florence B. Pomares taught me how to analyze the (f)MRI data and supervised my analyses. Jessica Di Sante assisted in recruitment and the data collection. I participated in the recruitment, the data collection, the (f)MRI analysis, and I wrote up the paper. Dr. Linda Booij designed the study, supervised the data collection and the statistical analysis, provided feedback on all the versions of the manuscript and submitted the latter for publication. All co-authors reviewed and approved the manuscript prior to submission. Manuscript was published in *Frontiers in Human Neuroscience* (Ismaylova et al. Front Hum Neurosci. 2018; doi: 10.3389/fnhum.2018.00168).

Associations between Daily Mood States and Brain Gray Matter Volume, Resting-state Functional Connectivity and Task-based Activity in Healthy Adults

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Abstract

Numerous studies have shown differences in the functioning in the areas of the frontal-limbic circuitry between depressed patients and controls. However, current knowledge on frontal-limbic neural substrates of individual differences in mood states in everyday life in healthy individuals is scarce. The present study investigates anatomical, resting-state and functional neural correlates of daily mood states in healthy individuals. We expected to observe associations between mood and the frontal-limbic circuitry and the default-mode network [DMN]. Forty-two healthy adults (19 men, 23 women; 34 ± 1.2 years) regularly followed for behavior and psychosocial functioning since age 6, underwent a fMRI scan and completed a daily diary of mood states and related cognitions for five consecutive days. Results showed that individuals with smaller left hippocampal gray matter volumes experienced more negative mood and rumination in their daily life. Greater resting-state functional connectivity [rsFC] within the DMN, namely between posterior cingulate cortex [PCC] and medial prefrontal cortical [MPFC] regions as well as between PCC and precuneus, was associated with both greater negative and positive mood states in daily life. These rsFC results could be indicative of the role of the DMN regional functioning in emotional arousal, irrespective of valence. Lastly, greater daily positive mood was associated with greater activation in response to negative emotional stimuli in the precentral gyri, previously linked to emotional interference on cognitive control. Altogether, present findings might reflect neural mechanisms underlying daily affect and cognition among healthy individuals.

Keywords: Daily mood, fMRI, left hippocampus, Default-Mode Network, emotion

Introduction

The function and structure of frontal-limbic brain regions play a major role in the regulation of mood. Most of the evidence stems from anatomical and functional magnetic resonance imaging [(f)MRI] studies conducted in individuals with major depressive disorder [MDD]. Compared to healthy controls, individuals with MDD displayed smaller gray matter [GM] volume in such regions as dorsal lateral prefrontal cortex [LPFC] (e.g., Shad et al., 2012; Grieve et al., 2013) and hippocampus (e.g., Zou et al., 2010; Stratmann et al., 2014). Individuals with MDD also display greater neural responses to negative emotional stimuli in limbic regions including amygdala and hippocampus (e.g., Victor et al., 2010; Hall et al., 2014), as well as lower resting-state functional connectivity [rsFC] between amygdala and such (pre)frontal regions as dorsal LPFC, ventral medial prefrontal cortex [MPFC] and anterior cingulate cortex [ACC] (e.g., Pannekoek et al., 2014; Connolly et al., 2017). Additionally, several studies indicated that MDD is characterized by resting-state functional hypoconnectivity between dorsal LPFC and parietal regions, which are involved in attending to the environmental cues, as well as hyperconnectivity among MPFC, ACC and hippocampus, implicated in self-referential processes (e.g., Kaiser et al., 2015; Northoff, 2016). In MDD, this connectivity "imbalance" would contribute to shifting focus on self-oriented thoughts, potentially resulting in rumination (e.g., Kaiser et al., 2015; Northoff, 2016).

While MDD-related maladaptive affect and cognition has been associated with altered frontal-limbic brain processes, transient changes in mood can also transiently alter neural functioning in these networks. This has been demonstrated by experimental studies in healthy individuals, combining fMRI with experimental mood-induction techniques such as emotional videos/images/music and autobiographical recall of emotional events (e.g., Harrison et al., 2008; Subramaniam et al., 2016). In healthy adults, experimental induction of negative mood has been associated with heightened brain activity in amygdala and hippocampus and various prefrontal regions including the orbitofrontal cortex [OFC], MPFC, ventral LPFC and ACC, as well as with lowered rsFC between posterior cingulate cortex [PCC] and MPFC and greater ACC-insula rsFC (e.g., Pelletier et al., 2003; Habel et al., 2005; Harrison et al., 2008).

Induction of positive mood in healthy adults has been associated with greater neural activity in MPFC, dorsal LPFC and PCC (e.g., Habel et al., 2005; Subramaniam et al., 2016).

Several (f)MRI studies have investigated the neural correlates of mood reactivity in daily life using ecological assessment methods. Most of these studies focused on patients with psychotic disorders or at-risk samples. For instance, one brain morphometry study showed that (relative to controls) greater emotional reactivity to daily stress in patients with schizophrenia and at-risk first-degree relatives was associated with smaller hippocampal GM volumes (Collip et al., 2013). Using task-based fMRI, Tully and colleagues (2014) indicated that within individuals with schizophrenia (relative to controls), higher dorsal LPFC activity during cognitive control of negative emotional information was associated with positive mood following highly-distressing interpersonal conflict. In a study of couples free of mental health problems, Hooker and colleagues (2010) showed that lower ventral LPFC response to negative emotional stimuli was associated with greater daily negative mood and rumination following stressful interpersonal conflict with their partner.

Additionally, individual differences in resting-state functional connectivity have been linked to the well-being/positive lifestyle, such as life satisfaction, self-realization or pleasure attainment (Smith et al., 2015; Luo et al., 2017). Noteworthy, PCC-based rsFC with PFC, precuneus, parahippocampal and superior temporal gyri – all part of the so-called Default-Mode Network (Greicius et al., 2003; Fransson, 2005, 2006; Buckner et al., 2008) – has been linked to experimentally induced sadness (Zamoscik et al., 2014; Renner et al., 2017). We are not aware of any study examining the association between these brain processes and the daily-life mood in healthy individuals. The link between PCC-based resting-state functional connectivity and daily mood is of particular interest since it might be informative of how mood states in the everyday life echo in the functioning of the brain not strained by any specific task.

The aim of the present study is to examine, in a sample of healthy adults, anatomical, resting-state and functional neural correlates of daily mood states, namely negative mood, rumination and positive mood. We expected these associations to occur in brain regions that are part of

the frontal-limbic neural circuitry and the Default-Mode Network, namely prefrontal cortex, anterior and posterior cingulate cortices, precuneus, insula, hippocampus and amygdala.

Materials and Methods

Participants

Participants were members of two longitudinal cohorts of individuals, followed since their kindergarten year (Van Bokhoven et al., 2006; Rouquette et al., 2014). Careful screening for eligibility in the brain-imaging component was based on the absence of any prior or current Axis I disorder, neurological disorder, medical illness and medication intake as well as on the absence of irremovable foreign metals in the body (e.g. braces, piercings). Presence/absence of past and current Axis I disorders was assessed by conducting the Structured Clinical Interview for DSM-IV [SCID-IV] (First et al., 2002), whereas the other (neuroimaging-related) exclusion criteria were verified by means of a Montreal Neurological Institute [MNI] in-house questionnaire. After thorough screening for eligibility and availability, 47 individuals underwent an (f)MRI session at the MNI. Participants also completed the Beck Depression Inventory [BDI] (Beck et al., 1988) as well as the Neuroticism scale (12 items) of the Eysenck Personality Questionnaire (Birley et al., 2006), neither of which have been found to be associated with any brain processes in the current study. After the fMRI session, participants were asked to fill out a daily online questionnaire for five consecutive days.

Five participants did not fill out the daily online questionnaire despite multiple reminders. Therefore, the final sample of the present study was composed of 42 healthy adults (19 men, 23 women; age range 32 to 36 years). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki (World Medical Association, 1991). The study protocol was approved by the ethics review boards of Sainte-Justine Hospital and of MNI, Montreal, Canada.

Image Acquisition

All forty-two participants were scanned on a 3T Siemens TIM Trio Scanner (www.medical.siemens.com) using a 32-channel head-coil. Following a brief localizer, the

scan sequence included, respectively, a 9-minute-long anatomical scan (MPRAGE sequence; 176 slices in sagittal plane; TR=2300ms, TE=2.98ms, FA=9°, FOV=256mm, matrix size=256x256, voxel size=1mm x 1mm x 1mm), a 7-minute-long resting state scan (gradient EPI sequence; 180 whole-brain volumes; TR=2300ms, TE=30ms, FA=30°, FOV=224mm, matrix=64x64, 43 slices in axial plane, voxel size=3.5mm x 3.5mm x 3.5mm,) during which participants were asked to stay awake while keeping their eyes closed (Beliveau et al. 2015), and lastly a 15-minute-long event-related functional scan during which an emotion-processing task was performed (gradient EPI sequence; 400 whole-brain volumes; TR=2300ms, TE=30ms, FA=30°, FOV=224mm, matrix=64x64, 43 slices in axial plane, voxel size=3.5mm x 3.5mm x 3.5mm). During the emotion-processing task, adapted from Canli and colleagues (2005), 120 Ekman facial emotional expressions (happy, sad, angry, fearful and neutral) (Ekman and Frisen, 1976; Keltner and Ekman, 2003) were presented to participants randomly for 2 seconds, followed by a fixation cross (1 second) and a question asking participants to choose whether the face belonged to a man or a woman. Activation following exposure to happy (versus neutral) and sad (versus neutral) was studied in the context of the present study.

Daily Diary of Mood States

Following the brain scan, participants were instructed to fill out online a daily diary of mood states, at the end of the day for five consecutive days. This online diary allowed participants to provide precise information on the mood they have experienced in their natural context (e.g. family life, marital life, social life), within the 24-hour period for five consecutive days. All the responses were in the form of multiple choices, ranging from “never” to “repeatedly”.

Positive and Negative Affect subscales were formed based on the Positive And Negative Affect Schedule [PANAS] (Watson et al., 1988), assessing the extent to which participants had experienced, respectively, positive emotional states characterizing sharp and alert mind (happy, enthusiastic, energetic, determined) and negative emotional states with negative connotations (irritable, sad, nervous, embarrassed) over a period of twenty-four hours, in a form of a multiple choice ranging from “never” (score “0”) to “repeatedly” (score “4”). Therefore, the total score for each scale ranged from 0 to 16.

Repetitive negative thoughts (rumination) were assessed with the items of the Ruminative Responses Scale of the Response Style Questionnaire [RSQ] (Nolen-Hoeksema et al., 1999). Relative to the reflective pondering subtype of rumination (involving active attempts to gain insight into problems), brooding subtype of rumination (implying passive comparison of one's current situation with an unachieved standard) and worry have been consistently associated with negative mood in healthy and clinical populations (e.g., Burwell and Shirk, 2007; Raes and Hermans, 2008; Verhaeghen et al., 2014). Therefore, we constructed a subscore focusing solely on the brooding and worry components, as potential contributors to the maintenance and intensification of mood states. Eight RSQ-derived items describing brooding rumination and worry were used for the construction of the current scale (i.e. "Negative thoughts came to mind throughout the day", "I analyzed recent events to try to understand why I was sad or upset", "I wondered why I always react this way", "I wondered why I cannot seem to cope better with events", "I imagined how I would have liked things to happen", "I worried about saying or doing something wrong", "I worried about what others would think of me", " I worried about being criticized for something I said or did"). For each statement, the participants were asked to indicate how many times they felt this way in a form of a multiple choice, ranging from "never" (score "0") to "repeatedly" (score "3"). Therefore, the total score for this scale ranged from 0 to 24.

Total scores for each subscale were averaged for the period of five consecutive days, indicating individuals' average experience of daily mood, and used in the analyses (Bolger et al., 2003). Cronbach alpha's for the subscales of the daily diary averages across 5 days were 0.99 for negative mood, 0.91 for rumination and 0.99 for positive mood, indicating excellent internal stability.

Statistical Analyses

Statistical Parametric Mapping ([SPM12] v6470, Wellcome Department of Cognitive Neurology, London UK) implemented in MATLAB R2010a (Mathworks, Sherborne, MA) was used for anatomical and functional analyses.

Voxel-based morphometry [VBM] analyses were computed using the CAT12 toolbox (http://www.neuro.uni-jena.de). T1-weighted images were normalized to MNI space and segmented into gray matter [GM], white matter [WM] and cerebrospinal fluid [CSF] based on intensity distribution of the image and using tissue probability maps. Then, normalized GM segments were modulated with the resulting Jacobian determinant maps and smoothed with an 8-mm full width at half maximum [FWHM] Gaussian kernel. VBM, not biased to one particular structure, allows a balanced comprehensive assessment of anatomical differences throughout the brain (Ashburner and Friston, 2001). Whole-brain analysis was conducted, followed by a region of interest [ROI] analysis using a mask encompassing frontal-limbic regions (PFC, ACC, insula, hippocampus and amygdala) obtained from Anatomical Automatic Labeling [AAL] atlas within the Wake Forest University Pick Atlas utility [version 3.0.5] (Maldjian et al., 2003, 2004). For the anatomical analyses, the brain regions were examined at a voxel-wise threshold of $p<.001$, with cluster-wise family-wise error rate [FWE]-correction for multiple comparisons (Shaffer, 1995) threshold set at $p<.05$, and corrected for smoothness non-uniformity (Hayasaka et al., 2004). All peak coordinates are reported in MNI format.

Using CONN functional connectivity toolbox [version 16.b] pipeline (Whitfield-Gabrieli and Nieto-Castanon, 2012) and based on SPM12 preprocessing, resting-state functional images were spatially realigned to correct for interscan movement and normalized to the MNI space, while structural images were segmented and separately normalized to the MNI space with non-linear transformation. Next, ART-based scrubbing was done, which consisted in a detection of outlier functional scans (defined as points exceeding the default threshold set with a conservative 95% percentile, including a global brain activation signal of $z>3$ and linear motion parameters>0.5mm). Any outlier functional scans were, then, entered as a covariate during the denoising step to control for potential confounding effect. Finally, all the images were smoothed using an 8-mm FWHM Gaussian kernel. A denoising procedure including the component-based correction method (Behzadi et al., 2007) followed by the first-level analysis, was applied in order to remove motion artifacts and other artefactual effects from the fMRI signal. Next, considering that PCC-based – one of the Default-Mode Network's nodes (Greicius et al., 2003) – connectivity has been previously associated with mood states (e.g.,

Zamoscik et al., 2014; Renner et al., 2017), second-level seed-based analysis was conducted using PCC as a seed, which was defined using AAL atlas (MNI coordinates: -6, -52, 40) (Fox et al., 2005). For the connectivity analyses, the brain regions were examined at a voxel-wise threshold of $p<.001$, with cluster-wise FWE-correction for multiple comparisons (Shaffer, 1995) threshold set at $p<.05$. All peak coordinates are reported in MNI format. When significant results were observed, the individual connectivity values for the regions showing up in the seed-to-voxel analysis were extracted using the CONN v.16.b software. These connectivity values were, then, transferred in an Excel document in order to generate a scatter-plot for visualization purposes.

Preprocessing of the functional data acquired during the emotion-processing task (slice timing, realignment, coregistration, stereotaxic spatial normalization, and smoothing with 8-mm FWHM Gaussian kernel) was followed by intra-individual first level analyses, performed in order to calculate (emotion minus neutral) contrasts for each emotion at each voxel. Next, whole-brain analysis was performed, followed by ROI analysis using a mask encompassing all frontal-limbic regions (see earlier) (Maldjian et al., 2003, 2004). For the functional analyses, the brain regions were examined at a voxel-wise threshold of $p<.001$, with cluster-wise FWE-correction for multiple comparisons (Shaffer, 1995) threshold set at $p<.05$. All peak coordinates are reported in MNI format.

We performed multiple regression VBM and fMRI analyses, with separately negative mood, rumination and positive mood as second-level variables in the imaging analyses. The same variables of interest were separately implemented to test for voxel-wise correlations between them and PCC-based resting-state connectivity. A total of nine analyses were performed in the present study, for which we applied the cluster-wise FWE correction for multiple comparisons.

Results

Descriptive Analyses

Table 1 shows the characteristics of the included sample. BDI and EPQ Neuroticism scale scores were generally considered low compared to normative scores (Beck et al., 1988; Birley et al., 2006). As expected, daily negative mood was found to be positively correlated to daily rumination (r=0.73, p<.001). Daily positive mood was not found to be significantly correlated to either daily negative mood (r=-0.24, p=.12) or daily rumination (r=-0.29, p=.06). Furthermore, BDI scores (as assessed at the time of scanning) were found to be positively correlated to daily negative mood (r=0.45, p=.003) and daily rumination (r=0.32, p=.04). In the same vein, EPQ Neuroticism scale scores were found to be positively correlated to daily negative mood (r=0.51, p=.001) and daily rumination (r=0.40, p=.009). No significant association was found between daily positive mood and BDI score and EPQ Neuroticism scale score (p>.11). There were no significant associations between daily mood and sex (F(1,40)=0.10-1.60; p>.213).

