Genetics and Epigenetics of Suicidal Behavior

by

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Abstract

According to the latest World Health Organization report (2012), suicide was the second leading cause of death among people. Approximately 90% of suicide victims or suicide attempters are diagnosed with a psychiatric disorder. Schizophrenia patients have been shown to be at an increased risk for suicide. Although many risk factors have been identified, few of these factors have a significant clinical impact on predicting suicide. Whether genetic and epigenetic alterations are potential risk factors for suicidal behavior in schizophrenia has yet to be investigated. A well-characterized sample of schizophrenia patients with a distinct suicide attempt history was recruited to evaluate the influence of genetic and epigenetic alterations as potential risk factors for suicidal behavior. Sociocultural and clinical variables were also examined. Selected genes were genotyped to test whether independently or combined, specific clinical variables and DNA variants can accurately predict suicidal behavior. Our study found no evidence that genetic or epigenetic factors have a significant influence on the risk for suicidal behavior in schizophrenia.

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Contributions

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Chapter 4 was reproduced with permission from Neuropsychobiology 2016; 73(1): 10-15. Ali Bani-Fatemi collected the participant data, performed epigenetic techniques, analyzed the clinical, genetic, and epigenetic data, and wrote the manuscript. Aaron Howe and Michelle Matmari assisted in participant recruitment and data collection. Arthur Koga and John Strauss assisted in statistical analysis. Vincenzo De Luca, Clement Zai, and John Strauss provided expert advice and aided in proof-reading the manuscript. Vincenzo De Luca developed the study design.

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5-HIAA = 5- hydroxyindoleacetic acid

5-hmC = 5-hydroxymethylcytosine

5-HT = 5-hydroxytryptamine

5-HT1B = 5-hydroxytryptamine receptor 1B

5-HTP = 5-hydroxytryptophan

5-HTTLPR = 5-hydroxytryptamine transporter-linked polymorphic region

5-mC = 5-methylcytosine

AADC = Aromatic amino acid decarboxylase

AAO = Age-at-onset

ACC = Anterior cingulate cortex

ACN = Accumbens nucleus

ADP = Adenosine diphosphate

AMA = American Medical Association

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Amy = Amygdala

ASM = Allele-specific methylation

BA = Brodmann area

BD = Bipolar Disorder

BDNF = Brain-derived neurotrophic factor

BHS = Beck’s hopelessness scale

BSS = Beck scale for suicide ideation

C-SSRS = Columbia suicide severity rating scale

CAMH = Centre for addiction and mental health

CASV = Childhood-abused suicide victims

CC = Corpus callosum

cDNA = Complementary DNA

Ce = Cerebellum

CEU = Utah Residents with European ancestry

CHB = Han Chinese in Beijing

CNS = central nervous system

CNV = Copy number variation

COMT = Catechol-*o*-methyl transferase

CPu = Caudate-putamen

CRH = Corticotrophin-releasing hormone

CRHBP = Corticotrophin-releasing hormone binding protein

CRHR = Corticotrophin-releasing hormone receptor

CSF = Cerebrospinal fluid

Ct = Cycle threshold

CTQ = Childhood trauma questionnaire

Cx = Cortex

DARPP-32 = Dopamine- and cAMP-regulated neuronal phosphoprotein

DH = Dorsal horn spinal cord

DNA = Deoxyribonucleic acid

Dnmt = DNA methyltransferase

DRN = Dorsal raphe nucleus

DSM IV= Diagnostic and Statistical Manual of Mental Disorders 4th edition

DTI = Diffusion Tensor Imaging

ELAs = Early life adversities

EWAS = Epigenome-wide association studies

Fcx = Frontal cortex

FEP = First-episode psychosis

FIGS = Family interview for genetic studies

fMRI = Functional magnetic resonance imaging

GABA = Gamma-Aminobutyric acid

GABRG2 = Gamma-aminobutyric acid receptor subunit gamma-2

GI = Gastrointestinal tract

GR = Glucocorticoid receptor

GWAS = Genome-wide association study

H3K27 = Histone 3 Lysine 27

hGR = Hippocampus glucocorticoid receptors

Hip = Hippocampus

HPA = Hypothalamic–pituitary–adrenal

HTR1A = 5-HT1A = 5-hydroxytryptamine receptor 1A

HTR1B = 5-HTT = 5-hydroxytryptamine transporter

HTR2A = 5-HT2A = 5-hydroxytryptamine receptor 2A

HTR4 = 5-hydroxytryptamine receptor 4

HWE = Hardy-Weinberg equilibrium

Hyp = Hypothalamus;

IPN = Interpeduncular nucleus

JPT = Japanese in Tokyo

LC = Locus coeruleus

LD = Linkage disequilibrium

LS = Lateral septum

MAOA = Monoamine oxidase A

MC2R = Melanocortin-2 receptor

miRNA = micro RNA

miRNP = microRNA ribonucleoprotein complex

MRI = Magnetic resonance imaging

MRN = Median raphe nucleus

mRNA = Messenger RNA

N = Nucleus

NCASV = Nonchildhood-abused suicide victims

NEO = Neuroticism, Extraversion, Openness

NMDA = N-methyl-D-aspartate

NR3C1 = Nuclear Receptor Subfamily 3 Group C Member 1

NSA = Non-suicide attempt

NSSI = Non-suicidal self-injury

NTS = Nucleus of the solitary tract

OAZ1 = Ornithine decarboxylase antizyme 1

OB = Olfactory bulb

OFC = Orbitofrontal cortex

PAG = Periaqueductal gray

PCA = Principal component analysis

PCR = Polymerase chain reaction

PET = Positron emission tomography

piRNA = piwi-interacting RNA

PMI = Postmortem interval

POE = Parent of origin effect

PTSD = Post-traumatic stress disorder

QTDT = Quantitative and discrete traits

RGS4 = Regulator of G-protein signaling 4

RISC = RNA-induced silencing complex

RMg = Raphe magnus nucleus

RNAi = RNA interference

RNase = Ribonuclease

RO = Raphe obscurus nucleus

Rpa = raphe pallidus

RPo = Raphe pontis nucleus

rRNA = ribosomal RNA

SA = Suicide attempt

SAT1 = Spermine/spermidine N1-acetyltranferase

SCA = Schizoaffective disorder

SCID = Structured clinical interview for DSM

SCZ = Schizophrenia

SD = Standard deviation

siRNA = small interfering RNA

SLEs = Sftressful life events

SLESQ = Stressful life events screening questionnaire

SMOX = Spermine oxidase

SMS = Spermine synthase

SN = Subtantia nigra

SNP = single-nucleotide polymorphism

SPECT = Single-photon emission computed tomography

SVS = SNP & variation suite

TET = Ten–Eleven Translocation

Tha = Thalamus

TNF = Tumor necrosis factor

TPH = Tryptophan hydroxylase

TrkB = Tyrosine kinase B

TrkB.T1 = Tropomyosin-related kinase B

UTR = Untranslated region

VBM = Voxel-based morphometry

VH = Ventral horn

VNTR = Variable number tandem repeat

VTA = Ventral tegmental area

WHO = World health organization

YRI = Nigeria in Western Africa

Chapter 1
Introduction

Suicide is a deliberate act of self-injurious behavior that results in mortality (World Health Organization [WHO], 2015). Suicide is a complex behavior and a major public health concern, ranking as the 17th leading cause of death worldwide (Hawton et al., 2009, WHO, 2015). Suicide in Canada remains a concern as 2611 individuals died by suicide in 2007 alone, which accounts for approximately 10.2 deaths per 100,000 Canadians (Statistics Canada, 2012; WHO, 2015). The incidence of suicide is most notable among young adults (aged 15-29) who are also vulnerable to the onset of various neuropsychiatric disorders (Värnik, 2012; Turecki, 2014). Although suicide is preventable, it continues to be a global phenomenon where research has focused on developing improved surveillance and prevention strategies to reduce the associated mortality rates (Hawton et al., 2009).

Suicide is generally defined along a continuum that includes suicidal ideation, deliberate self-harm with minimal intent (non-suicidal self-injury or NSSI), suicide attempt (higher levels of intent and medical endangerment), and deliberate self-inflicted death (i.e. suicidal completion) (Hawton et al., 2009). Suicidal behavior includes contributions from genes, life adversities, and psychopathology. Other notable contributions influencing suicide include social factors and the accessibility to the methods of suicide. Each of these contributions has varying individual and synergistic impact on the genesis of suicidal behavior.

Suicidal behavior has a strong association with several neuropsychiatric disorders (e.g., schizophrenia, bipolar and major depressive disorder) and is one of the leading causes of preventable death among these disorders (Fliege et al., 2009; Palmer et al., 2005). In particular, individuals with schizophrenia (SCZ) have a noticeably shorter lifespan that is partly attributable

to elevated suicide rates as a result of the interplay of adverse life events and genetic predisposition (Laursen et al., 2014). Approximately 40–50% of psychiatric patients report at least one distinct episode of suicidal ideation in their lifespan (Montross et al., 2005). A recent study by Palmer et al. places the lifetime risk of suicide at 4.9%, with a higher frequency of suicide attempts occurring near the onset of the mental health disorder (Palmer et al., 2005). Suicide rates are 12 times greater among individuals with SCZ, and these patients remain four times more likely to commit suicide even a decade after diagnosis (Dutta et al., 2010). Up to 50% of patients with bipolar disorder (Jamison et al., 2000) and about 30% of patients with SCZ will attempt suicide in their lifetime, with slightly higher rates found in schizoaffective disorder (SZA) than SCZ (Radomsky et al., 1999). Patients with psychiatric illness are treated with pharmacological agents that can reduce suicide rates, but issues of treatment adherence and treatment-resistance can make suicide prevention problematic.

Although more patients with mood disorders may attempt suicide than those with other psychiatric disorders (Jamison et al., 2000; Alberdi-Sudupe et al., 2011; Mann, 2003), suicide can be seen as an independent heritable trait which can be assessed across diagnoses (Mann, 2003). When stratifying for sex, there is a disparity among suicide attempts between males and females with or without a diagnosable mental health disorder. It has been shown that females commit twice as many suicide attempts as their male counterparts; however; the vast majority of the fatal attempts (~80%) are in the male population (Alaraisanen et al., 2009; Hawton et al., 2005; McGirr et al., 2006). This paradox gives further evidence that the neurobiological underpinnings of suicide are multi-factorial.

1 Literature Review

1.1 Epidemiology of Suicidal Behavior

A study by Olfson et al. (2017) showed that the rate of suicide attempt in adults older than 21 years significantly increased between 2005 and 2013 in the United States. They found this increase mainly in people with lower socioeconomic status, depression, antisocial personality, substance abuse, and a history of violence. The American Medical Association (AMA) reported a change in US suicide rates per 100,000/year between 2000 and 2015. They showed that the suicide rates grew from 17.67 to 21.48 in men and from 3.95 to 6.25 in women.

Studies investigating suicide risk factors have identified gender as a contributing factor. It is already well-established that there are differences in suicidal behavior between males and females, regardless of whether or not they have been diagnosed with a mental illness. In fact, in the general population, men die by suicide up to four times more than women, while women attempt suicide much more frequently (Värnik, 2012; Chang, Gitlin, & Patel, 2011). The reported difference in suicide rates for males and females is partially a result of the lethality of the methods used by males and females. Although females attempt suicide at a higher rate, they are more likely to use methods that are less lethal (Canetto & Sakinofsky, 1998). Males frequently complete suicide using highly lethal methods such as hanging or firearms. This is in contrast to females, who tend to rely on drug overdoses. This is commonly known as the gender paradox of suicidal behavior (Canetto & Sakinofsky, 1998). A major exception to this trend is found in China, where females have one of the highest suicide rates globally and is the only country where females complete suicide at a higher rate than men (Värnik, 2012).

A study by Jokinen et al. (2017) also showed that the life expectancy after the first suicide attempt is different between males and females. They found a reduction of 18 years in life expectancy in men who attempt suicide at the age of 20. On the other hand, life was shortened by 11 years in women who attempt suicide when they were 20-year-old. However, the aforementioned study indicated that this gender difference in life expectancy is less evident in patients who committed their first suicide attempt at an older age especially after the age of 70. Among the world's populations, suicide rates are similar between men and women in the Eastern Mediterranean, while the highest rate of suicide among females is in South Korea (Värnik, 2012). Notably, the difference in gender suicide rates appears to be less apparent in developing countries where the use of lethal agricultural pesticides by young women is more prevalent.

1.2 Neurobiology of Suicidal Behavior

The present understanding of suicide is that subjects with a genetic predisposition are particularly vulnerable to stressful environmental inputs. The biological basis of suicide has been investigated in several studies to better understand its precipitating factors. As a result, there have been several biological hypotheses. Recent studies have explored the genetic impact on suicidal behavior through Genome-wide association studies (GWAS). However, many GWAS suggested associations that did not reach genome-wide significance, pointing out that there are multiple genomic targets that need further investigations in suicidal behavior. A recent study by Galfalvy et al. (2015) identified several genes involved in neural functions such as neurodevelopment (cellular assembly, function, and organization), cell death, survival mechanisms, and immunological/inflammatory mechanisms. The use of GWAS has been limited to the investigation of common genetic variants. However, more recent studies have explored the influence of environmental input on gene function. The associations of early life adversities (ELAs) and suicide have suggested interaction with genetic liability leading to the stress-diathesis model of suicide (Figure 1-1). Studies on functional genomics have consistently identified brain regions implicated in both psychiatric disorders and suicide such as the prefrontal cortex, anterior cingulate cortex, amygdala, and the hippocampus. These brain regions have been implicated in both structural and functional alterations from the investigation of post-mortem tissue of suicide completers and imaging studies of suicide attempters.

In a deeper search into the neurobiology of suicidal behavior, research has also looked at the neurotransmitter systems within the brain regions that may be implicated in suicidality. Monoaminergic systems have widespread innervations that connect neurons located in the raphe

nucleus and locus coeruleus to neurons within the prefrontal cortex, anterior cingulate cortex, amygdala, and the hippocampus.

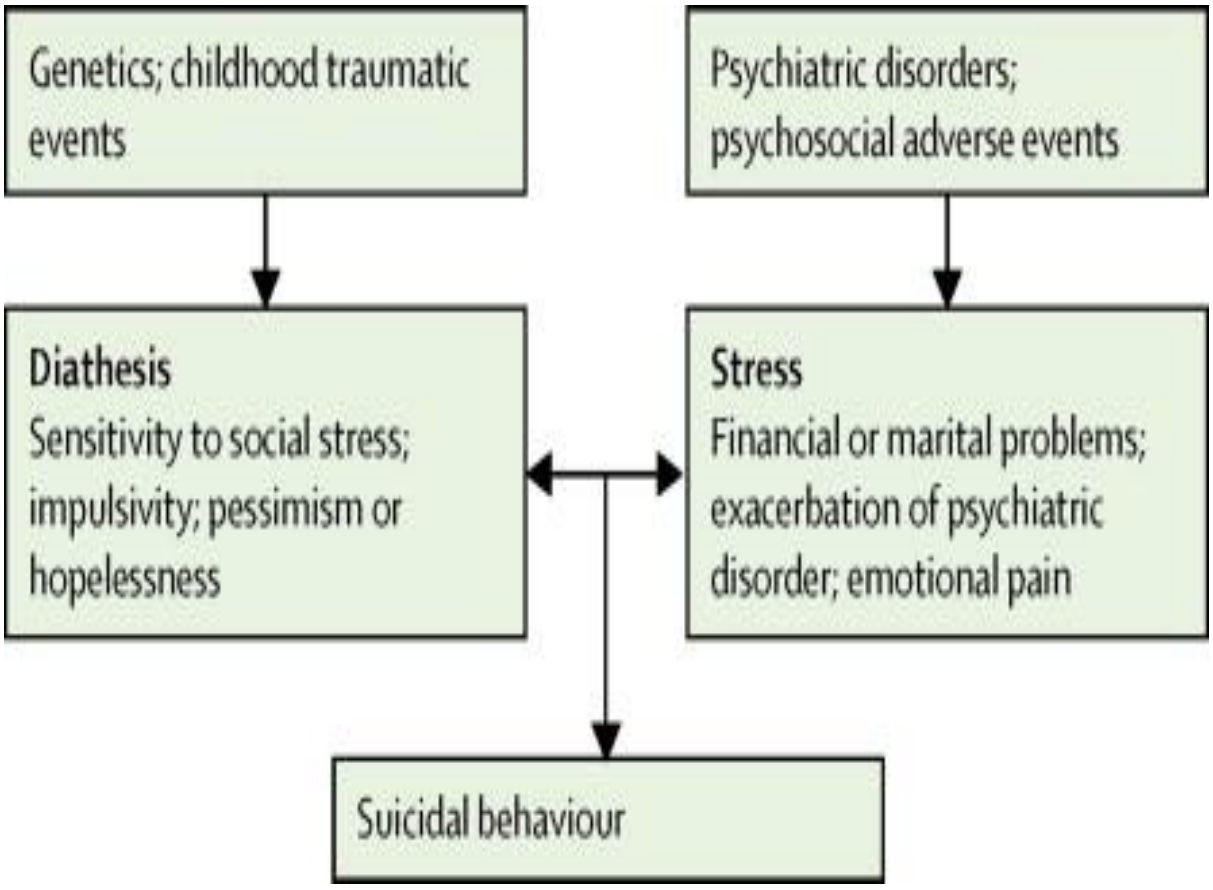

Figure 1 - 1. The stress-diathesis model of suicidal behavior. Figure adapted (with permission) from Kees van Heeringen and John Mann (2014). The Lancet Psychiatry.

The prefrontal cortex is commonly involved in neural connections associated with executive function, and the anterior cingulate-amygdala-hippocampal pathway is involved in emotional regulation, stress response and integration of emotional, cognitive schemes. Considering the emotional valence associated with suicidal ideation and attempt, these structures play an important role in the regulation of suicidal behavior. Suicide studies have found dysregulation of monoaminergic pathways including serotonergic, noradrenergic, GABAergic, and most recently the glutamate pathway. Ketamine has recently drawn attention due to effectiveness in treating treatment-resistant depression and its rapid onset of action on depressive symptoms (Berman et al., 2000; McGirr et al., 2015; Zarate et al., 2006). The antidepressant effect of ketamine appears to lie within its activation of AMPA signaling within NMDA receptors, which is the cause of downstream effect on regulation of microRNAs linked to the serotonergic and neurotrophin pathways (Zanos et al., 2016).

There is strong evidence suggesting the involvement of the monoaminergic systems in suicidal behavior; however, owing to the complexity of suicide, it can be asserted that additional neurotransmitter systems are also involved. Researchers have focused on the underlying neuropsychological and neurobiological processes to understand suicidal behavior and try to predict the individuals who are at risk. Evidence of a neurobiological basis of the vulnerability for suicidal behavior is increasing (van Heeringen and Mann, 2014). From a neurochemical point of view, the serotonin neurotransmitter system and the hypothalamic–pituitary–adrenal axis (HPA axis) system appear to be crucially involved. At a neuroanatomical level, neuroimaging studies show changes in a number of areas in the brain in association with vulnerability to suicidal behavior. At the forefront, it appears that the dysfunctions associated with the frontal, temporal, and parietal cortices are implicated in suicidal behavior (Jollant, 2016). However, it is

difficult to determine whether these changes are specific to the underlying psychiatric disorders or to suicidality.

Suicide brains have shown altered mRNA levels of *BDNF* and its receptor, tyrosine kinase B (*TrkB*) (Dwivedi et al., 2003). Epigenetic studies have shown that the modulation of DNA methylation of *BDNF* is similar in post-mortem brains of suicide victims and in subjects with suicidal behavior and depression. (Keller et al., 2010; Kang et al., 2013; Roth et al., 2009). There is also evidence that peripheral lipid metabolism has an association with suicidality. Studies have reported an association between low levels of cholesterol and suicides; however, negative findings have been reported in the literature (Lalovic et al., 2007). Cholesterol has also been shown to have implications in neurotransmitter signaling, neurosteroid assembly, and precursors to lipid molecules (Dietschy et al., 2009).

The polyamine system and HPA axis system have also been investigated in suicidal behavior. Activation of polyamines increases in the presence of physical, hormonal or emotional stress (Gilad et al., 2003; Karssen et al., 2007). The sensitivity of polyamine and HPA axis to stress has been well documented in studies of early life adversities (ELAs) in animal models and humans (Heim et al., 2010). Stress causes glucocorticoid release that potentiates a negative feedback loop to activate glucocorticoid receptors in the hypothalamus. Epigenetic repression and activation have shown differential expression of glucocorticoid receptors in response to chronic and acute stressors (Francis et al., 1999; Liu et al., 1997; Weaver et al., 2004). Given the link between depression, stress and suicidal behavior, the examination of these pathways has led to further insights into the impact of stressful life events in triggering suicidal behavior in psychiatric populations.

1.3 Serotonergic System

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that modulates neural activity and a wide range of neuropsychological processes. It is produced from the amino acid tryptophan in a two-step synthetic pathway. In the first step, tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). Tryptophan hydroxylase has two isoforms, TPH1 and TPH2. The isoform TPH1 is primarily responsible for 5-HT synthesis in the periphery, while TPH2 is responsible for 5-HT synthesis in CNS (Muller et al., 2016). In the second step, 5-HTP is converted to 5-HT by the aromatic amino acid decarboxylase (AADC) (Figure 1-2). Several studies investigated 5-HT within gastrointestinal, cardiovascular, pulmonary, and genitourinary systems as well as the central nervous system (CNS) (Roth et al., 2007).

Over 90% of the human body serotonin is found outside the CNS (Gershon et al., 2007). Enterochromaffin cells in the gastrointestinal (GI) tract are the primary source of peripheral 5-HT (Anderson et al., 1987). GI-derived 5-HT is unable to pass the blood brain barrier and interact with neural tissue (Hardebo and Owman, 1980). Brain 5-HT is produced by TPH2-expressing serotonergic neurons in the midbrain and hindbrain (Muller et al., 2016). Serotonergic neurons innervate a broad collection of brain regions (Figure 1-3). Although serotonin modifies substantially all of human behavioral processes, it is produced by less than one in a million CNS neurons (Berger et al., 2009). Serotonin modulates the behavioral and neuropsychological processes such as mood, perception, reward, anger, aggression, appetite, memory, sexuality, and attention (Berger et al., 2009). Brain and peripheral 5-HT exert their effects through 14 receptor subtypes (Hannon and Hoyer, 2008; Millan et al., 2008). Each serotonin receptor is expressed in

different brain regions and probably contributes to the regulation of different behavioral processes (Berger et al., 2009). This may explain why medications targeting specific serotonin receptors have effects on several behavioral processes (Giorgetti and Tecott, 2004).

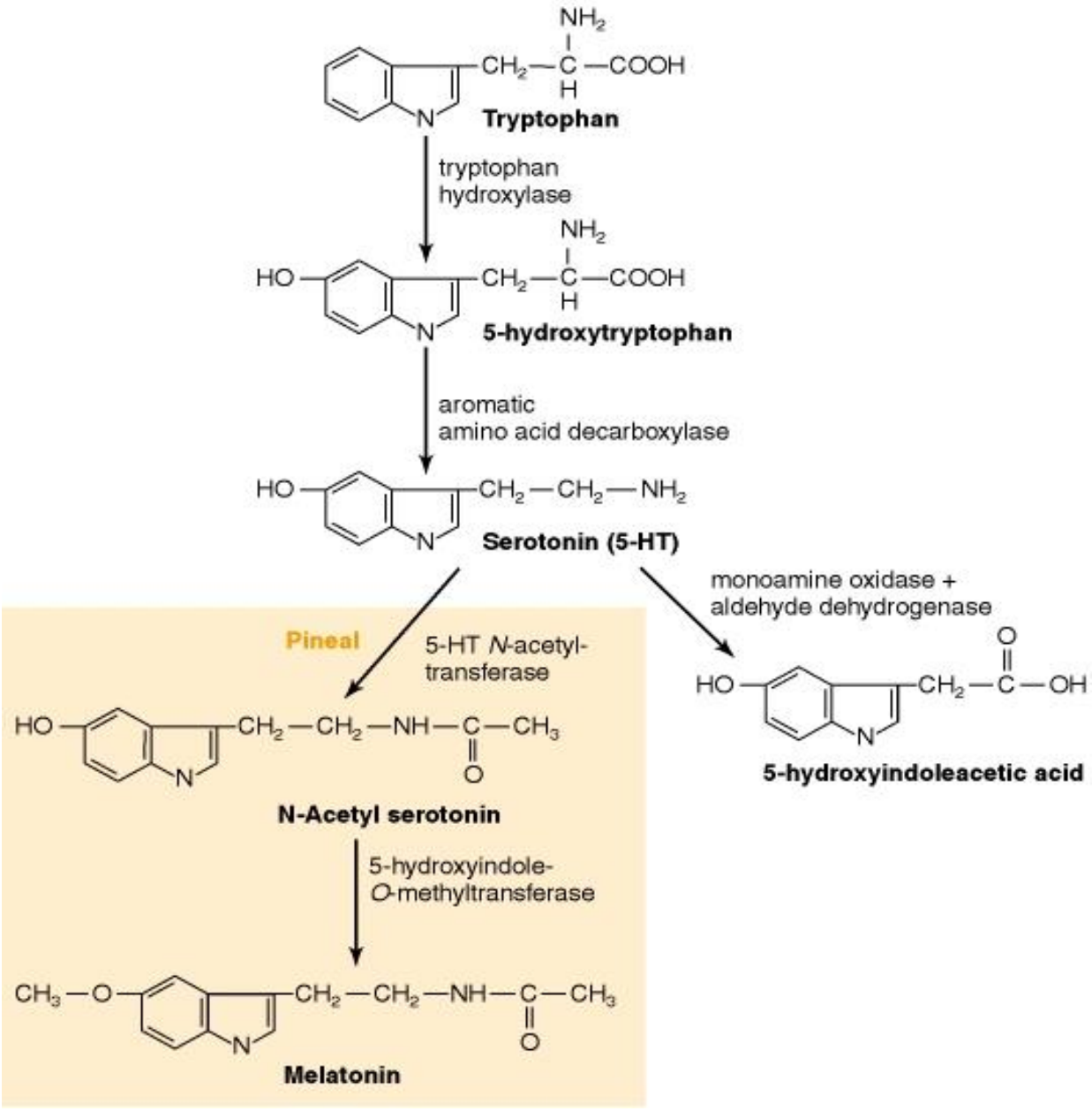

Figure 1 - 2. The biosynthesis and catabolism of serotonin. Figure adapted (with permission) from Frazer A, Hensler JG. Serotonin. Lippincott-Raven; 1999.