Imaging Analyses

Brain morphometry. No significant associations were found at the voxel p<.001 level following the cluster-wise FWE-correction. The exploratory results of positive and negative VBM GM correlations significant at the uncorrected voxel p<.001 level without the cluster-wise FWE-correction are indicated in Table 2. Greater negative mood in daily life was associated with smaller left hippocampal GM volume (k=110; t=4.02; cluster p=0.16; voxel p<.001; (peak MNI coordinates: -24, -18, -9)) (Figure 1). Greater levels of rumination were also associated with smaller left hippocampal GM volume (k=215; t=4.54; cluster p=.06; voxel p<.001; (peak MNI coordinates: -24, -18, -9)) (Figure 1). No other significant associations were found.

Resting-state functional connectivity. Significant results of positive and negative seed-to-voxel rsFC correlations are indicated in Table 3. Greater daily negative mood was associated with greater rsFC between PCC and MPFC, frontal poles and ACC (k=609, t=4.64, p_{FWE}<.001, voxel p<.001, (peak MNI coordinates: -4, 40, -6)), as well as between PCC and precuneus (k=939, t=5.02, p_{FWE}<.001, voxel p<.001, (peak MNI coordinates: -6, -54, 34)) (Figure 2A). Figures 2B and 2C contain scatter-plots depicting how the connectivity values of the PCC-MPFC, frontal poles, ACC cluster and PCC-precuneus cluster vary with daily negative mood.

Daily positive mood was also associated with greater seed-to-voxel rsFC between PCC and frontal poles and ACC (k=437, t=4.57, p_{FWE}<.001, voxel p<.001, (peak MNI coordinates: 2, 56, 12)), as well as between PCC and precuneus (k=1980, t=6.77, p_{FWE}<.001, voxel p<.001, (peak MNI coordinates: -10, -50, 38)) (Figure 3A). Figures 3B and 3C contain scatter-plots depicting how the connectivity values of the PCC-frontal poles, ACC cluster and PCC-precuneus cluster vary with daily positive mood. No other significant associations between daily mood and PCC-based resting-state functional connectivity were found.

Task imaging results. Greater daily positive mood was associated with greater bilateral precentral responses to sad stimuli (respectively for right precentral gyrus: k=82; t=4.13; cluster p_{FWE}=.016; voxel p<.001; (peak MNI coordinates: 8, -21, 63) and left precentral gyrus (k=82; t=3.87; cluster p_{FWE}=.02; voxel p<.001; (peak MNI coordinates: -6, -21, 60)). No other significant associations were found.

Discussion

In the present study, we examined neural correlates of daily mood states in healthy adults. Daily negative and positive mood were each associated with greater resting-state functional connectivity between PCC and such regions as ACC and precuneus. Furthermore, positive mood was associated with greater precentral responses to negative (i.e. sad) emotional stimuli. Lastly, we also found that daily negative mood and rumination were each associated with smaller left hippocampal GM volume (albeit only at the voxel-wise threshold of p<.001). Overall, these results seem to indicate not only mood-state-specific neural correlates but also that natural variation in daily mood is reflected in the frontal-limbic functioning and anatomy, relevant for understanding subtle changes in daily emotional life among healthy individuals.

In line with the previous research showing that individuals scoring higher in such positive personal indicators as positive affect and lifestyle satisfaction exhibited stronger rsFC patterns within the DMN including MPFC and parietal cortex (Smith et al., 2015), positive associations were observed between daily positive mood and Default-Mode Network resting-state connectivity between PCC and MPFC regions. Additionally, the finding that both positive and negative daily mood states were linked to the rsFC within the same DMN regions may suggest

that heightened PCC-MPFC rsFC could reflect an arousal (as opposed to valence) dimension of mood. In other words, individuals with increased PCC-MPFC functional connectivity at rest might experience more emotions on a daily basis, irrespective of valence. Further studies are necessary to replicate these findings as well as to examine potential physiological confounds, including heart rate.

Smaller left hippocampal GM structure has been repeatedly associated with various psychiatric symptoms, including trauma, MDD and schizophrenia (e.g., Seidman et al., 2002; Vythilingam et al., 2002; Steffens et al., 2011). Smaller right hippocampal GM volume has been repeatedly associated with childhood maltreatment and combat-related woes, particularly in individuals who later developed post-traumatic stress disorder (e.g., Bremner et al., 1995; Pavić et al., 2007). We may advance that in the present context, subtle variations in hippocampal GM volume – although on an uncorrected level – may be associated with individual differences in daily negative mood and ruminative response style in healthy adults. However, further studies are necessary to confirm these exploratory uncorrected results.

Lastly, a positive association was found between daily positive mood and brain activation in response to negative (i.e. sad) emotional stimuli in the precentral gyri. In addition to its role in motor behavior, heightened activity in precentral gyri – distinct structure in the posterior portion of the frontal lobe – has also been implicated in the retrieving and processing of (positive or negative) emotionally-valenced stimuli (Maratos et al., 2001; Kolesar et al., 2017). Also, precentral regional activity has been positively associated with emotional interference in cognitive and behavioral control, condition in which one's attention and goal-directed behavior is challenged by emotionally-salient stimuli (see meta-analysis by Song et al., 2017). Overall, current task-based activity in the precentral gyri (that are bound inferiorly by the cingulate cortices) as well as PCC-ACC resting-state connectivity may echo brain mechanisms involved in the assessment and cognitive regulation of emotions in everyday life. In light of these findings, current low between-subject scores and variation in daily negative mood and rumination, relative to daily positive mood, may reflect degrees of adaptive emotional reactivity and regulation in a healthy sample.

Strength of the study was that the current participant sample was part of a well-documented longitudinal sample followed for more than three decades, and participants for the present study were carefully selected on the basis of absence of psychopathology. This permitted to avoid any (residual) clinical symptoms or treatment as a potential confound. On the other hand, the careful screening for mental health problems may have led to low inter- and intra-individual differences in mood and low levels of negative affect and rumination. Additionally, the number of statistical tests may have increased the probability of finding false positives. Yet on the other hand, the relatively moderate sample size combined with a stringent testing could result in increasing probability of type II errors. Despite a restricted range in daily mood and a moderate sample size, significant associations were detected suggesting that individual differences in daily affect and cognition among healthy individuals are reflected in individual differences in frontal-limbic and default-mode network brain processes. Future research should include larger samples with a large range of daily mood levels and could include possible moderating factors such as genotype and sex.

In summary, this study showed in a sample of healthy adults that individual differences in left hippocampus GM volumes were associated with individual differences in negative affect in daily life. The observed association between both daily positive and negative mood and PCC-MPFC and precuneus rsFC might be indicative of the involvement of this DMN regional functioning in the emotional arousal in daily life, irrespective of valence. Future research should include samples with a larger inter- and intra-individual range of daily mood states.

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Table 1. Characteristics of the sample

Characteristics of the sample (N=42)	**Statistics**			
Variable	**Mean**	**SD**	**Median**	**Skewness**
Age (years)	33.95	1.15	34.00	0.189
Sex (Male/Female)	19/23	-	-	-
EPQ Neuroticism scale score (/12)	2.45	2.40	2.00	0.680
BDI total score (/63)	3.26	3.44	2.00	0.717
Daily negative mood score (/16)	2.35	1.46	2.20	0.907
Daily rumination score (/24)	3.10	2.74	2.00	1.058
Daily positive mood score (/16)	9.00	2.60	8.80	0.126

Note. SD=Standard deviation; EPQ=Eysenck Personality Questionnaire; BDI=Beck Depression Inventory.

Table 2. Exploratory gray matter findings of whole-brain voxel-based morphometry analyses and their associations with daily mood states

						MNI peak coordinates		
Variable	Region	k	Voxel t	Cluster *p*-value*	Voxel *p*-value*	x	y	z
Negative mood	left hippocampus	110	-4.02	.157	<.001	-24	-18	-9
Rumination	left hippocampus	215	-4.54	.061	<.001	-24	-18	-9

Note: MNI=Montreal Neurological Institute. *Uncorrected for multiple comparisons.

Table 3. Results of whole-brain seed-to-voxel resting-state functional connectivity analyses and their associations with daily mood states

							MNI peak coordinates		
Variable	Seed region	Target cluster	k	Voxel t	Cluster p_{FWE}-value	Voxel *p*-value	x	y	z
Negative mood	PCC	MPFC, frontal poles and ACC	609	4.64	<.001	<.001	-4	40	-6
	PCC	Precuneus	939	5.02	<.001	<.001	-6	-54	34
Positive mood	PCC	Frontal poles and ACC	437	4.57	<.001	<.001	2	56	12
	PCC	Precuneus	1980	6.77	<.001	<.001	-10	-50	38

Note: MNI=Montreal Neurological Institute. FWE=Family-wise error correction. PCC=posterior cingulate cortex. MPFC=medial prefrontal cortex. ACC=anterior cingulate cortex.

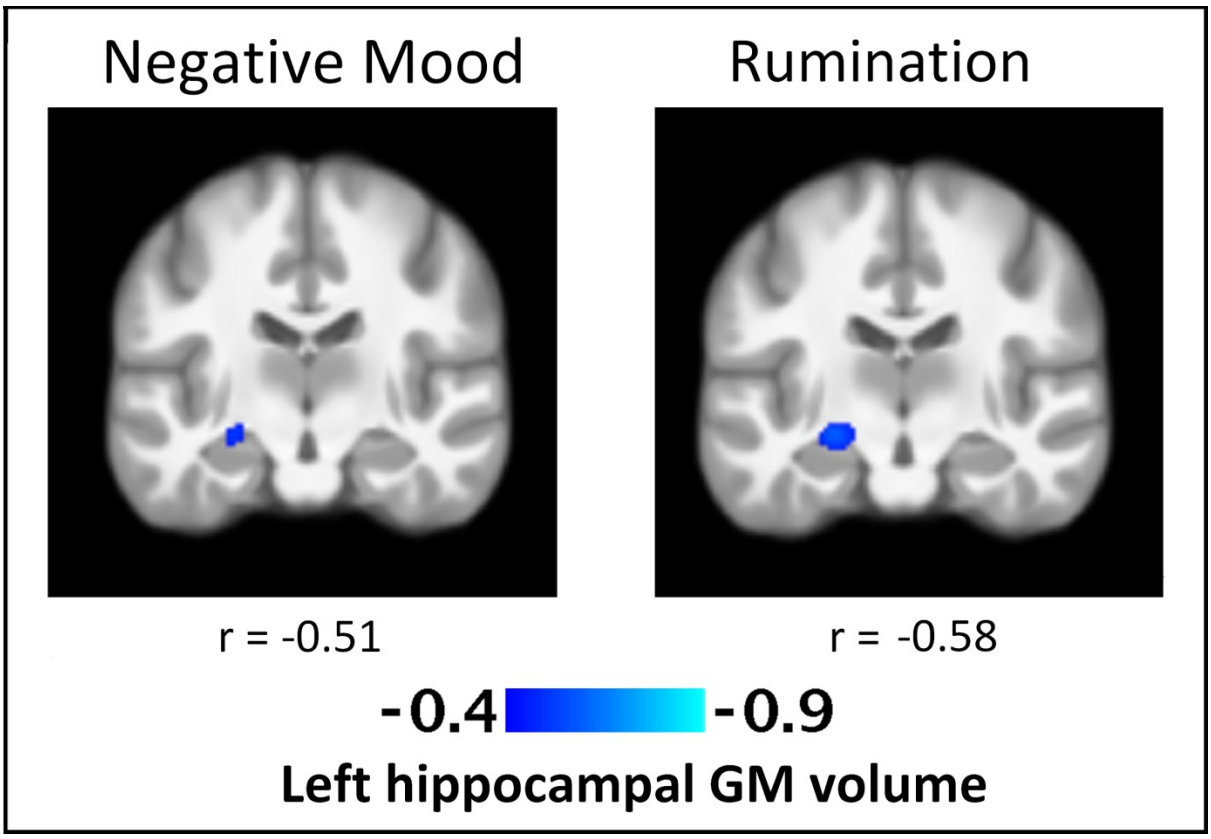

Figure 1. T-Statistic maps of the negative association between regional left hippocampal gray matter [GM] volume and daily negative mood and rumination. Both images were taken at peak Montreal Neurological Institute [MNI] coordinates: -24 -18, -9 and presented at the whole-brain level of voxel $p<.001$. There was no significant association after FWE correction.

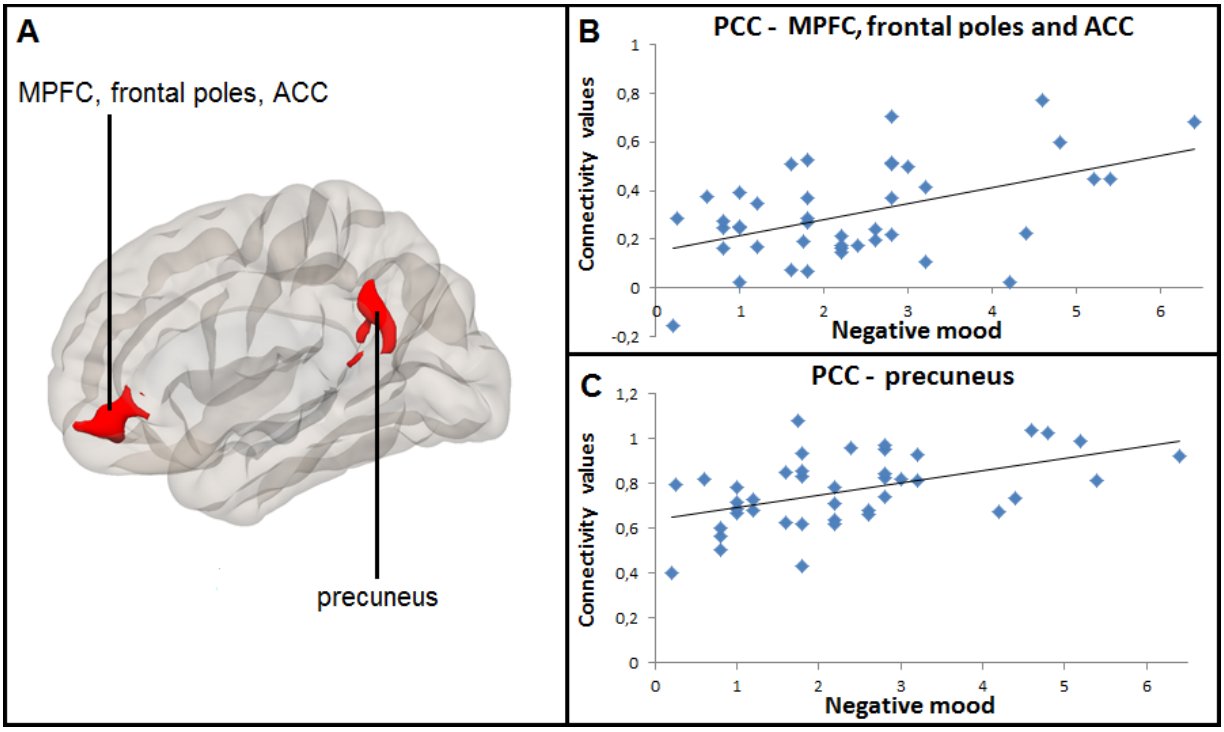

Figure 2. Positive correlation between daily negative mood and resting-state connectivity between posterior cingulate cortex [PCC] and medial prefrontal cortex [MPFC], frontal poles, anterior cingulate cortex [ACC], as well as between PCC and precuneus. PCC-based seed-to-voxel connectivity 3D map presented using CONN toolbox. **(A)** Left red cluster depicts MPFC, frontal poles and ACC positively coupled with PCC. Right red cluster depicts precuneus positively coupled with PCC. **(B)** Scatter-plot for visual inspection illustrates the result from the extracted mean connectivity values of the left cluster within the Default-Mode Network [DMN]. Data was taken at peak Montreal Neurological Institute [MNI] coordinates: -4, 40, -6; k=609; cluster p_{FWE}<.001. **(C)** Scatter-plot for visual inspection illustrates the result from the extracted mean connectivity values of the right cluster within the DMN. Data was taken at MNI coordinates: -6, -54, 34; k=939; cluster p_{FWE}<.001.

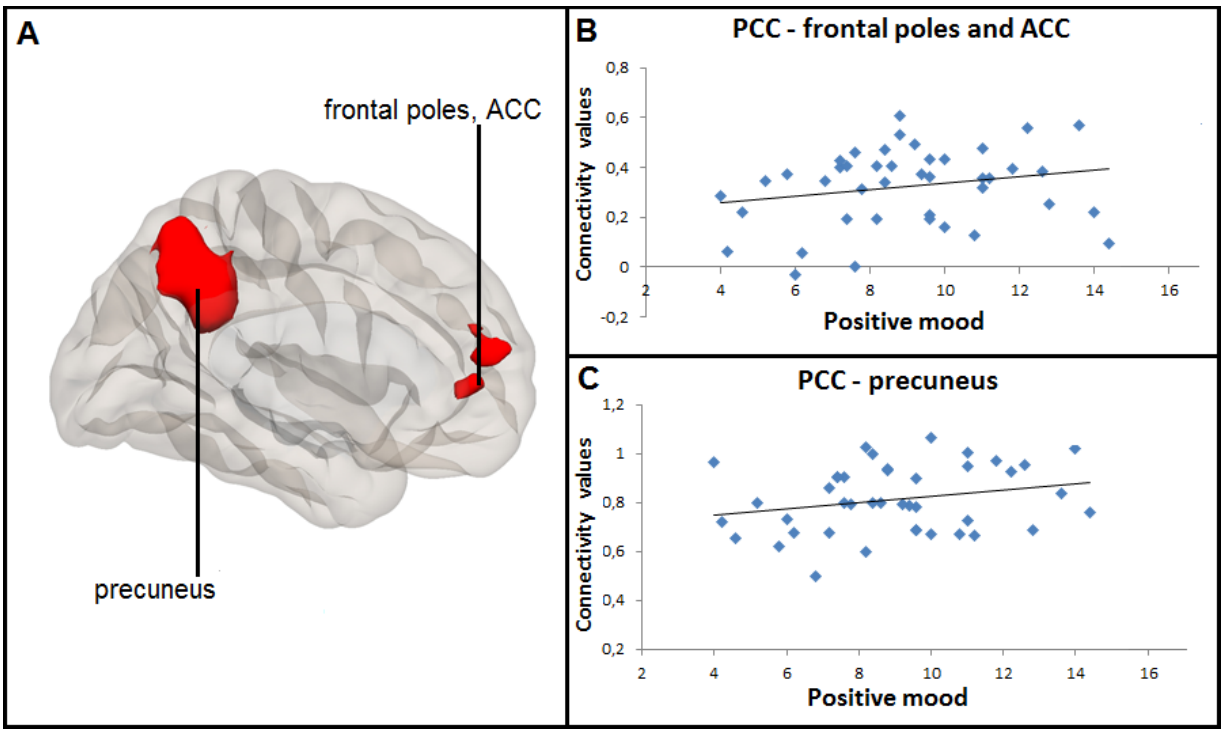

Figure 3. Positive correlation between daily positive mood and resting-state connectivity between posterior cingulate cortex [PCC] and frontal poles and anterior cingulate cortex [ACC], as well as between PCC and precuneus. PCC-based seed-to-voxel connectivity 3D map presented using CONN toolbox. **(A)** Right red cluster depicts frontal poles and ACC positively coupled with PCC. Left red cluster depicts precuneus positively coupled with PCC. **(B)** Scatter-plot for visual inspection illustrates the result from the extracted mean connectivity values of the right cluster within the Default-Mode Network [DMN]. Data was taken at peak Montreal Neurological Institute [MNI] coordinates: 2, 56, 12; k=437; cluster p_{FWE}<.001. **(C)** Scatter-plot for visual inspection illustrates the result from the extracted mean connectivity values of the left cluster within the DMN. Data was taken at MNI coordinates: -10, -50, 38; k=1980; cluster p_{FWE}<.001.

Chapter 2 – Tissue specificity: serotonin transporter gene methylation and brain processes

Foreword

In this second chapter, we assess the brain functioning in response to the emotional stimuli and during the resting state as well as the brain structure in the Quebec Longitudinal Study of Kindergarten Children (QLSKC) cohort at mean age of 33 years. Jessica Di Sante assisted in the recruitment and the data collection. Drs. Richard E. Tremblay and Frank Vitaro are responsible for the QLSKC cohort. The DNA samples were analyzed by Wei-Jo Yu and Dr. Zsofia Nemoda, under the supervision of Dr. Moshe Szyf. Dr. Florence B. Pomares supervised the (f)MRI analysis. I was involved in the recruitment, the data collection, and the (f)MRI analysis. Additionally, I wrote up the paper and submitted it for publication. Dr. Linda Booij designed the study, supervised the data collection and the statistical analysis, and provided feedback on all the versions of the manuscript. Laboratory of Dr. Gustavo Turecki was involved in the collection of the blood samples. Drs. Gabriella Gobbi, Frank Vitaro and Richard E. Tremblay provided feedback on the final version of the manuscript. All co-authors reviewed and approved the manuscript prior to submission. Manuscript was published in *European Neuropsychopharmacology* (Ismaylova et al. Eur Neuropsychopharmacol. 2017; doi: http://dx.doi.org/10.1016/j.euroneuro.2017.07.005).

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Serotonin transporter gene promoter methylation in peripheral cells in healthy adults: neural correlates and tissue specificity

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Abstract

Early adversity can influence gene expression via epigenetic mechanisms, including DNA methylation. Peripheral tissues are essential in psychiatric epigenetics, as methylation generally cannot be assessed in the living human brain. Several magnetic resonance imaging (MRI) studies show associations of peripheral serotonin transporter gene (SLC6A4) methylation with function and/or structure of frontal-limbic circuits and brain's resting-state. Commonly used samples are derived from blood, saliva or buccal cells. However, little is known regarding which peripheral tissue is most strongly associated with human brain processes. The aim of the current study was to compare the extent of the association between peripheral SLC6A4 promoter methylation and frontal-limbic function, structure and resting-state in healthy individuals across peripheral tissues. Forty healthy prospectively-followed adults underwent anatomical, resting-state and functional MRI. Saliva-, blood- and buccal-derived DNA methylation was assessed by pyrosequencing. Blood-derived SLC6A4 methylation was positively associated with superior frontal gray matter (GM) volume and with right lateral parietal area (RLP)-frontal pole regional resting-state functional connectivity (rsFC). Saliva-derived SLC6A4 methylation was positively associated with superior frontal GM volume. Buccal-derived SLC6A4 methylation was positively associated with superior and inferior frontal and anterior cingulate cortical (ACC) GM volumes, and with RLP-ACC, frontal pole and medial prefrontal regional rsFC. Current results confirmed the relevance of peripheral methylation for frontal-limbic processes in humans. Buccal cells may be the most sensitive cell type when studying SLC6A4 promoter methylation and its associated risk for neural vulnerability and resilience for psychopathologies in which serotonin is implicated. These data should be further validated in clinical populations.

Keywords: serotonin transporter, DNA methylation, frontal cortex, resilience, functional magnetic resonance imaging

1. Introduction

Early adverse environment has been associated with an increased risk for various mental health disorders later in life (Kessler et al. 2010). Albeit the exact mechanism underpinning this link is still unclear, a widely accepted hypothesis is that early adversity disrupts developmental trajectories and outcomes by interfering with individual neurobiological framework, a process known as biological embedding (Nelson, 2013). Various models, including the biopsychosocial pathway model, diathesis-stress model and epigenetic memory hypothesis advocate DNA methylation in genes essential for regulating neuronal function as a pivotal molecular mechanism underlying this process (Booij et al. 2015a; Levesque et al. 2016; Lutz et al. 2015). Among the genes associated with early-life adversity, brain development and mental health, those pertaining to the serotonin (5-hydroxytryptomaine; 5-HT) system appear to be particularly compelling since 5-HT system (dys)functioning has been associated with the pathogenesis of different psychiatric disorders as well as brain development (Booij et al. 2015a).

Among the different serotonergic genes, perhaps the most studied one is the serotonin transporter gene (SLC6A4). Following the findings of the role of SLC6A4 genetic variation in interaction with stressful life events in predicting risk for depression (Caspi et al. 2003, 2010), a number of studies have examined the role of DNA methylation in SLC6A4 gene and mental health. These studies rely on measuring DNA methylation in surrogate tissues, such as whole-blood, buccal cells or saliva, as DNA methylation generally cannot be assessed directly in the human brain. A number of studies have found positive associations or trends between peripheral SLC6A4 promoter methylation and major depressive disorder (MDD) symptoms (Kang et al. 2013; Philibert et al. 2008; van der Knaap et al. 2015; Zhao et al. 2013) or symptom severity (Okada et al. 2014), as well as stress sensitivity (Kang et al. 2013) and childhood abuse (Beach et al. 2010, 2011; Booij et al. 2015b; Kang et al. 2013; van IJzendoorn et al. 2010). In addition, neuroimaging studies conducted in a sample of depressed adult patients and healthy controls showed that higher whole-blood SLC6A4 promoter methylation was associated with smaller hippocampal volume (Booij et al. 2015b) and an increased insula response to emotional stimuli (Frodl et al. 2015). Greater whole-blood SLC6A4 methylation has also been associated with an increased amygdala response to threat-related stimuli in adolescents with depressive symptoms (Swartz et al. 2016) and in

adolescents with various mental health states (Nikolova et al. 2014). A positive association between amygdala reactivity in response to threat and peripheral SLC6A4 methylation has also been observed in young adults with various levels of mental health states when using saliva as the surrogate tissue (Nikolova et al. 2014). Furthermore, a brain volumetric study conducted in healthy adults carefully screened for absence of lifetime psychopathology showed that increased SLC6A4 methylation derived from venous blood was associated with greater amygdala, hippocampal and insula volumes (Dannlowski et al. 2014). Results of a resting-state functional connectivity (rsFC) study, that investigated the link between whole-blood SLC6A4 methylation and rsFC in healthy young adults, showed that increased peripheral SLC6A4 promoter methylation was associated with greater rsFC between amygdala and insula, as well as between amygdala and ACC (Muehlhan et al. 2015). These findings together support the relevance of SLC6A4 promoter methylation studies in peripheral tissues for the function and structure of human brain processes that play a role in emotion (dys)regulation, and may indicate a possible future use of peripheral SLC6A4 methylation levels in predicting diagnosis and treatment outcome for individuals with emotion dysregulation problems.

Although it is clear that DNA methylation is tissue-specific (Szyf, 2011), since studies that examined peripheral DNA methylation-brain processes relationships differ in terms of analytical approaches, utilized cell types and participant characteristics, it is presently unclear which peripheral tissue's methylation level correlates best with human frontal-limbic brain processes; i.e. is most sensitive to detect individual differences in frontal-limbic brain function. Therefore, the primary aim of the current study is to examine the association between human frontal-limbic brain processes and peripheral SLC6A4 promoter DNA methylation derived from commonly used surrogate tissues collected from the same healthy adults (blood, saliva, buccal cells). These associations were examined in a longitudinal prospective cohort of individuals followed for 30 years. Based on previous studies (Booij et al. 2015b; Frodl et al. 2015; Muehlhan et al. 2015; Nikolova et al. 2015), we hypothesized that blood-, saliva- and buccal-derived SLC6A4 methylation levels were associated with frontal-limbic volume and neural responses to negative stimuli in frontal-limbic regions (prefrontal cortex [PFC], anterior cingulate cortex [ACC], insula, hippocampus and amygdala), as well as with the resting-state functional connectivity in the Default-Mode Network. We compared the

extent of each association between frontal-limbic brain processes and methylation in each of the different biological samples.

2. Experimental procedures

2.1. Participants

Participants were recruited from the Quebec Longitudinal Study of Kindergarten Children, which has followed both females and males regularly since their kindergarten year in French-speaking schools in the 1980s (Rouquette et al. 2014). The cohort was followed yearly from age 6-12, at mid-adolescence (mean age = 15), in young adulthood (mean age = 21) and in adulthood (mean age = 27) on behavioral, social and family characteristics (Rouquette et al. 2014).

At the age 27 follow-up assessment, 1255 participants provided a peripheral venous blood sample for DNA isolation (Brezo et al. 2010). DNA from a subset of these participants (N = 300) were selected for DNA methylation analyses, as part of a larger project on the association between behavioral trajectories of internalizing symptoms, family adversity and whole-blood SLC6A4 promoter DNA methylation.

2.2. Procedures

At mean age of 34 years, three hundred adults (for whom whole-blood methylation data were available) were re-contacted and screened for the following exclusion criteria: (1) history and presence of any Axis I disorder, neurological disorder, head injury, or severe medical illness, (2) current medication intake, and (3) presence of any irremovable foreign metals in the body, such as piercings and braces. After a phone interview to determine initial eligibility and willingness to take part, participants were invited for a screening in person interview to further confirm eligibility. Personal history and presence/absence of Axis I disorders was assessed by conducting the Structured Clinical Interview for DSM-IV (SCID-IV) (First et al. 2015) and the Beck Depression Inventory (BDI) (Beck et al. 1988), whereas brain-imaging exclusion criteria were verified by means of an in-house questionnaire (Booij et al. 2010). After careful screening for eligibility, willingness and availability to take part in the study, 40 participants (14 men and 26 women) were included. Included cohort members underwent a functional

magnetic resonance imaging (fMRI) session. Following the fMRI scan, all participants were visited at home for the collection of saliva and buccal samples. A family adversity index, consisting of seven indices (familial composition as well as mother's and father's occupational prestige, age at birth of the first child and education level) prospectively collected each year from age 6 to 12 and at age 15, was available for members of the cohort (e.g., Haapasalo and Tremblay, 1994; Mâsse and Tremblay, 1999) and extracted from the database. Written informed consent was obtained from the participants. The study protocol was approved by the ethics review boards of Sainte-Justine Hospital and of the Montreal Neurological Institute, Montreal, Canada.

2.3. DNA methylation analyses

Four 6 ml blood samples at age 27 years, as well as one 2 ml saliva sample (collected by drooling into a tube) and two buccal epithelial samples (gathered by 3 cheek swabs per sample) have been collected from participants following the fMRI scan around the age of 34 years. Following DNA extraction, genomic DNA was converted with EZ DNA Methylation-Gold Kit (D5006, Zymo Research, Irvine, CA, USA). In order to obtain high reproducibility at pyrosequencing (as recommended by Tost and Gut, 2007), 20 ng of bisulfite-treated DNA was used in the PCR amplification using previously published primer set (Wang et al. 2012, Fw: 5'TTGTTAGGTTTTAGGAAGAAAGAGAGA-3', Rev: 5BiosgAAAAAAACTACACAAAAAAACAAATATAC-3') with EpiMark Hot Start Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA), optimized for bisulfite-treated (AT-rich) DNA template. CpG sites 5-14 were measured in the 28563060-28563221 region of chromosome 17 (according to hg19), since these sites have been shown to correlate with brain serotonin synthesis as assessed *in vivo* using Positron Emission Tomography (Wang et al. 2012) and shown to be informative of the brain functional and structural processes in healthy and clinical populations (Booij et al. 2015b; Frodl et al. 2015). The pyrosequencing was performed in triplicates on PyroMark Q24 (Qiagen, Venlo, Limburg, Netherlands) at CFI Imaging and Molecular Biology Platform at McGill University in the Department of Pharmacology and Therapeutics using 2 sequencing primers: 5'AAGAGAGAGTAGTTT-3' and 5'GTAGATTTTTGTGTG-3'. For each CpG site a mean score was computed after quality control of the technical triplicates. An average DNA methylation level of this promoter region

was calculated from the mean scores of the 10 CpG sites, and used as an outcome measure for the present study.

2.4. Image acquisition

All participants were scanned on a 3 Tesla Siemens TIM Trio Scanner (www.medical.siemens.com) located at the Montreal Neurological Institute (MNI) using a 32-channel head-coil. The scan included a brief localizer, followed by a 9-minute-long anatomical scan (MPRAGE sequence; 176 slices; TR=2300 ms, TE=2.98 ms, FA=9°, FOV=256 mm, matrix=256x256x176, voxel size=1mm x 1mm x 1mm), 6-minute-long functional resting-state scan (gradient EPI sequence; 180 whole-brain volumes; TR=2300 ms, TE=30 ms, FA=30°, FOV=224 mm, matrix=224x224x151, voxel size=3.5 mm x 3.5mm x 3.5 mm,) during which participants were asked to close their eyes, but not to fall asleep (Beliveau et al. 2015), and a 15-minute-long event-related functional scan during which an emotion-processing task was performed (gradient EPI sequence; 400 whole-brain volumes; TR=2300 ms, TE=30 ms, FA=30°, FOV=224 mm, matrix=224x224x151, voxel size=3.5mm x 3.5mm x 3.5mm). The emotion-processing task was adjusted from the task used in Canli et al. (2005) and consisted of series of 120 Ekman faces with different emotions (happy, sad, angry, fearful and neutral) from the Pictures of Facial Affect series (Ekman and Frisen, 1976; Keltner and Ekman, 2003). During this task, 18 blocks of emotional facial images were presented to the participants, each block consisting of six randomized trials (3 faces of men and 3 faces of women). For each trial, participants had to fixate a cross in the middle of the screen (1 second), followed by an emotional face (2 seconds). After a second-long pause, participants had to choose whether the face was of male or female nature by pressing the corresponding key.

2.5. Image analyses

Statistical Parametric Mapping (SPM12 v6470, Wellcome Department of Cognitive Neurology, London UK) implemented in MATLAB R2010a (Mathworks, Sherborne, MA) was used for anatomical and functional analyses.