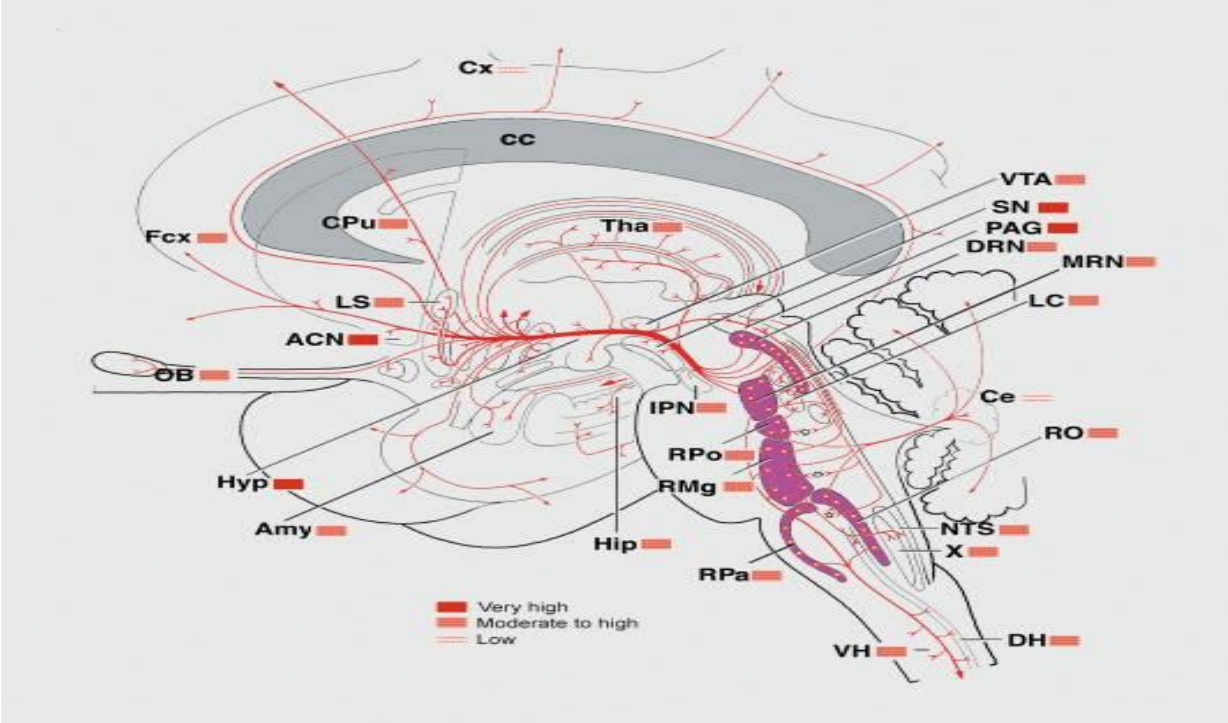

Figure 1 - 3. Schematic sagittal view of the human brain showing the distribution of the serotonergic systems. The raphe nuclei containing the majority of the serotonergic cell bodies appear in purple. It is readily seen that these nuclei are exclusively located in the brain stem. The axons issued from them are drawn in red. The trajectories and extensive branching of the axons until the main terminal areas are illustrated. The densities of the serotonergic axonal networks in these terminal areas are given by the colored boxes. Abbreviations: X, dorsal motor n of the vagus nerve; ACN, accumbens n; Amy, amygdala; cc, corpus callosum; Ce, cerebellum; CPu, caudate-putamen; Cx, cortex; DH, dorsal horn spinal cord; DRN, dorsal raphe n; Fcx, frontal cortex; Hip, hippocampus; Hyp, hypothalamus; IPN, interpeduncular n; LC, locus coeruleus; LS, lateral septum; MRN, median raphe n; n, nucleus; NTS, n of the solitary tract; OB, olfactory bulb; PAG, periaqueductal gray; RMg, raphe magnus n; RO, raphe obscurus n; Rpa, raphe pallidus; RPo, raphe pontis n; SN, subtantia nigra; Tha, thalamus; VH, ventral horn; VTA, ventral tegmental area. Figure Adapted (with permission) from Nieuwenhuis R. Springer Verlag.

1.4 The Role of Serotonergic System in Suicidal Behavior

The neurotransmission of serotonin is regulated by a network of receptors and the 5-HT transporter (5-HTT) (Figure 1-4). 5-HT neurons have been implicated in depressive disorders and suicide with substantial evidence suggesting impaired serotonergic function (Boldrini et al., 2008; Bach et al., 2012; Kaufman et al., 2015) as summarized in recent comprehensive reviews (Oquendo et al., 2014; Mann, 2013; Antypa et al., 2013; Sudol et al., 2017). Clinical and post-mortem studies suggest that reduced serotonergic input precipitates vulnerability to suicidal behavior in individuals with or without psychiatric illness. These studies show reduced 5-HT transporter binding in the prefrontal cortex of suicide completers and low levels of 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid of individuals diagnosed with depression who have attempted suicide. Among other findings, studies found that depressed suicide completers show in dorsal raphe nucleus decreased levels of the serotonin metabolite, 5-HIAA, as well as more 5-HT neurons (Mann, 2003; Arango et al., 2001) and increased mRNA expression and protein levels of tryptophan hydroxylase (Boldrini et al., 2005; Bach-Mizrachi et al., 2006; Drevets et al., 2007; Bach-Mizrachi et al., 2008). Upregulation of tryptophan hydroxylase activity and increased numbers of 5-HT neurons have been interpreted as mechanisms compensating for an overall reduction in 5-HT transmission, a finding that has been supported by imaging studies (Sullivan et al., 2015; Wang et al., 2016).

Small changes in serotonin transporter and HTR1A receptor expression have also been observed in suicide. Neuroendocrine challenges studies have also shown that decreased serotonergic activity has led to blunted prolactin responses in patients with depression and suicidal behavior. Studies have reported reduced amounts of neurons in the anterior cingulate, hippocampus and prefrontal cortex in psychiatric disorders and suicidal behavior, suggesting that

neuronal deficiency could be potentially due to the downstream effect of impaired serotonin signaling.

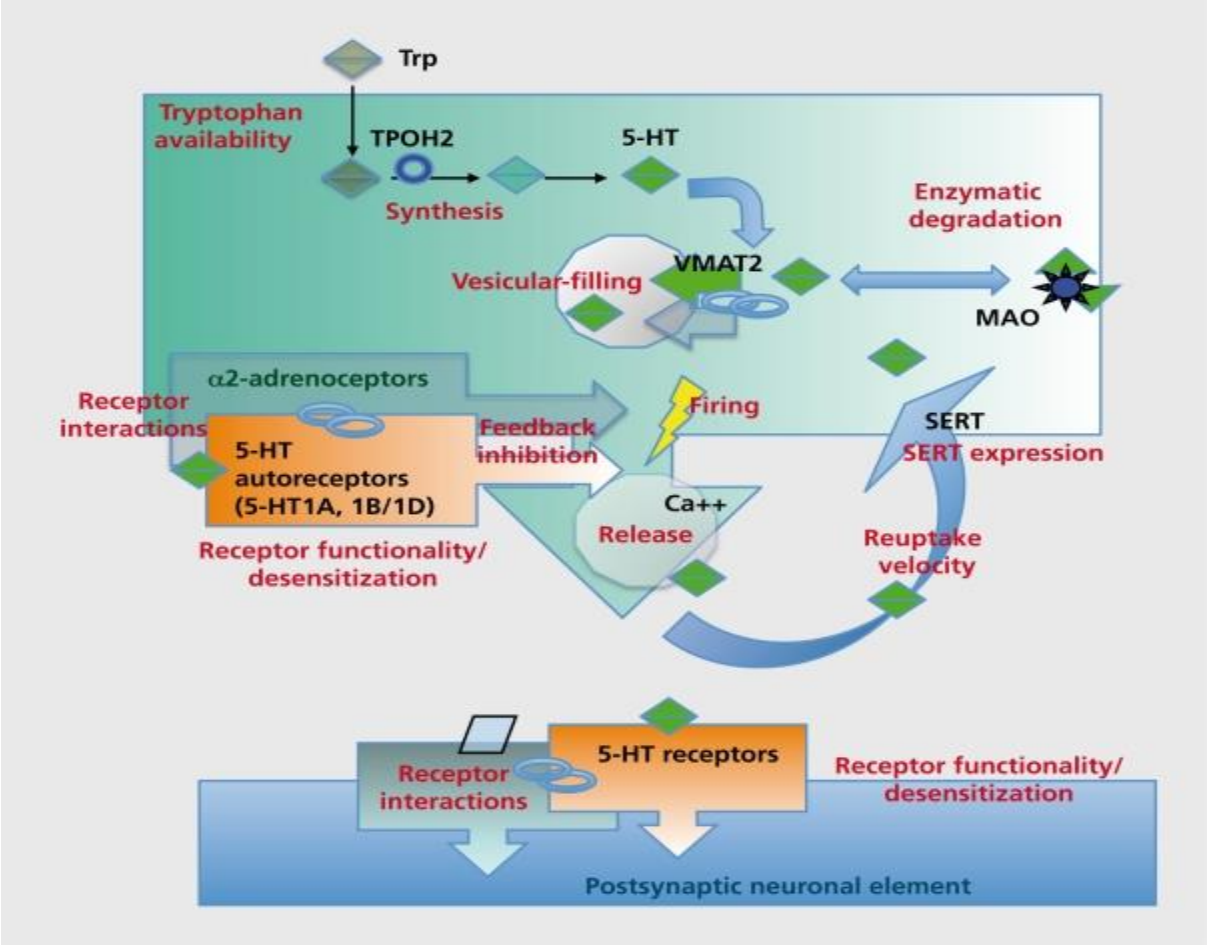

Figure 1 - 4. The serotonergic neurotransmission depends on serotonin (5-HT) levels present in the extracellular space and on membrane receptors triggering functional changes in neighboring neuronal elements. 5-HT synthesis, release, and reuptake are regulated by several mechanisms including feedback inhibition by 5-HT1A, 5-HT1B/1D autoreceptors, and α-2 adrenoceptors. Other mechanisms of regulation are receptor dimerization and desensitization affecting their trafficking and functionality. Adapted (with permission) from Yves Charnay, Lucienne Leger. (2010). Dialogues Clin Neurosci, 2010. 12(4):471-487.

1.5 Genetics of Suicidal Behavior

Adoption, family and twin studies strongly suggest a genetic basis of suicidal behavior (Brent and Mann, 2005; Melhem et al., 2007). Twin studies demonstrate a high concordance in suicidal behavior even with a proper account of confounding variables (Roy, et al. 1995). Moreover, monozygotic twins show a greater concordance of suicide compared with dizygotic siblings (Roy et al., 2001). One estimate suggests that 43% of the variability of suicidal behavior may be due to the genetic influence (Bondy et al., 2006). In addition, the genetic component of suicide is correlated with the susceptibility to psychiatric illness and impulsive aggression (Brent and Mann, 2005).

Despite the reported genetic influence on suicidal behavior, GWA studies have only used samples recruited to investigate the genetic vulnerability of mood disorders. Schosser et al. (2011) evaluated suicide attempt in 2023 depression cases, but the identified variants did not achieve genome-wide significance and could not be replicated in two additional cohorts. Perlis et al. (2010) reported five loci associated with suicide attempt but failed to replicate findings in an independent cohort. In bipolar disorder (BD) population, Willour et al. (2011) compared individuals with and without a history of a suicide attempt but no variants survived multiple testing corrections.

1.6 Epigenetic Mechanisms of Suicidal Behavior

Epigenetic approaches investigate the influence of environmental stimuli on the genome such as stressors, life adversity, and biological processes (Bender, 2004; Iwamoto et al., 2009; Nagy et al., 2012). The term epigenetics ("epi" meaning beyond) refers to the study of reversible mitotically, and in some cases meiotically, heritable changes in the genome through non-DNA sequence coding processes that alter gene expression (Petronis, 2001). The epigenome and epigenetic modifications present in the genome originate at pre-conceptual gametogenesis and gestational periods (Champagne, 2008; Puri et al., 2010). Parental exposures to the environment and stochastic errors during gametogenesis and gestational periods have been demonstrated to influence the offspring's epigenome (Weaver et al., 2004).

Epigenetic modifications that occur in the mammalian genome include DNA methylation, histone modifications [methylation, acetylation (Grunstein, 1997; Marmorstein et al., 2001), phosphorylation, ubiquitination, sumoylation, deimination, ADP ribosylation, and proline isomerization)], RNA interference (miRNA, siRNA, piRNA), histone variants, and nuclear organization (Fraser et al., 2007; Fuks et al., 2003; Tchurikov, 2005). These epigenomic modifications allow our genome to respond and adapt, through the variations in gene expression, to intrinsic and extrinsic environmental factors (e.g., childhood trauma and life adversity). The epigenomic changes present in the mammalian genome are based on three founding epigenetic principles (Chen et al., 2002; D'Aquila et al., 2013). First, a DNA sequence can have variable expressivity patterns depending on the chromatin state (Harper, 2005). Second, epigenetic modifications are reversible, and in a sense this modification is a dynamic process, allowing the cell to become more responsive to environmental stimuli (Lorincz et al., 2004). Third, the possibility of meiotic inheritance allows for the transmission of epigenetic modifications from

parent to offspring, which in the context of suicidal behavior may confer additional suicide risk beyond the genetic code (Champagne, 2008; Harper, 2005; Meaney, 2010; Puri et al., 2010; Szyf, 2009).parapgraph.

1.6.1 DNA methylation

DNA methylation, the most studied epigenetic mechanism, involves a covalent biochemical modification that results from the addition of a methyl group by DNA methyltransferases from S-adenyl methionine to the aromatic ring of cytosine (5′-mC) located 5′ to a guanosine (CpG) (Aapola et al., 2000; Baylin et al., 2001; Bellizzi et al., 2012; Eden et al., 2003; Klose et al., 2006; Razin, 1998) (Figure 1-5). Many human genes have large clusters of CpGs (cytosine and guanine separated by a phosphate) associated with regulatory regions called CpG islands (genomic sequences of >500 nucleotides in length, with a cytosine and guanosine content >55% and an observed CpG over expected CpG ratio > 0.65) (Schilling et al., 2009). The level of methylation at C nucleotides within CpG islands generally reflects the levels of gene expression, with hypermethylation associated with gene silencing (and in some cases activation) and hypomethylation with gene activation.

DNA methylation is assumed to be complementary on both sex chromosomes in the mammalian genome (Hellman et al., 2007; Schilling et al., 2009). However, in diploid mammalian genomes, parental alleles can exhibit different methylation patterns (allele-specific methylation or ASM), which have been documented in the imprinted regions.

Recent works have provided a large candidate list of imprinted genes, but it is suspected to be a small fraction of the total number of imprinted genes (Hata et al., 2002; Luedi et al., 2007). An example of ASM has recently been reported, whereby genotype (mainly single nucleotide polymorphisms) exerts an influence upon DNA methylation in cis (Schilling et al., 2009). A very simple way to look at ASM is to study CpG SNP loci in heterozygous subjects, where it can be hypothesized that these heterozygous loci function as a flag for a more complex epigenetic mechanism. The biological importance of methylation is clear as disturbances of known methylation patterns are linked to numerous complex diseases and possibly to suicidal behavior. In fact, a number of CpG islands have been found to exhibit inter-individual methylation variation, and it has been proposed that the methylation levels of these genes are especially sensitive to differential exposure to environmental factors. A recent study has reported the ability of the Ten–Eleven Translocation (TET) Proteins to oxidize 5-mC to 5-hydroxymethylcytosine (5-hmC), which is rapidly being considered as another significant epigenetic marker (the "sixth" base), because of its critical role in the regulation of tissue-specific gene expression (Li et al., 2011).

Recently, several studies (Ernst et al., 2009b; Guipponi et al., 2009; Keller et al., 2010, 2011; McGowan et al., 2008, 2009) have investigated DNA methylation patterns in suicide victims. However, to date, only a few studies have investigated DNA methylation state in suicide, and no studies have investigated the role of the polymorphic CpG sites in suicidal behavior. Interestingly, Murphy et al. (2013) reported a significant association between functional single-nucleotide polymorphisms (SNPs) in epigenetic regulatory genes, DNA methyltransferases (DNMT1 and DNMT3b), and suicide attempts in psychiatric patients. The rs2424932 functional SNP that resides in the 3′ UTR of the DNMT3b gene was associated with

suicide attempt. In terms of DNA methylation, the same authors reported significantly higher levels of global DNA methylation in psychiatric patients with a history of suicide attempts compared to controls.

A genetic association study by Polsinelli et al. (2013) reported that potential methylation of polymorphisms rs6873382 and rs13182501 in the serotonin receptor 4 gene (HTR4) was not associated with suicide attempt. However, the association between serotonin genes and suicidal behavior has been inconsistent across different studies.

McGowan et al. (2008) reported that the ribosomal RNA (rRNA) promoter was hypermethylated throughout the regulatory region in the hippocampus of 18 postmortem brains of suicide victims. They tested 26 CpG sites and found that 21 of them were significantly heavily methylated in the suicide group compared to the controls. They also tested the methylation levels in the rRNA promoter of the cerebellum and found no significant methylation patterns. Ribosomal RNA expression was found to be significantly higher in controls, indicating that rRNA expression is downregulated in postmortem brains of suicide attempters. These findings suggest that methylation patterns in the genome may influence suicidal behavior.

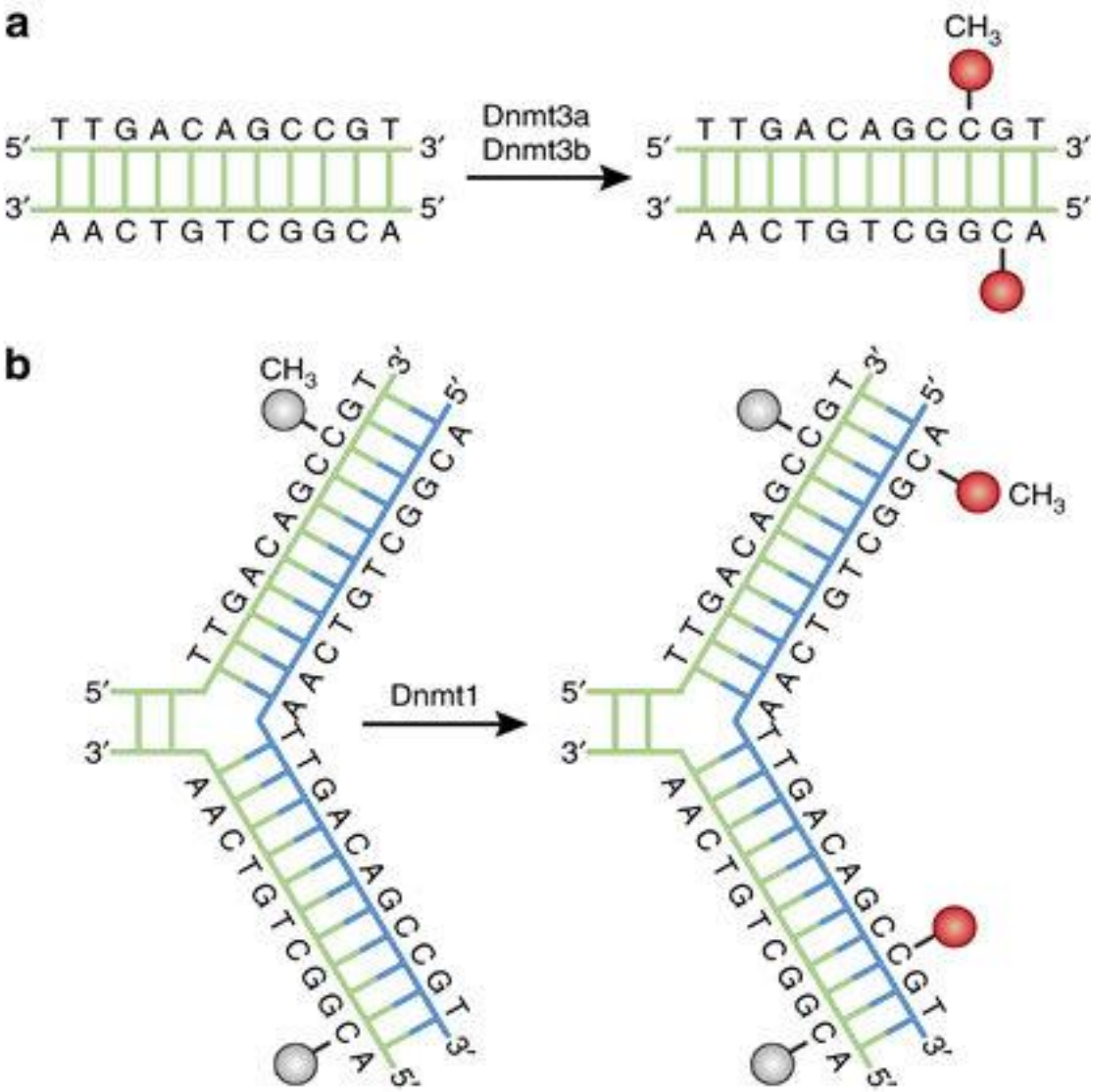

Figure 1 - 5. DNA methylation pathways. A family of DNA methyltransferases (Dnmts) catalyzes the transfer of a methyl group from S-adenyl methionine (SAM) to the fifth carbon of cytosine residue to form 5-methylcytosine (5mC). (a) Dnmt3a and Dnmt3b are the de novo Dnmts and transfer methyl groups (red) onto naked DNA. (b) Dnmt1 is the maintenance Dnmt and maintains DNA methylation pattern during replication. When DNA undergoes semiconservative replication, the parental DNA stand retains the original DNA methylation pattern (gray). Dnmt1 associates at the replication foci and precisely replicates the original DNA methylation pattern by adding methyl groups (red) onto the newly formed daughter strand (blue). Adapted from Lisa D Moore, Thuc Le & Guoping Fan (2013). Neuropsychopharmacology, 38: 23–38.

1.6.2 Histone modification

Histones are involved in linking nucleosomes together, resulting in the formation of a higher-order structure of chromatin (Bannister et al., 2011; Barski et al., 2007). Chromatin is composed of DNA wound around a core of proteins known as histones, which associate with each other to pack DNA into the nucleus (Bannister et al., 2011; Burgess et al., 2010). In the past, it was believed that histone proteins provided simply a static DNA packaging device; however, it is now becoming clear that histones, through a number of chemical modifications, play a very active role in remodeling the chromatin structure and regulating gene activity (Baylin et al., 2001; Sun et al., 2013). There are two functional structures of chromatin that are facilitated by histones: the euchromatin – which is active and associated with gene transcription, and the heterochromatin – which is inactive and associated with silencing (Labonte et al., 2010; Lippman et al., 2004).

To date, eight types of histone modifications have been characterized: methylation, acetylation (Machado-Vieira et al., 2011), phosphorylation, ubiquitination, sumoylation, deimination, ADP ribosylation, and proline isomerization (D'Aquila et al., 2013). These histone modifications induce changes in the nucleosome structure and function. However, acetylation and methylation have been the most studied histone modifications. Histone acetylation refers to the transfer of acetyl groups by histone acetyltransferase from Acetyl Co-A to the lysine residues of histones. This modification results in chromatin decondensation and transcriptional activation. Histone deacetylation is the process of removing acetyl groups from histones, conversely repressing transcriptional activity. Histone methylation is conducted by histone methyltransferases on lysine residues with the ability to transfer three methyl groups per site. Methylation on the H3 protein decreases transcription of DNA close to the histone complex.

As a result, histone methylation can result in the inhibition of transcriptional processes, thereby increasing the complexity of epigenetic control on the chromatin structure.

An epigenetic study by Ernst et al. (2009a) reported a significant correlation between increased H3K27 (Histone 3 Lysine 27) methylation and decreased TrkB.T1 (Tropomyosin-related kinase B) expression in the orbitofrontal cortex of 20 suicide completers. According to the authors, only heavy methylation of H3K27 can produce observable and quantifiable effects on transcription. Therefore, their findings provide limited evidence of the contribution of histone methylation on the epigenome. A study by Fiori et al. (2012) reported that increased levels of H3K4me3 (transcriptional active chromatin biomarker) in the promoter region significantly upregulates the activity of OAZ1 (ornithine decarboxylase antizyme 1) in the brains of suicide completers. OAZ1 is primarily involved in polyamine synthesis, and its upregulation suggests that epigenetic mechanisms in the polyamine system are also involved in suicidal behavior. Arginase 2 (another polyamine system enzyme) and OAZ1 had positive correlations with gene expression and H3K4me3 levels, indicating the critical role of histone modifications on gene regulation in the polyamine system (Gilad et al., 1998). It is becoming more evident that epigenetic modifications may contribute to the complexity of the suicide phenotype (Fiori et al., 2012; Gross et al., 2013).

1.6.3 RNA interference

RNA interference (RNAi) is a process by which organisms regulate the production of messenger RNA (mRNA) through small noncoding RNAs known as small interfering RNA (siRNA), PIWI-interacting RNA (piRNA) and microRNA (mi-R) (Castel & Martienssen, 2013).

RNAi begins with a double-stranded RNA molecule that folds itself into a stem-loop structure and is processed into small RNA duplexes by ribonuclease III (RNaseIII) (Djupedal et al., 2009; Grewal et al., 2007). The formed duplex is unwound, and one strand of siRNA becomes bonded with a protein complex known as RNA-induced silencing complex (RISC). This complex finds potential target RNAs by directing an endonuclease that is present in RISC to cleave the messenger RNA with sequence specificity to the guiding strand of siRNA. Once the target is found, the guiding strand binds to the target mRNA and marks it for degradation.

In terms of suicidal behavior, there are no relevant studies that link the epigenetic effects of siRNA and piRNA to alterations in transcriptional activity in suicidal behavior. However, siRNA and piRNA have been implicated as possible precursors to degradation of mRNA involved in transcriptional activity in neuropsychiatric disorders.

mi-R regulate post-transcriptional modifications of gene expression in the genome (Castel et al., 2013; Flowers et al., 2013; Grewal et al., 2007; Mack, 2007). mi-R are short sequences (18–25 nucleotides) of RNA found in the cytoplasm of a genomic cell (Kosik, 2006; Schratt, 2009). Currently, there are approximately 2,042 mature human mi-R sequences known according to the mi-R database. Studies have predicted that mammalian mi-R participates in the regulation of transcriptional activity of ~50% of all protein-coded genes (Serafini et al., 2012). mi-R can be encoded by both protein-coding processes and noncoding transcriptional units, which allow them to be located in both exons and introns. mi-R is formed through the transcription of DNA in the cell nucleus by using a host promoter (intragenic) or by using their own promoters within CpG islands, TATA box sequences, initiation elements, and histone modification (collectively known as intergenic). The primary stem-looped RNA molecule becomes modified by a sequential series of enzymatic reactions to produce a mature, short,

single-stranded mi-R molecule. The mature mi-R molecule binds to a stabilizing protein to form complexes called RNA-induced silencing complex (RISC) which is also known as a microRNA ribonucleoprotein complex (miRNP). These complexes eventually collect in the cell's cytoplasm and become p-bodies, which ultimately regulate transcriptional activity within the cell.

mi-R regulates transcriptional activity by base pairing to mRNA and inhibiting translation into polypeptides or by inducing deadenylation and degradation of the target mRNA (Dwivedi, 2011; Tardito, Mallei et al., 2013).

mi-R is highly variable, in terms of expressivity, causing variable concentrations of protein derivatives to be produced. However, the variability of mi-R expression has not yet been fully understood. The effects of intracellular, extracellular, and external environments are thought to have important effects on the mi-R mechanism. This characteristic of mi-R makes it a candidate for gene-environment interaction studies investigating suicidal behavior.