T1-weighted images were initially segmented into gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF) in order to compute voxel-based morphometry (VBM) analyses using the CAT12 toolbox (http://www.neuro.uni-jena.de). First, T1-weighted images

were normalized to MNI space. Second, they were segmented into 3 tissue classes (GM, WM and CSF), based on intensity distribution of the image, and using tissue probability maps. Following these steps, the normalized gray matter (GM) segments were modulated with the resulting Jacobian determinant maps and smoothed with an 8-mm full width at half maximum (FWHM) Gaussian kernel. Statistical analyses used the general linear model (GLM) to identify regions of GM volume that are significantly related to a specific variable (Ashburner and Friston, 2001). We conducted a whole-brain analysis, followed by a region of interest analysis. Frontal-limbic masks for the regions of interest (PFC, ACC, insula, hippocampus and amygdala), the structure and function of which have been consistently associated with peripheral SLC6A4 methylation (e.g., Booij et al. 2015; Dannlowski et al. 2014; Frodl et al. 2015; Swartz et al. 2016), were obtained from Anatomical Automatic Labeling (AAL) Atlas within the Wake Forest University Pick Atlas utility [version 3.0.5] (Maldjian et al. 2003, 2004). VBM is not biased to one particular structure, which permits an even-handed and comprehensive assessment of anatomical differences throughout the brain (Ashburner and Friston, 2001). For the anatomical analyses, the brain regions were examined at a voxel-wise threshold of T>4 corresponding to $p<.05$ uncorrected, only clusters with a spatial extent of at least 30 contiguous voxels were considered (with the exception of the amygdala for which was threshold was set at 5 contiguous voxels), and smoothness non-uniformity correction was applied. All peak coordinates are reported in Montreal Neurological Institute (MNI) format.

The CONN functional connectivity toolbox [version 16.b] was utilized for resting-state functional connectivity analysis (Whitfield-Gabrieli and Nieto-Castanon, 2012). Using CONN toolbox pipeline (Whitfield-Gabrieli and Nieto-Castanon, 2012) and based on SPM12, functional images were spatially realigned to correct for interscan movement, while structural images were segmented and normalized to the Montreal Neurological Institute (MNI) reference brain. Functional images were also normalized to the reference brain, followed by the outlier detection (ART-based scrubbing). All the images were then smoothed using an 8-mm FWHM Gaussian kernel. A denoising procedure including the component-based correction method (Behzadi et al. 2007) followed by the first-level analysis was applied in order to remove motion artifacts and other artefactual effects from the fMRI signal, with ART-detected outliers included as first-level nuisance covariates. Lastly, because of the importance of Default Mode Network (DMN) during resting state (Fransson et al. 2005; Greicius et al.

2003), seed-to-voxel analyses with four DMN seeds [posterior cingulate cortex (PCC), medial PFC (mPFC), right lateral parietal (RLP) and left lateral parietal (LLP) areas] were performed. For the connectivity analyses, the brain regions were examined at a voxel-wise threshold of $p<.001$, with cluster-wise false-discovery rate error correction (FDR)-correction for multiple comparisons (Shaffer, 1995) threshold set at $p<.05$. All peak coordinates are reported in MNI format.

Functional data acquired during the emotion-processing task were preprocessed in SPM12: images of all participants were first corrected for slice timing differences in acquisition, realigned to the first image to correct for small head movements, co-registered with their anatomical scan, spatially normalized into an EPI stereotactic Space (MNI template) and smoothed with an 8-mm FWHM Gaussian kernel in order to improve the signal-to-noise ratio and correct for minor heterogeneity in neural anatomy across participants. Next, intra-individual first level analyses were performed in order to calculate (emotion minus neutral) contrasts for each emotion at each voxel. Then, second-level whole-brain analysis was performed, followed by a region of interest analysis. Frontal-limbic masks for the regions of interest (PFC, ACC, insula, hippocampus and amygdala) were obtained from AAL within the Wake Forest University Pick Atlas utility [version 3.0.5] (Maldjian et al. 2003, 2004). For the functional analyses, the brain regions were examined at a voxel-wise threshold of $p<.001$, with cluster-wise family-wise error correction (FWE)-correction for multiple comparisons (Shaffer, 1995) threshold set at $p<.05$. Only clusters with a spatial extent of at least 30 contiguous voxels were considered (with the exception of the amygdala for which was threshold was set at 5 contiguous voxels), and smoothness non-uniformity correction was applied. All peak coordinates are reported in MNI format.

We performed regression VBM and fMRI analyses, with separately blood-, buccal-, and saliva-derived SLC6A4 promoter DNA methylation as second-level variables in the imaging analyses. The same three variables of interest were implemented to test for voxel-wise correlations between them and seed-to-voxel resting-state connectivity.

3. Results

3.1. Participants

Table 1 shows the characteristics of the included sample. BDI scores were considered low (compared to normative scores, Beck et al. 1988). The level of family adversity was considered low-medium (mean 0.23, SD=0.27, range 0-1), based on the criteria in our previous work (Mâsse and Tremblay, 1999). The sample did not statistically differ from the full cohort sample in terms of gender distribution or levels of family adversity. Average DNA methylation levels of the assayed SLC6A4 promoter region were relatively similar across the three biological tissues (i.e., blood, saliva, buccal cells) (Table 1 and supplementary figure 1), albeit correlations were low (p>.48), possibly due to the limited variance in these variables. There were no significant correlations between peripheral SLC6A4 promoter DNA methylation and levels of family adversity (r between -0.20 and 0.01; p>.24), BDI scores (r between - 0.035 and 0; p>.84), neither were there any associations with sex (F between 0 and 2.44; p>.13).

3.2. Imaging analyses

3.2.1. Brain morphometry

Greater average SLC6A4 promoter DNA methylation level derived from whole-blood, saliva and buccal epithelial cells was associated with greater prefrontal GM volumes at whole-brain level [right superior frontal gyrus for blood t=4.73, k=171, cluster p=.006, voxel p<.001, (peak MNI coordinates: 30, -2, 66)); left superior frontal gyrus for saliva (t=4.75, k=194, cluster p=.010, voxel p<.001, (peak MNI coordinates: -30, 50, 42)); left superior frontal (t=4.30, k=109, cluster p=.089, voxel p<.001, (peak MNI coordinates: -18, 56, 14)) and left inferior frontal (t=4.50, k=300, cluster p=.023, voxel p<.001, (peak MNI coordinates: 53, 3, 21)) for buccal cells] (Figure 1). Also, greater buccal-derived SLC6A4 promoter methylation was associated with greater right ACC GM volume (t=4.21, k=150, cluster p=.019, voxel p<.001, (peak MNI coordinates: 8, 24, 12)). For the results of whole-brain VBM analyses, see Table 2. ROI analysis did not yield any significant results.

3.2.2. Resting-state functional connectivity

Greater blood-derived SLC6A4 promoter DNA methylation was associated with greater resting-state functional connectivity between RLP and bilateral frontal poles and superior

frontal gyri (t=5.02, k=322, p_{FDR}=.001, voxel p<.001, (peak MNI coordinates: -2, 54, 44)), as well as between RLP and left lateral occipital cortex (t=5.93; k=276; p_{FDR}=.001, voxel p<.001, (peak MNI coordinates: -26, -84, 50)). Greater buccal-derived SLC6A4 promoter DNA methylation was associated with greater resting-state functional connectivity between RLP and right lateral occipital cortex and right angular gyrus *(*t=6.39; k=633; cluster p_{FDR}<.001, voxel p<.001, (peak MNI coordinates: 50, -68, 34)) as well as between RLP and ACC, right frontal pole and mPFC (t=4.64, k=156, cluster p_{FDR}=.015, voxel p<.001, (peak MNI coordinates: 6, 54, -4)) (Figure 2A). Figure 2B contains a scatter-plot depicting how the connectivity values of the latter cluster vary with the average DNA methylation level in the SLC6A4 promoter measured from buccal swab samples. No associations between saliva-derived SLC6A4 promoter methylation and resting-state functional connectivity were found.

3.2.3. Functional imaging

The average DNA methylation level in the SLC6A4 promoter region (of either blood, saliva, or buccal samples) was not associated with neural responses to negative or positive emotional stimuli (relative to neutral stimuli) either on whole-brain level or in the investigated ROIs.

4. Discussion

In this study, we examined whether peripheral SLC6A4 promoter DNA methylation is associated with frontal-limbic brain structure and function as well as Default-Mode Network (DMN) resting-state functional connectivity similarly across different tissues in healthy adults. Results of the present study showed evidence for peripheral SLC6A4 promoter DNA methylation cross-tissue convergent association with superior frontal gray matter (GM) structure. Specifically, greater blood-derived DNA methylation of SLC6A4 promoter was associated with greater superior frontal GM volume as well as with greater RLP-frontal pole and RLP-occipital regional resting-state functional connectivity. Greater saliva-derived DNA methylation of SLC6A4 promoter was associated with greater superior frontal GM volume. In addition to greater superior frontal GM volume and greater RLP-frontal and RLP-occipital regional (including angular gyrus) resting-state functional connectivity, buccal-derived SLC6A4 promoter DNA methylation was also associated with greater inferior frontal and ACC GM volumes as well as with greater resting-state functional connectivity between RLP

and ACC and mPFC. Worth mentioning, although occipital cortex and angular gyrus were not part of our frontal-limbic-driven hypothesis, they constitute an integral part of the DMN (Seghier et al. 2013; Uddin et al. 2009), involved in brain activity during rest (Fransson, 2005; Greicius et al. 2003), which might explain our resting-state functional connectivity findings in these regions. Overall, peripheral methylation associations with frontal-limbic brain processes were strongest in buccal swab samples. These findings are relevant for the design of future DNA methylation studies where blood sampling is not feasible or difficult (e.g. studies with young children or long-distance DNA collection).

Using the same methylation assay and genetic location, in previous work that included individuals with depressive symptoms, we found that peripheral SLC6A4 promoter DNA methylation level was primarily linked to activation in ventral limbic brain regions (i.e., regions involved in the production of affective state) (Frodl et al. 2015). We also found in these samples links with a smaller hippocampal volume (Booij et al. 2015b). In the present study of a healthy sample, our associations between peripheral SLC6A4 promoter DNA methylation and frontal-limbic brain structure were mostly in regions involved in cognitive control; i.e. greater average DNA methylation of SLC6A4 promoter region was associated with greater superior frontal volumes, (when methylation was assessed in whole-blood and buccal cells) with greater RLP-frontal pole resting-state functional connectivity, and (when methylation was assessed in buccal cells) with greater inferior frontal and anterior cingulate cortical volumes as well as with greater resting-state connectivity between RLP and such (pre)frontal regions as ACC, frontal pole and mPFC. Yet, these findings are of particular interest given that these brain regions have been increasingly linked to adaptability and resilience for psychiatric disorders. Specifically, a number of studies reported that greater superior frontal GM volume is associated with more optimal self-regulation and cognitive control in healthy adults (e.g., Falquez et al. 2014; Schilling et al. 2014) and in adolescents who experienced high levels of adversity but displayed positive adaptation (Burt et al. 2016). Correspondingly, frontal pole activity at rest has been shown to be involved in processing of social and emotional cues and cognitive regulation of behavior in response to these cues (Zhou et al. 2015). In addition, results of a longitudinal fMRI study, investigating risk and resilience endophenotypes for major depression in adults with mean age of 25 years, illustrated, among others, a distinct pattern of neural activity during self-regulatory task that included the superior

frontal and anterior cingulate regions as part of a brain-based resilience endophenotype (Peterson et al. 2014). In a similar vein, greater functional coupling of medial prefrontal (of which ACC is an integral part) and parietal regions were associated with greater self-awareness/self-reflection (Lou et al. 2004), metacognitive processes implying a better cognitive insight into personal goals, behavioral and affective states (Moeller and Goldstein, 2014), that have been linked to improved mental health states (Ganzer and Zauderer, 2013). Likewise, a lesion study has shown that the left inferior frontal cortex is particularly important for response inhibition (Swick et al. 2008). Together these findings suggest that greater peripheral SLC6A4 promoter DNA methylation in healthy individuals may reflect optimized neural regulation in brain regions that are involved in self-regulatory control, positive adaptation and resilience. Indeed, at the behavioral level, our present sample appeared to be mentally healthy and resilient; in addition to absence of lifetime psychiatric history, as assessed carefully for the past 3 decades, current depressed mood scores were in the lower range even for healthy individuals (e.g., BDI scores <10 are considered as absent or minimal depressive symptoms, Beck et al. 1988). Moreover, their age was beyond the typical onset age for psychiatric disorders (Moffitt et al. 2007) in which the SLC6A4 plays a major role (Booij et al. 2015a), such as major depression and anxiety disorders. Although speculative, together these findings may indicate that individuals in our present study who have higher peripheral DNA methylation of the SLC6A4 promoter may represent a highly resilient sample, and that the greater prefrontal volumes and increased resting-state prevented, at least in part, the development of mental health problems over the past 3 decades. Such hypothesis is in line with results of a previous fMRI study, in which we used an emotion-processing task that also had a cognitive evaluative component. We found that healthy volunteers without any lifetime psychopathology but who had higher levels of peripheral SLC6A4 promoter DNA methylation showed greater neural activation to negative stimuli specifically in those brain regions that are involved in cognitive control (Frodl et al. 2015), thereby supporting the finding of a resilience mechanism. Longitudinal studies in healthy at-risk samples (e.g. first-degree relatives of family members with mental health problems) will help to further investigate such hypothesis in more detail.

Our finding that DNA methylation obtained from non-blood surrogate tissues are most strongly associated with brain processes in living humans is in line with a previous study

comparing DNA methylation patterns of blood and saliva samples in living humans and comparing them with post-mortem brains' DNA methylation data (Smith et al. 2015). This latter genome-wide DNA methylation study showed that, relatively to blood samples, DNA methylation in saliva cells with high buccal epithelial cell content appeared to be more similar to methylation patterns observed in post-mortem brain samples (Smith et al. 2015). Our finding also fits with the result of an epigenome-wide study, examining whether blood or buccal cells represent the most suitable tissue for non-blood-based phenotypes/diseases, showing that DNA obtained from buccal cells may be a better surrogate tissue compared to blood (Lowe et al. 2013). The present study extended these findings to normal human brain processes as assessed by fMRI. Common origin of buccal epithelial and neuronal cells enrooting from the ectodermal layer during early development as well as previously-reported similarity between DNA methylation patterns in peripheral surrogate samples, containing higher portion of buccal (as opposed to blood) cells, and various brain areas including prefrontal cortex (Smith et al. 2015) further support the relevance of buccal cells methylation for the understanding of the human brain.

The findings from the current study should be considered in light of limitations. Firstly, given the relatively modest number of participants, the present sample size did not allow us to study the moderating role of genotype in the associations. The modest number of participants also may have elicited false negatives as well as limited our ability to detect the existing subtle variation in GM volume corrected for multiple comparisons across the brain imaging analyses, including ROI analyses. In addition, the time gap between the collection of the blood samples and the collection of the other biological samples and brain scans may have affected blood cells sensitivity in predicting current brain processes.

Furthermore, DNA methylation levels in the selected SLC6A4 region were low. This may imply that this promoter region – similarly to other CpG-islands – is not the best candidate in terms of technical measurements; however, it might be one of the best candidates in terms of potential biological functions. As we showed in our previous study using an *in vitro* model (Wang et al. 2012), higher levels of DNA methylation of this promoter region significantly decreased the transcriptional activity as measured by luciferase reporter assay, confirming the regulatory role of DNA methylation of this region. Since DNA methylation is a binary signal (i.e. an allele could be either methylated or unmethylated at a particular CpG

site), the percentage change in DNA methylation indicates the fraction of cells that had potentially experienced a complete change in methylation at a particular site. Thus, a small increase in SLC6A4 promoter methylation of a tissue sample may imply that few cells are possibly turning off their expression. We applied the pyrosequencing method to measure DNA methylation of the SLC6A4 promoter region because this technique can (likely most) reliably assess regions with low DNA methylation levels. However, a limitation of this technique is the pre-treatment of genomic DNA (i.e. bisulfite conversion) which does not differentiate between methylated and hydroxymethylated cytosines; therefore, only the overall modified cytosine level can be measured with this method. This issue could pose a significant problem analyzing brain samples where hydroxymethylation epigenetic mark is abundant (Jin et al. 2011), but probably less important issue in peripheral tissues (see comparative data provided by Nestor et al. 2012).

Moreover, as indicated above, the present study was conducted in healthy volunteers, carefully screened for absence of lifetime psychopathology. An advantage is that the results were unlikely to be due to clinical confounding factors like medication or symptomatology. Yet, it would be important to replicate our results in a large-scale (sub)clinical population. Indeed, while we did not found any association between peripheral SLC6A4 promoter DNA methylation and neural responses to negative stimuli, using a similar emotion-processing task, another group showed that higher DNA methylation level in the SLC6A4 promoter was associated with greater amygdala responses to threat (Nikolova et al. 2014). Alternatively, in addition to possible greater variation in behavioral (non)clinical phenotypes in Nikolova and colleagues' study (Nikolova et al. 2014) compared to our study, discrepancies in results due to differences in methodology between studies cannot be ruled out (e.g. emotional faces > shapes activation contrast in Nikolova et al. 2014 as opposed to emotional faces > neutral faces in the current study; location of the investigated SLC6A4 genetic region; and sample size differences (larger in Nikolova et al. 2014).