Recent epigenetic studies have investigated the link between mi-R and completed suicide. An epigenetic study by Maussion et al. (2012) investigated the role of mi-R in the regulation of TrkB.T1 expression. The authors found that a significant increase in Hsa-miR-185 expression is associated with low TrkB.T1 cortical expression in four suicide victims. The study was replicated with 38 male suicide victims, and Hsa-miR-185 expression was reported to be significant in the frontal cortex, but not in any other areas of the brain. It can be suggested that an increase in Hsa-miR-185 expression in the frontal cortex may play a vital role in suicidal behavior. A study by Smalheiser et al. (2012) reported downregulation of mi-R in the prefrontal cortex of postmortem brains of depressed suicide subjects. Interestingly, the authors suggest that the networks of mi-R are implicated in the pathogenesis of suicide. mi-R networks are

responsive to changes in environmental stimuli, and contextual cues producing alterations to protein expression that may be involved in suicidal behavior. These studies have provided the framework for further studies to elucidate the epigenetic contributions of mi-R to the suicide phenotype.

1.7 Epigenetic studies of suicide: Candidate genes of suicidal behavior

Recent epigenetic studies have elucidated several epigenetic mechanisms as potential epigenomic markers of gene alteration in suicidal behavior. The purpose of epigenetic studies of suicidal behavior has been to understand how the alteration of gene expression and the inhibition or activation of transcriptional activity influence risk for suicidal behavior. These studies have presented evidence of the current knowledge of epigenetic mechanisms and their links to suicidal behavior. They address the candidate genes and whole genome studies of suicidal behavior from an epigenetic perspective.

Genetic association studies are the most simple and popular means of associating genetic factors to suicidal behavior in psychiatric populations and brains of suicide victims (Brezo et al., 2008). Molecular studies have mainly focused on the dysfunction of the serotonergic system (mainly the class of 5-hydroxytryptamine receptors) in the relationship to suicidal behavior (Arango et al., 2001; Du et al., 2001). Other significant neurotransmitter systems (and related candidate genes) possibly involved in suicidal behavior include dopaminergic system [tyrosine hydroxylase and catechol-o-methyl transferase (COMT)], noradrenergic system [monoamine oxidase A (MAOA), and adrenergic receptor alpha 2a], gamma-aminobutyric acid receptors, brain-derived neurotrophic factor and hypothalamic–pituitary–adrenal axis genes (corticotropin-releasing hormone, corticotropin-releasing hormone receptors, corticotropin-binding protein, and FK506-binding protein 5) (Dwivedi et al., 2006; Murphy et al., 2011). Many other genes have also been investigated such as P450 system genes, cholecystokinin, and lipoprotein lipase. However, few of these candidate genes of suicidal behavior have been explored from an epigenetic perspective.

1.7.1 5-Hydroxytryptamine receptor 2A

5-Hydroxytryptamine receptor 2A (HTR2A) variant in exon I, T102C, has been extensively studied in relation to suicidal behavior in subjects with psychosis and mood disorders (Du et al., 2000; Mann et al., 2000; Polesskaya et al., 2002). However, the function of the T102C gene variant remains unclear and its link to suicidal behavior has provided inconsistent results (Correa et al., 2007; Khait et al., 2005; Zalsman et al., 2005). It is possible that the T102C gene variant can directly influence mRNA expression levels by methylation of the C allele of a CpG dinucleotide in exon I of HTR2A.

Recently, Abdolmaleky et al. (2011) investigated the methylation of the entire HTR2A promoter region in the frontal lobe of suicide victims with major psychoses using 105 brain samples. It was found that dysregulation of the HTR2A promoter was linked to hypermethylation of DNA found in postmortem brains of suicide victims with major psychoses. This could have important implications as this study also found an inverse relationship between membrane-bound-COMT and HTR2A expression in suicide brains. It can be concluded that hypermethylation of the DNA encoding the HTR2A promoter leads to a downregulation of gene expression, which has downstream effects on other suicide candidate genes (e.g., COMT) Also, De Luca et al. (2009) investigated the methylation patterns in the HTR2A allele 102C in postmortem brains of suicide victims. De Luca et al. (2009) reported significant hypermethylation of the C allele in peripheral leukocytes in suicide attempters. However, the level of methylation between leukocytes and brains was strikingly different, with the peripheral leukocytes being more heavily methylated. However, the methylation differences did not suggest that the C allele influences completed suicide. Thus, the variability in methylation may be

attributed to specific environmental stimuli and can be the reason for inconsistent results produced in genetic association studies of HT2RA and suicidal behavior.

1.7.2 Gamma-aminobutyric acid type A receptor

Gamma-aminobutyric acid type A (GABAA) receptors have been studied in fewer genetic association studies of suicidal behavior compared to 5-HT genes (Baca-Garcia et al., 2004; Merali et al., 2004). However, Poulter et al. (2008) investigated the methylation patterns in the frontopolar cortex of postmortem brains in suicide victims at the GABAA receptor promoter region. In 14 brains of suicide victims, it was found that downregulation occurred in the DNA methyltransferase-1 (DNMT1) mRNA expression. DNA methyltransferase-3b (DNMT3b) was also found to be downregulated in the amygdala. The GABAA receptor was reported to be hypermethylated in the frontopolar cortex in ten brains of suicide victims. However, upregulation of DNMT3b expression was significantly associated with reduced GABAA expression. The involvement of GABAA in DNMT3b expression provides plausible evidence for the involvement of the GABAergic system with suicidal behavior. Further epigenetic studies are required to elucidate the contributions of the GABAA receptor (and GABAergic system) to the suicide phenotype.

1.7.3 Glucocorticoid receptor

Numerous hypothalamic–pituitary–adrenal (HPA) axis studies have implicated HPA axis dysfunction in individuals engaging in suicidal behavior (Liu et al., 1997). The glucocorticoid receptor (GR) has mainly been associated with negative feedback regulation of HPA axis activity

in response to elevated glucocorticoid levels (Presul et al., 2007; Turner et al., 2010; Turner et al., 2008). Recently, epigenetic modifications of the GR have been implicated in suicidal behavior. A study conducted by Labonte et al. (2012) investigated methylation patterns in GR noncoding exons 1b, 1c, and 1h in postmortem brains of childhood-abused (CASV) and nonchildhood-abused suicide victims (NCASV). The authors observed significantly decreased total expression of hippocampus glucocorticoid receptors 1b (hGR1b), 1c (hGR1c), and 1h (hGR1h) in the child-abused group compared to controls and in the non-abused suicide victims. Hypermethylation was found in both CASV and NCASV compared to controls at CpG6, CpG8, and CpG11 in hGR1h. Methylation levels at hGR1c were low, with significant methylation differences at CpG8, CpG9, CpG12, and CpG13 in CASV compared to the NCASV and the controls. Hypermethylation was observed at CpG8 and CpG9 in CASV compared to NCASV and controls, whereas hypomethylation was observed at CpG13 in CASV compared to NCASV and controls. Significant hypomethylation levels at hGR1h were observed for CASV at CpG2, CpG5, and CpG1. The hGR1b and hGR1c promoter global CpG methylation was negatively associated with hGR expression whereas the hGR1h promoter global CpG methylation was positively associated with hGR expression. The low methylation levels reported in the promoter regions are indicative of transcriptional regulation of hGR expression in CASV.

McGowan et al. (2009) conducted a similar study involving epigenetic regulation of GR. The authors reported an association between decreased levels of GR mRNA, including the 1F splice variant, and increased cytosine methylation of a GR promoter in CASV brains. It seems that childhood abuse has downstream effects on transcriptional activity in the HPA axis, contributing to the complexity of suicidal behavior.

1.7.4 Spermine/spermidine N1-acetyltransferase gene

Spermine and Spermidine are synthesized by spermine and spermidine synthases through the transfer of aminopropyl groups from decarboxylated S-adenosylmethionine (SAM) and are catabolized by the spermine/spermidine N1-acetyltransferase gene (SAT1) reaction. Downregulation of SAT1 expression has been found in suicide victims (Sequeira et al., 2006). Recent investigations of the polyamine system have provided insight on genetic and epigenetic modifications that regulate gene expression in the suicide phenotype that were not previously known.

An epigenetic study by Fiori et al. (2010) examined CpG methylation in the promoter regions of spermine synthase (SMS), spermine oxidase (SMOX), and H3K27 in the prefrontal cortex of suicide victims and controls. The authors reported no significant effects of the low CpG methylation of the promoter haplotypes in SMS and SMOX on expression levels in the prefrontal cortex. H3K27 modifications were also analyzed for levels of methylation at the SMS and SMOX promoter regions, but no difference between suicide victims and controls was found. The epigenetic mechanisms (CpG methylation and H3K27 histone modification) do not appear to significantly influence the regulation of expression of SMS and SMOX genes in the prefrontal cortex of suicide victims. A subsequent epigenetic study by Fiori et al. (2011) used prefrontal tissue from 10 postmortem brains of male suicide victims to determine the role of epigenetic modifications in the expression of SAT1. Tri-methylated H3K27 and promoter CpG methylation levels were investigated due to their role in gene suppression in the genome. It was reported that no haplotypes had significant differences in methylation patterns, but CpG methylation of the entire SAT1 excluding exon I was associated with decreased SAT1 expression in BA 8/9 region. Interestingly, there was no significant difference in global methylation and site-specific

methylation between suicide victims and controls. In terms of the H3K27 modification, there was no significant difference between controls and suicide victims and gene expression was not correlated with H3K27 levels.

1.7.5 Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) belongs to a family of proteins called neurotrophins, which include neurotrophin-3, -4, and -5 (Aid et al., 2007; Dwivedi, 2009; Dwivedi et al., 2003). BDNF is involved in development, survival, and maintenance of neurons in the central and peripheral nervous systems (Aid et al., 2007). Previous evidence suggests involvement of BDNF in behavior and disorders such as social stress, aggression, and anxiety (Roth, Lubin et al., 2009). Previous suicide research on BDNF had found abnormal BDNF expression levels in both suicide attempters and victims. However, the BDNF polymorphism, Val66Met, has been proven to be inconsistent across the association studies with suicidal behavior (Zai et al., 2012).

A recent study by Keller et al. (2010) reported increased BDNF promoter (exon IV) methylation levels in the Wernicke area of 44 suicide completers compared to non-suicide controls. The hypermethylation of the BDNF promoter (exon IV) in the Wernicke area of the suicide brains may contribute to the downregulation of BDNF expression in the genome. Genome-wide methylation analyses indicated that DNA methylation levels were significantly low across the genome, but not in the promoter. The hypermethylation of the BDNF promoter reveals a novel link between epigenetic mechanisms in the brain and alterations of genes implicated in suicidal behavior. A study by De Luca et al. (2011) examined the differential

allelic expression of the BDNF functional polymorphism Val66Met in suicide victims. They found no significant differences in BDNF Val/Met allele mRNA levels of suicide victims with major psychoses compared to non-suicide controls. Val/Met allelic ratios were significantly higher in Brodmann area 9 than Brodmann area 46, but no association with suicide was reported. It may be of particular interest to explore histone variants epigenetic mechanisms and mi-R implicated in BDNF gene expression, as there is some evidence of epigenetic alteration present in the BDNF gene.

1.8 Suicidal Behavior in Schizophrenia

Schizophrenia (SCZ) is a complex psychiatric illness marked by deficits in emotional responsiveness, deteriorating cognition and a loss of contact with reality (Hui et al., 2012; Lopez-Moríñigo et al., 2012). Schizophrenia is one of the most severe psychiatric disorders affecting approximately 1% of the population worldwide. Individuals with SCZ generally experience a range of symptoms, such as delusions, sensory hallucinations, bizarre behavior, flat or blunted emotions, and disorganized thoughts and speech (Phan et al., 2013).

Many individuals that attempt suicide often experience severe despair frequently attributed to a psychiatric disorder. In fact, schizophrenia (SCZ), a common psychiatric disorder, has been shown to increase an individual's risk for suicide (Saha et al., 2007). In Canada, we are arguably witnessing an increase in the rate of SCZ and higher prevalence and incidence rates compared to some international populations (Dealberto, 2013). SCZ may potentially arise due to a combination of environmental and genetic factors. Generally, SCZ onset is around late adolescence and early adulthood, producing a functional decline and then chronic impairment (Holder et al., 2014). SCZ, which is a major cause of disability, has severe consequences on patients and their relatives such as economic, health, and personal issues, especially in those with no sufficient treatment.

Individuals with SCZ generally experience a range of symptoms, such as delusions, sensory hallucinations, flat or blunted emotions, and disorganized thoughts and speech (Fletcher et al., 2009; Phan et al., 2011; Hovington et al., 2012). Accordingly, SCZ is associated with a significantly increased risk for self-injurious and suicidal behaviors (Brown et al., 2000). In particular, suicide attempts and suicide completion are one of the largest contributors to the increased morbidity and mortality rates in SCZ, respectively (Hawton et al., 2005). Recent epidemiological studies have identified that the current suicide risk for patients with SCZ is

approximately 4.9% (Palmer et al., 2005) or 0.24 per 100 people per year (Fleischhacker et al., 2014). It has also been proposed that approximately between 20 and 40% of patients diagnosed with SCZ will attempt suicide during the duration of their illness (Pompili et al., 2007). Sadly, attempting to identify individuals with SCZ at the highest risk for suicide remains a major obstacle. Some studies have proposed that an increased incidence of SCZ is associated with some ethnic minorities and migration status (Kirkbride et al., 2014; Fearon et al., 2006; Leão et al., 2006).

1.8.1 Risk for suicide in schizophrenia

Many individuals who attempt suicide often experience severe despair frequently attributed to their underlying predicaments, including psychiatric disorder. The most critical issue within schizophrenia is the alarmingly high rate of suicidal behavior (Palmer et al., 2005; Tandon, 2005). In fact, SCZ has been shown to substantially increase an individual's risk for suicide (Brown et al., 2000). The SCZ symptoms may be associated with an increased risk for self-injurious and suicidal behaviors (Brown et al., 2000). In particular, suicide attempts and suicide completion are among the largest contributors to the increased morbidity and mortality rates in SCZ (Hawton et al., 2005). Recent epidemiological studies have identified that the currently revised suicide risk for patients with SCZ is approximately 4.9% (Palmer et al., 2005). Many studies have proposed a host of risk factors that may contribute to suicide risk in SCZ; however, their ability to accurately predict suicide risk in individual patients is limited.

Discrepancies in the ability to identify suicide risk factors in SCZ are largely attributed to differences in research methodologies and target samples that attempt to assess single risk factors for suicide. This inconsistency may be attributed to epidemiological studies focusing on patients

at different stages of the illness: first-admission individuals with schizophrenia are at a higher risk for suicidal behavior compared to chronic patients (Alaraisanen et al., 2009). The risk for suicidal behavior in SCZ is greater during the early years of disease onset, typically during the first 5 years following a diagnosis (Heilä et al., 2005). Despite a relative decline in the suicide rate among older adults with SCZ, it is still greater than among the general population (Cohen et al., 2010; Montross et al., 2008). Studies have been shown that patient demographics, illness-related factors, comorbid substance abuse, previous suicide attempt(s), lifetime Stress, and biological risk factors are the robust risk factors that potentiate suicidal behavior in SCZ.

1.8.1.1 Patient demographics

Many studies have attempted to identify key demographic traits that are predominantly shared among those with SCZ who also demonstrate suicidal behavior. Age, gender, marital and employment status of these individuals have all been found to contribute to the likelihood that they will attempt suicide.

It appears that there are two major peaks in age for when suicide risk is highest. Previous studies have demonstrated that being of a younger age group leads to an increased predisposition for suicide, compared to the general population (Hor et al., 2010). Interestingly, completed suicide occurs at a younger age in SCZ (at around 30 years old) compared to other psychiatric disorders (Gómez-Durán et al., 2012). Another study suggested that there is a higher risk for suicide among patients over the age of 50 (Hor et al., 2010).

Individuals with SCZ typically develop symptoms at a younger age. According to van Os and Kapur (2009), the peak period for the onset of SCZ is during late adolescence and early

adulthood. Additionally, as the risk for suicide also appears to peak around five years following illness onset (Carlborg et al., 2010) it is not surprising that suicide risk is associated with younger age. Particularly, individuals with first-episode psychosis (FEP) with longer duration of first hospital treatment, the presence of depressive symptoms, and nonadherence to treatment in early phases of follow up after FEP predict future suicide attempts (Togay et al., 2015). When diagnosed at a young age, an FEP patient who believes that having schizophrenia may lead to a deteriorating mental state and lower quality of life can react differently to someone with high insight but a more optimistic outlook on the course of the disorder (Melle et al., 2012). Several studies indicate that beliefs about psychosis can influence insight and aspects related to suicide risk, as patients with high insight and low levels of stigmatizing beliefs report lower levels of depression, more hope, better self-esteem and better quality of life than patients with high insight and high levels of negative beliefs (Linden et al., 2007; Staring et al., 2009).

Young adulthood is a critical period in an individual's social and career growth and development (Addington et al., 2007). The debilitation from the onset of SCZ during these formative years may lead to poor employment rates and being single, which are also independent risk factors for suicide in SCZ. Previous reports have found that being single, unemployed, and unable to work generally increases the risk for suicide in the overall population and among individuals with mental illnesses such as SCZ (Popovic et al., 2014).

Further studies on patient demographic risk factors for suicide in SCZ have also identified gender as a contributing factor. It is already well established that there are differences in suicidal behavior between men and women, regardless of whether or not they have been diagnosed with SCZ (Carlborg et al., 2010). In fact, in the general population, men die more often by suicide than females, while females attempt suicide much more frequently. In contrast,

among the SCZ population, women have been described as displaying male tendencies for suicide whereby they act impulsively and aggressively and choose lethal methods for suicide (Caldwell et al., 1990). The odds ratio for suicide in SCZ in men compared to women is approximately 1.574, suggesting a less extreme gender difference in suicide risk than seen in the general population. Nevertheless, while less frequently reported, other studies have suggested that women have a higher risk for suicide in SCZ or that there is no difference in suicidal behavior between genders (Popovic et al., 2014).

Considering all reported controversial demographic factors that contribute to suicide risk, some studies have attempted to assign a diagnostic profile for the average suicide attempter with SCZ. Fenton et al. (2000) characterized a typical patient with SCZ at high risk for suicide as a young male, with good pre-morbid functioning and late age of first hospitalization, high IQ, paranoid subtype, and retention of abstract thinking. While these variables do contribute to the suicide phenotype in SCZ, the relative contributions of each in determining suicide risk remains ambiguous. An updated review by Pompili et al. (2007) characterized a high-risk suicide phenotype as typically a young Caucasian male, never married, with good pre-morbid functioning, post-psychotic depression, history of substance abuse, and previous suicidal behavior (attempt, self-harm, ideation). Many of these identified risk factors are considered in today's risk assessments; however, further investigations have taken into account the role of more recently reported risk factors.

1.8.1.2 Illness-related factors

Schizophrenia is a debilitating mental disorder that entails a range of positive and negative symptoms that are often exacerbated by comorbid affective disorders or symptoms, and treatment complications. Given the degree of dysfunction and life disruption that an average individual with SCZ endures, it is not surprising that a significant proportion of these individuals attempt or consider suicide.

One of the most controversial illness-related risk factors for suicide in SCZ is the impact of insight by a patient into their illness. Insight is generally described in psychiatry as being able to recognize one's own mental illness and its consequences. Insight can have major repercussions on many related clinical aspects over the course of the illness, such as recognizing the need for treatment and complying with medication recommendations (Amador et al., 2004). Intact insight in SCZ is associated with medication adherence, lower symptomatology, and better treatment outcomes. At the same time, greater insight is linked with hopelessness, depression, and suicide (Amador et al., 2004). Generally, insight has been proposed to be a factor which increases the risk for suicide in SCZ; however, it has not been sufficiently investigated and conflicting results have been reported. In a meta-analysis by Hawton et al. (2005), there was no statistically significant association between the presence of insight and risk for suicide. This finding, however, is likely attributed to the low sample size of the studies used to investigate insight and suicide risk.

The major argument in favor of insight leading to higher suicide risk in SCZ is that it is often correlated with a sense of hopelessness, demoralization, and depression. Furthermore, feeling hopeless is one of the most consistently reported risk factors for suicide in SCZ and other psychiatric disorders (Amador et al., 2004). On the other hand, other studies have found that

individual insight is the first step in gaining mastery over their illness and is usually associated with improved treatment compliance (López-Moriñgo et al., 2012). Notably, the meta-analysis by Hawton et al. (2005) considered insight and compliance as separate entities and found a significant association for poor compliance as a risk factor for suicide. Therefore, it can also be argued that better insight may reduce suicide risk via improved treatment compliance.

Whether or not it is accepted that hopelessness, self-depreciation, and depression are the direct consequence of clinical insight by the patient, it remains clear that these affective symptoms pose a high risk for suicide in individuals with SCZ. Hopelessness has often been proposed as an important risk factor for suicide, perhaps even more than depression (Friedman et al., 2004). Patients who feel hopeless about their situation as a result of their illness and life circumstances are often the ones that resort to suicide. In fact, hopelessness is a key risk factor that interacts with illness insight. Some studies have suggested that insight into the illness is only associated with suicide if it is followed by hopelessness (Amador et al., 2004). For that reason, hopelessness can be seen as the deciding factor in the debate as to whether or not illness insight leads to increased suicide risk. When individuals' insight into their illness is high but they do not have severe feelings of hopelessness, it is more likely that they will experience the benefits of good insight. On the other hand, if individuals with SCZ have clear insight into their illness and see their situation as hopeless, they more likely develop suicidal behavior.

Depression is, of course, another major factor in predicting suicide risk. Previous studies have demonstrated that many SCZ patients experience a mood disorder during their lifetime (Hawton et al., 2005). In fact, many of these individuals experience depressive symptoms concurrently with suicidal behavior (Hawton et al., 2005; Pompili et al., 2013). Specifically, having a comorbid depressive disorder is believed to elicit suicidal behavior in patients with

SCZ. Unfortunately, many studies have failed to take into account the timing and severity of depressive symptoms in assessing suicide risk (Carlborg et al., 2010). It appears that one of the periods of highest suicide risk occurs during the 6 months post-discharge phase following hospitalization, when the illness remains active and during which the relationship between depression and suicide is proposed to be highest (Heilä et al., 2005).

Hospital admissions are an important risk factor for suicide in SCZ as they demonstrate important evidence regarding the mental state of the patient. Previous findings have suggested that suicide risk is highest when considering the time of hospitalizations (Qin et al., 2005). Approximately one-third of SCZ and general inpatient suicides occur during admission or one week following hospital discharge. There is also evidence that suggests that the risk for suicide remains high for as long as one year following discharge (Kao et al., 2011). As expected, the number of hospitalizations is also indicative of the severity of the SCZ illness. This may represent the cumulative extent to which an individual experience on any debilitating positive symptoms, leading to previously described feelings of hopelessness and depression.

When assessing the risk for suicide in SCZ, it is necessary to examine the extent to which psychotic symptoms associated with the illness play a role. Historically psychosis has been associated with increased suicidal behavior in SCZ (Fenton et al., 2000; De Hert et al., 2001). At first glance, this may be explained by the tendency for irrational thinking during periods of psychosis. Despite any initial logical reasons for why someone with SCZ would want to attempt suicide, experiences from the content of delusions or thought disorders may be the deciding factor. Another way in which psychotic symptoms may act as risk factors for suicide is that they may drive the patients to inflict self-injury without suicidal intent but their impaired judgment leads them to miscalculate the probability of death from their action (Fenton et al., 2000).

Additionally, these individuals may be experiencing delusions in which they feel that they are impervious to physical harm or experiencing hallucinations that distract them from this risk (Wong et al., 2013). Even though these are plausible explanations for the association between psychosis and suicide risk, there is little substantive evidence for these in the literature.

Another explanation for an increase in suicide risk during psychosis is the idea that individuals respond to command hallucinations that instruct them to kill or harm themselves. Command hallucinations are cases in which patients hear voices which tell them to perform certain acts, occurring in 18-50% of the SCZ population, including the command to kill themselves (Wong et al., 2013). However, there is limited and conflicting evidence for command hallucinations being associated with suicide in SCZ. In one of the first controlled studies investigating an association between suicidal behavior and command hallucinations, Hellerstein et al. (1987) found no significant differences in suicide ideation in SCZ. On the contrary, however, additional studies have shown an association between having command hallucinations and a history of suicide (Wong et al., 2013).

Previously, studies have suggested that psychotic symptoms per se may not be the actual cause for increases in suicide risk in SCZ, but the distressed and low mood that follows (Hu et al., 1991). This concept has been observed in previous reports in which more severe levels of psychosis are associated with suicide. This pathway to suicidal behavior in SCZ represents another way in which multiple risk factors interact.

More recent research regarding psychosis and suicide risk in SCZ has attempted to observe whether specific symptoms, namely positive and negative symptoms, are more likely to lead to increased suicide risk (Pješčić et al., 2014). Positive symptoms typically include

hallucinations, delusions, and disorganization, while negative symptoms usually involve flat affect, anhedonia and a lack of motivation. In contrast to analyzing command hallucinations only, studies considering all positive symptoms in its risk for suicide have found more promising results (De Hert et al., 2001). Even though there is little evidence for command hallucinations playing a significant role in suicide risk, several studies have suggested that active positive symptoms are associated with attempted suicide (De Hert et al., 2001). Furthermore, the total number of positive symptoms is also associated with suicide risk.

Among the illness-related factors, it is not easy to determine what factor is at highest risk for suicide in schizophrenia. However, the systematic review of Hawton et al. (2005) stated that the clinical factor with the highest relative risk for suicide in schizophrenia is the fear of mental disintegration.

1.8.1.3 Comorbid substance abuse

Substance abuse is commonly associated with SCZ and is often believed to influence suicide risk in these individuals. In a previous study, comorbid substance abuse was found more often among younger individuals with SCZ who exhibited suicidal behavior (Pompili et al., 2012). While substance abuse is often considered when assessing suicide risk in SCZ, there is not necessarily a difference in the frequency of drug abuse between individuals with SCZ who attempt and do not attempt suicide (Harkavy-Friedman et al. 2004). On the other hand, individuals with SCZ who are substance abused may have experienced more positive symptoms, particularly hallucinations, and correspondingly more suicide attempts compared to patients with

no substance use (Sokya et al., 2000). Notably, hallucinations have been found to increase the incidence of suicide attempts in SCZ independently from alcohol or drug abuse/dependence.

As expected, substance abuse is associated with impulsiveness and thus suicidal behavior. However, in general, this association is not unique in SCZ. It appears that substance abuse, while not directly influencing suicide risk, does, in fact, impact many other risk factors for suicide that may increase the risk for suicidal behavior in SCZ, such as treatment noncompliance, loss of self-control, violence, and economic difficulties (Harkavy-Friedman et al. 2004). These risk factors may thus become stressors which trigger suicidal behavior later. Given these examples, current research on suicide in SCZ takes into account the role of substance abuse, not as an independent factor, but as a modulating risk factor that may increase the risk for suicide. While reports of alcohol abuse and suicide in SCZ have been conflicting, a recent study by Zai et al. (2014) demonstrated the interaction between specific genetic variants and suicidal behavior that was only significant when alcohol abuse or dependence was considered in the analysis.