Worth noting, a previous study showed that for the SLC6A4 gene, only 2 out of 14 studied CpG sites showed significant correlations between the levels obtained from saliva and blood (Smith et al. 2015), a finding corresponding with the low cell type correlations observed in the present study. Low non-significant correlations ought to be expected in the present

study because of the limited variance in these variables for the selected sample. Furthermore, childhood adversity levels were generally in the low-medium range.

Strengths of the study include the use of a well-documented longitudinal sample followed for more than three decades, thereby having prospective measures of their life history and having careful control over non-confounding factors. Moreover, the investigated SLC6A4 region and primary CpG sites were chosen *a priori*, based on previous work and validation *in vivo* and *in vitro* in independent samples (Booij et al. 2015b; Frodl et al. 2015; Wang et al. 2012).

In conclusion, the present findings support the relevance of peripheral SLC6A4 promoter DNA methylation for frontal-limbic brain processes in healthy individuals, and provided some support for convergence across tissues. Buccal epithelial cells may be most sensitive surrogate samples in DNA methylation studies to detect frontal-limbic brain associations. Albeit results need to be confirmed in clinical populations, DNA methylation levels obtained from buccal epithelial cell in the SLC6A4 promoter region may be a promising marker to detect individual differences in frontal-limbic brain processes and neural vulnerability and resilience for psychiatric disorders in which serotonin plays an important role.

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Table 1. Characteristics of the sample

Characteristics of the sample (N=40)	Statistics	
Variable	Mean	Standard Deviation
Age	33.70	0.94
Sex (Female/Male)	26/14	-
Mean DNA methylation level of SLC6A4 promoter in blood samples	3.85	1.42
Mean DNA methylation level of SLC6A4 promoter in saliva samples	2.98	0.73
Mean DNA methylation level of SLC6A4 promoter in buccal samples	2.50	0.55
Beck Depression Inventory total score	2.68	2.37
Family adversity	0.23	0.27

The assayed region in the SLC6A4=serotonin transporter gene promoter consisted of 10 CpG sites in the location of chr17:28563060-28563221 according to hg19 numbering.

Table 2. Results of whole-brain VBM analyses and its association with SLC6A4 methylation

						MNI peak coordinates		
Direction of effect: Variable	Region	k	Voxel t	Cluster p-value	Voxel p-value	x	y	z
Positive: SLC6A4 blood methylation mean	right superior frontal	171	4.73	.006	<.001	30	-2	66
Positive: SLC6A4 saliva methylation mean	left superior frontal	194	4.75	.010	<.001	-30	50	42
Positive: SLC6A4 buccal methylation mean	left inferior frontal	300	4.50	.023	<.001	53	3	21
	left superior frontal	109	4.30	.089	<.001	-18	56	14
	right ACC	150	4.21	.019	<.001	8	24	12

MNI=Montreal Neurological Institute. SLC6A4 = serotonin transporter gene. VBM = voxel-based morphometry. ACC=anterior cingulate cortex.

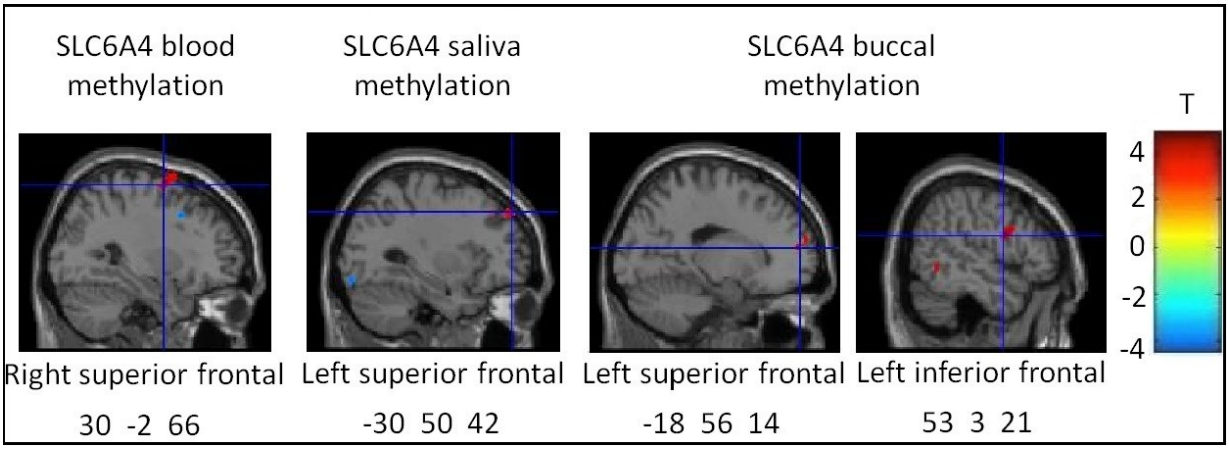

Figure 1. T-statistics maps of the positive association between regional prefrontal gray matter volume and average DNA methylation level of *SLC6A4* promoter derived from whole-blood, saliva and buccal cells, respectively. Results are presented on a whole-brain voxel level of $p<.001$.

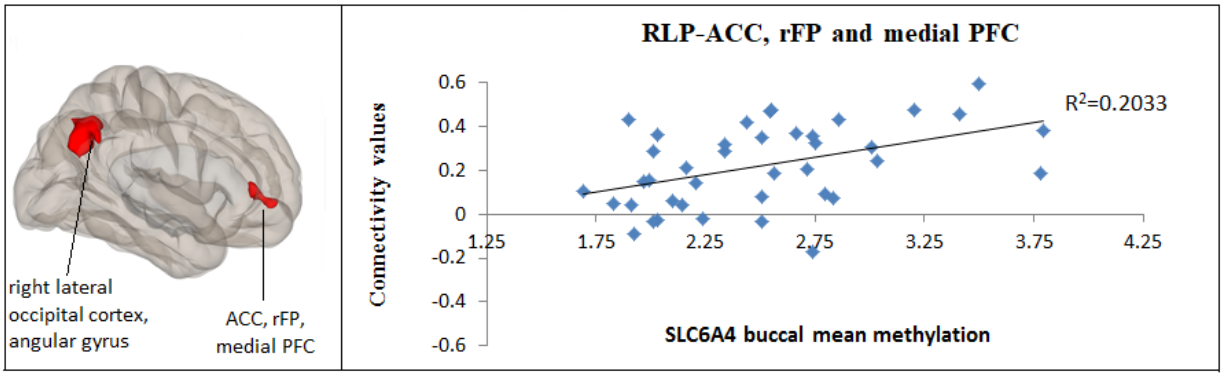

Figure 2. Positive correlation between right lateral parietal (RLP) area and anterior cingulate cortex (ACC), right frontal pole (rFP) and medial prefrontal cortex (mPFC) seed-to-voxel resting-state functional connectivity and buccal average DNA methylation level of SLC6A4 promoter. Seed-to-voxel connectivity 3D map presented using CONN toolbox. (A) Right red cluster depicts ACC, rFP and mPFC regions positively coupled with RLP. (B) Scatter-plot for visual inspection illustrates the result from the extracted mean connectivity values of that cluster within the Default-Mode Network. Both images were taken at MNI coordinates: 6, 54, -4; k=156; cluster p_{FDR}=.015, voxel p<.001.

Chapter 3 – Genes and environment: serotonin transporter gene methylation and brain functioning

Foreword

In this third chapter, we assess the brain functioning in response to the emotional stimuli in the Quebec Newborn Twin Study (QNTS) at 16 years of age. Drs. Richard E. Tremblay, Mara Brendgen, Ginette Dionne, Frank Vitaro and Michel Boivin are responsible for the twin cohort. Dr. Cherine Fahim performed the initial fMRI analyses. Dr. Melissa L. Lévesque assisted in the submission to the ethics board and in the data collection as well as performed the preprocessing and the analysis of the fMRI data. Dr. Florence B. Pomares also performed the preprocessing and the analysis of the fMRI data. The analysis of the DNA samples was conducted by Dr. Zsofia Nemoda in the laboratory of Dr. Moshe Szyf. I took part in the recruitment, the data collection, the fMRI analysis and I wrote up the paper. Dr. Linda Booij designed the study, supervised the data collection and the statistical analysis, provided feedback on all the versions of the manuscript and submitted the latter for publication. All co-authors reviewed and approved the manuscript prior to submission. Manuscript was published in *Translational Psychiatry* (Ismaylova et al. *Transl Psychiatry*. 2018; doi: 10.1038/s41398-018-0195-6).

Serotonin transporter promoter methylation in peripheral cells and neural responses to negative stimuli: A study of adolescent monozygotic twins

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Abstract

Several studies have examined associations between peripheral DNA methylation patterns of the serotonin transporter gene (*SLC6A4*) promoter and symptoms of depression and anxiety. The *SLC6A4* promoter methylation has also been associated with frontal-limbic brain responses to negative stimuli. However, it is unclear how much of this association is confounded by DNA sequence variations. We utilized a monozygotic-twin within-pair discordance design, to test whether DNA methylation at specific CpG sites in the *SLC6A4* promoter of peripheral cells is associated with greater frontal-limbic brain responses to negative stimuli (sadness and fear), independently of DNA sequence effects. Forty-eight pairs of healthy 15-year-old monozygotic twins from the Quebec Newborn Twin Study, followed regularly since birth, underwent functional magnetic resonance imaging while conducting an emotion-processing task. The *SLC6A4* promoter methylation level was assessed in saliva samples using pyrosequencing. Relative to the co-twins with lower SLC6A4 promoter methylation levels, twins with higher peripheral *SLC6A4* methylation levels showed greater orbitofrontal cortical (OFC) activity and left amygdala-anterior cingulate cortex (ACC) and left amygdala-right OFC connectivity in response to sadness as well as greater ACC-left amygdala and ACC-left insula connectivity in response to fearful stimuli. By utilizing a monozygotic-twin design, we provided evidence that associations between peripheral *SLC6A4* promoter methylation and frontal-limbic brain responses to negative stimuli are, in part, independent of DNA sequence variations. Although causality cannot be determined here, *SLC6A4* promoter methylation may be one of the mechanisms underlying how environmental factors influence the serotonin system, potentially affecting emotional processing through frontal-limbic areas.

Introduction

Animal and human studies suggest that environmental exposures can affect our epigenome and these epigenetic marks can persist through cell divisions (1). Epigenetic modifications can change gene expression without altering the genetic sequence. These epigenetic modifications play an important role in setting up tissue- and cell type-specific gene expression programs during development and are crucial in maintaining normal cellular physiology (2). DNA methylation – one of these possible epigenetic mechanisms – implies a covalent modification of the DNA molecule itself through enzymatic addition of a methyl group to (mostly) cytosine bases (2). DNA methylation, involved in epigenetic regulation of gene expression, at critical regulatory regions such as promoters and enhancers, can lead to permanent silencing of the gene, either directly by blocking transcriptional factors from binding to the DNA sequence or by attracting proteins to form corepressor complexes (3, 4). DNA methylation patterns are shaped during development and cellular differentiation but are also responsive to environmental signals, particularly during gestation (5). Indeed, even in newborns, variations in DNA methylation levels can be detected across twins, suggesting that environmental factors might be influencing the normal developmental trajectories of DNA methylation pattern (6). DNA methylation alterations in response to early environmental exposure also appear to be stable, at least in some gene regions, for many years. For example, a study in humans has shown that individuals who were exposed to the Dutch famine in the perinatal period had, six decades later, altered DNA methylation in particular sites compared to their non-exposed siblings (7).

One of the most widely studied genes in relation to DNA methylation and mental health is the serotonin transporter gene, *SLC6A4*. The serotonin transporter is of interest as it has been shown to play an important role in mood, cognition and psychophysiology, as well as brain development (8). Following results of association studies showing a link between *SLC6A4* methylation assessed from peripheral cells and early life environment and later behavior in human clinical and nonclinical populations (9, 10), a number of studies have now investigated possible associations between peripheral *SLC6A4* methylation and brain processes. For instance, Wang et al. (11) found that *SLC6A4* promoter methylation in T lymphocytes of adults was associated with lower *in vivo* Positron Emission Tomography (PET) measures of brain serotonin (5-hydroxytryptamine; 5-HT) synthesis in the orbitofrontal

cortex and higher childhood aggression. Furthermore, a recent study found associations between *SLC6A4* methylation and *in vivo* PET measures of serotonin transporter availability (12). We also previously reported that *SCL6A4* methylation obtained from white blood cells was significantly associated with activation in response to negative emotional stimuli in the insula (13) and lower hippocampal volume (14) in depressed patients and controls. Using functional magnetic resonance imaging (fMRI), greater peripheral *SLC6A4* methylation from saliva and whole-blood DNA samples has also been associated with an increased amygdala response to threat-related stimuli in adolescents and young adults (15, 16). In one (f)MRI study, we reported that greater peripheral *SLC6A4* methylation from whole-blood, saliva and buccal DNA samples have been associated with greater (superior) prefrontal cortical GM volume and parietal-frontal regional functional connectivity at rest in healthy adults (17). Together, these findings support the relevance of peripheral *SLC6A4* methylation measures for frontal-limbic brain processes and support the notion that *SLC6A4* methylation level changes may be an underlying mechanism of how environmental factors can influence aspects of the 5-HT system and, consequently, emotion processing.

However, these associations could be confounded by the effects of variations in DNA sequence. Indeed, certain genotypic variants have been shown to have different DNA methylation patterns than others in the context of similar exposures and experiences (18). For instance, lower methylation was found in association with unresolved trauma or loss at individuals with the s/s genotype of the *SLC6A4* promoter polymorphism, while an inverse correlation was found in the l/l genotype group (18). Furthermore, the influence of DNA sequence on methylation status may depend on genomic location, cell type, and developmental stage (19).

Monozygotic (MZ) twin study designs are unique in their ability to control for DNA sequence differences because each pair of MZ twins shares essentially the same genetic sequence. Thus, differences within twin pairs in gene expression and phenotype, including brain processes, can be attributed to environmental effects rather than DNA sequence influences.

In the present study, we utilized an MZ design to test, in a sample of healthy adolescents, whether DNA methylation at the *SLC6A4* promoter in peripheral cells is associated with greater frontal-limbic activation and connectivity in response to negative

stimuli, irrespective of DNA sequence differences. Specifically, we focused on sad and fearful stimuli because neural responses to these negative emotional facial expressions have been relatively consistently associated with *SLC6A4* genetic and epigenetic variation (13, 15, 20).

Subjects and Methods

Participants

Participants were 96 monozygotic twins (48 pairs: 21 male and 27 female pairs; age 15 years) from the Quebec Newborn Twin Study (QNTS) (21, 22), followed longitudinally since birth, in the area of Montreal, Canada, between April 1995 and December 1998. They were all physically healthy, free of any medication liable to affect brain function and free of current and past psychopathology. Mental health status was confirmed by the Schedule for Affective Disorders and Schizophrenia for School-Age Children- Present and Lifetime Version (K-SADS) (23). Furthermore, we used the Dominic Interactive (Adolescent version), a computerized questionnaire to assess subclinical levels of internalizing symptoms (phobias, generalized anxiety and depression) (24). The appropriate ethics committees (CHU Sainte-Justine and Montreal Neurological Institute) approved the research protocol. Written assent was obtained from all participants and written consent from parents of all participants.

DNA methylation protocol

Whole saliva was collected using the Oragene TM DNA self-collection kit following manufacturer instructions (DNA Genotek Inc., 2004, 2006). Participants did not eat, chew gum, or drink anything but water for 30 minutes prior to saliva collection. Once extracted, whole-saliva DNA was converted with EZ DNA Methylation-Gold Kit (D5006, Zymo Research, Irvine, CA, USA). Following recommendation by Tost and Gut (25), 20 ng of bisulfite-treated DNA was used in the PCR amplification to reach high reproducibility of pyrosequencing, using previously published primer sets optimized for bisulfite-treated DNA template (11, Fw: 5'TTGTTAGGTTTTAGGAAGAAAGAGAGA-3', Rev: 5Biosg-AAAAAAACTACACAAAAAAACAAATATAC-3') with EpiMark Hot Start Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA). Ten CpG sites (numbered as 5-14) in the 28563060-28563221 region of chromosome 17 (according to hg19) were assayed.