1.8.1.4 Previous suicide attempt(s)

Previous suicide attempts or ideation have a strong positive correlation with completed suicide in SCZ patients and have been among the strongest predictors of suicidal behavior in patients with FEP (Hor et al., 2010). Moreover, the strongest predictor for suicide attempt in SCZ is a past history of deliberate self-harm behavior that includes past suicide attempting and acts in which other motives may have been more prominent (Hawton et al., 2005).

A history of suicide attempt appears to elevate the risk for completed suicide threefold (McGirr et al., 2008; Reutfor et al., 2009). McGirr et al. (2008) found that individuals with SCZ

who were admitted for attempted suicide had the highest risk (of all variables studied) of committing suicide. Reutfor et al. (2009) reported that the proportion of suicide patients with an earlier suicide attempt was 32%. This is lower than the 40-71% estimated in previous studies (Heilä et al., 2005). However, Reutfor's finding of a five-fold higher suicide risk associated with a history of a prior suicide attempt is in the same range as several previous findings, and comparable to the risk estimate obtained in the meta-analysis by Hawton et al. (2005).

Patients with a history of suicidal behavior (attempt, ideation, self-harm) may also be suffering from emotional dysregulation that may be related to affective symptoms or a general inability to express emotion in an empathetic way (Hawton et al.,2005). Suicidal ideation often fluctuates and many patients may think about suicide (McGirr et al., 2008). Although active suicidal ideation is considered a significant risk factor of suicidal behavior, it may act as a deterrent in some cases (when not associated with a command hallucination) (Nordentoft et al., 2004). More specifically, it may be a coping factor for SCZ patients to engage in thoughts about the emotional significance of suicide and the reasons for performing the behavior. Nevertheless, it is important that clinicians monitor active suicidal ideation in patients that have attempted suicide in the past.

1.8.1.5 Lifetime stress

One of the most highly studied risk factors for suicide currently is a history of stressful life events (SLEs), particularly considering childhood abuse and trauma (Enns et al., 2006). Unfortunately, only few studies have examined SLEs and suicidal behavior in SCZ, and the vast majority of them are association analyses for suicide completion. SLEs frequently precede

suicide and they were identified in almost half the cases of suicide in SCZ in a Finnish national psychological autopsy study (Hor et al., 2010). Furthermore, another study (Enns et al., 2006) found that suicide in SCZ is more likely to occur following a severe life event associated with aggressive behavior or psychiatric impairment, and a severe life event of a humiliating nature.

In a retrospective cohort study by Melle et al. (2013), life dissatisfaction was found to be a significant predictor of suicide in FEP patients. It is possible that they are in a period of their life that is accompanied by high stress, and the burden of a chronic illness may interact with predisposing suicide traits (Fenton et al., 2000). In a retrospective report by Cohen, Abdallah and Diwan (2010), suicide risk in older adults with SCZ was associated with the lifetime traumatic and victimization scale, revealing that lifetime stressful events do contribute to the suicide phenotype. In particular, higher rates of early sexual and physical abuse were modestly associated with suicide attempt history (Cohen et al., 2010).

Roy (2005) analyzed the effects of childhood trauma on 50 chronic schizophrenic patients with and without a history of suicide attempts. The author found that schizophrenic patients who attempted suicide had significantly higher scores for the Childhood Trauma Questionnaire (CTQ) subscales of emotional, physical and/or sexual abuse, and emotional and/or physical neglect. The suicide phenotype was also associated with a higher CTQ total score. Despite the clear association between childhood adversity and suicidal behavior, this association may have been a result of increased psychopathology in SCZ patients that attempted suicide (Funahashi et al., 2000). Ucok and Bikmaz (2007) found that childhood trauma was associated with increased psychopathology and suicide attempts before admission in patients with FEP. This finding is interesting because previous studies have found an association between early life stress and development of mental illnesses. Therefore, it is not conclusive that early life stress

contributes to the suicide phenotype directly, but rather indirectly through its effects on illness severity (Cohen et al., 2010).

1.8.1.6 Biological risk factors

Clinical studies showing the effectiveness of clozapine in reducing suicidal behavior in SCZ (Meltzer et al., 2003) pointed out on the involvement of biological factors related to the mechanism of action of the atypical antipsychotics in influencing suicidal behavior in SCZ.

Therefore, in addition to the previously described psychosocial risk factors, there is also evidence for a biological basis for suicide.

Among the studies investigating the biological basis of suicidal behavior, genetic studies have had a prominent role. In fact, familial studies, adoption studies, and twin studies suggest that suicide is both genetic and heritable (Fu et al., 2002; Glowinski et al., 2001). Previous studies also suggest that the heritability of suicidal behavior is 43% (Bondy et al., 2006; McGuffin et al., 2001). Notably, monozygotic twins show a greater concordance rate for attempted and completed suicide compared to dizygotic siblings (Roy and Segal, 2001). Schizophrenia patients who are relatives of individuals who committed suicide have increased risk of attempting suicide likely caused by familial aggregation (Qin et al., Trémeau et al., 2001). The familial aggregation of suicide and schizophrenia diagnosis also extends to siblings of schizophrenia patients who have a significantly increased risk for suicide (Alaraisanen et al., 2009). For instance, suicide completion was eight times higher in the offspring of fathers with SCZ. Furthermore, Ljung et al. (2013) found that the risk of having SA or suicide completion in offspring of SCZ parents was two times higher. Given the aforementioned evidence, it is possible

that the genetic risk profile for suicidal behavior in SCZ is similar to the genetic risk for developing SCZ.

Early attempts at uncovering biomarkers for suicide tried to evaluate serotonin dysfunction in suicidal brains by measuring cerebrospinal fluid (CSF) monoamine metabolites and serotonin receptor-binding in fronto-orbital brain tissue. While low levels of indices of serotonin activity were associated with suicide in the general population, this was not replicated in a SCZ population (Chistiakov et al., 2012). Only one study found a significantly lower concentration of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the CSF of individuals with SCZ who completed suicide (Mann, 1999). While the serotonergic system is one of the most extensively studied in terms of suicidal behavior, inconclusive evidence for several serotonin receptors and candidate genes has been widely reported Anguelova et al., 2003; Buttenschøn et al., 2013; Judy et al., 2012).

Continuing onward, many studies began looking for potential genes or genetic markers that may influence suicidal behavior in SCZ. A large portion of these studies focused on candidate genes in an attempt to identify specific genetic variants or single nucleotide polymorphisms (SNPs) that may indicate a risk for suicide. The most investigated genes believed to be associated with suicidal behavior in SCZ are those of the serotonergic system. One of these major candidate genes is the serotonin 2A receptor (HTR2A) (Serretti et al., 2007). More than 200 SNPs along the gene have been characterized, but only two SNPs [rs6313 (T102C), rs6311 (A1438G)] have been extensively investigated in suicidal behavior (Tsai et al., 2011). It has been repeatedly shown that rs6313 may not be involved in suicide among SCZ patients. Both Ertugrul et al. (2004) and Tan et al. (2002) did not find an association between the T102C polymorphism and suicidal behavior. Correspondingly, an A allele, A/- genotypes of the

polymorphism rs6311 G>A, and a T allele, T/- genotypes of the polymorphism rs6313 C>T, were found to be less frequent in SCZ patients with a history of suicide attempt than among patients with SCZ without a history of suicide attempt, thus suggesting their protective role in the development of suicidal behavior.

In addition to HTR2A, tryptophan hydroxylase (TPH) genes were among the first serotonergic genes to be investigated regarding suicidal behavior, specifically TPH1 and TPH2. An early study by Paik et al. (2000) examined a Korean sample of individuals with SCZ who had and had not attempted suicide, in addition to controls. They looked at the A218C variant in the gene TPH1. They found that the C allele was more frequently observed among individuals with SCZ who attempted suicide. In a later study by Saetre et al. (2010), A218C as well as A779C were tested among a sample of SCZ patients with and without a history of suicide attempt. This study, however, found no significant associations for both polymorphisms when comparing attempters and non-attempter patients. A polymorphism in the promoter region of TPH2 was also examined for a relationship with suicidal behavior in SCZ (De Luca et al., 2005). TPH2 has one polymorphism (−473T>A) in the putative promoter and many highly polymorphic variants in introns within the gene. Allele and haplotype frequencies for this promoter polymorphism and other variants demonstrated no significant difference among a cohort of SCZ patient with and without a history of suicidal behavior (De Luca et al., 2005).

The serotonin transporter (5-HTT) is crucial in regulating the amount of serotonin in the synapse of the brain. Not surprisingly, the serotonin transporter gene (SLC6A4) and the common functional promoter polymorphism (5-HTTLPR) have been extensively investigated in many suicide studies. The 5-HTTLPR promoter contains an insertion/deletion polymorphism that leads to either a long (L) or short allele (S). In an early report, Bayle et al. (2003) demonstrated

genotypic and allelic differences for the 5-HTTLPR polymorphism between violent and non-violent suicide attempters. Furthermore, violent attempters also differed from non-attempters according to genotype distribution. In both associations, the S allele was more frequently represented among violent suicide attempters. However, this study found no significant difference between suicide attempters (both violent and non-violent) and non-attempters according to genotype and allele distributions. In a Chinese sample of SCZ patients, rather than the short allele, it was the L allele that was more frequently represented in male suicide attempters compared to those with no suicidal history (Hung et al., 2011). This was also seen in Božina et al. (2012) in which the L allele was more frequently carried by suicide attempters. In addition to the conflicting evidence, a negative association was found for this polymorphism (Contreras et al., 2010); however, the suicidal behavior they investigated was not clearly indicated. Finally, in a Scandinavian sample which consisted of 837 SCZ patients, Carlstrom et al. (2012) tested seven polymorphisms in SLC6A4 for suicidal behavior. In this study, an association between a polymorphism in intron 1 (rs16965628) of SLC6A4 and suicide attempters was found. While studies of 5-HTTLPR and suicide in SCZ provide inconclusive results, preliminary findings for SLC6A4 appear promising though require further replication.

Candidate genes other than those of the serotonergic system have been investigated, though not as extensively, as potential players in modulating suicide risk in SCZ. Genetic variants for monoamine oxidase A (MAOA) and catechol-o-methyl transferase (COMT), for example, have been investigated for their potential in conferring risk for suicide in SCZ. De Luca et al. (2006) looked at the promoter VNTR polymorphism in the MAOA gene and three COMT polymorphisms (-287A>G, Val/Met, and 3'UTR C Insertion/Deletion) among 270 SCZ patients. However, no association between suicide attempt and the polymorphisms were found, including

haplotype analyses. An inhibitory neurotransmitter, γ-Aminobutyric acid (GABA), was also tested for an association with suicide attempt when taking alcohol use into account (Zai et al., 2014). Haplotypes for rs183294 and rs209356 in the GABA subunit GABRG2 gene were significantly associated with a history of suicide attempt in a SCZ sample. This study suggests that GABRG2 may be able to predict suicide attempters in SCZ among those with a history of alcohol abuse or dependence. Lastly, the hypothalamic-pituitary-adrenal (HPA) pathway is becoming increasingly implicated in suicidal behavior. As the only study to date to examine the role of HPA axis genes and suicide attempt exclusively in SCZ, De Luca et al. (2010) analyzed and tested six HPA genes (CRH, CRHR1, CRHR2, CRHBP, MC2R, NC3R1) for an association with previous suicide attempt history. Genotype analyses revealed a significant association between CRHBP and suicide attempt, and interaction analyses demonstrated a significant interaction between CRHR1 and CRHBP with suicide attempt.

Beyond traditional genetics and candidate gene studies, researchers have attempted to uncover whether differences in transcription and gene expression represent a marker for suicidal behavior in SCZ. Dopamine-and-cAMP-regulated neuronal phosphoprotein (32 kDa) (DARPP-32) is expressed in brain regions receiving dopaminergic projections and is implicated in the pathophysiology of schizophrenia. Feldcamp et al. (2008) found differences in DARPP-32 mRNA expression levels between SCZ patients who died by suicide and patients who died of other causes. Suicide completers with SCZ also demonstrated differences in gene expression compared to controls with no history of psychosis. Garbett et al. (2008) attempted to measure gene expression in the frontal cortex of SCZ patients who died by suicide and patients who died of other causes using DNA microarray expression profiling. Expression analyses revealed a significant prefrontal cortical downregulation of the HTR2A transcript in suicide completers

compared to controls and non-suicidal SCZ patients. This approach further led credence to the belief that the serotonin system is implicated in suicide in SCZ.

1.9 Structural and functional studies of suicidal brain

Functional magnetic resonance imaging (fMRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT) are non-invasive techniques which offer an in vivo estimation of regional brain functioning with an acceptable spatial resolution (Desmyter et al., 2011). With perfusion tracers such as 99mTc-ECD or 99mTc-HMPAO, or metabolism tracers such as 18F-FDG, functional brain imaging can provide insight into the brain regions and the circuitry involved during the pharmacological challenge studies with serotonergic agents. Through the use of radioligand tracers with specific affinity for the serotonin transporter (5-HTT) and the serotonin 1A receptor (HTR1A), functional brain imaging offers the possibility to investigate the binding index of these tracers to transporters or receptors. The six recent reviews on neuroimaging studies of the suicidal brain (Desmyter et al., 2011, Jollant et al., 2011, van Heeringen et al., 2011, Cox Lippard et al., 2014, Zhang et al., 2014, Jollant, 2016) agreed on the involvement of particular brain areas in suicidal behaviour, including the dorsolateral and orbitofrontal cortex. Moreover, van Heeringen et al. (2014a) outlined six structural and functional areas of vulnerability: the left superior temporal gyrus, rectal gyrus, and caudate nucleus were associated with structural vulnerability while the right cingulate gyrus, the anterior cingulate and posterior cingulate were associated with functional vulnerability. However, no final picture emerged from the reviews with regard to other cortical regions and subcortical involvement. Moreover, sample sizes of studies published within that time were small, hampering the interpretation of findings.

1.9.1 Neuroimaging studies of suicidal behavior in schizophrenia patients

Brain imaging is a non-invasive and in vivo direct estimation of detailed brain structure, regional brain functioning and estimation of molecular processes in the brain.

Structural studies of the suicidal brain in SCZ have found abnormalities in the frontal, temporal, and subcortical brain regions. A surface-based T1-weighted MRI technique was used by Besteher et al. (2016) to study the cortical folding and thickness of the brain in a group of SCZ patients, in which 14 attempted suicide and 23 did not. They found cortical thinning in the right dorsolateral prefrontal cortex, superior temporal lobe, middle temporal lobe, and the insular cortex in the suicidal patients compared to the non-suicidal patients. Moreover, they included 50 healthy controls to compare the suicidal SCZ patients and found significant cortical thinning of the lateral orbitofrontal and superior frontal gyri, bilateral superior caudal middle frontal gyrus, right caudal ACC, left pars orbitalis, pars opercularis and triangularis. However, in these areas, there were no differences between the suicidal SCZ group and the non-suicidal SCZ group.

In a large cohort study (Giakoumatos et al., 2013) of patients with SCZ and bipolar I disorder, SCZ attempters had lower left thalamus volume compared to non-attempters and healthy controls, but this difference was not significant. Interestingly, in high lethality attempters with SCZ, the volumes of the left lingual, right cuneus and lateral occipital, and bilateral pericalcarine were reduced. A history of low lethality was associated with decreased volumes of left supramarginal gyrus, thalamus and the right insula compared to non-attempters.

In another whole-brain Voxel-based morphometry (VBM) study with SCZ patients, suicide attempters had lower grey matter volumes in the left superior temporal lobe and left orbitofrontal cortex (OFC), compared to non-attempters. Interestingly, significant differences in

the amygdala volumes were not reported, which could be attributed to the small sample size (13 SCZ attempters) and inherent heterogeneity of suicide attempters with SCZ (Aguilar et al., 2008). However, an increased volume of the right amygdala was seen in SCZ patients with a previous suicide attempt, as compared to non-attempter SCZ patients and healthy controls (Spoletini et al., 2011).

A study using VBM (Rüsch et al., 2008) found that greater white matter volumes were revealed bilaterally in the posterior orbital and inferior frontal gyri in suicide attempters compared to non-attempters. Interestingly, no other regions showed white matter volume differences between the two groups.

Chapter 2
Rationale, Hypothesis, and Objectives

2 Studies 1-4

2.1 Study 1

2.1.1 Rationale

Few GWAS have analyzed suicide attempt in SCZ compared to several GWA studies of suicidality in mood disorders. To date, no studies have investigated DNA methylation and polymorphic CpG sites as potential DNA methylation regarding suicide attempt in schizophrenia patients.

2.1.2 Hypothesis

We hypothesized that there is a significant association between CpG SNPs and suicide attempt in schizophrenia.

2.1.3 Objectives

This study has two main objectives: the primary aim is to compare suicide attempters and non-attempters in regards of 384 SNPs across the genome. The secondary aim is to develop a new methodology to select and analyze CpG SNPs. This technique could be applied to different phenotypes other than suicide and schizophrenia.

2.2 Study 2

2.2.1 Rationale

Despite previous investigations examining the polymorphisms of serotonin receptors in suicidal behavior, little is known about the relationship between DNA methylation level and CpG SNP variation of 5-HT genes in suicide.

2.2.2 Hypothesis

DNA methylation and CpG SNP in HTR2A may be associated with a risk of suicide attempt in SCZ patients.

2.2.3 Objectives

The aim of the present study is the combined analysis of HTR2A methylation and CpG SNP in HTR2A to ascertain the contributions of HTR2A in conferring a risk for suicide attempt in SCZ.

2.3 Study 3

2.3.1 Rationale

Methylation analysis of HTR2A in suicidal behavior has not been fully investigated across different tissues, and little is known about the correlation of DNA methylation of HTR2A in peripheral tissues.

2.3.2 Hypothesis

DNA methylation of HTR2A exon I will be associated with attempted and completed suicide in schizophrenia.

2.3.3 Objectives

This study has two objectives. The main aim of the study is to investigate DNA methylation in HTR2A exon I CpG sites as possible risk factors for suicidal behavior. The second aim is to analyze the methylation level in three different tissues as epigenetic mechanisms are tissue specific.

2.4 Study 4

2.4.1 Rationale

Parent of origin effect (POE) of serotonin genes in suicidal behavior has been investigated by some studies; however, no studies have examined POE of the HTR1B gene. Moreover, previous studies have failed to find alleles in 5-HT genes that are significantly over transmitted in individuals who have a history of suicide attempt.

2.4.2 Hypothesis

Our hypothesis is that differential allele-specific expression of the rs6296 SNP is associated with suicidal behavior.

2.4.3 Objectives

The main objective of this study is to investigate the role of allelic imbalance of the C861G SNP (rs6296) in HTR1B within the frontal cortex of suicide victims.

Chapter 3
Analysis of CpG SNPs in 34 genes: association test with suicide attempt in schizophrenia

3 Abstract

Background: Suicide is the act of intentionally causing one's own death. The lifetime suicide risk in schizophrenia is 4.9% and 20% to 50% of patients with SCZ will attempt suicide during their life. The other risk factors for suicidal behavior in schizophrenia include prior history of suicide attempts, active psychosis, depression, and substance abuse. To date, there are no robust genetic or epigenetic predictors of suicide or suicide attempt in this specific population.

Methods: We collected detailed clinical information and DNA samples from 241 schizophrenia patients and performed the genetic analyses in suicide attempters and non-attempters, among these patients. Using the structured research interview, we determined the presence of suicide attempt lifetime and then we tested 384 DNA variants in candidate genes supposed to be involved in the neurobiology of schizophrenia. We applied a novel mapping analysis using a specific bioinformatic tool that analyzed only the polymorphic CpG sites in our SNP panel. This analysis looked at the presence or absence of methylation sites affected by the SNP allele. The SNPs in the candidate genes were studied under a different perspective considering their direct contribution to the availability of methylation sites within the gene of interest. The level of potential methylation was compared using a linear model in attempters and non-attempters.

Results: Among the 384 SNPs selected from the Illumina Bead Chip only the rs2661319 in the RGS4 gene was significantly associated with suicide attempt (p=0.002). There were 119 CpG SNPs in the aforementioned panel. The gene-wise potential methylation level of RGS4 was 55% in the attempters and 65% in the non-attempters with a p-value of 0.005. The total level of

potential methylation in the overall panel (119 SNPs combined) was not associated with suicide attempt. However, when considering the potential methylation at chromosome 1, we found that suicide attempt ($p=0.036$) was associated with lower methylation.

Discussion: The overall results showed no strong association between CpG SNPs and suicide attempt but the information regarding the CpG SNP potential methylation could be used as a covariate in future methylation analysis.

3.1 Introduction

Schizophrenia (SCZ) is one of the most severe psychiatric disorders affecting approximately 1% of the population worldwide. The symptoms include delusions, hallucinations, disorganized speech, bizarre behavior, and emotional blunting around others. The lifetime suicide risk is 4.9% in SCZ (Palmer et al., 2005) and 20% to 50% of patients with SCZ will attempt suicide (Radomsky et al., 1999).

Adoption, family and twin studies strongly suggest genetic basis of suicidal behavior (Brent and Mann, 2005; Bondy et al., 2006).

There are no Genome-Wide Association Studies (GWAS) analyzing suicide attempt in SCZ compared to several GWAS studies for suicidality in mood disorders.

Recently, several studies (McGowan et al., 2008; Poulter et al., 2008; Ernst et al., 2009; Guipponi et al., 2009; McGowan et al., 2009; Keller et al., 2010) that have investigated DNA methylation patterns in suicide victims have been published. Interestingly, there are several DNA methylation studies of completed suicide in mood disorders, that have reported altered DNA methylation patterns in the brain of suicide completers with both hyper and hypomethylation in gene promoters across the genome (Labonte et al., 2012; Gross et al., 2013; Labonté et al., 2013), but few studies in schizophrenia, that have suggested lower methylation in HTR2A in completed suicide compared to non-suicide controls (De Luca et al., 2009). The biological importance of methylation is clear as disturbances of known methylation patterns are linked to complex diseases (Robertson, 2005; Eggermann, 2009). Furthermore, a number of CpG islands have been found to exhibit inter-individual methylation variation and it has been proposed that the methylation levels of these high-density CpG clusters are especially sensitive to differential

exposure to environmental factors. DNA methylation is assumed to be complementary on both chromosomes (paternal and maternal) across the genome. However, in diploid mammalian genomes, parental alleles can exhibit different methylation patterns (allele-specific methylation DNA methylation or ASM), which have been documented in the imprinted regions. The number of currently known imprinting genes is suspected to be a small fraction of the total number of imprinted genes. Recent work has provided a list of potentially imprinted genes (Luedi et al., 2007). Even less is known about the genes that fall into the broader ASM category. Another example of ASM has recently been reported, whereby genotype (mainly SNPs) exerts an influence upon DNA methylation in cis (Schilling et al., 2009). Interestingly, Fiori and Turecki found that the expression of spermidine/spermine N (1)-acetyltransferase (SAT1) showed a negative correlation with the promoter methylation of a polymorphic CpG site in the brain DNA of suicide victims (Fiori and Turecki, 2011). Aiming to investigate ASM regions in bipolar disorder, Chuang et al. (2013) have selected SNPs, reanalyzing GWAS data, based on a previous publication that tested the SNPs in relationship of methylation level in the brain. However, to date, only few studies have investigated DNA methylation state in suicide attempt and schizophrenia and no studies have investigated the role of the polymorphic CpG sites.

The primary aim of this study was to compare suicide attempters and non-attempters in regards of 384 SNPs across the genome. The secondary aim was to develop a new methodology to select and analyze CpG SNPs that could be applied to different phenotypes of complex disorders and traits.

3.2 Materials and Methods

3.2.1 Sample

Patients were recruited from the Centre for Addiction and Mental Health in Toronto. The sample was described in our previous publication (Teo et al., 2012). Subjects diagnosed with schizophrenia according to DSM IV criteria (APA, 1994) were included. We administered the Structured Clinical Interview for DSM-IV (SCID) and the Family Interview for Genetic Studies (FIGS). Age at onset, diagnostic subtype, current treatment, co-morbid Axis I diagnoses, family history of psychiatric disorders, history of alcohol or drug abuse, smoking status, and the presence or absence of suicide attempt lifetime were collected. The clinicians who conducted the interviews were blind to the hypothesis tested. If required, the information was supplemented with medical case notes. The Research Ethics Board of the Centre for Addiction and Mental Health reviewed and approved the study. Written informed consent was obtained from all participating subjects.

This cross-sectional DNA sample comprises of 241 subjects with a diagnosis of SCZ. We included 172 males and 69 females (μ age=36.41±10.42) and 53 were classified as suicide attempters and 188 as non-attempters. The age ranged from 17 to 65, and the mean age at the time of the assessment was 38.0±9.8 for the attempters and 36.3±10.6 for the non-attempters. The age at onset in the attempters and non-attempters was 19.0±4.0, and 20.9±6.1, respectively. The mean duration of illness was 16.2±9.9years. There were 197 White Caucasian and 44 subjects from mixed background.

3.2.2 DNA analysis

Genomic DNA was obtained from peripheral leukocytes, using high salt extraction methods (Lahiri et al., 1991). We genotyped 384 SNPs selected from genes that are supposed to be involved in the neurobiology of SCZ (Table 1 – 1) using a customized Illumina Bead Chip.

3.2.3 Genotyping and quality control analysis

Only markers that passed quality control thresholds were included in the analysis as independent variables. Quality control analysis consisted of the exclusion of subjects with more than 5% missing SNPs. In addition, SNPs with a call rate lower than 85% and SNPs with minor allele frequencies smaller than 5% were excluded. Markers were also tested for departures ($p<0.01$) from Hardy-Weinberg equilibrium (HWE). For HWE, we applied a less conservative approach to avoid excluding informative SNPs.

3.2.4 Controlling for sample stratification

The principal component analysis (PCA) was performed to check for genetic subgroups inside the sample, potentially able to produce false-positive findings. The analysis excluded A/T and C/G SNPs because they are difficult to align. Afterward, the dataset pruned was merged with the HapMap Phase 3 data. The merged file was used as input for the PCA using SVS/Golden Helix software. Default parameters were used and we considered two components.

3.2.5 Association analysis

The SNP quality control was tested using PLINK (Purcell et al., 2007) for association with the suicide attempt outcome. The Wald test was used to check for additive SNP effect. The two principal components and duration of the disease (in years) were included in the model as covariates. False positives were controlled by using the Q–Q plot.