Site-specific methylation analyses were performed by pyrosequencing using PyroMark

Q96 (Qiagen, Venlo, Limburg, Netherlands) using the CFI Imaging and Molecular Biology Platform at McGill University in the Department of Pharmacology and Therapeutics using 2 sequencing primers: 5'AAGAGAGAGTAGTTT-3' and 5'GTAGATTTTTGTGTG-3'. The DNA methylation level at each CpG site was computed after quality control of the triplicates. If a sample did not pass quality control at a certain CpG site, the mean DNA methylation value for that CpG site was calculated from the remaining two replicates. We then computed the average methylation level from our ten CpG sites of interest (CpG sites 5-14). In line with other studies that linked brain processes with *SLC6A4* methylation (13, 16, 17, 26), average methylation levels across the investigated CpG sites values were calculated and used in the analyses. The average value of promoter methylation level was used in the analyses rather than the methylation level of each individual CpG site to limit the number of statistical comparisons. The choice to calculate the mean of the ten studied CpG sites was further based on our previous work in healthy and clinical samples, showing associations between average *SLC6A4* methylation levels across these 10 sites and *in vivo* measures of brain 5-HT synthesis in the frontal-limbic brain circuits as well as frontal-limbic functional and structural processes (11, 13, 17).

Neuroimaging

Participants were scanned on a 3T Siemens TIM Trio Scanner located at the Montreal Neurological Institute (MNI) with a 32-channel head coil. The scan included a magnetization-prepared rapid acquisition gradient-echo (MPRAGE) 9 min sequence (176 slices, 1mm thickness, TR=2300ms, TE=2.98ms, TI=900ms, FA=9°, FOV=256x256mm, matrix size 256x256) to assess brain anatomy. Next, a 15-minute functional scan during an event-related emotion-processing task, adapted from the task used in Canli and colleagues (27), was performed. Briefly, the task consisted of a series of 120 Ekman faces with different emotions (happy, sad, angry, fearful and neutral) from the Pictures of Facial Affect series (28). Faces were presented randomly for 2 seconds and followed by a fixation cross (approximately 2 seconds; jittered) and a question asking whether the face belonged to a man or a woman. Two hundred and twenty-six functional whole-brain images (multi-slice gradient echo EPI with 3.5 mm isotropic resolution and TR/TE = 2110/30ms, 40 slices, FOV=224x224mm, matrix size 64x64, interleaved descending slice acquisition) were acquired.

Image processing

Task-based fMRI

Pre-processing steps (slice timing and motion correction, coregistration with the anatomical scan, normalization into an EPI stereotactic Space (MNI template)) and spatial smoothing at 7mm FWHM with a Gaussian Kernel were performed in SPM8 (Wellcome Department of Cognitive Neurology, London UK). For each emotion, activation during neutral stimuli was subtracted from the activation during the different emotions. Voxel-wise sad minus neutral and fear minus neutral contrasts were generated at the first level. We calculated within-pair discordance by subtracting the activation contrast of the twin with higher *SLC6A4* methylation levels from the activation contrast of the co-twin with lower *SLC6A4* methylation levels (29). Discordance values were then used in the regression analyses, by regressing discordance in brain activation in response to sadness > neutral and fear > neutral independently onto discordance in methylation. Whole-brain analysis was conducted. The threshold p value was set at $p < 0.001$. Only clusters showing a spatial extent of at least 10 contiguous voxels were considered (except for the amygdala, in which we required at least 5 contiguous voxels). We corrected for multiple comparisons at the cluster level using the family-wise error rate (FWE) (30).

Functional connectivity

Following the pre-processing steps, functional connectivity analyses were performed using the CONN Functional Connectivity toolbox v174 (http://www.nitrc.org/projects/conn). We controlled for the possible confounding effects of head motion artifacts using the ComCor strategy and default preprocessing parameters (31, 32). In addition, we applied the following denoising options in CONN: detrending, which removes linear/quadratic/cubic trends within each functional session, and despiking, which applies a squashing function to reduce the influence of potential outlier scans (32).

We conducted ROI-to-ROI analyses to assess functional connectivity during sad and fearful conditions. The following ROIs were chosen, namely bilateral amygdala, bilateral orbitofrontal cortex (OFC), anterior cingulate cortex (ACC) and bilateral insula. Amygdala was chosen in light of the link between peripheral *SLC6A4* methylation and amygdala responses to fearful stimuli in (non-twin) adolescents (16). OFC was of particular interest in light of the results of our study in healthy (non-twin) individuals showed an association

between lower brain serotonin synthesis in the OFC and greater peripheral *SLC6A4* methylation (11). ACC was chosen given the findings of activation in this region during sadness in (non-twin) individuals with depressive symptoms (33, 34). Insula was chosen based on our previous study showing an association between insula response to negative stimuli and peripheral *SLC6A4* methylation in (non-twin) healthy and depressed adults (13). Mean BOLD time series were extracted from each ROI and correlated with the BOLD time-series signal of every other ROI in the network to create a ROI-to-ROI connectivity matrix (connectome) showing connectivity between each region within the network.

In order to assess discordance between ROI-to-ROI connectivity values based on peripheral *SLC6A4* promoter methylation discordance, ROI-to-ROI connectivity for twins with greater *SLC6A4* methylation level was compared to their co-twins with lower methylation level. Given the sample size and the correlational nature of functional connectivity and to balance the risk for type I and type II errors, we set the statistical threshold for each voxel at $p < 0.05$, uncorrected.

Results

DNA methylation

The mean *SLC6A4* promoter methylation level was 3.74% (SD=2.37, range: 1.23-14.37%). This level is comparable to that found in other peripheral *SLC6A4* promoter methylation studies, derived from various biological tissues including saliva, using the same CpG sites (11, 13, 17). Within pair, the average discordance in *SLC6A4* promoter methylation varied from 0.23-8.58%. There were no sex differences in mean *SLC6A4* methylation levels ($F(1,94)=0.01$, $p=0.98$), nor in *SLC6A4* methylation discordance levels ($F(1,46)=2.53$, $p=0.12$). Mean level of internalizing symptoms on the Dominic scale was 10.30 (SD=6.16, range 1-27 out of maximum total score of 52), indicative of absent/low likelihood of internalizing symptomatology. Within pair, the average discordance in symptoms was 4.37 points (SD=3.39, range 0-15). *SLC6A4* promoter methylation did not correlate with internalizing symptoms (as assessed by Dominic Interactive questionnaire) neither globally ($r=0.12$, $p=0.25$) nor within pair ($r=0.12$, $p=0.42$).

fMRI results

Sad condition

Relative to their co-twin with lower methylation, twins with higher *SLC6A4* promoter methylation levels had greater left OFC activation (k=206, t=5.11, cluster p_{FWE}=.03) (Figure 1) as well as greater functional connectivity between left amygdala and ACC and between left amygdala and right OFC (Figure 2 and Table 1). No other significant fMRI results were found in the sad condition.

Fearful condition

There was no significant association with neural activity in the fearful condition. However, twins with higher *SLC6A4* promoter methylation level had greater functional connectivity between ACC and left amygdala and between ACC and left insula, relative to the co-twin with lower *SLC6A4* methylation (Figure 3 and Table 1). No other significant fMRI results were found in the fearful condition.

Discussion

The aim of the present study was to assess potential neural correlates of peripheral *SLC6A4* promoter methylation during processing of negative facial expressions, i.e., sadness and fear. While an emerging number of studies have now shown that peripheral *SLC6A4* methylation correlates with frontal-limbic processing of negative emotions, it is not clear whether this association is due to the environment or to genetic factors. Here, we utilized a within-pair MZ-twin design to control for the putative role of DNA sequence in this association. We found that relative to the co-twins with lower *SLC6A4* promoter methylation, twins with greater *SLC6A4* methylation levels had greater left OFC activation, greater amygdala-OFC and left amygdala-ACC connectivity in the sad condition, as well as greater ACC-amygdala and ACC-insula connectivity in the fearful condition. In spite of relatively low within-pair variability, current findings are of particular interest given the implication of these brain regions in emotion regulation. Indeed, regional functioning of OFC, ACC, insula and amygdala, all part of the frontal-limbic circuitry, has previously been associated with emotion regulation (35, 36) and *SLC6A4* methylation (16). Our findings do not only support the previously suggested hypothesis that peripheral *SLC6A4* promoter methylation shares potentially functional relevant information about frontal-limbic functioning and emotion-

processing, but, most importantly, demonstrate for the first time that these associations occur, at least in part, independently of DNA sequence.

The finding of within-pair differences in neural processing of sad stimuli in the OFC regions fits well with the results of our previous study in which we compared neural activation to sad stimuli between 8-year-old monozygotic (MZ) and dizygotic (DZ) twins, allowing separation of genetic factors from environmental factors shared between twin pairs and non-shared environmental factors that are unique for each twin member (37). Among other brain areas, neural activation during processing of sad stimuli in Broadmann Area (BA) 47, a key region in the OFC, was found to be fully driven by non-shared/unique environmental factors (37). Interestingly, results of a positron emission tomography (PET) study, investigating the neural basis of various emotions (sadness, happiness, anger and fear) in healthy individuals, the orbitofrontal regional activity was found to peak solely during the sadness condition (38), emphasizing the role of the OFC in the detecting and processing of sad stimuli. That being said, in addition to the OFC, Damasio and colleagues (38) reported increases in insula and ACC activity during sad condition (and, respectively, during fearful and angry condition). The absence of these insula-ACC regional responses to sad or fearful cues in the current study may be, in part, explained by differences in methodology (e.g., use of current implicit emotion-processing task versus self-generated emotional conditions by recall). Furthermore, we previously found – using *in vivo* PET measures of brain 5-HT synthesis – that serotonergic synthesis in the OFC is not only modulated by DNA sequence (39) but also by early environmental factors (40). The environmental influences on serotonin functioning in the OFC observed in the present study also fits well with our previous PET study showing that greater peripheral *SLC6A4* methylation correlated with lower *in vivo* brain serotonin synthesis in the OFC region (11). Current findings are also in line with the results of a study by another research group reporting positive association between peripheral *SLC6A4* promoter methylation and resting-state functional connectivity between amygdala, insula and ACC in healthy adults (26). Notably, OFC, ACC, insula and amygdala constitute an integral part of the salience functional network, involved in affective processes (41). Interestingly, results of an fMRI study examining neural correlates of emotion regulation in healthy individuals, showed that functional connectivity between amygdala and several (pre)frontal regions, including OFC and ACC, was associated with attenuated emotional response to negative emotional

stimuli (41), thereby underscoring the involvement of frontal-limbic functional connectivity in processing and regulation of emotions. Interestingly, salience network has been found to show relatively low cross-twin correlation in both MZ and DZ twins (42), indicative of scarce evidence for either genetic or non-shared environmental effect on this network. Results of the present study suggest that this *SLC6A4*-related salience network is, at least in part, independent of variation in DNA sequence. Altogether the findings suggest an important role for OFC, ACC and amygdala in the processing of sadness and fear, which appear to be largely driven by *SLC6A4*-modulated non-shared/unique environmental factors.

Some laterality effects were observed as well. Notably, the finding that the association in the amygdala was specific for the left side is in line with other research showing that the left amygdala is more frequently activated in response to emotional stimuli than the right amygdala (see 43 for meta-analysis). Furthermore, stronger functional connectivity between left (but not right) amygdala and bilateral ACC and OFC has been associated with decreased negative affect induced by negative emotional content in healthy individuals (44). Besides, conjoint activity of ACC and left (but not right) insula has been linked to increased awareness of one's own emotions (45). Interestingly, greater *SLC6A4* promoter methylation has been previously linked to greater left (but not right) amygdala and insula activation in response to negative emotional stimuli (13, 16). In light of these findings, we might advance that these lateralized regional activities reflect serotonin functioning-related neurobiological processing of the (sad or fearful) emotionally salient content. However, additional *SLC6A4* methylation fMRI studies of the connectivity of amygdala, OFC and insula subdivisions are necessary to deepen the understanding of the laterality.

While we found significant associations between peripheral *SLC6A4* promoter methylation discordance and neural processing of negative stimuli, we did not find any correlations between depressive symptoms and peripheral *SLC6A4* methylation. However, the levels of symptomatology variation were very low. The observation of an association between *SLC6A4* methylation and neural processing of negative stimuli in the absence of an association with anxiety- or depression-related symptoms is consistent with studies in healthy adults (e.g., 16). Thus, variation in frontal-limbic brain activation, as a function of variation in *SLC6A4*, occurs in the absence of behavioral variation. This supports the idea that *SLC6*A4 methylation may reflect a marker for neural regulation of negative stimuli, but additional factors may be

necessary for an overt behavioral phenotype to be expressed (8). Nevertheless, it would be of particular interest to test MZ twins who are highly discordant in levels of internalizing symptoms and in *SLC6A4* promoter methylation and link their discordance to differences in frontal-limbic processes.

Furthermore, peripheral *SLC6A4* promoter methylation levels and methylation differences within MZ twin pairs were low. However, considering the binary (either methylated or unmethylated allele at a particular CpG site) nature of the DNA methylation, the percentage change in DNA methylation indicates the fraction of cells having possibly experienced a complete change in methylation at a given site (17). In other words, a small increase in *SLC6A4* promoter methylation of a tissue sample may suggest that few cells are potentially turning off their expression. Indeed, using an *in vitro* model, higher DNA methylation levels in the selected *SLC6A4* promoter region have been previously shown to lower its transcriptional activity (11), thereby corroborating the regulatory role of DNA methylation of this region. Additionally, peripheral DNA methylation in this particular gene region has been shown to be a potential marker of serotonin synthesis in the OFC (11).

The strengths of this study include the use of a well-characterized, prospective, homogeneous longitudinal sample of adolescents. All twins were 15 years of age and carefully screened for absence of psychiatric history. Furthermore, the investigated *SLC6A4* region and primary CpG sites were chosen a-priori, based on previous work and validation *in vivo* and *in vitro* in three independent samples (11, 13, 14, 17). However, the present findings should also be considered in the context of certain limitations. First, methylation was assessed from peripheral tissue (i.e. saliva), as DNA methylation generally cannot be assessed in the living human brain. However, studies combining epigenetics and various fMRI measures have shown substantial correlations between methylation assessed from peripheral tissue and brain functioning (13-17, 46). Moreover, future studies will be necessary to investigate potential moderating roles of age, sex and various psychosocial variables, as well as to identify individual participant characteristics that account for the within-pair variation. Furthermore, we will continue to follow these twins in the future to assess the clinical relevance of these alterations in peripheral *SLC6A4* promoter methylation and processing of negative emotional stimuli. In spite of these limitations, the findings in our MZ twin study provide evidence that previously reported associations between peripheral *SLC6A4* promoter methylation and frontal-limbic processing of negative stimuli are

detectable while controlling for DNA sequence. These associations may have implications for the future use of non-invasive DNA methylation markers in diagnosis, treatment and risk prediction in mental health problems in which serotonin plays an important role.

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Table 1. Significant within-pair fMRI associations with *SLC6A4* promoter methylation

Direction of effect: Condition	Region	*t*	*p*-value
Positive: Sad	left amygdala-ACC	1.83	0.036
	left amygdala-right OFC	1.71	0.047
Positive: Fearful	ACC-left amygdala	2.79	0.004
	ACC-left insula	1.85	0.035

fMRI=functional Magnetic Resonance Imaging. *SLC6A4*=serotonin transporter gene. OFC=orbitofrontal cortex. ACC=anterior cingulate cortex.

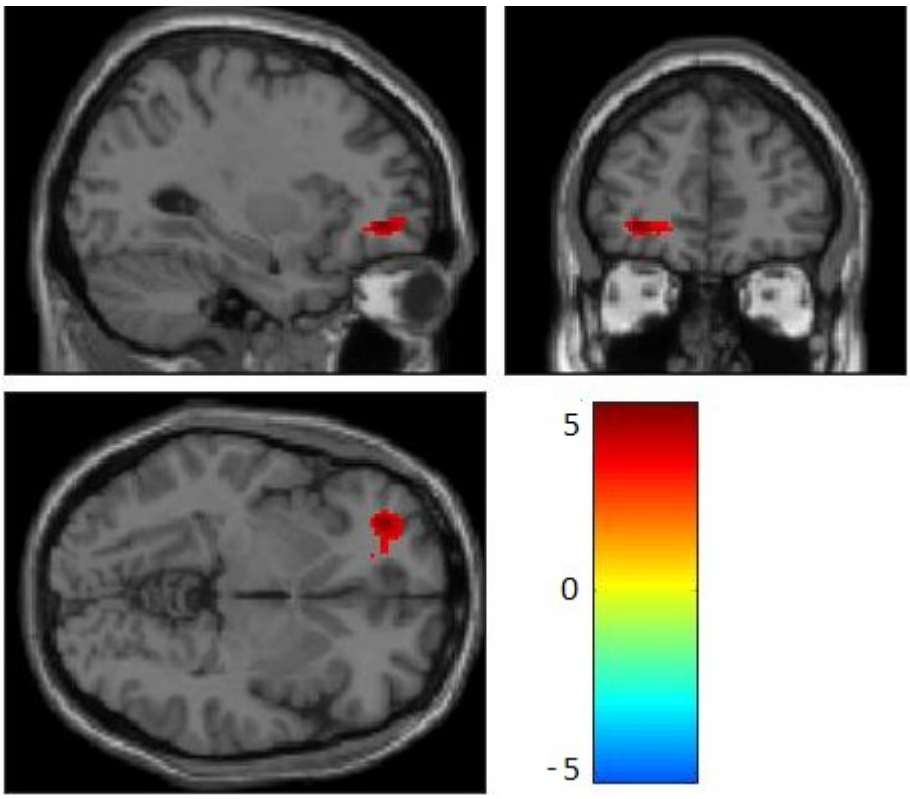

Figure 1. Greater within-pair difference in peripheral *SLC6A4* promoter methylation was associated with greater responses to sad stimuli in left orbitofrontal cortex (T=5.11, pFWE=0.032). The color bar represents T values.