3.2.6 CpG statistics

We performed a novel analysis using a specific bioinformatic tool 'CpG SNP Recode' that we developed. The script is available on request. This tool selected a list of SNPs that are CpG SNPs from the 384 SNP panel that we used in this study and created a table listing the CpG SNPs and non-CpG SNPs (Figure 2 - 1). This bioinformatic tool detected the SNPs that are affecting the polymorphic CpG among the 384 SNPs genotyped in this study. Furthermore, the program calculated the presence/absence of a methylation site as affected by the SNP allele. Therefore, each subject in our sample had two, one or no methylation sites for each SNP locus that in turn was translated in potential methylation level of 100, 50 or 0%. The direct effect of the SNP allele can be translated in the availability or the percentage of methylation sites because in case the CG dinucleotide sequence is disrupted by the SNP, the methylation cannot occur resulting in 0% potential methylation. On the other hand, if the SNP allele conserves the CG dinucleotide sequence, the methylation can occur resulting in 50% or 100% potential methylation in heterozygosity or homozygosity respectively. The analytical procedure for analyzing the potential methylation is summarized in Figure 2 - 1. The CpG analysis has been performed at locus and gene level since this is a candidate gene study; however, we calculated

the chromosome-wise and global potential methylation as part of the novel analytical pipeline, even though they are not very informative in testing our candidate genes.

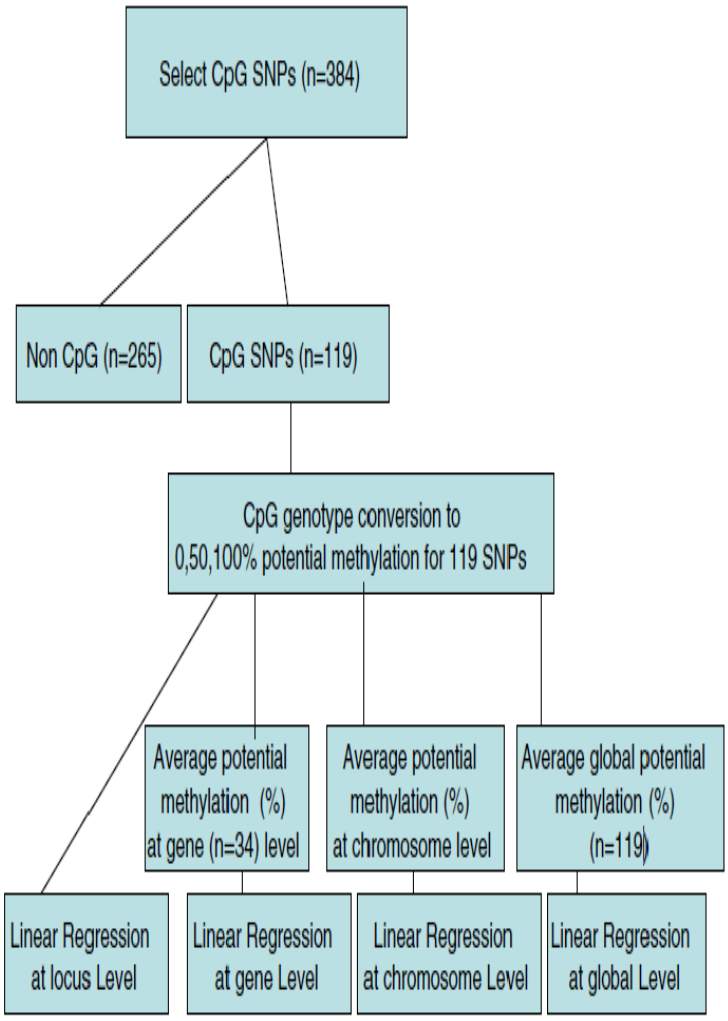

Figure 2 - 1. CpG SNP mapping analysis: flow chart. The global analysis was included to show the total potential methylation of all 119 CpG SNPs.

3.3 Results

3.3.1 Analysis of clinical variables

We tested the following clinical covariates in regard of suicide attempt: gender, age, ethnicity, age at onset, length of illness, current treatment with typical antipsychotics, current clozapine treatment, alcohol use disorder, smoking and positive family history of psychiatric disorders. The only trend we found was the clozapine treatment ($p=0.078$) protecting against suicide attempt.

3.3.2 Genetic association

Initially, 384 SNPs were included in the customized chip (see the list in the online supplemental material). Twenty SNPs were monomorphic in our population and they were excluded from the analysis; for three SNPs the genotyping failed; all subjects were heterozygous for the SNPs rs2227846 and rs16888561, therefore, these two SNPs were excluded from the analysis. At the end when we filtered our genotypes for a call rate higher than 85% and minor allele frequency higher than 5% and a Hardy-Weinberg p-value higher than 0.01, a total of 311 SNPs passed the quality control test.

Only 234 subjects had more than 95% SNPs genotyped, therefore, seven subjects were excluded from the analysis.

We generated a two-component plot generated from 191 markers genotyped in the HapMap phase III for the CHB, JPT, YRI, CEU populations chosen among the original 384 markers that were genotyped in our sample (Figure 2 - 2).

The association analysis was performed adjusting for the effects of duration of the disease and the first two principal components. The genomic control inflation factor (λ) showed a value equal to 1.0 across the 311 biallelic markers in attempters/non-attempters, indicating that there is no sub-structure in the sample due to ancestry issues.

The genotype test under additive model after the PCA correction showed that the SNP rs2661319 had the best p-value (p=0.0006) in the regulator of G-protein signaling 4 gene (*RGS4*). The frequency of the allele A was 60.1% in the suicide attempters and 42.1% in non-attempters. We show the p-values of our SNPs using a Manhattan Plot to highlight our top SNPs (Figure 2 - 3). Other two SNPs (rs2842030 and rs10799897) in the RGS4 gene were nominally associated with suicide attempt. However, the adjusted P-value became non-significant after the Bonferroni multiple testing. The observed p-value of the SNP rs2661319 was above the expected value, suggesting that this is a true positive result (Figure 2 - 4).

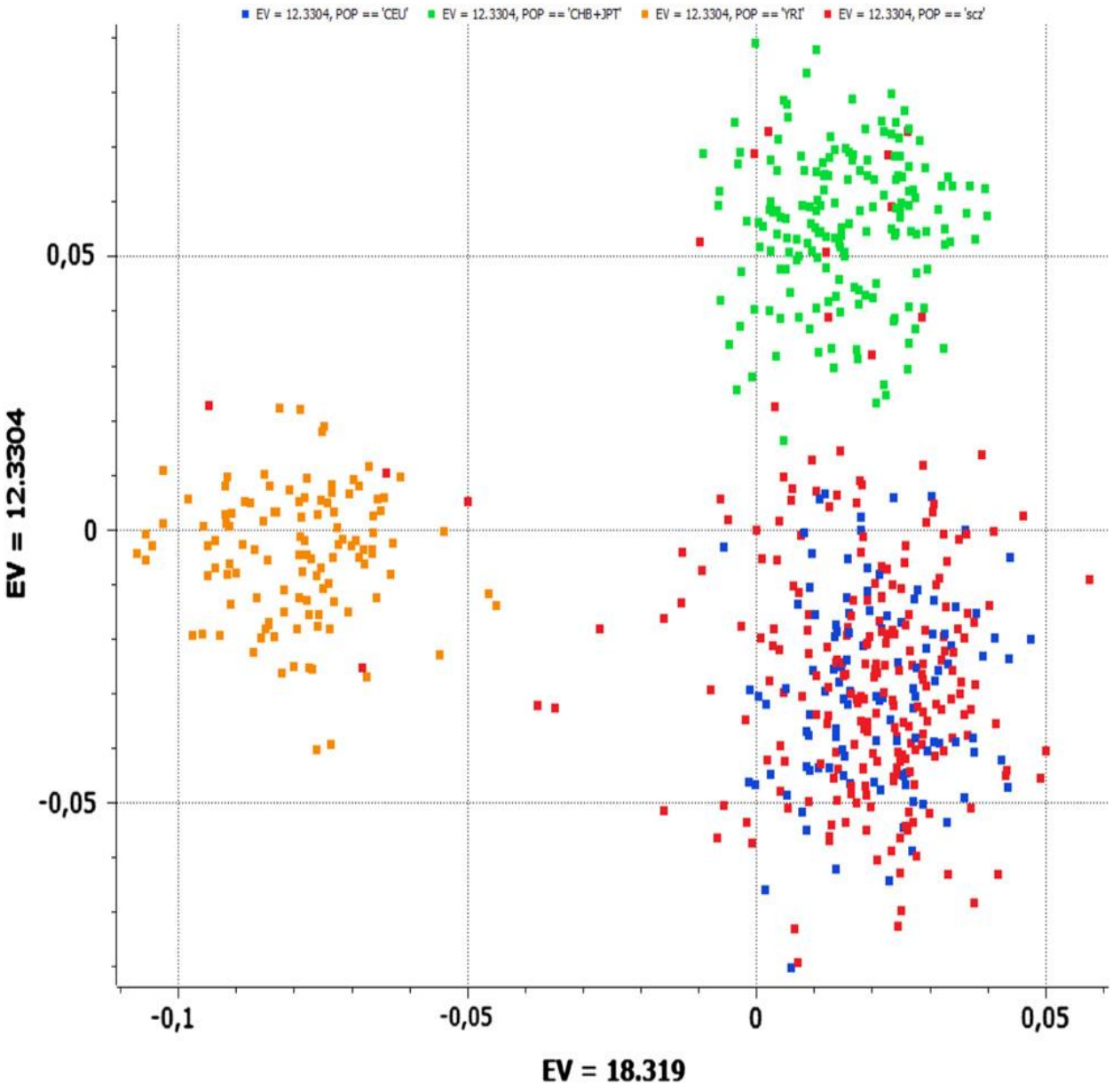

Figure 2 - 2. PCA analysis using 191 informative markers. Analysis of 191 SNPs: Green: Han Chinese and Japanese from HapMap; Orange: African from Nigeria HapMap; Blue: White Caucasians from Utah HapMap; Red: Schizophrenia Sample. Axis X = Principal Component 1 (Eigen Vector = 18.3); Axis Y = Principal Component 2 (Eigen Vector = 12.3).

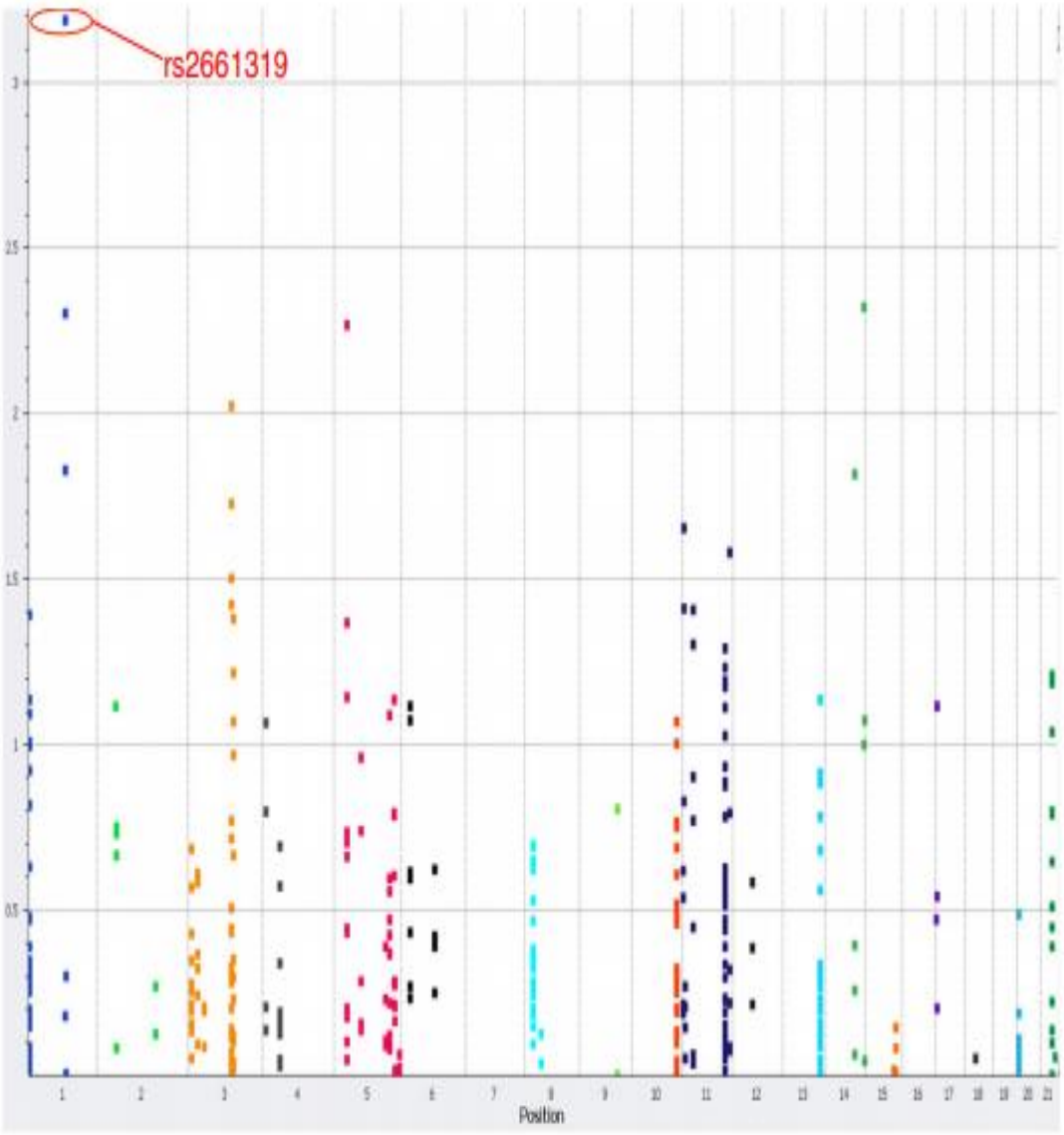

Figure 2 - 3. Manhattan plot conventional GWAS analysis (384 SNPs displayed). The RGS4 SNP rs2661319 on chromosome 1 is highlighted.

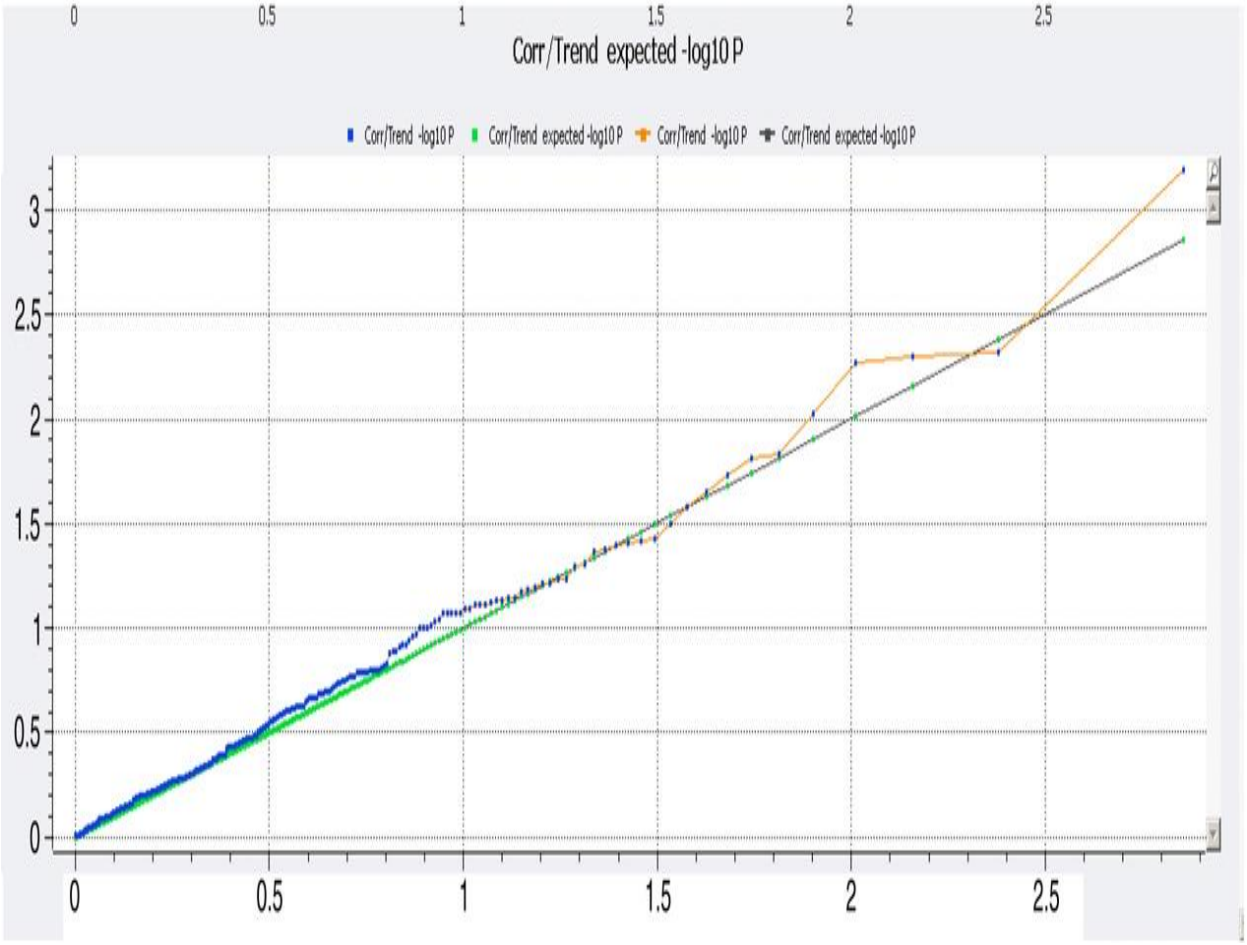

Figure 2 - 4. Q–Q plot. Y-axis observed and x-axis expected 384 SNP analyses.

3.3.3 CpG SNP analysis

When we applied the script CpG Recode, we found 119 SNPs out of 384, spanning 34 genes on 17 chromosomes located in CpG sites (Table 1- 1). The SNP rs2661319 that was significantly associated with suicide attempt in the conventional analysis, was one of the 119 CpG SNP selected by CpG Recode. When we applied the CpG SNP analysis converting the genotypes of the polymorphic methylation sites, the top SNP was rs2661319 with a p-value of 0.005 after correcting for the two principal components and with a regression coefficient of −0.016 and lower potential methylation associated with suicide attempt.

3.3.4 Analysis of polymorphic CpG SNPs (gene-wise)

We had data available for 51 attempters and 183 non-attempters in the schizophrenia sample for 21 schizophrenia candidate genes (Figure 2 - 5) and 13 neurotransmission genes (Figure 2 - 6). We considered as neurotransmission genes only those expressed in the synaptic cleft of the major neurotransmitters.

We found a significant difference for *RGS4* (p=0.005) (Table 1 - 1). Potential methylation level of *RGS4* was 55% in the attempters and 65% in the non-attempters (Table 1 - 1). The other genes were showing negative results.

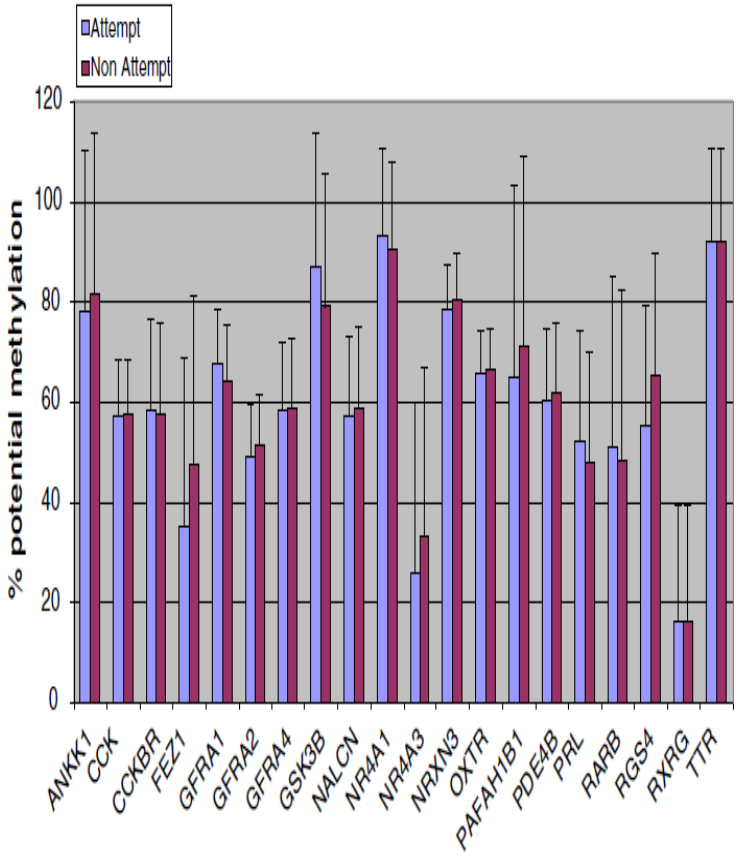

Figure 2 - 5. Potential methylation analysis gene-wise. Data are described as mean and Standard Deviation (SD).

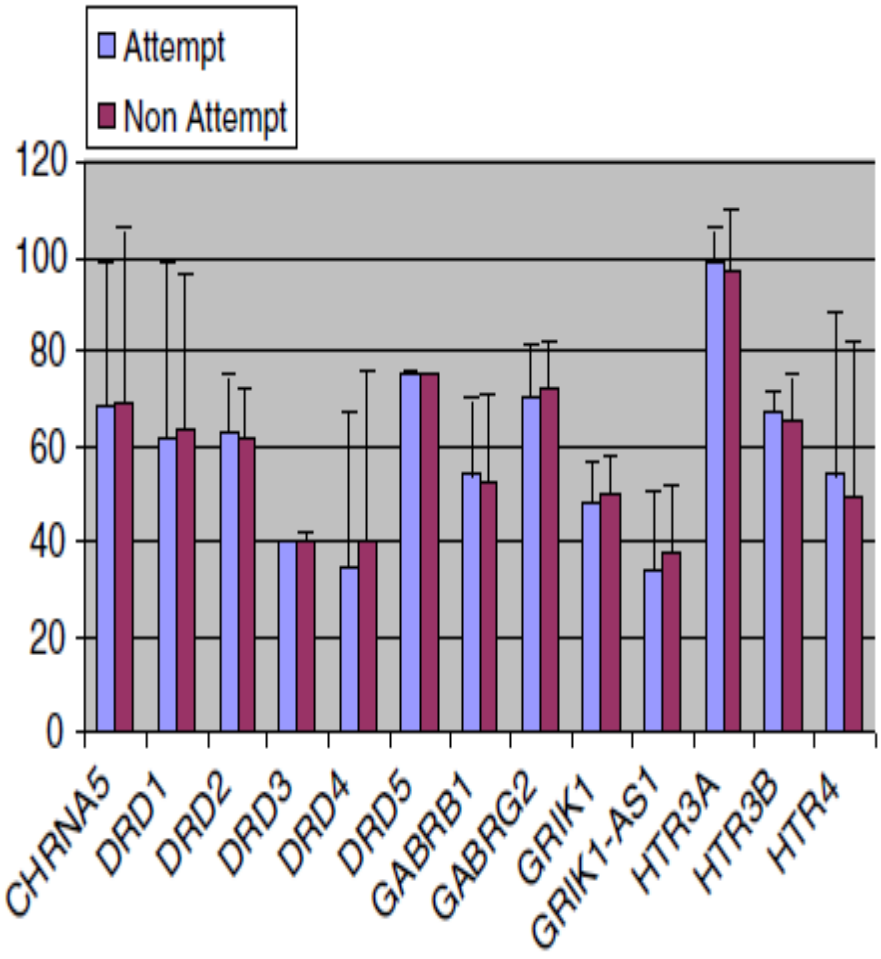

Figure 2 - 6. Potential methylation analysis of synapse genes. We considered as synapse genes, only the molecules present in the main neurotransmitter synaptic clefts (cholinergic, dopaminergic, GABAergic, glutamatergic, serotonergic).

Potential methylation average per gene.

Chr	Start position	End position	Gene	Transcript #	Strand	NonAtt M	NonAtt SD	Att M	Att SD	P-val
11	112763723	112776350	ANKK1	NM_178510	+	81.522	26.362	78.000	32.198	0.339
3	42274322	42280443	CCK	NM_001174138	−	57.201	13.033	57.000	11.339	0.850
11	6237542	6249932	CCKBR	NM_176875	+	57.201	18.082	58.000	18.516	0.834
15	76644917	76674666	CHRNA5	NM_000745	+	69.565	36.557	69.000	30.119	0.970
6	88906304	88911775	CNR1	NM_033181	−	76.359	30.448	80.000	30.305	0.379
5	174800281	174803769	DRD1	NM_000794	−	63.859	32.405	62.000	37.198	0.862
11	112785527	112851211	DRD2	NM_000795	−	61.491	10.673	63.333	11.610	0.192
3	115330247	115380589	DRD3	NM_000796	−	39.946	2.217	40.000	0.000	0.859
11	627305	630705	DRD4	NM_000797	+	40.278	35.492	34.375	32.870	0.221
4	9392356	9394731	DRD5	NM_000798	+	75.027	0.369	75.100	0.707	0.379
11	124820851	124871416	FEZ1	NM_005103	−	47.283	33.359	35.000	33.882	0.023
4	46728052	47123204	GABRB1	NM_000812	+	52.717	18.083	53.800	16.149	0.532
5	161427226	161515123	GABRG2	NM_000816	+	71.943	10.122	70.500	10.938	0.529
10	117806432	118021809	GFRA1	NM_145793	−	64.286	12.890	67.571	10.723	0.145
8	21593810	21702292	GFRA2	NM_001165038	−	51.178	10.922	49.167	10.275	0.237
20	3587939	3592046	GFRA4	NM_022139	−	58.696	13.293	58.000	13.777	0.668
21	29831125	30234153	GRIK1	NM_175611	−	49.728	8.442	48.333	8.748	0.242
21	30042365	30058196	GRIK1-AS1	NR_027021	+	37.908	14.309	34.000	16.568	0.081
3	121023492	121295954	GSK3B	NM_001146156	−	79.076	28.337	87.000	26.361	0.052
11	113351007	113366244	HTR3A	NM_000869	+	97.011	12.985	99.000	7.071	0.231
11	113280799	113322493	HTR3B	NM_006028	+	65.308	9.839	67.667	3.998	0.077
5	147810788	147996817	HTR4	NM_001040169	−	48.913	33.452	54.000	34.759	0.346
13	100504131	100866814	NALCN	NM_052867	−	58.696	13.558	57.000	16.036	0.445
12	50702883	50739558	NR4A1	NM_001202233	+	90.217	21.219	93.000	17.525	0.343
9	101623958	101636162	NR4A3	NM_173199	+	33.152	34.098	26.000	33.866	0.139
14	77939846	79400513	NRXN3	NM_004796	+	80.503	8.714	78.350	9.001	0.126
3	8767095	8786300	OXTR	NM_000916	−	66.440	9.319	65.750	8.174	0.448
17	2443673	2535659	PAFAH1B1	NM_000430	+	70.924	33.219	65.000	38.132	0.353
1	66030781	66612850	PDE4B	NM_001037341	+	61.793	15.061	60.400	13.845	0.475
6	22395456	22405709	PRL	NM_000948	−	47.962	21.849	52.000	21.923	0.245
3	25444758	25614426	RARB	NM_016152	+	48.098	38.541	51.000	34.241	0.485
1	161305020	161313216	RGS4	NM_001102445	+	65.217	23.069	55.000	24.223	0.005
1	163636783	163681216	RXRG	NM_006917	−	15.761	24.991	16.000	23.561	0.967
18	27425728	27432984	TTR	NM_000371	+	91.848	19.941	92.000	18.516	0.909

Results are sorted by gene name.

Table 1 - 1. Potential methylation average per gene.

3.3.5 Chromosome potential methylation analysis

We surveyed 17 autosomes for potential methylation (Figure 2 - 7) and we found a slight significance on chromosome 1 (p=0.036) (Table 1 - 2).

3.3.6 Total potential methylation

We considered the total methylation of all 119 SNPs in attempters and non-attempters and we found no significant difference (p=0.787). In fact, the potential methylation was 60.0±2.66% in the attempters and 59.9±2.66% in the non-attempters.

Potential methylation average per chromosome.

Chromosome	Attempters		Non attempters		p-val	OR	LCI	UCI
	mean	SD	mean	SD				
Chr1	53.309	10.559	56.957	11.104	0.036	0.970	0.942	0.998
Chr3	53.991	4.299	53.567	4.727	0.562	1.020	0.954	1.091
Chr4	65.615	7.441	64.806	8.187	0.524	1.013	0.974	1.052
Chr5	51.569	7.314	51.985	7.519	0.724	0.992	0.952	1.035
Chr6	65.768	14.321	61.339	16.033	0.072	1.019	0.998	1.040
Chr8	49.183	10.173	51.184	10.951	0.237	0.982	0.954	1.012
Chr9	25.490	33.723	33.333	34.103	0.139	0.993	0.983	1.003
Chr10	67.227	10.896	64.364	12.882	0.145	1.019	0.993	1.045
Chr11	64.661	5.638	64.270	6.018	0.676	1.011	0.959	1.066
Chr12	93.137	17.377	90.164	21.264	0.343	1.008	0.991	1.025
Chr13	57.003	15.875	58.704	13.595	0.445	0.991	0.970	1.014
Chr14	78.382	8.914	80.505	8.738	0.126	0.973	0.939	1.008
Chr15	69.608	30.130	69.399	36.588	0.970	1.000	0.991	1.009
Chr17	65.686	38.066	70.765	33.240	0.353	0.996	0.987	1.005
Chr18	92.157	18.365	91.803	19.987	0.909	1.001	0.985	1.017
Chr20	44.363	10.098	45.014	10.959	0.701	0.994	0.966	1.024
Chr21	48.203	8.710	49.772	8.444	0.242	0.978	0.943	1.015

Results are sorted by chromosome number.

Table 1 - 2. Potential methylation average per chromosome

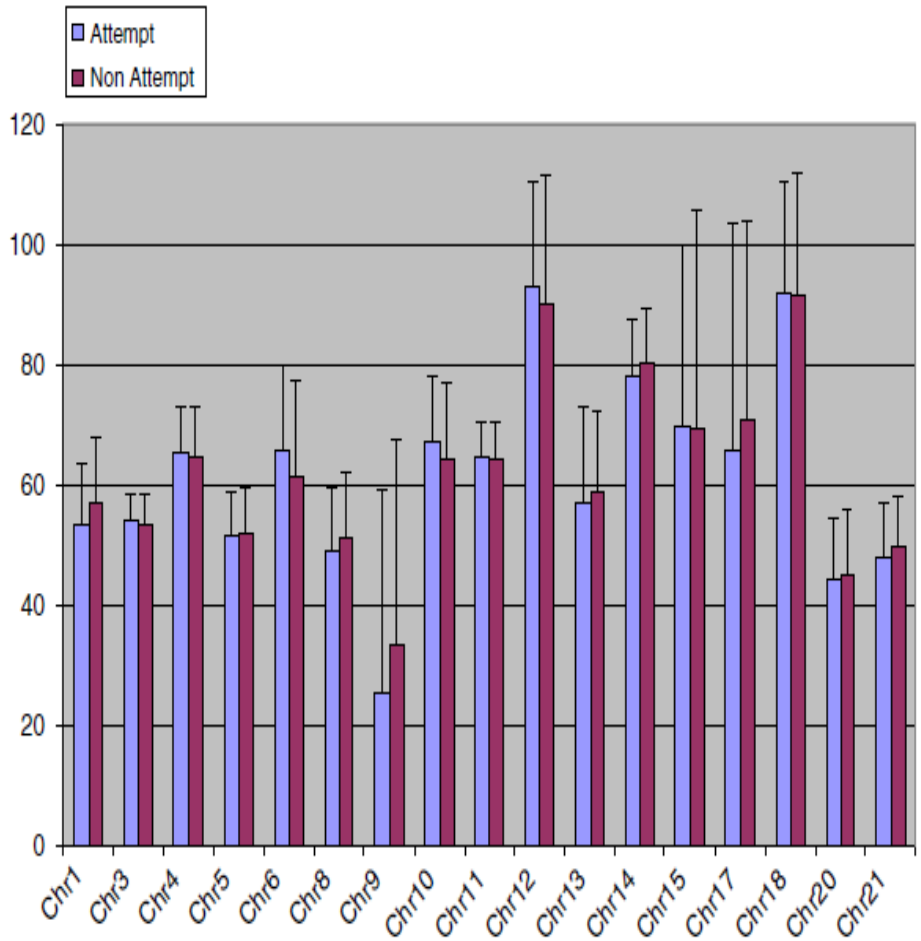

Figure 2 - 7. Potential methylation analysis chromosome-wise. Data are presented as mean and SD.

3.4 Discussion

This study did not found a robust association between the candidate genes we tested and suicide attempt in schizophrenia. However, the small sample size represents the main limitation of the study that could have resulted in false negative findings.

Furthermore, we analyzed the polymorphic CpG sites using a customized Illumina Bead Chip; we screened 384 SNPs in the chip for the presence of CpG effect. Only 119 SNPs were located in CpG sites. When we analyzed only the CpG sites located on chromosome l, we found significance for lower potential methylation in the attempters; however, this association did not survive the multiple corrections. The nominal p-value of the *RGS4* rs2661319 was significant, however, after multiple corrections was not significant, consistently with the GWAS studies of mood disorder and suicide attempt (Perlis et al., 2010; Willour et al., 2012), where no SNP survived the genome-wide correction. On the other hand, *RGS4* seems to be an interesting candidate gene in determining vulnerability to schizophrenia (Talkowski et al., 2006) and in determining outcomes such as response to antipsychotics (Lane et al., 2008) and risk for suicide attempt.

The overall results show no association between CpG SNPs and suicide attempt, however, the information of the SNP CpG methylation analysis can be used as a covariate in future methylation analysis. Our analysis is different from other approaches for investigating ASM regions as proposed by Chuang et al. (2013) since our SNPs are selected based on the flanking sequence including only those that are affecting the CpG sites.

The CpG analysis of a single locus produces almost the same p-value of the traditional genotype analysis with the advantage that provides more functional insight, specifying whether the disease risk is associated with higher or lower potential methylation.

The potential methylation gene and chromosome analysis did not show a significant difference, however, in large-scale studies, the potential methylation data can be used to identify the chromosomes and the genes that can be targeted in methylation studies. Furthermore, future studies should investigate the correlation between potential methylation and global methylation across the genome.

From our analysis, we estimate that one-third of the SNPs analyzed in a SNP-array chip is affecting CpG sites. Therefore, the contribution of these SNPs to DNA potential methylation is significant. The coverage of the traditional GWAS chips maybe inadequate for experiments aimed to link sequence and methylation variation in the DNA. Therefore, we propose this new analytical technique to select and analyze CpG SNPs. The analysis of heterozygous CpG SNPs is a simple way to look at ASM and it is possible that these heterozygous loci function as a flag for more complex epigenetic mechanisms.

In summary, this study did not show significant associations between suicide attempt and candidate genes in schizophrenia but suggests a new analytical technique to analyze the CpG SNPs in the genome.

Chapter 4
Interaction between methylation and CpG single-nucleotide polymorphisms in the HTR2A gene: association analysis with suicide attempt in schizophrenia

4 Abstract

Dysfunctional mechanisms in the serotonergic system have been implicated in suicidal behavior among patients with schizophrenia. However, previous association analyses of major serotonin genes have provided inconsistent findings regarding their role in suicidal behavior. The goal of the current study was to identify single-nucleotide polymorphisms (SNP) within *HTR2A* that directly affect CpG methylation sites in schizophrenic patients with suicidal behavior. Furthermore, direct methylation analysis was performed using genomic DNA from peripheral leukocytes employing bisulfite pyrosequencing to assess the contributions of six CpG sites in *HTR2A* exon I in 67 schizophrenia patients assessed for lifetime suicide attempt. Potential methylation in 25 CpG SNPs across the entire *HTR2A* gene was analyzed considering their direct contribution to methylation. When we compared direct methylation between attempters and non-attempters, we found that only the polymorphic T102C (rs6313) was significantly different between the two groups ($p = 0.02$). Furthermore, in the potential methylation analysis, we found a nominal association with suicide attempt for six of the 25 SNPs analyzed, i.e. rs2770293 ($p = 0.045$), rs6313 ($p = 0.033$), rs17068986 ($p = 0.029$), rs4942578 ($p = 0.024$),

rs1728872 ($p = 0.014$), and rs9534511 ($p = 0.003$). The results of this investigation provide preliminary evidence that the combined analysis of CpG SNPs and methylation may be useful for investigating the genetic and epigenetic factors involved in suicidal behavior.

4.1 Introduction

Family, twin, and adoption studies have provided a considerable amount of evidence for a significant genetic contribution to suicidal behavior. A recent review clearly demonstrated that suicidal behavior has both a familial and a heritable component (Brent et al., 2005; Smith et al., 2012). Previous twin studies have estimated that the heritability of suicide attempt is between ~ 17 and 55% (Statham et al., 1998; Glowinski et al., 2001; Fu et al., 2002). Despite the evidence from previous family-based studies, molecular genetic approaches have failed to elucidate any robust associations between candidate genes and suicide attempt (Tsai et al., 2011).

The neurobiology of suicidal behavior appears to be influenced by dysregulation of serotonergic neurotransmission (Tsai et al., 2011; Ernst et al., 2009). Some studies have suggested that suicidal behavior may be associated with hyposerotonergic neurotransmission (Abdolmaleky et al., 2011). However, previous research has failed to ascertain the specific, direct link between reduced serotonergic activity and suicidal behavior (Ernst et al., 2009). There has been inconclusive evidence for several serotonin receptors and candidate genes (Anguelova et al., 2003; Judy et al., 2012; Buttenschøn et al., 2013). Conversely, allelic analyses of the serotonin transporter and suicidal behavior in a meta-analysis found an association between the ‘s’ allele and suicidality (Anguelova et al. 2003).

One of the major 5-HT candidate genes in studies of suicidal behavior is the gene encoding the serotonin 2A receptor (HTR2A) (Serretti et al., 2007). Only 2 HTR2A SNPs, i.e. rs6313 (T102C) and rs6311 (A1438G), have been extensively investigated in suicidal behavior (Tsai et al., 2011). Molecular genetic association studies of HTR2A have provided equivocal findings on the relationship between the variants and suicidal behavior (Arias et al., 2001; De

Luca et al., 2007; De Luca et al., 2008; Saiz et al., 2008; Vaquero-Lorenzo et al., 2008; Ertugrul et al., 2004; Yoon et al., 2008; Zalsman et al., 2005). A meta-analysis analyzing the A1438G SNP found an association with suicidal behavior in a dominant model (genotypes AA and AG combined) (Li et al., 2006). A meta-analysis analyzing the T102C variant found no association with suicidal behavior (Anguelova et al., 2003). An updated meta-analysis on the same SNP (González-Castro et al., 2013) confirmed the lack of association with suicidal behavior.

Recent studies investigating gene-environment effects of the T102C SNP have reported significant associations between stressful life events per T102C and suicidal behavior (Ben-Efraim et al., 2013; Shinozaki et al., 2013). This gene-environment relationship suggests a possible epigenetic influence on HTR2A variants and the suicide phenotype.

Studies investigating methylation levels of the HTR2A gene in schizophrenia (SCZ) have been limited. Two studies (Abdolmaleky et al., 2011; Ghadirivasfi et al., 2011) have reported significant associations between the methylation level of HTR2A T102C and psychosis. In those studies, hypomethylation and a reduced gene expression of HTR2A were observed in the frontal cortex of C allele carriers in psychotic patients compared to controls. Methylation analysis of HTR2A in suicidal behavior has not been fully investigated in the literature. In our previous report (De Luca et al., 2009), we found a nominal trend for hypomethylation of the C allele in the T102C polymorphism in brain tissue of suicide completers with major psychoses and significant hypermethylation in peripheral leukocytes of SCZ suicide attempters.

Despite previous investigations examining the expression and polymorphisms of 5-HT receptors in suicide, little is known about the relationship between methylation level and CpG SNP variation of 5-HT genes in suicide. Thus, the aim of the present pilot study is the combined

analysis of HTR2A methylation and CpG SNP in HTR2A to ascertain the contributions of HTR2A in conferring a risk for suicide attempt in SCZ.

4.2 Methods

This sample included 67 white European Caucasian participants with a diagnosis of SCZ ascertained using the Structured Clinical Interview for DSM-IV (SCID-I/P). All patients were carefully assessed for lifetime suicide attempt by means of the Columbia Suicide Severity Rating Scale (C-SSRS) and the Beck Scale for Suicide Ideation (BSS). The Family Interview for Genetic Studies (FIGS) was administered to classify familial cases if patients had a history of psychiatric disorders in first-degree relatives. We administered the Childhood Trauma Questionnaire (CTQ) and the Stressful Life Events Screening Questionnaire (SLESQ) to document the presence of childhood and lifetime trauma. The level of current stress at the time of the assessment was measured using the Perceived Stress Scale.

Information about current or past treatments was also recorded from clinical charts. The patients were recruited from the Centre for Addiction and Mental Health (CAMH), and the CAMH Research Ethics reviewed and approved this study. Genomic DNA was extracted from white blood cells using standard high-salt methods (Lahiri et al., 1991). We first analyzed the SNPs within the *HTR2A* region, applying the conventional strategy to evaluate the markers using an additive model with logistic regression. We screened the entire gene region including the promoter region and the 3′ region. Then, we conducted a new analysis selecting only the polymorphic CpG sites in the *HTR2A* gene. This analysis looks at the presence/absence of a methylation site as affected by the SNP allele. According to this analysis, each subject can have 2, 1, or no methylation site for each SNP locus that in turn can be translated in a methylation level of 0, 50, or 100% (table 2 - 1). In this model the SNPs in the *HTR2A* gene were studied from a different perspective, considering their direct contribution to the availability of potential

methylation sites. We selected 25 CpG SNPs in *HTR2A* from those present in the Illumina Omni 2.5 array, and we genotyped the 67 subjects using automated methods.

The bioinformatic tool, SNP Recode (Bani-Fatemi et al., 2013), was used to select and convert the *HTR2A* CpG SNPs.

Methylation patterns of CpG sites located in the exon I of the HTR2A gene were compared between attempters and non-attempters.

The white blood cell genomic DNA was treated with sodium bisulfite using an EZ DNA Methylation ^{TM} Kit (Zymo Research, Irvine, Calif., U.S). Bisulfite treatment converts non-methylated cytosine (C) to uracil (U). The degree of methylation was analyzed as a C/T SNP using the formula: C (%) = C/(C + T).

The pyrosequencing reactions were performed according to the manufacturer's standard protocol. For the pyrosequencing analysis, we amplified a 256-bp fragment encompassing six polymorphic CpG sites in HTR2A exon I. We designed our sequencing primers on the minus strand. The height of the pyrosequencing output traces was converted to a percentage of methylation for each CpG.

The methylation percentages of six CpG sites in exon I (i.e. +44, +102, +141, +154, +188, and +191) were compared between suicide attempters and non-attempters. The CpG sites were named considering their distance from the ATG.

We analyzed first the association between clinical predictors and suicide attempt. We analyzed all SNPs using a binary logistic regression under an additive model. Then, we used binary logistic regression to test the association between suicide attempt and the potential

methylation level in the selected CpG SNPs in the HTR2A gene identified by CpG Recode. Furthermore, we averaged all CpG SNPs across the gene to calculate the average potential methylation of HTR2A. We employed the same test for the association with suicide attempt using direct methylation of the six CpG sites in HTR2A exon I.

The analysis for the +102 CpG site was conducted using the potential methylation of the rs6313 as a covariate. Finally, the average methylation level in exon I was compared between attempters and non-attempters. All analyses were performed using IBM SPSS Statistics version 20.

SNP genotype	SNP (C/T) Flanking Sequence	Potential Methylation (%)
CC	CCG/CCG	100
CT	CCG/CTG	50
TT	CTG/CTG	0

Table 2 - 1. CpG SNPs conversion to potential methylation level (0, 50, 100%)

4.3 Results

The sample consists of 46 males and 21 females with a mean age at the time of the interview of 47 ± 12.4 years; 32 patients in the sample had attempted suicide at least once in their lifetime. Table 2 - 2 shows the demographic characteristics of suicide attempters and non-attempters.

There was no gender difference between attempters and non-attempters ($p = 0.987$). The age at the time of the assessment was not different between attempters and non-attempters ($p = 0.928$).

The duration of illness was not different between the two groups ($p = 0.708$); however, the age at the onset of psychosis was significantly different ($p = 0.040$), with an earlier onset in attempters (21.2 vs. 25.2 years).

When we measured the presence and intensity of suicidal ideation using the C-SSRS, at the time of the assessment, we did not find evidence of a confounding effect ($p = 0.912$). There were no differences in SLESQ scores between attempters and non-attempters ($p = 0.525$). However, when we considered the early-life adversities measured by means of the CTQ, we found that the trauma score was higher in attempters than in non-attempters ($p = 0.041$).

Regarding the potential methylation analysis, CpG Recode identified 25 CpG SNPs from those in the HTR2A gene included in the Illumina Omni 2.5-8 platform. The genotypes were then converted considering the percentage of methylation sites available at each SNP (i.e. 0 or 50 or 100%).

After the conversion, the potential methylation level was included as a predictor in the logistic regression model and we found that the following six CpG SNPs were associated with suicide attempt: rs2770293 ($p = 0.045$), rs6313 ($p = 0.033$), rs17068986 ($p = 0.029$), rs4942578 ($p = 0.024$), rs1728872 ($p = 0.014$), and rs9534511 ($p = 0.003$) (Figure 3 - 1). Suicide attempt was associated with lower potential methylation at loci rs2770293, rs17068986, rs4942578, and rs9534511, and with higher methylation at loci rs6313 and rs1728872. When we compared the overall potential methylation in the entire HTR2A gene, we found no significant difference ($p = 0.732$), with average potential methylations of 54.1% in attempters and 54.5% in non-attempters (Figure 3 - 2). When we tested the sex effect on HTR2A potential methylation, we did not find any confounding effect ($p = 0.427$).

Our methylation analysis of 32 suicide attempters and 35 non-attempters showed that there was no significant difference between suicide attempters and non-attempters in HTR2A exon I, except for the polymorphic CpG at position 102 (rs6313), which was significantly hypermethylated ($p = 0.029$) in suicide attempters (Figure 3 - 3). However, this significant association disappeared when the potential methylation at this position was incorporated into the analysis ($p = 0.713$).

When we considered the average methylation in exon I, the level was 75.1% in attempters and 74.4% in the non-attempter group, and we did not find a statistical difference ($p = 0.662$) (Figure 3 - 4). Interestingly, the overall methylation level of HTR2A exon I showed a strong positive correlation with the potential methylation in the entire HTR2A gene ($r = +0.388$, $p = 0.002$) (Figure 3 - 5). When we looked at the confounder effect on exon I methylation, we found that sex was not associated with the overall methylation ($p = 0.279$); however, age showed a negative correlation ($r = –0.279$; $p = 0.02$).

	All subjects	Attempters	Non-Attempters
Participants, n	67	32	35
Age, years	47 ± 12.40	47 ± 12.22	47 ± 12.75
Female:male ratio	21:46	10:22	11:24
Age at Onset, years	23.25 ± 7.89	21.22 ± 7.26	25.21 ± 8.09
Duration of Illness, years	24.16 ± 12.78	24.78 ± 12.86	23.6 ± 12.87
CTQ score	39.64 ± 14.94	40.93 ± 15.38	38.44 ± 14.65
SLESQ score	3.02 ± 2.65	3.26 ± 2.75	2.82 ± 2.58

Table 2 - 2. Demographic and diagnostic information on subjects with schizophrenia (Values are presented as means ± SD unless otherwise stated).

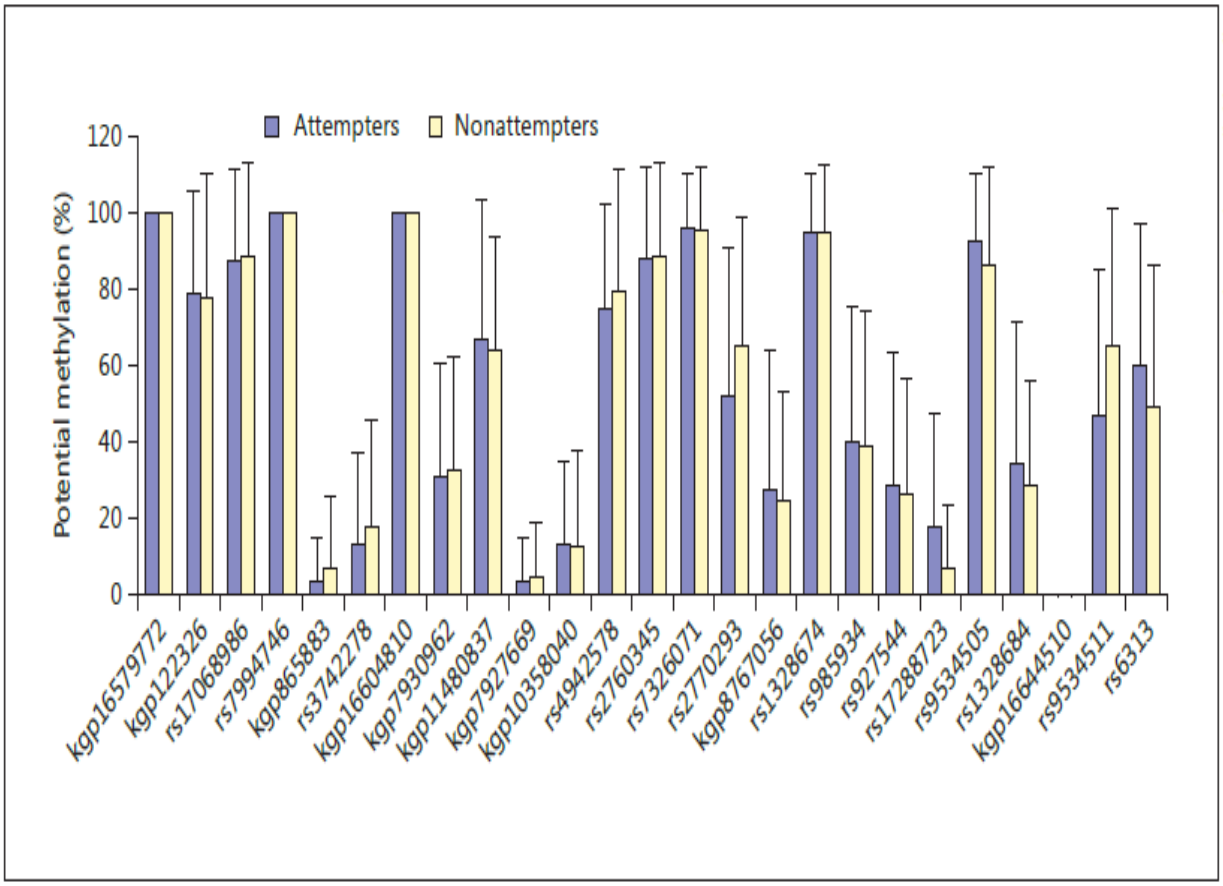

Figure 3 - 1. Potential methylation for 25 CpG SNPs across the entire HTR2A gene. Results are shown as means and SD.

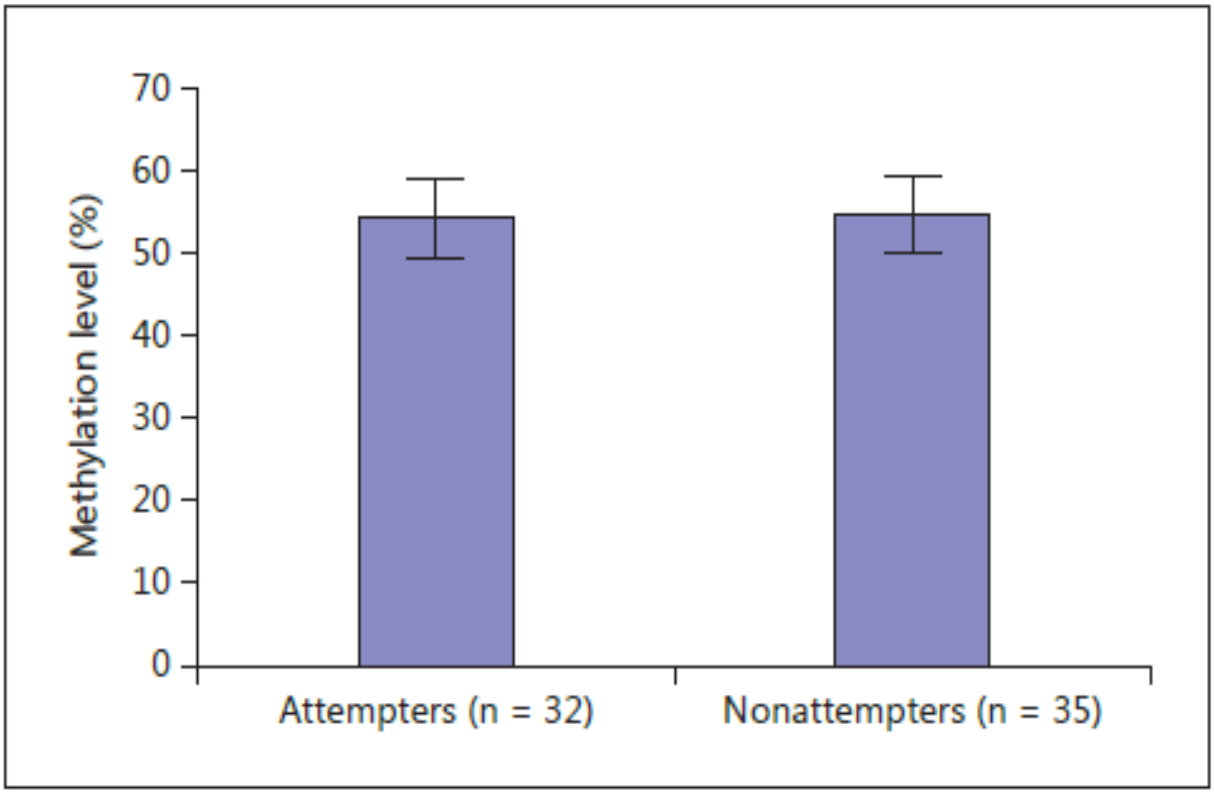

Figure 3 - 2. Overall potential methylation of CpG SNPs in the HTR2A gene. Data are presented as means and SD.