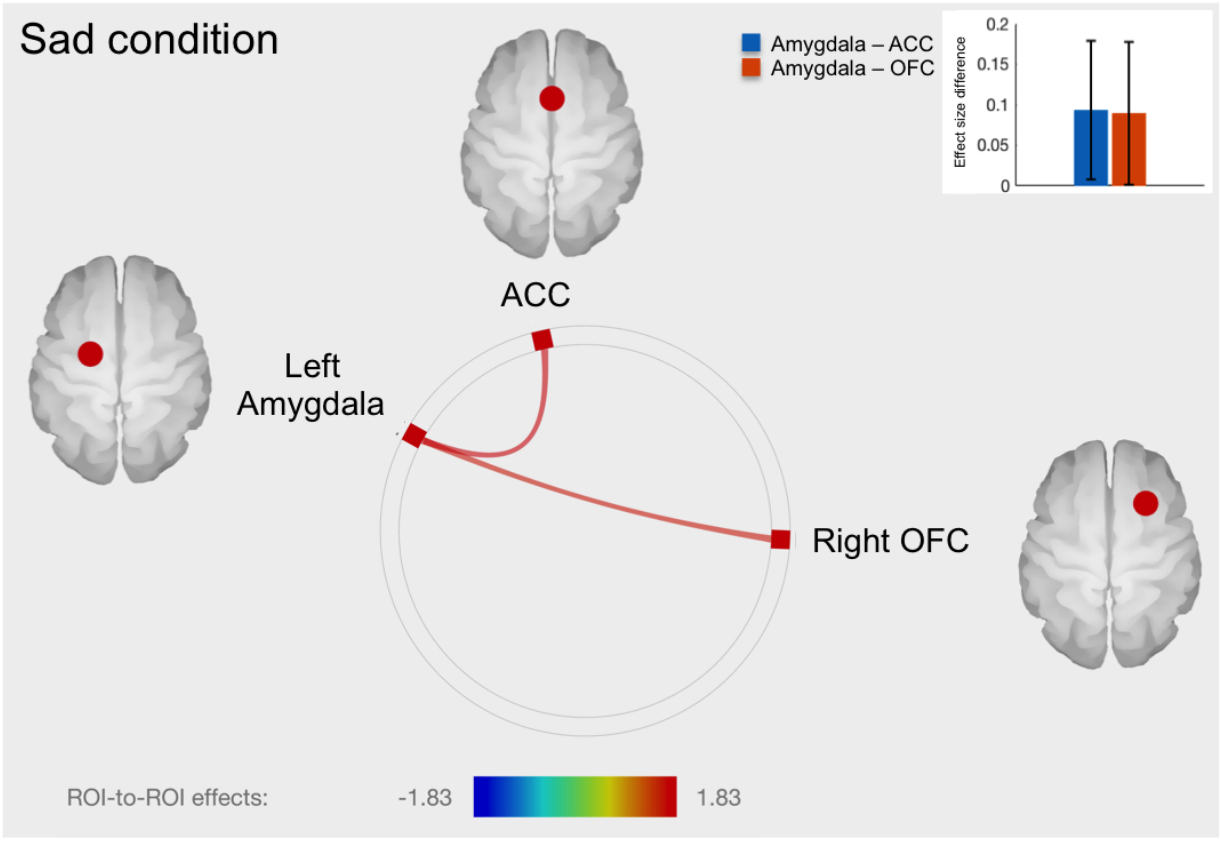

Figure 2. Connectome ring representation of ROI-to-ROI connectivity between amygdala, anterior cingulate cortex (ACC), orbitofrontal cortex (OFC) and insula in the sad condition. Only significant connectivity discordances are represented: greater ROI-to-ROI connectivity in the sad condition for twins with greater methylation level compared to their co-twins with lower methylation level, between left amygdala and ACC (T=1.83, p=0.036), as well as between left amygdala and right OFC (T=1.71, p=0.047). The color bar represents T values. The bars plot in the top right-hand corner, represents differences in effect sizes (difference in Fisher-transformed correlation coefficients) between twins with greater methylation level and their co-twins with lower methylation level, for each connection.

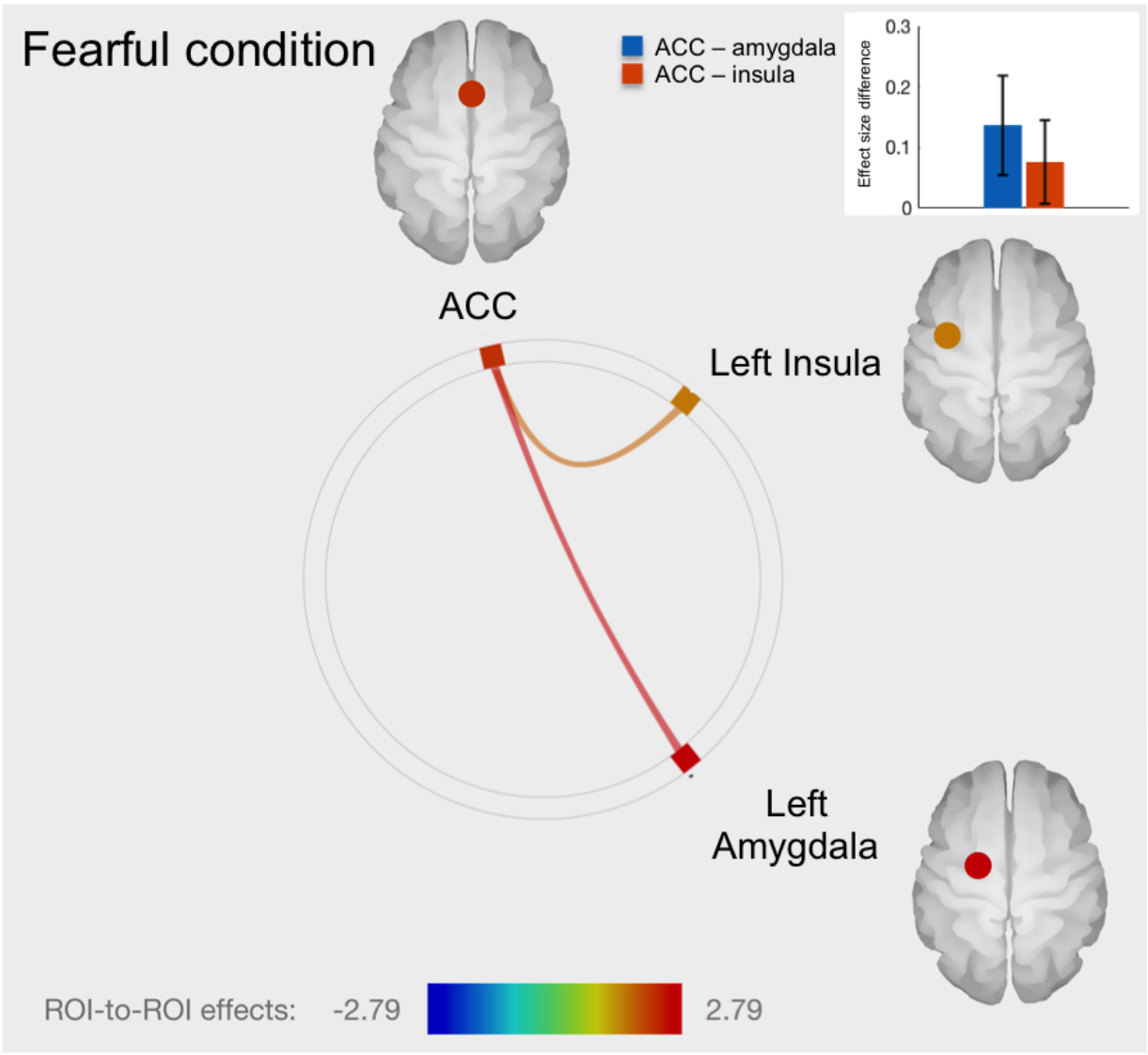

Figure 3. Connectome ring representation of ROI-to-ROI connectivity between anterior cingulate cortex (ACC), left amygdala and insula in the fearful condition. Only significant connectivity discordances are represented: greater ROI-to-ROI connectivity in the fearful condition for twins with greater methylation level: between ACC and left amygdala (T=2.79, p=0.004), as well as between ACC and left insula (T=1.85, p=0.035). The color bar represents T values. The bars plot in the top right-hand corner, represents differences in effect sizes (difference in Fisher-transformed correlation coefficients) between twins with greater methylation level and their co-twins with lower methylation level, for each connection.

Conclusion

In the present thesis, three research questions were addressed. Firstly, we investigated the association between the daily mood in healthy individuals and their neural activity in response to emotional stimuli, the GM volume and the resting-state functional connectivity within the frontal-limbic neurocircuitry. For this purpose, healthy adult members of a prospective longitudinal cohort, followed regularly since kindergarten, completed a daily diary of mood states – namely, negative mood, positive mood and rumination – providing information on the mood they have experienced in the everyday context. Doing so allowed a careful documentation and investigation of individuals' environment as well as their history of emotional well-being. Results showed that individuals with smaller left hippocampal GM volumes experienced more negative mood and rumination in the everyday life. Smaller left hippocampal GM structure has been observed in the individuals with various psychiatric symptoms, including trauma-related and MDD symptoms [173-177]. However, the participants of the present study have not presented any lifetime psychopathology. Hence, we might argue that the subtle variations in the left hippocampal GM volume (although on an uncorrected level) might be associated with the individual differences in the negative mood and the ruminative response style in the everyday life. Nonetheless, further studies are warranted to confirm these exploratory uncorrected results. Furthermore, we found that greater rsFC within DMN – between the PCC and the ACC as well as between the PCC and the precuneus – was associated with both greater negative and positive mood in the everyday life. This finding accentuates how the dynamic brain processes may echo changing internal states of humans. Additionally, this result might suggest that a heightened PCC-based rsFC with the ACC and the precuneus might reflect the arousal, as opposed to the valence, dimension of emotion. That is to say, the individuals with an increased PCC-ACC and precuneus rsFC may experience more emotions in the everyday life, regardless of the valence. Notably, medium-strength correlations observed between the daily negative mood and the rsFC in these aforementioned DMN regions may suggest the negative mood as more sensitive mood state for studying the daily-life emotional reactivity in healthy brains. Supporting this idea are the results of a resting-state fMRI [rsfMRI] mood induction study, showing that following a

negative mood induction, healthy (as opposed to depressed) individuals presented an increase in the rsFC between the PCC, the anterior prefrontal regions and the precuneus, but lower rsFC between the PCC, the parahippocampus and the (superior, inferior) temporal regions [178]. Further rsfMRI studies are necessary to verify that, similar to the induced mood, the daily mood-related resting-state neural processes vary across the DMN components. In addition to the PCC-ACC and precuneus rsFC, daily positive mood was associated with greater activation in response to negative (sad) emotional stimuli in the precentral gyri. Besides its role in the motor functioning, heightened activity in the precentral gyri has been linked to the emotional interference in the cognitive and the behavioral control, condition in which one's attention and behavior is challenged by emotionally-salient stimuli [179]. We may argue that current task-based activity in the precentral gyri (that are bound inferiorly by the cingulate cortices) as well as the PCC-ACC rsFC might reflect brain mechanisms involved in the assessment and regulation of emotions on a daily basis. Together, these findings might reflect frontal-limbic brain mechanisms underpinning the natural mood fluctuation in the everyday life of the healthy adults. Considering Mayberg's and Phillips' neural models of emotion dysregulation [51, 52] involving an impairment in the coordinated interactions of frontal-limbic regions, it might be of interest for the future (f)MRI studies to examine neural substrates of the daily mood states in a clinical population. Additionally, daily negative mood has been argued to be moderated by variation in the epigenetic-regulatory genes such as the DNA methyltransferase 3A gene [180]. It might be of interest for the future research to examine whether the functioning of other genes moderates the relationship between the daily mood states and the frontal-limbic processes in the healthy as well as in the clinical populations. The *SLC6A4* gene may be of particular interest because of its environmental sensitivity. For instance, carriers of the short (i.e. vulnerability) allele of the serotonin transporter gene polymorphism were found to be most likely to benefit from the enriching experiences and to suffer from the adverse environmental conditions (see [181] for meta-analysis). In other words, individuals with such serotonin transporter genetic make-up would be concurrently sensitive to both negative and positive experiences [177]. However, before investigating a potential peripheral *SLC6A4* gene methylation modulatory role in various developmental outcomes, it is important to examine the notion of peripheral tissue specificity.

The second research question we addressed concerned the peripheral *SLC6A4* gene methylation derived from which tissue – blood, saliva or buccal cells – corresponds best to the neural activity in response to emotional stimuli, GM volume and rsFC within the frontal-limbic neural circuitry in healthy members of a prospective longitudinal cohort whose environmental conditions and mental health have been followed since kindergarten. The saliva-derived *SLC6A4* gene methylation was positively associated with the (superior) PFC GM volume. Blood-derived *SLC6A4* methylation was positively associated with the (superior) PFC GM volume and with rsFC within the DMN, including the parietal and frontal regions. In addition to greater (superior) prefrontal GM volume and greater parietal-frontal rsFC, buccal-derived *SLC6A4* methylation was also positively associated with the ACC GM volume as well as with the rsFC between the parietal areas, the ACC and the medial PFC. These results provided evidence for the peripheral *SLC6A4* gene methylation cross-tissue convergent association with the prefrontal GM structure. These results vary from the findings of the previous studies, employing epigenetic analysis in the same gene and at the same sites, which showed an association between the peripheral *SLC6A4* gene methylation and limbic brain processes involved in the production of the affective states (i.e. increased insula activity [165]; smaller hippocampal GM volume [163]). Current findings are mostly located in the ACC and the PFC regions linked to the cognitive regulation of emotion [183, 184]. Greater superior PFC GM volume has also been linked to resilience to the adverse events in youth [137]. At the heart of both adversity and resilience lies the development of effective responses to stress [185], which might be reflected in the altered frontal-limbic brain processes. Humans have the ability to modulate their emotional reactivity through various techniques, which lie in the core of resilience-related behaviors. Among affect labelling (also known as "putting feelings into words" [186]) and self-distraction from an emotion-eliciting stimulus [187], cognitive reappraisal is probably one of the most extensively studied emotion regulation strategies [188-192]. Cognitive reappraisal, characterized by an effortful change of the interpretation of a stimulus to modify its ability to elicit an emotion reaction, has been argued to regulate the psychological and physiological aspects of the stress response and to affect mental health in the long run [193]. For instance, healthy adults exposed to sexual trauma (relative to those who developed PTSD) displayed greater neural activity during the reappraisal of negative stimuli in various brain regions including the ACC and the (superior and medial) PFC [194].

The connectivity between the medial PFC with other (e.g., parietal) brain regions has been found to be positively associated with an enhanced awareness of one's own mental states [195], which in turn has been linked to the improved mental health states [196]. Hence, we may argue that these regional processes may reflect a potential brain-based resilience endophenotypes.

Therefore, current findings not only confirmed the relevance of peripheral *SLC6A4* gene promoter methylation for the frontal-limbic brain processes, but also showed evidence that buccal cells may be a sensitive cell type for studying DNA methylation of the *SLC6A4* gene and its potential link with brain-based resilience for mental health disorders in which the 5-HT plays a crucial role.

Finally, we tested the hypothesis that the peripheral saliva-derived *SLC6A4* gene methylation would be associated with frontal-limbic functional activity and connectivity in response to emotional stimuli in the healthy adolescent MZ twins, regardless of their DNA sequence. We found that adolescent twins with higher (relative to their co-twins with lower) peripheral *SLC6A4* gene methylation exhibited greater OFC activity as well as greater connectivity between such frontal-limbic regions as the amygdala, the ACC, the OFC and the insula in response to negative (i.e. sad and fearful) emotional stimuli. In addition to confirming previous findings of the link between frontal-limbic (i.e. amygdala and insula) responses to emotional cues and peripheral *SLC6A4* gene methylation [165, 167], current study extends these findings by demonstrating that this link might be independent of the DNA sequence. Similar within-pair differences in the OFC regional response to sad stimuli have also been reported in 8-year-old twins [197], thereby emphasizing the importance of the environment in accounting for variability in brain processing of sadness. Taken together, these findings suggest an important contribution of the OFC, the ACC, the insula and the amygdala in the processing of sadness and fear, which appear to be largely driven by the *SLC6A4*-modulated environmental factors.

Altogether, current results indicate that greater peripheral *SLC6A4* gene methylation is associated with greater frontal-limbic structure as well as functional activity and connectivity at rest and during emotion processing in a healthy general population. In particular, (superior

and medial) PFC and ACC appear to constitute brain regions the increased structure and function of which are consistently linked to greater peripheral *SLC6A4* gene methylation.

Past research has shown that peripheral (blood-derived) *SLC6A4* gene methylation modified the effect of stressful events on mental health, irrespective of *SLC6A4* genotype [198]. Specifically, middle-aged adults exposed to traumatic events were found to be at greater risk for PTSD only at lower peripheral *SLC6A4* gene methylation levels [198]. Conversely, traumatized adults with greater methylation levels appeared to be "shielded" from PTSD [198]. Considering the role of ACC and (medial and superior) PFC neural processes in resilience to adverse life events in youth and adulthood [136, 137, 199], we might argue that *SLC6A4* gene methylation-related ACC and (superior and medial) PFC neural processes could contribute to development of resilience skills. Moreover, neural structures typically emerging in the fMRI studies of cognitive reappraisal of the negative emotional content include the ACC as well as the dorsolateral and medial PFC regions, such as superior and medial frontal gyri [200-205]. For instance, depressed (relative to healthy) individuals were found to display lower activity in the superior frontal gyrus, the ACC, the PCC and the precuneus in response to negative (sad) emotional stimuli at baseline, followed by an increase in this regional activity after cognitive-behavioral therapy [CBT] [204]. These findings suggest that the functioning of these frontal-limbic regions is involved in cognitive-affective alterations.

Overall, our results are relatively in line with the previous findings of associations between peripheral *SLC6A4* gene methylation and frontal-limbic processes in healthy individuals [49, 164, 166, 167] and corroborate this relationship while controlling for the genetics in a prospective longitudinal MZ twin design. We also extended previous investigations of the peripheral *SLC6A4* gene methylation by assessing DNA methylation in three different biological tissues (blood, saliva and buccal cells), comparatively to one or two tissues measured in the previous studies [163-168]. Additionally, this is the first study so far providing evidence for the link between peripheral *SLC6A4* gene methylation and rsFC within the Default-Mode Network.

That being said, current findings are not entirely consistent with previously reported association between childhood environment and peripheral *SLC6A4* gene methylation in adults independent of their diagnosis [163, 165]. Indeed, we have not found an association between childhood socioeconomic conditions and peripheral *SLC6A4* gene methylation. This difference

might be, in part, due to the methodological differences (e.g., current sample of 40 subjects was smaller relative to 60-69 subjects in the previous studies [163, 165]) and different conceptualization of childhood environment (i.e. family socioeconomic conditions in childhood relative to childhood maltreatment, which was the focus of the previous studies [163, 165]). Moreover, while we expected to observe frontal-limbic activation in response to the sad and fearful emotional stimuli, we observed only the OFC response to sadness. That being said, results of a PET study, examining the neural basis of four emotions in healthy individuals – sadness, happiness, anger and fear – reported greater OFC regional activity only during the sadness condition [206], underscoring the contribution of the OFC in detection and processing of sad stimuli. In addition to OFC activity, increases in insula and ACC activation have also been reported during sad condition (and, respectively, during fearful and angry condition) [206]. The absence of these ACC and insula regional responses to the sad or fearful stimuli in the present study might be partly explained by the use of differential neuroimaging task (implicit emotion-processing task in the current study relative to previously employed self-generated emotional conditions by recall [206]).

Similarities are noticeable in adolescents and adults in terms of peripheral *SLC6A4* gene methylation-related frontal-limbic regional functioning, particularly in the ACC area. Indeed, positive associations were found between the peripheral *SLC6A4* gene methylation and functional connectivity (during the emotion processing and at rest) of ACC, respectively with limbic regions in adolescents and with the parietal area in adults. Additionally, greater (resting-state) functional connectivity in the ACC (with PCC) was positively associated with the daily-life mood. Together, these findings might reflect the role of ACC functioning in emotional well-being, in which serotonin might play an important role. These findings might be relevant for the intervention efforts to prevent the emergence of psychiatric disorders in which 5-HT is implicated. For instance, Roberts and colleagues [207] have measured peripheral (blood-derived) *SLC6A4* gene promoter methylation in adolescents with an anxiety disorder pre- and post-CBT administration. Results demonstrated that, while driven by one CpG site, the DNA methylation levels presented significant change during treatment, such that responding (relative to non-responding) youth exhibited an increase in their DNA methylation following CBT sessions [207].

Limitations and Strengths

Current studies should be considered in light of several limitations and strengths. Firstly, the relatively modest number of participants did not allow us to study the moderating role of genotype in the *SLC6A4* gene methylation-brain relationship. Nonetheless, using the MZ-twin design, we were able to show that the association between peripheral *SLC6A4* gene methylation and frontal-limbic brain processes holds regardless of individuals' genetic variation. Despite the benefits of the MZ-twin design, the utilization of a longitudinal sample of healthy monozygotic twins carefully screened for the absence of lifetime psychopathology might also limit the generalizability of the present findings. That being said, the independent variables, namely the level of DNA methylation and daily mood, have been subjected to the same analyses. Indeed, in addition to gender differences, which were examined (and not found) in all of the independent variables, the relationship of the latter with the brain processes has been systematically examined. With respect to the brain imaging data, the same preprocessing, first-level and second-level analyses have been carried out across all the studies. The sole exception to the rule would be the MZ twins' study that required particular analyses, notably the within-pair analysis. The modest number of participants might have also limited our ability to detect the subtle variation in GM volume corrected for multiple comparisons across the brain imaging (including regions-of-interest) analyses. Additionally, the relatively moderate sample sizes did not always permit taking a conservative statistical threshold for multiple comparisons [208]. Therefore, we might argue that some of the present findings could be due to false positives (e.g., see discussion about multiple comparisons in the fMRI research [209]). On the other hand, the number of performed statistical tests may have resulted in an increased probability of findings "false positives". The rationale for applying a relatively liberal threshold was to balance the risk for Type I and Type II errors [210]. Notwithstanding, in the growing field of psychiatric epigenetics in which the neuroimaging techniques are being increasingly employed, it would be essential to replicate current findings in larger samples which would allow the application of more conservative statistical thresholds [208].

Also, while we did not find any significant association between the peripheral *SLC6A4* gene methylation and frontal-limbic brain responses to negative emotional stimuli in adults,

another group – employing a similar emotion-processing task – showed that greater peripheral *SLC6A4* gene methylation was associated with greater amygdala response to negative (angry) emotional stimuli [167]. Discrepancies in results due to differences in statistical neuroimaging methodology and in location of the investigated *SLC6A4* genetic region between studies cannot be ruled out. That being said, the aforementioned amygdala responses to negative stimuli were reported in 11-15-year-old and 19-20-year-old individuals [167]. Additionally, the relationship between the peripheral *SLC6A4* gene methylation and the amygdala reactivity appeared to be stronger in adolescents [167]. In light of the currently-observed positive association between peripheral *SLC6A4* gene methylation and amygdala-based functional connectivity with the ACC and the OFC as well as the OFC activity in response to negative (sad) emotional stimuli in 15-year-olds, we might argue that such functional imaging results can be found in younger population. The age of 15 years is of particular interest, from the developmental point of view, as it corresponds to the middle of the adolescence, after the puberty onset and before the more mature period of early adulthood. Developmental differences in brain activation in response to emotional content between adolescents and adults have been examined in the past [211]. When passively viewing negative emotional (fearful) faces, adolescents were found to display greater neural activation in the amygdala, the ACC and the OFC regions, relative to the adults [211]. On the other hand, comparatively to the adolescents, the adults were found to exhibit greater OFC activity when they had to report their subjective feelings in response to negative (fearful) emotional faces that they were viewing [211]. Such differences in neural activity were argued to echo differential efficiency in attentional control, whereby adolescents would present lower ability to concentrate on personal cognitive-affective processes when confronted with emotionally engaging stimuli [211]. Results of another fMRI study showed that age was negatively associated with amygdala activation during the viewing of negative (fearful) emotional faces [212]. Amygdala, one of the most archaic brain regions, has been consistently linked to the detection of emotional significance to the environmental stimuli as well as to the contribution to emotional learning and responsivity [213, 214]. We might argue that brain development processes occurring between adolescence and adulthood might also entail an enhanced capacity to deploy cognitive control when confronted to the negative emotional stimuli.

Worth mentioning is the fact that adolescent DNA methylation was derived solely from the saliva samples. As we aimed to test all the twins – born between 1995 and 1998 – at the same age, the data collection of the twins was spread out over three years, with the first waves of data collection occurring before the study in adults. At the time, less was known about the validity of buccal cells to assess methylation. Yet both buccal cells and saliva collection are characterized by the low invasiveness, easy sampling and low maintenance. In fact, studies published after we started the data-collection in the twins using either buccal cells, saliva or blood inspired us to compare the different cell types and its relation to brain processes.

Another limitation consists in the low non-significant correlations found between the peripheral tissues. A previous epigenetic study demonstrated that for the *SLC6A4* gene, only 2 out of the 14 examined CpG sites showed significant correlations between the levels obtained from saliva and blood [215], which corresponds with the low cell type correlations observed in the current sample of adults. Therefore, the low correlations between tissues were expected due to the limited variation in cross-tissue *SLC6A4* gene methylation levels in the selected sample. Similar to relatively low levels of the peripheral *SLC6A4* gene methylation in the adults, methylation levels and differences within the MZ twin pairs were also quite low. However, these subtle differences were still sufficient to detect an association between within-pair discordance in the peripheral *SLC6A4* gene methylation and the frontal-limbic functioning. Moreover, currently-studied *SLC6A4* promoter region and specific CpG sites were chosen *a priori*, based on the previous work and validation *in vivo* and *in vitro* in different samples [162, 163, 165].

Additionally, an interesting future avenue would be to study the individuals exposed to the stress occurring before, around or shortly after the time of birth. These periods represent critical windows during which negative exposures might influence the individual's development. The epigenetic plasticity in relation to these early exposures might underpin the observed phenotypical traits through cumulative subtle alterations in gene expression. Various studies using peripheral DNA, from blood or saliva of infants, children and adolescents, have indicated increased peripheral methylation levels of various genes in response to stress shortly after birth. Indeed, increased methylation levels of the *SLC6A4* and the glucocorticoid receptor [*NR3C1*] genes have been observed in response to hospitalization and exposure to maternal

depression within the first three months of the individual's life [216-218]. In line with dynamic epigenetic patterns is the result of the study, using an MZ-twin design to investigate the relationship between the epigenome and the environment [219]. Specifically, in spite of their identical genetic background, monozygotic twins were found to gradually diverge in their global DNA methylation levels as a consequence of different environments [219]. The environment, especially during earlier developmental stages, is perhaps the most important factor that contributes to the DNA methylation pattern. Already during pre-implantation, embryos have been found to undergo massive DNA demethylation for future cell differentiation [220]. In other words, prenatal period appears to be crucial for establishing cell-specific epigenetic patterns for subsequent developmental stages. That is not say that postnatal period is not of consequence. Findings of a genome-wide study of DNA methylation, derived from infant MZ twins' buccal cells, showed an increase in within-pair discordance of global methylation levels between birth and 18 months of age [221]. Notably, the results are driven by one third of the sites in the genome-wide promoter regions [221]. Therefore, further research examining the DNA methylation patterns in specific genes is necessary to deepen the understanding of these epigenetic changes. Findings of a recent animal study indicated that environmental conditions, to which the pregnant dams were exposed, appeared to have a bigger effect on the adult mouse brain epigenome (including *NR3C1* methylation) than the postnatal environmental conditions [222], emphasizing the importance of the prenatal period for the epigenome formation. Indeed, maternal stress during pregnancy (resulting from a co-occurring natural disaster) has been found to be associated with children's DNA methylation levels in various genes, mainly related to the immune functioning [223]. The relationship between exposure to prenatal maternal hardship and offspring's DNA methylation levels was found to be similar whether methylation was derived from blood or saliva cells [224]. Yet, relationship between the daily-life demands and DNA methylation in the adults, in particular that of stress-related genes (specifically, *NR3C1*) has been shown to hold when the methylation was derived from buccal (not saliva) cells in the adults [225]. Here, in addition to the gene and tissue specificity, it is important to consider the developmental window during which (prenatal relative to daily) stress might influence differently the DNA methylation patterns. Moreover, saliva- (but not buccal-) derived *NR3C1* gene methylation was positively associated with the rsFC within medial prefrontal cortical regions, including ACC [225].

Results of another recent study, investigating peripheral (blood-derived) DNA methylation patterns in stress-related genes in depressed and healthy individuals, indicated that peripheral (blood-derived) *NR3C1* gene methylation was found to be positively associated with cortisol concentrations and childhood emotional abuse severity in the clinical group [226]. That being said, results of a study investigating the joint effect of prenatal and postnatal environmental exposures showed that peripheral (saliva-derived) *NR3C1* gene promoter methylation was elevated in the presence of maternal postnatal depression following prenatal depression [227]. Combined with the current results, these findings call for the future research to determine whether postnatal environmental stress interacts with, moderates or adds up to the effect of prenatal environmental stress to produce changes in DNA methylation and, possibly, in the health outcomes.

Moreover, the term "resilience" used throughout the thesis should be more suggestive of a "protective" factor, characterized by the individual potential ability to cope adequately with the stressful environment [228]. Future research is necessary to examine the resilience by recruiting the individuals exposed to a wide range of the early- or daily-life stress who would develop a mental health disorder as well as those who would remain healthy.

An advantage of our studies is that they were conducted in a well-documented prospective longitudinal sample of healthy (adolescent or adult) individuals carefully screened for the absence of lifetime psychopathology. Therefore, current results were unlikely to be due to clinical confounding factors like medication or symptomatology. That being said, such thorough screening for psychiatric disorders might have steered toward a low within- and between-individual differences in daily-life mood. Nonetheless, (exploratory) associations between daily-life mood and frontal-limbic neural processes were detected, suggesting that individual differences in daily affect in healthy individuals is echoed in individual differences in frontal-limbic brain processes.

Future research should include larger longitudinal samples with a wider range of the peripheral *SLC6A4* gene methylation and the daily mood as well as include potential moderating factors such as sex. Moreover, it would be of interest to replicate our results in a clinical population. This would allow deepening the investigation of the relationship between frontal-limbic regional processes and daily affect and cognition. Similarly, it would be of particular interest to test the MZ twins highly discordant in levels of affective symptomatology

and in the peripheral *SLC6A4* gene methylation and link their discordance to differences in the frontal-limbic processes.

Integrative summary

The studies of the present thesis demonstrated the association between cross-tissue peripheral *SLC6A4* gene methylation and frontal-limbic brain processes in healthy individuals, with buccal cells seemingly the preferable biological tissue for the future DNA methylation brain imaging studies. This thesis also showed (for the first time) that the association between brain processes and peripheral *SLC6A4* gene methylation also occurs irrespective of the DNA sequence.

Additionally, peripheral *SLC6A4* gene methylation was found to be positively associated with such frontal-limbic features as the ACC GM volumes, ACC GM volume as well as with the functional connectivity (at rest or during an emotion-processing task) of ACC with parietal areas or amygdala. Functional connectivity of ACC with other brain regions, namely PCC, was also associated with both daily positive and negative mood. Results of this thesis showed that ACC-related functional connectivity reflects emotional arousal, regardless of the valence.

Considering the role of ACC functioning in resilience to adverse life events [136, 137, 204], we might argue that ACC regional neural processes, with potential epigenetic underpinning, might contribute to the development of the resilience skills. In the long run, these skills might "shield" individual's emotional well-being from incoming stressors. In fact, cognitive-behavioral therapy administered to the individuals presenting internalizing symptoms has been associated with an increase in ACC and superior frontal activity in response to negative emotional content [205] as well as with an increase in peripheral *SLC6A4* gene methylation and symptoms' decrease [207]. That being said, the studies of the present thesis highlighted that further methodological studies combining psychiatric epigenetics and brain imaging are needed to deepen the understanding of the role of peripheral measures of DNA methylation in the context of risk and resilience to psychopathology. It might be of interest to conduct such research in the individuals, exposed to a wide range of prenatal, postnatal or daily-life environmental stress, who remained healthy and in those who did

develop a mental health disorder. Altered expression in relevant genes (e.g. *SLC6A4*, *NR3C1*) might underpin the alterations in the frontal-limbic brain processes and, potentially, in health outcomes. Measuring such epigenetic changes might help identify a target for intervening to attenuate the health outcomes related to the environmental stressors. A prospective longitudinal study with multiple methylation assessments to examine the dynamics of epigenetic changes in the relevant genes would also be important. Analysis of epigenetic marks might be a useful indicator of the efficacy of potential therapies for stress-related psychiatric disorders.

To sum up, current findings might have promising implications for the future use of non-invasive DNA methylation markers in identifying a risk prediction and protective factors for mental health problems in which serotonin plays an important role.

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