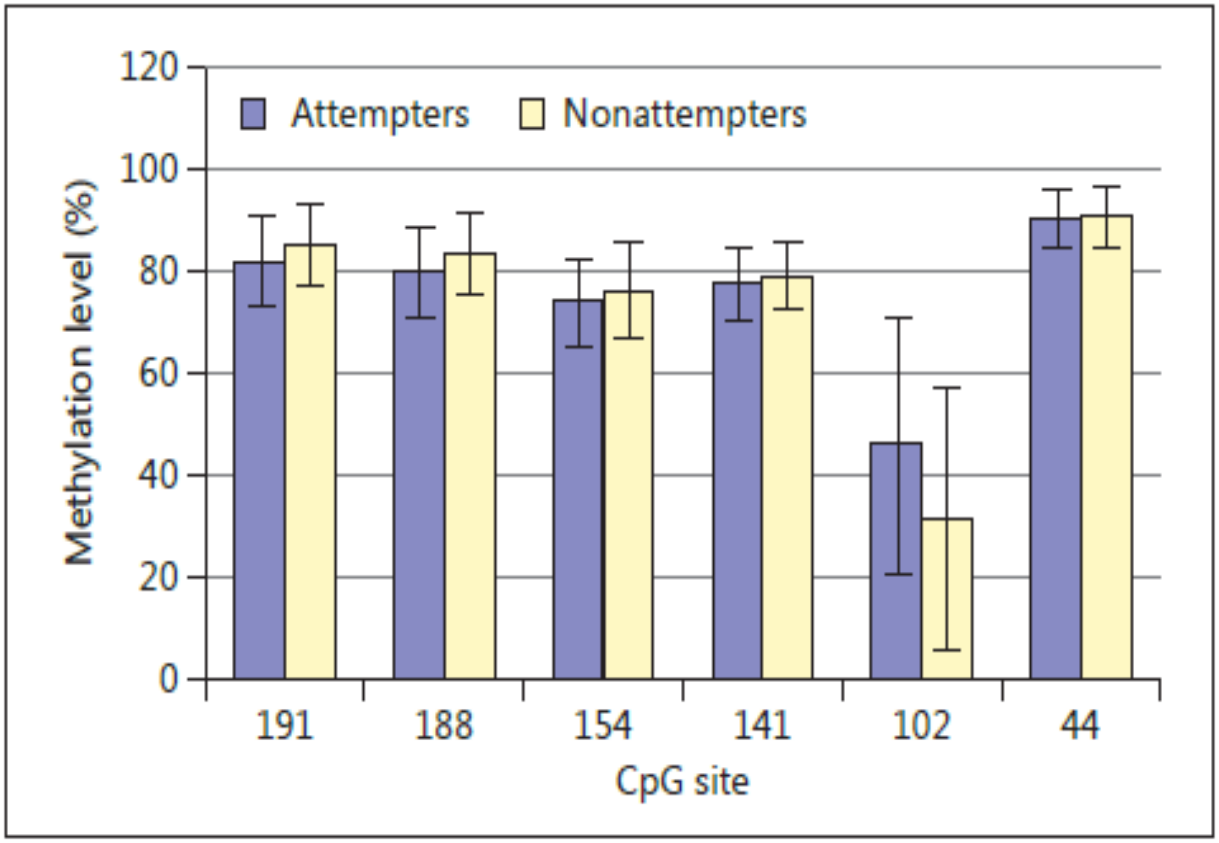

Figure 3 - 3. Methylation levels of CpG sites in HTR2A exon I. Data are presented as means and SD.

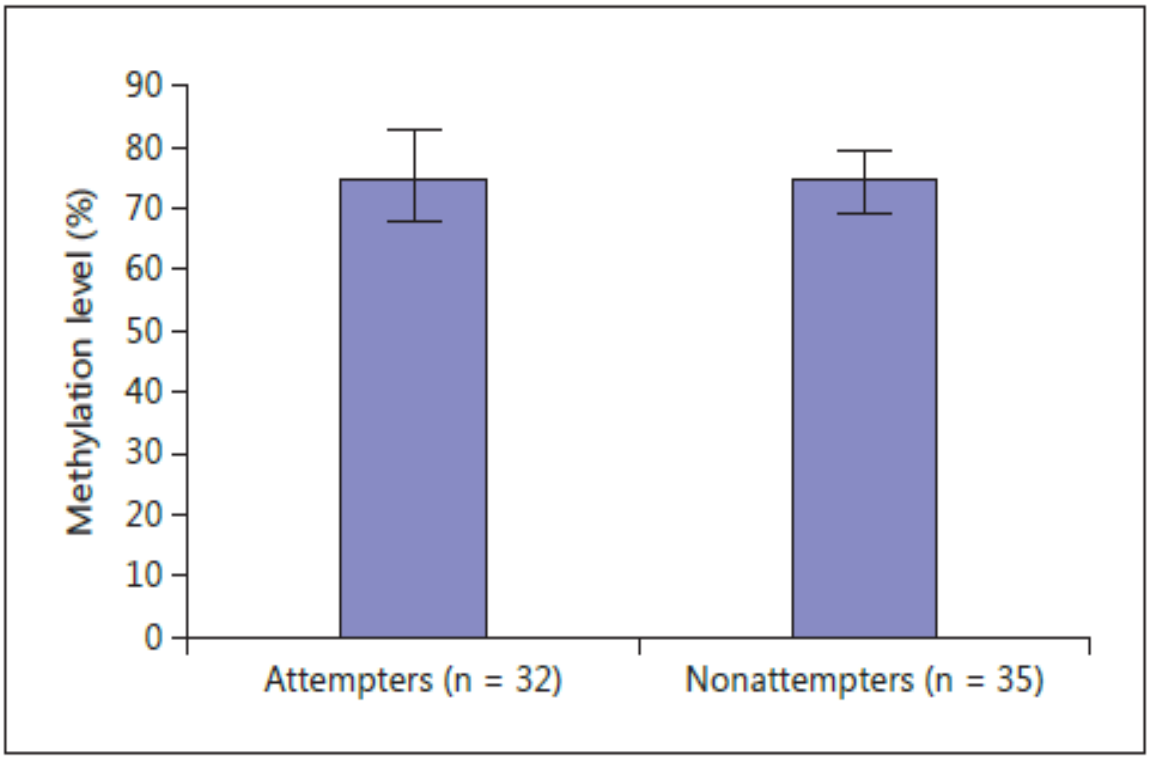

Figure 3 - 4. Overall direct methylation of CpG SNPs in HTR2A exon I. Data are presented as means and SD.

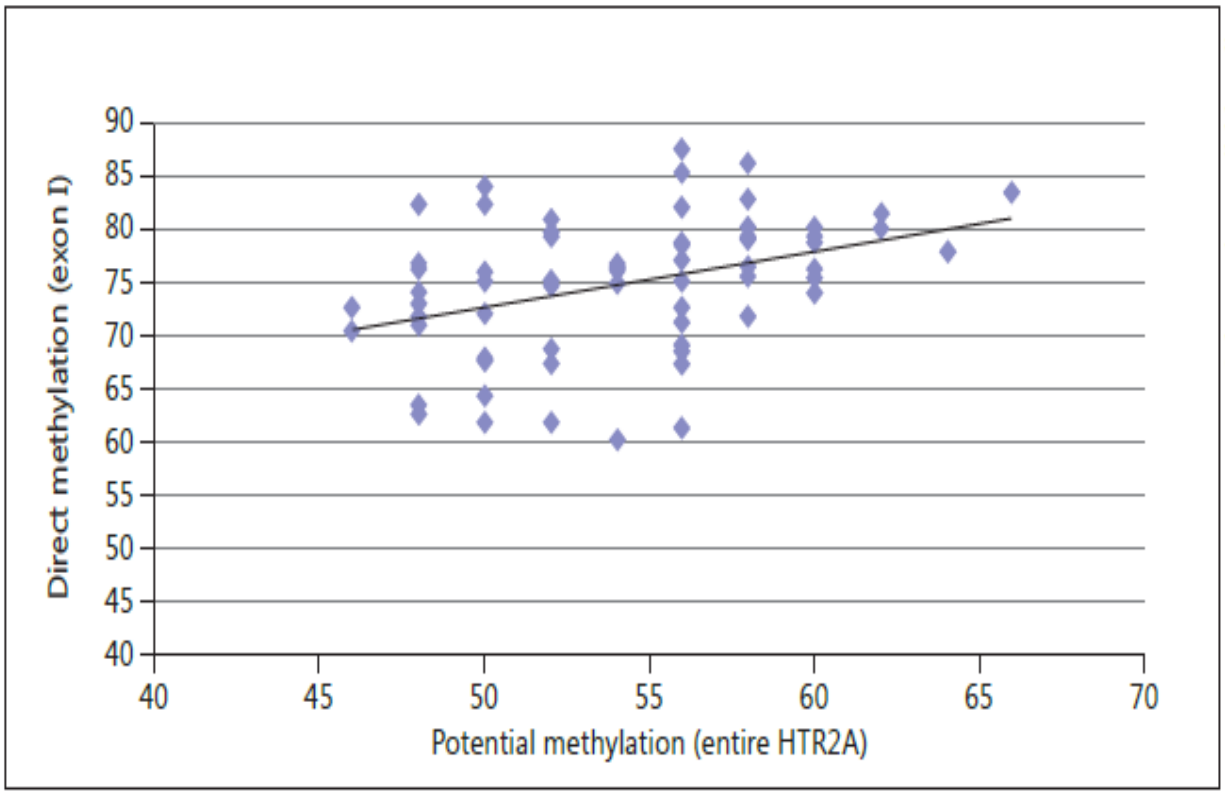

Figure 3 - 5. Correlation between potential methylation in the HTR2A gene and direct methylation in exon I of HTR2A (overall methylation).

4.4 Discussion

The present analysis assessed whether DNA methylation and CpG SNP in the *HTR2A* gene are associated with a risk of suicide attempt in SCZ patients. We assessed potential methylation for 25 CpG SNPs across the entire *HTR2A* gene, as well as direct methylation at the CpG sites of HTR2A exon I. The SNP selection in this study is somewhat new; in fact, the majority of previous association studies of the *HTR2A* gene and suicidal behavior in SCZ have focused on tag SNPs.

This study did not find a robust association between the *HTR2A* gene and suicide attempt in SCZ. However, we found an association between potential methylation in six CpG SNPs in *HTR2A* not previously investigated in suicide studies.

In the direct methylation analysis of 32 suicide attempters versus 35 non-attempters, our results showed that the polymorphic CpG rs6313 was significantly hypermethylated in suicide attempters. However, this could be the effect of the C/T polymorphism; in fact, after correcting for potential methylation, this CpG site lost significance. Furthermore, in the potential methylation analysis, we did find a significant difference for this polymorphic CpG site between suicide attempters and non-attempters; therefore, the association with methylation at this locus seems to be driven by the confounding effect of the 102T allele in the pyrosequencing reaction.

Nevertheless, the results of the *HTR2A* 102CpG methylation replicate the previous findings by De Luca et al. (2009), which indicated that hypermethylation of C102T may be associated with suicide attempt in SCZ patients.

The strong correlation between overall direct and potential methylation in *HTR2A* suggests that the methylation process is influenced by the SNPs directly affecting CpG sites.

Thus, the combined potential-direct methylation methods described here may be applied to other 5-HT receptor genes that have been linked to suicide to better understand the relationship between DNA methylation and suicidal behavior.

Regarding the clinical factors, our sample showed that age at onset may be associated with an increased risk of suicide attempt in SCZ; however, the other demographic factors such as gender, age, and current suicidal ideations did not seem to be confounding factors. Moreover, the small sample size represents the main limitation of our study and could have resulted in false-negative findings.

In conclusion, we did not find strong evidence for epigenetic changes in *HTR2A* associated with suicidal behavior; however, there was a synergistic effect of DNA methylation and potential methylation at the rs6313 locus in conferring a risk of suicide attempt when there is less availability of methylable cytosines and methylated cytosines. Future studies should investigate the correlation between potential methylation and direct methylation in other candidate gene systems for suicidality.

Chapter 5
Multiple tissue methylation analysis of HTR2A exon I in suicidal behavior

5 Abstract

The main aim of the current study was to investigate epigenetic alterations in serotonin 2A receptor (*HTR2A*) exon I CpG sites as possible risk factors for suicidal behavior. We also aimed to analyze the epigenetic alterations in two different tissues as epigenetic mechanisms are tissue specific. These epigenetic changes may lead to a better prediction of suicidal behavior. Direct CpG methylation analysis was carried out on genomic DNA from the saliva of 20 schizophrenia suicide attempters and 27 non-attempters, and from post-mortem brain tissues of nine suicide victims and 11 controls. We used bisulfite pyrosequencing to assess the contributions of six CpG sites including the rs6313 (C102T) site in the first exon of *HTR2A* in suicide attempters and suicide victims. DNA methylation analysis did not find a significant difference in CpG methylation between suicide attempters and non-attempters ($P=0.759$) or between suicide victims and controls ($P=0.189$). We found a strong positive correlation between CpG methylation levels in blood and saliva ($r=0.547$, $P<0.001$). DNA methylation analysis confirmed that the overall methylation level of *HTR2A* exon I was around 80% for DNA extracted from saliva and almost 30% in the frontal cortex DNA. The results of this investigation do not support the evidence that methylation analysis of the *HTR2A* gene may be useful for investigating the epigenetic factors involved in suicidal behavior.

5.1 Introduction

There are several risk factors associated with suicide. We may consider the risk factors for suicide in two different dimensions: (i) distal factors, which form the individual's diathesis for suicide such as neurobiological disturbances, personality characteristics, perinatal and early-life experiences, and genetic loading. (ii) Proximal factors, which represent the stress component such as psychiatric disorders, environmental or societal factors, and acute stressful life events (Antypa et al., 2013b). A significant genetic influence in suicidal behavior has been largely affirmed. The data suggest that the heritability of suicidal behavior is 43% (McGuffin et al. 2001; Bondy, et al., 2006). In addition, the genetic component of suicide is correlated with the susceptibility to psychiatric illness, impulsivity, and aggression (Brent and Mann, 2005). Familial studies, adoption studies, and twin studies suggest that suicide is both genetic and heritable. Suicidal behavior seemingly clusters within families (Melhem et al., 2007).

Evidence shows that suicide attempt and completed suicide can be inherited because of transmission of distal factors, being independent of the transmission of major psychiatric disorders, as genetic susceptibility can be passed from one generation to another (Mann and Currier, 2010). It is still unclear what genetic and neurobiological systems relate to suicide, but the serotonin (5-hydroxytryptamine) system has received considerable attention as disruptions in its functionality lead to abnormal cortical function, which can result in mood disorders or suicidal behavior (Antypa et al., 2013a; Dean et al., 2014). The serotonergic system also possibly plays a role in schizophrenia (SCZ), a neuropsychiatric disorder with a high potential risk for suicide attempt and a considerable lifetime risk for suicide completion (Polsinelli et al., 2013). The alterations in the serotonin system, including *HTR2A* expression (the regulation of which occurs in the promoter region), can be related to childhood experiences or even genetic and/or

epigenetic factors (Mann and Currier, 2010; Abdolmaleky et al., 2011; Ghadirivasfi et al., 2011). Epigenetic events affect the gene expression and can vary from one copy to another. One of the mechanisms by which this regulation occurs is DNA methylation, which involves a covalent biochemical modification that results from the addition of a methyl group by DNA methyltransferases from S-adenyl methionine to the aromatic ring of cytosine (5-methylcytosine) located 5′ to a guanosine (CpG) (Bellizzi et al., 2012).

Haghighi et al. (2014) reported an increased DNA methylation level in the brain of suicide victims. The results of their study showed that the number of methylated CpG sites in the brain of suicide victims was eight-fold greater than that in the brains of the control group. Studies have shown that the majority of the DNA methylation variation in *HTR2A* occurs within the promoter region and exon I and only little DNA methylation variation occurs in the other regions of this gene (Cheah et al., 2017). In another study, Abdolmaleky et al. (2011) showed that *HTR2A* promoter CpG sites were hypermethylated around the −1438A/G (rs6311) polymorphic site in SCZ and bipolar disorder (BD) patients compared with controls. However, they reported the T102C polymorphism and near CpG sites in exon I were hypomethylated in SCZ and BD compared with controls. There is strong evidence that DNA methylation in the *HTR2A* promoter and exon I alter *HTR2A* mRNA expression (Abdolmaleky et al., 2011; Paquette and Marsit, 2014). Methylation regulations differ from tissue to tissue, meaning that the best tissue for epigenetic analysis in mental diseases would be the brain. However, the brain is not accessible in living patients; thus, the identification of biomarkers needs to be performed with samples from other tissues. Evidence suggests that DNA methylation levels in the saliva can reflect and be correlated to the methylation levels in the brain, possibly representing the disease state in patients compared with healthy controls or a baseline (Ghadirivasfi et al., 2011).

The focus of the current study is the epigenetic alterations, specifically DNA methylation, of the serotonin 2A receptor (*HTR2A*) in suicide attempters compared with non-attempters in a sample of SCZ patients. We also compared DNA methylation levels in saliva and blood as potential alternatives for brain samples for suicidal behavior studies. We selected the functional C102T single-nucleotide polymorphism and the neighboring CpG sites of exon I as they are methylation sites that may affect the *HTR2A* expression in suicide attempters and suicide victims.

5.2 Participants and methods

In this study, we carried out the methylation analysis of six CpG sites in *HTR2A* exon I in 20 suicide attempters versus 27 non-attempters (using genomic DNA extracted from saliva) and nine suicide victims versus 11 controls (using genomic DNA extracted from the brain). The participants were recruited from the Great Toronto Area with the approval of the Centre for Addiction and Mental Health institutional research ethics board. The sample that we used for methylation analysis in suicide attempt was a subsample of our previous research (Bani-Fatemi et al., 2016). This sample consists of 47 Caucasian European Caucasian participants with a diagnosis of SCZ ascertained using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (SCID-I/P) (First et al., 2002).

All patients were assessed carefully for lifetime suicide attempt using the Columbia Suicide Severity Rating Scale (C-SSRS) (Posner and Brown et al., 2011) and the Beck Scale for Suicide Ideation (BSS) (Beck et al., 1991). The Family Interview for Genetic Studies was administered to classify familial cases if patients had a history of psychiatric disorders among first-degree relatives. We administered the Childhood Trauma Questionnaire (CTQ) (Bernstein et al., 1994) and the Stressful Life Events Screening Questionnaire (SLESQ) (Goodman et al., 1998) to document the presence of childhood and lifetime trauma. The level of current stress at the time of the assessment was measured using the Perceived Stress Scale. Information on current or past treatments was also recorded from clinical charts.

Genomic DNA was extracted from saliva (Oragene; DNA Genotek Inc., Ottawa, Ontario, Canada). We also matched nine suicide victims for sex, age of death, and post-mortem interval with the controls, who died from other causes rather than suicide, in our sample. The brain

sample contains genomic DNA from 10 SCZ, one major depression, seven BD patients, and two unaffected controls.

Genomic DNA from the saliva and brain was treated with sodium bisulfite using an EZ DNA Methylation Kit (Zymo Research, Irvine, California, USA). Bisulfite treatment converts nonmethylated cytosine (C) into uracil (U). The degree of methylation was analyzed as a C/T single-nucleotide polymorphism using the following formula: C (%) = C/(C +T). The pyrosequencing reactions were performed using PyroMark Q24 (Qiagen, Hilden, Germany). For the pyrosequencing analysis, we amplified a 256 bp fragment encompassing six CpG sites in HTR2A exon I. We used the following PCR primers (forward primer: TTTTTTTTGGAGATGAAGTAAGGA and reverse primer: AGAATTTGTAGTAGATGAGGTGTAG). Three sequencing primers were designed on the antisense strand FS1: TTTGGAGATGAAGTAAGG, FS2: TTTGGTGTTATTATTTAATT, and FS1re: AGTTAAATGTATTAGAAGTG). The height of the pyrosequencing output traces was converted into a percentage of methylation for each CpG. The methylation percentages of six CpG sites in exon I [+ 44 (chromosome 13:47 469 998), + 102 (rs6313; chromosome 13:47 469 940), + 141 (chromosome 13:47 469 901), + 154 (chromosome 13:47 469 888), + 188 (chromosome 13:47 469 854), and + 191 (chromosome 13:47 469 851)] were compared between suicide attempters and non-attempters and also between suicide victims and controls. The CpG sites were named considering their distance from the start codon.

Using an independent-sample t-test, we first analyzed the association between clinical variables and suicide attempt. The analysis in patients with the SCZ spectrum diagnosis (SCZ and schizoaffective depressive type) was carried out with the following test variables: sex, age, duration of illness (DOI), age at onset (AAO), stressful life events (SLESQ), and childhood

trauma (CTQ). We also analyzed the CpG sites using a binary logistic regression under an additive model. Then, we tested the association between suicide attempt/completed suicide and direct methylation level in the selected CpG sites in the HTR2A exon I. Furthermore, we calculated the overall methylation percentage of each CpG sites within the exon I of the HTR2A gene. The overall methylation level in exon I was compared between suicide attempters and non-attempters and between suicide victims and controls. Finally, we checked the correlation of the overall methylation in our saliva sample with the matched sample of blood from the same participants using Pearson's correlation analysis. All analyses were carried AQ5 out using IBM SPSS Statistics, version 20.

5.3 Results

Table 3 - 1 shows the demographic characteristics of suicide victims and controls, whereas Table 2 shows the demographic characteristics of suicide attempters and non-attempters. In the analysis of the demographic data for the brain sample, we did not find any significant difference between suicide victims and controls in age (P =0.341), AAO (P= 0.99), DOI (P = 0.614), post-mortem interval (P = 0.837), and brain pH (P = 0.799) (Table 3 - 1). The sample for analysis of attempted suicide consists of 35 men and 12 women (47 totals) with a mean age at the time of the interview of 48.23 ± 11.30 years; 19 patients in the sample had a history of suicide attempt at least once in their lifetime (Table 3 - 2).

Test variables	Suicide victims (mean ± SD)	Non-suicide (controls) (mean ± SD)	*P* value	Mean difference	95% confidence interval of the difference	
Sex (male/female)	2/7	4/7	-	-	-	-
Schizophrenia (*N*)	5	5	-	-	-	-
Bipolar disorder (*N*)	4	3	-	-	-	-
Major depressive disorder (*N*)	0	1	-	-	-	-
Normal control (*N*)	0	2	-	-	-	-
Age at death	38.55 ± 9.23	42.54 ± 8.95	0.341	3.989	−4.59	12.57
AAO	24.22 ± 6.64	24.22 ± 11.16	1	0	−9.18	9.18
DOI	14.33 ± 8.03	16.33 ± 8.45	0.614	2	−6.24	10.24
PMI	32.66 ± 17.76	31.45 ± 6.94	0.837	−1.212	−13.42	10.99
Brain pH	6.39 ± 0.34	6.43 ± 0.24	0.799	0.034	−0.24	0.31

AAO, age at onset; DOI, duration of illness; PMI, post-mortem interval.

Table 3 - 1. Demographic characteristics of suicide victims and controls (n=20)

Test variables	Suicide attempters (mean ± SD)	Non-attempters (mean ± SD)	*P* value	Mean difference	95% confidence interval of the difference	
Sex (male/female)	14/6	21/6	-	-	-	-
Age at interview	44.90 ± 12.51	51.70 ± 9.46	0.039	6.803	0.354	13.253
AAO	20.55 ± 4.94	24.85 ± 7.94	0.038	4.301	0.238	8.365
DOI	23.65 ± 11.58	26.81 ± 11.64	0.361	3.164	−3.740	10.069
SLESQ	2.94 ± 2.51	2.73 ± 2.89	0.807	−0.210	−1.942	1.521
CTQ total	42.25 ± 16.64	37.73 ± 16.75	0.368	−4.519	−14.535	5.496

AAO, age at onset; CTQ, Childhood Trauma Questionnaire; DOI, duration of illness; SLESQ, Stressful Life Events Screening Questionnaire.

Table 3 - 2. Demographic characteristics of suicide attempters and non-attempters (n-47)

We did not find any significant differences in sex (P =0.545) and the DOI (P =0.608) between suicide attempters and non-attempters; however, the age at the time of the assessment (P = 0.039) and the AAO of psychosis, with an earlier onset in suicide attempters (P =0.038), were significantly different between the two groups (P = 0.025). There were no differences in SLESQ scores (P= 0.807) and CTQ scores (P =0.388) between suicide attempters and non-attempters (Table 2). The methylation analysis of CpG sites in exon I of the HTR2A gene in 20 suicide attempters and 27 non-attempters showed that there was no significant difference between suicide attempters and non-attempters. Moreover, the average methylation level of exon I was 77.6% in suicide attempters and 78.2% in non-attempters, and we did not find a significant difference between them (P =0.759) (Figure 1). To test whether the methylation levels were altered because of the medication, we compared the methylation level of these CpG sites in participants who were using medications that affect DNA methylation such as clozapine or its derivatives at the time of the interview. We only found one CpG site (+141) significantly hypomethylated in clozapine users (P= 0.029). Furthermore, we examined the methylation percentage of rs6313 only in the patients who were C102T heterozygous. The results of this analysis did not indicate any significant differences between suicide attempters and non-attempters (P = 0.128). The results of *HTR2A* exon I methylation analysis in nine suicide victims and 11 non-suicide controls also did not show any significant differences between these two groups (Figure 2). In our correlation analysis, the overall methylation level of *HTR2A* exon I in the saliva sample showed a strong positive correlation with the matched blood sample from the same participants (r =0.547, P <0.001) (Figure 3). Finally, the results of this methylation analysis in different tissues indicated that overall methylation in saliva and blood was around 80%; however, overall methylation in the brain was around 30% (Figure 4).

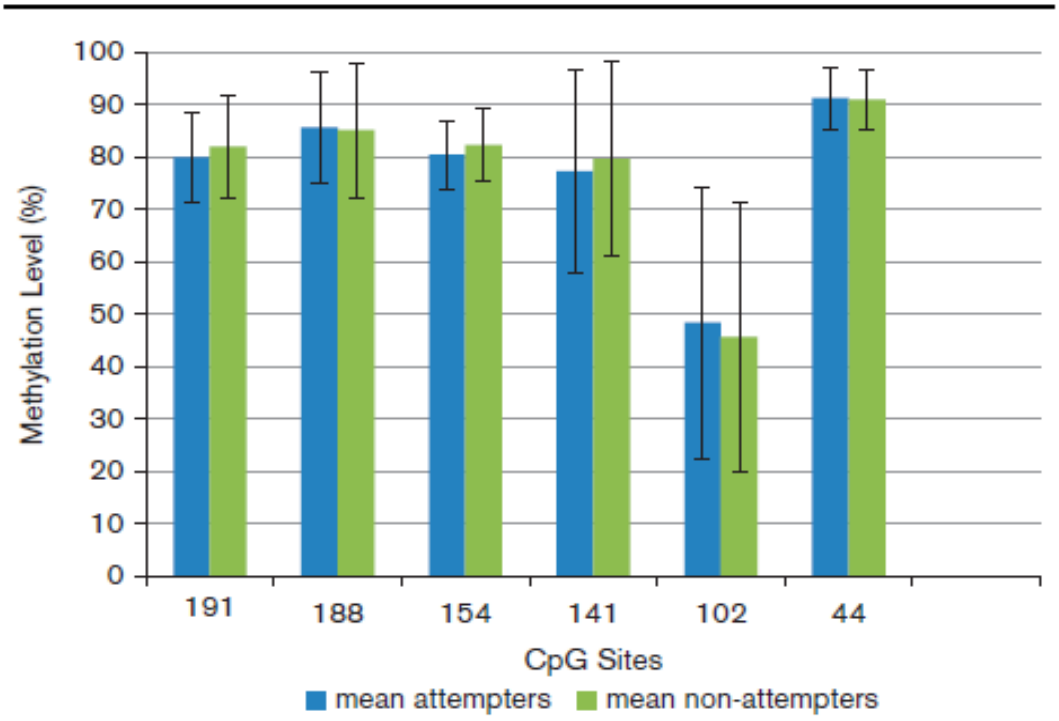

Figure 4 - 1. The methylation level of CpG sites of HTR2A exon I in suicide attempters and non-attempters. Data are presented as mean and SD.

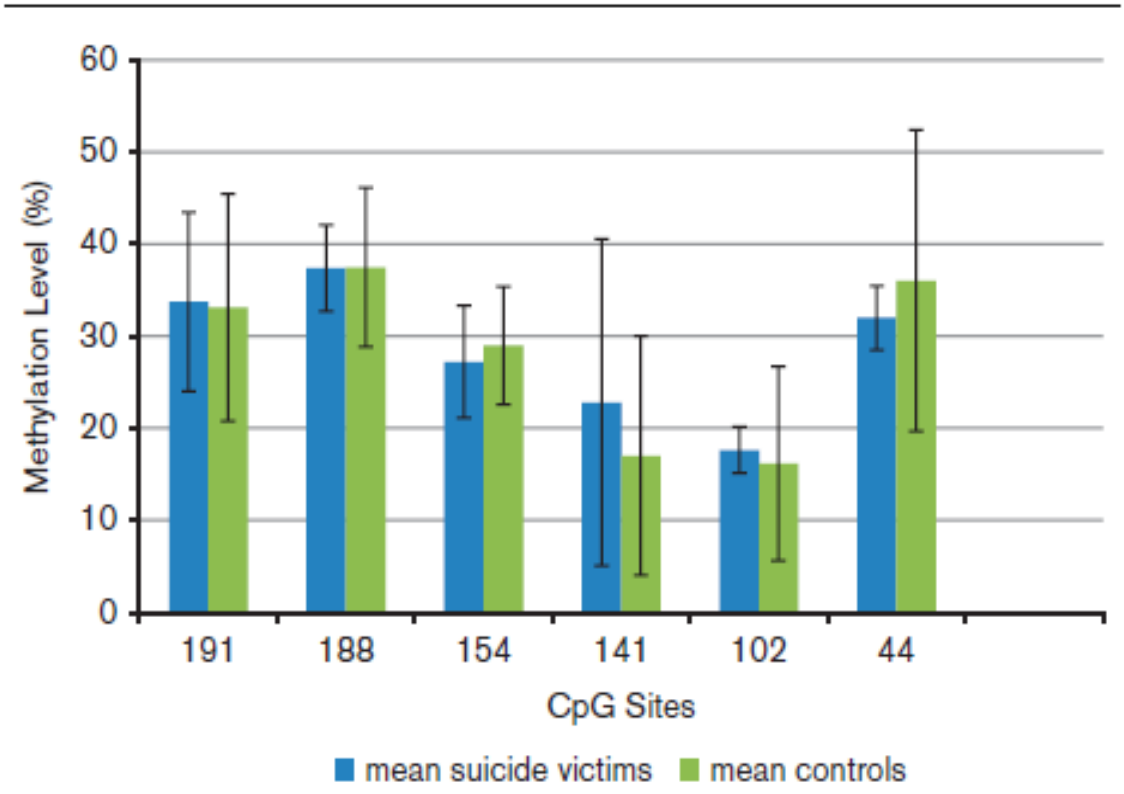

Figure 4 - 2. Methylation level of CpG sites of HTR2A exon I in suicide victims and controls. Data are presented as mean and SD.

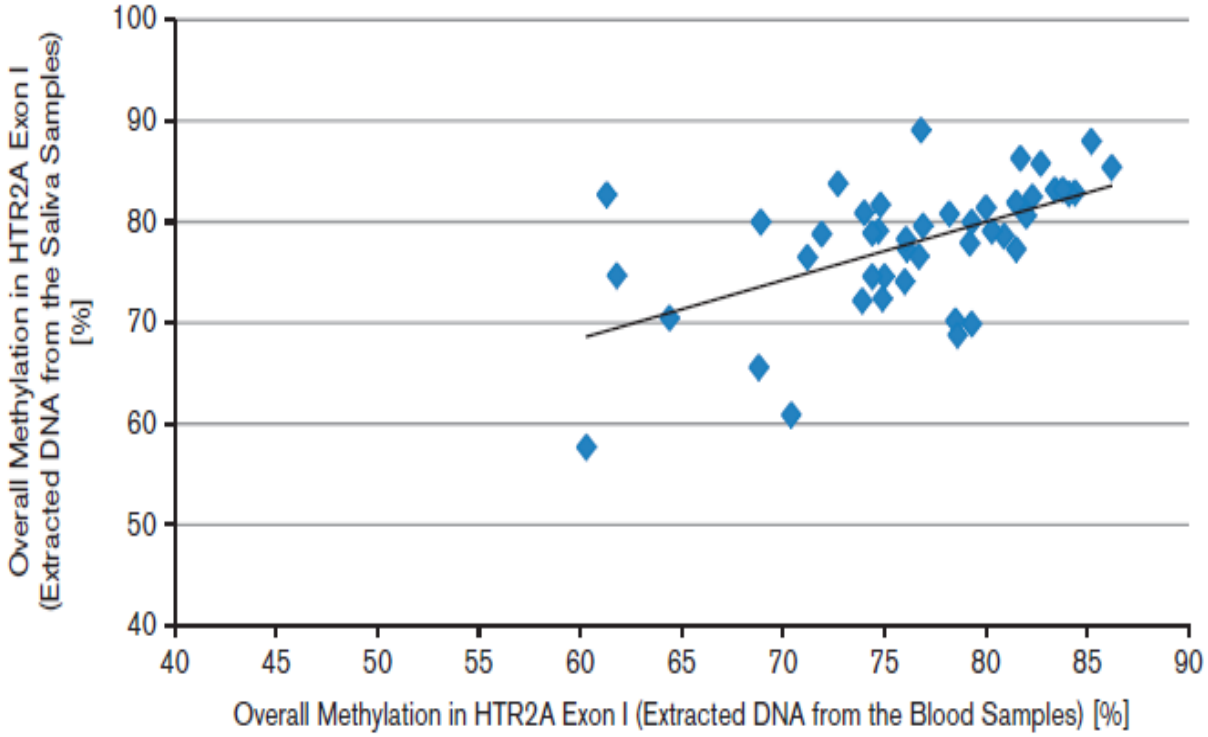

Figure 4 - 3. Correlation between methylation level in saliva samples and the matched blood samples from the same schizophrenia participants (r = 0.55, P=0.00007).

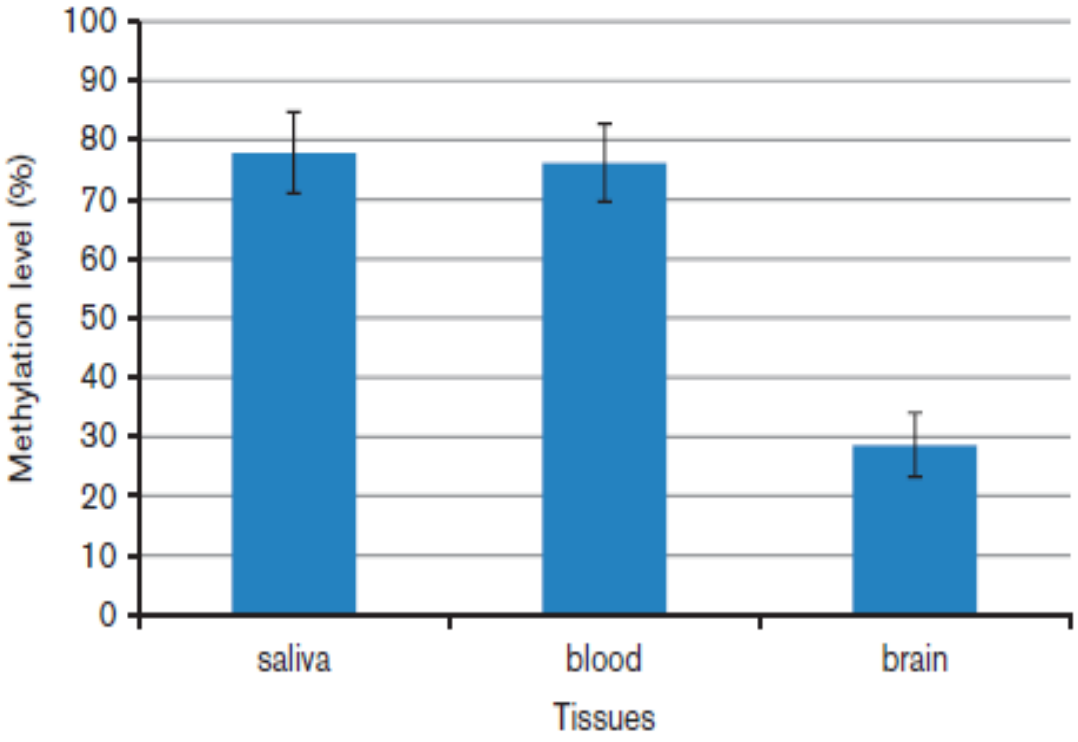

Figure 4 - 4. Overall methylation levels in different tissues.

5.4 Discussion

As most genetic studies did not find robust predictors for suicidal behavior, there has been an interest in investigating the role of epigenetic mechanisms. The current analysis assessed whether DNA methylation in the *HTR2A* gene is associated with a risk of suicide attempt or completed suicide. We assessed direct methylation at six CpG sites of *HTR2A* exon I in DNA samples extracted from saliva and brain. Despite the findings of our previous study for an association between Methylation level of *HTR2A* gene and suicide attempt using DNA extracted from blood (Bani-Fatemi et al., 2016), the current study did not find any association between this gene and suicide attempt or completed suicide. In our previous report, the direct methylation analysis of DNA extracted from the blood in 32 suicide attempters versus 35 non-attempters showed that the polymorphic CpG rs6313 was significantly hypermethylated in suicide attempters. The reason why the current results could not replicate the findings of our previous study may be the fact that we had a smaller sample size. The results of the *HTR2A* C102T (rs6313) methylation analysis also did not replicate the previous findings by de Luca et al. (2009), which indicated that hypermethylation of C102T may be associated with suicide attempt in SCZ patients. This inconsistency may be because of differences in the molecular techniques that we used in our study. In addition, de Luca et al. (2009) selected only heterozygous C102T in their study, whereas we had both heterozygous and homozygous C102T in our sample. However, even when we selected only the participants heterozygous for the C102T polymorphism in our sample, we could not replicate the findings of de Luca et al. (2009).

The results of this study also showed that the DNA methylation levels in saliva are strongly and positively correlated to those in blood. According to the previous findings by Ghadirivasfi et al. (2011), which showed that DNA methylation levels in saliva can reflect and

be correlated to the methylation levels in the brain, we may use either blood or saliva, which is accessible and less challenging to obtain, to approximate the DNA methylation level in the brain. In the clinical analysis of suicide attempters and non-attempters, our results indicated that younger age and having an earlier onset may lead to an increased predisposition for suicide. The small sample size represents the main limitation of our study, which could have resulted in false-negative findings. In fact, the sample size necessary to achieve 80% power is much larger than our current sample (G*Power 3.1.9.2) (Faul et al., 2009). We tested the effect of clozapine on DNA methylation in our sample; however, the majority of our patients were using antipsychotics and as these antipsychotics may decrease the methylation level of *HTR2A* exon I at polymorphic C102T (Ghadirivasfi et al., 2011), this could be another limitation of this study. Moreover, the brain samples are from different diagnostic groups; therefore, we could not rule out the confounding effect of the mental disorders on methylation level in *HTR2A* exon I in influencing the correlation between DNA methylation in peripheral samples with DNA methylation in the brain. Furthermore, we combined SCZ and BD patients, which may be problematic as suicide could be a different phenomenon in these disorders. Future studies should investigate the correlation between DNA methylation in blood, brain, and saliva in a larger sample. The proposed approach may be applied to other serotonin system genes or other candidate gene systems that have been linked to suicide, such as genes involved in the stress response and in the glucocorticoid pathway. The proposed studies should also consider antipsychotic-free individuals as a control group to rule out the effect of these medications on DNA methylation.

Chapter 6
Differential Allelic Expression of *HTR1B* in Suicide Victims: Genetic and Epigenetic Effect of Cis-Acting Variants

6 Abstract

Objectives: In the present study, we tested the allelic imbalance of the C861G single nucleotide polymorphism (SNP) of HTR1B in the frontal cortex of suicide victims.

Methods: The study was conducted using 3 sets of samples. First, C861G allele-specific mRNA levels in the frontal cortex were compared between suicide (n = 13) and non-suicide controls (n = 13) from the Stanley Medical Research postmortem brain collection. Second, we tested common variants in the HTR1B promoter for linkage disequilibrium (LD) with the C861G variant in an unrelated sample of suicide attempters (SA; n = 38) and non-SA (NSA; n = 42). Finally, we performed a family-based association study of the C861G and promoter variants in 162 nuclear families using suicidal behavior severity scores as phenotype.

Results: We observed no alterations in the C/G expression ratio in suicide victims compared to non-suicide controls (p = 0.370). When comparing the LD between the C861G and cis-acting SNP, we did not find any differences in SA and NSA. There was no association between preferential transmission of cis-acting SNP and suicidal behavior severity scores in both maternal and paternal meiosis.

Conclusions: We found several promoter variants in LD that may potentially influence the allelic imbalance in the C861G variant. However, no evidence of allelic imbalance or parent-of-

origin effects of the C861G variant was observed in suicidal behavior. Further research is required to assess this marker in larger cohorts.

6.1 Introduction

Suicide is a public health concern due to its dramatically high incidence in young adults and psychiatric populations. The relative contributions of environment and genetics has yet to be fully characterized in the suicide phenotype, but twin, adoption, and family-based studies suggest the involvement of a genetic component in the risk for suicidal behavior (Statham et al., 1998; Glowinski et al., 2001; Fu et al., 2001).

A recent review by Smith et al. (Smith et al., 2012) demonstrated that suicidal behavior has both a familial and heritable component, with heritability estimates ranging from 17 to 55%. Despite the evidence from family-based studies, molecular genetic approaches have failed to elucidate any robust associations between candidate genes and suicide attempt history (Tsai et al., 2011). This lack of association can be partly explained through the influences of phenotypic heterogeneity in defining suicidal behavior, genetic heterogeneity, and possibly epigenetic mechanisms.

The neurobiology of suicidal behavior appears to be influenced by dysregulation of serotonergic neurotransmission (Tsai et al., 2011; Ernst et al., 2009). Studies have stated that suicidal behavior might be associated with reduced levels of 5-hydroxytryptamine (5-HT) (Abdolmaleky et al., 2011; Antypa et al., 2013). However, previous genetic association studies have failed to ascertain a specific and direct link between serotonergic genes and the risk for suicidal behavior (Ernst et al., 2009).

Conflicting evidence for genetic associations with suicidality was reported for serotonin transporters in 2 meta-analyses. The “short” allele of the serotonin-transporter-linked polymorphic region (5HTTPLR) was found to confer a risk in one meta-analysis (Anguelova et

al., 2003a, Anguelova et al., 2003b) and to be protective in another (Li and He, 2007). Functionally, the short allele reduces transcriptional activity of the serotonin transporter, and, thus, it may represent an endophenotype of reduced serotonergic activity in individuals with a history of suicidal behavior (Antypa et al., 2013).

Equivocal evidence has been reported for several serotonin receptors as candidate genes for suicidality (Anguelova et al., 2003a; Anguelova et al., 2003b; Judy et al., 2012; Buttenschøn et al., 2013). Few studies have examined the association between variations in the serotonin receptor 1B (*HTR1B*) gene and suicidal behavior. The main effect of rs11568817 and the haplotype rs11568817- rs130058 effect were observed in conferring risks for suicidal ideation in a cohort of patients of Asian ancestry with major depressive disorder (Wang et al., 2009). Conversely, Zupanc et al. (2010) reported no association of *HTR1B* variants rs6296 and –161A/T (rs130058) in 373 suicide victims of European ancestry. The discrepancy between both studies may be influenced by the effects of population stratification in risk allele determination. However, most *HTR1B* association studies did not account for environmental and epistatic effects, which was recently examined in a study by Murphy et al. (2011), where a significant 3-locus gene interaction (rs6296× rs4755404 × rs1659400) was predictive of suicide attempt after controlling for clinical covariates. In the same study, rs6296 was also significant in the single nucleotide polymorphism (SNP) association analysis.

Based on these findings, there appears to be a contribution of *HTR1B* common variants to suicidal behavior, but the magnitude of the contribution is still poorly understood. On the other hand, the involvement of serotonergic epigenetic mechanisms in suicidal behavior still remains equivocal due to the paucity of epigenetic studies.

In the present study, we examined the existence of allelic imbalance in the C861G SNP (rs6296) of *HTR1B* in the frontal cortex of suicide victims. Furthermore, we investigated the genetic and the epigenetic effect of the *HTR1B* cis-acting variants testing their linkage disequilibrium (LD) and parent-of-origin effect (POE) with regard to suicide phenotypes.

6.2 Experimental Procedures

6.2.1 Postmortem Study

We matched 13 suicide victims for genotype, gender, and age of death with 13 non-suicide controls, who died from other causes than suicide. Table 1 shows demographic and clinical data of suicide and non-suicide subjects in Stanley Array and Stanley Neuropathology Collections included in this study. The Stanley Array Collection contains mRNA from the dorsolateral prefrontal cortex (Brodmann area 46) from 2 schizophrenia (SCZ) and 3 bipolar disorder (BD) patients, and 1 unaffected control (3 heterozygous suicide matched with 3 heterozygous non-suicide controls). The Stanley Neuropathology Collection contains RNA from the frontal cortex (Brodmann area 9) from 5 SCZ, 5 major depressive disorder, and 5 BD patients, and 5 unaffected controls (10 heterozygous suicide and 10 heterozygous non-suicide matched controls).

6.2.1.1 Gene Expression Analyses

Complementary DNA (cDNA) was synthesized following the Omniscript protocol for reverse transcription (Qiagen). Allele-specific of HTR1B mRNA was measured with quantitative PCR employing a TaqMan ® assay. Differential allele-specific expression analysis was carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems Inc., Foster City, CA, USA).

All reactions were performed in triplicate. Differential allele expression was calculated by subtracting real-time PCR threshold cycle (Ct) values for C and G alleles (ΔCt). However, assays based on allele-specific probes may generate heterozygote ratios that deviate from 1 as a result of differential binding efficiencies of the probes rather than differences in expression

(Hoogendoorn et al., 2000; Bray et al., 2003). In this regard, to correct for this potential bias, the average ΔCt of the genomic DNA sample was subtracted from the ΔCt of the respective cDNA sample (ΔΔCt). Relative levels of C to G were calculated as $2^{-\Delta\Delta Ct}$. The differential expression ratio for each diagnostic group was described as a mean ± SD.

Table 4 - 1. Post-Mortem Brain Sample: Clinical and demographics.

	Suicide Subjects	**Non-Suicide (Controls)**	**Total**
Stanley array/ Stanley neuropathology	3/10	3/10	6/20
Sex (M/F)	5/8	5/8	10/16
Schizophrenia	4	3	7
Bipolar disorder	5	3	8
Major depressive disorder	4	1	5
Healthy control	0	6	6
Age at death (Mean ± SD)	42.30 ± 11.76	44.69 ± 10.53	
AAO (Mean ± SD)	28.16 ± 11.23	22.57 ± 11.26	
DOI (Mean ± SD)	14.30 ± 11.65	26.42 ± 13.72	
Antipsychotics at the time of death (Y/N)	2/1	1/2	3/3
Mood stabilizer at the time of death (Y/N)	2/1	1/2	3/3
Antidepressants at the time of death (Y/N)	2/1	1/2	3/3
Anticholinergic at the time of death (Y/N)	1/2	0/3	1/5
Valproate at the time of death (Y/N)	2/1	1/2	3/3

6.2.1.2 Statistical Analyses

Group differences were evaluated using independent and paired t-tests. Agonal status is one of the major limitations of postmortem brain studies (Iwamoto and Kato, 2006), and thus we examined the effect of brain pH and postmortem interval (PMI) on the allelic ratio. The correlation between mRNA expression and confounding factors was calculated by the Pearson coefficient (PMI and pH) and, where appropriate (alcohol and drug abuse), by nonparametric Spearman correlations. All test and p values were two-tailed. Considering an effect size (dx) between 0.30 (moderate) and 0.50 (large), in our brain sample consisting of 13 suicide victims and 13 non-suicide controls with α set at 0.05 (two tails), we expect a power ranging from 31 to 69% using G * Power 3.1.9.2 (Faul et al., 2009).

6.2.2 Suicide Attempter Study

The sample consisted of 80 (n = 53 males and n = 27 females) unrelated patients. This sample included participants that were recruited from the Centre for Addiction and Mental Health (CAMH) in Toronto, ON, Canada, with the approval of the CAMH Institutional Research Ethics Board. The sample consisted of 38 suicide attempters (SA) and 42 non-SA (NSA), all white European Caucasians with a diagnosis of SCZ or schizoaffective (SCA) disorder ascertained using the Structured Clinical Interview for DSM-IV (SCID-I/P). Inclusion criteria included a diagnosis of SCZ or SCA while our exclusion criteria included evidence of intellectual disability, the presence of neurodegenerative disorders, a history of brain injury trauma with loss of consciousness, and a history of major substance abuse prior to the onset of illness. Written informed consent was obtained for participation in the study. The patients were carefully assessed for lifetime suicide attempt using the Columbia Suicide Severity Rating Scale

(C-SSRS) and the Beck Scale for Suicide Ideation (BSS). They were classified as SA (individuals who have attempted suicide at least once in their lifetime) and NSA (those who never attempted suicide in their lifetime). To document the presence of childhood and lifetime trauma, we administered the Childhood Trauma Questionnaire and the Stressful Life Events Screening Questionnaire.

6.2.2.1 Genetic and Statistical Analyses

We selected 78 SNP upstream the C861G variant genotyped using Illumina Omni 2.5. The SNP were analyzed first in the traditional way using Haploview, and then the pattern of rs6296 LD (r^2) in SA and NSA were compared using an independent t-test. For the classic genotypic association test, to control for the expected proportion of discoveries that are false, we performed false discovery rate using SNP and Variation Suite (online suppl. Table 4 - 1; see www.karger.com/doi/10.1159/000456010 for all online suppl. material). Considering the G-allele frequency of 30% and an odds ratio ranging from 1.2 to 1.9 with a population risk of 30% (Lahiri and Nurnberger, 1991), in our sample consisting of 38 SA and 42 NSA, we expected a power ranging from 12 to 76% under an additive model with α set at 0.05 calculated using Quanto version 1.2.4.

6.2.2.2 LD of HTR1B Cis-Acting Variants with the C861G Polymorphism in SA and NSA

We analyzed 80 unrelated subjects with regard to 78 *HTR1B* SNP located upstream the C861G variant, and we compared the LD patterns in SA and NSA. We calculated the degree of LD between 5′ variants and the C861G separately in SA and NSA. We first performed a standard

genetic test comparing the genotypic counts in SA and NSA. Then, the genetic differences between SA and NSA were tested by comparing the D′ and r^2 within the two groups.

6.2.3 Epigenetic Effect of *HTR1B* Cis-Acting SNP: POE of Suicidal Behavior Severity Scores

The participants were recruited in Toronto with the approval of the CAMH Research Ethics Board. The sample consisted of 487 individuals in 162 families. Genomic DNA was extracted from white blood cells using a standard high salt method (Lahiri and Nurnberger, 1991). Our sample consisted of 162 nuclear families with at least 1 subject affected by BD. DSM-IV diagnoses were confirmed using the SCID and, when necessary, telephone interviews with family members. SNPs were genotyped using the Affy chip 1.0. Sex-specific parent analysis of the transmission of the 78 cis-acting variants was conducted using the quantitative trait LD test (QTDT) package (Abecasis et al., 2000). This analysis allows us to detect POE against a quantitative trait.

6.3 Results

6.3.1 Differential Allelic Expression in Suicide Victims and Controls

The examination of the raw Ct value for the "C" and "G" alleles (not normalized by subtraction from the average genomic DNA sample) confirmed lower performance of the "C" probe in the overall sample (n = 26). The average Ct was 31.87 ± 1.06 for the "C" allele and 31.07 ± 0.93 for the "G" allele (t = –9.877; 25 df; p < 0.001). The average Ct in the genomic DNA was 25.78 ± 0.05 for the "G" allele and 26.95 ± 0.08 for the "C" allele (t = –32.450; 2 df; p=0.001). However, comparisons of the raw measurements of Ct for the two alleles should be interpreted with caution since differences in PCR amplification efficiency could affect this parameter.

PMI was not different between the suicide and the non-suicide group (t = –0.456; 11 df; p = 0.132). ΔCt in the genomic DNA was 1.17 ± 0.06. The C/G allele cDNA level ratio in the 13 heterozygous suicide victims was 1.25 ± 0.15 after the correction for the calibrator (genomic DNA ΔCt). The ΔCt in suicide victim cDNA was 0.86 ± 0.36, significantly lower than the expected ΔCt of 1.17 (t = –3.055; 12 df; p = 0.01). The ΔCt was <1.17 in cDNA from all 26 heterozygous subjects (t = –4.604; 25 df; p < 0.001). The relative expression of the "C" to "G" allele in heterozygous non-suicide controls was 1.27 ± 0.36 after the correction. Also, this group ΔCt (0.86 ± 0.36) was significantly lower than the expected 1.17 (t = 8.65; 12 df; p < 0.001). The mean ratios for the suicide and non-suicide groups were not significantly different (t = –1.044; 12 df; p = 0.317). The allele ratio was not associated with demographic variables such as gender (t = 0.157; 23 df; p = 0.877), age at death (t = –0.476; 12 df; p = 0.642), DSM-IV diagnosis (f = 0.129; 3 df; p = 0.942), PMI (t = –0.456; 11 df; p = 0.132), or brain pH (t = 0.568; 24 df; p =

0.301). Furthermore, the C/G ratio was not influenced by having a psychiatric diagnosis (healthy controls vs. other psychiatric diagnosis: t = 0.354; 24 df; p = 0.726), age at onset of illness (r = 0.202; 24 df; p = 0.407), or different sample collections (t = 1.148; 24 df; p = 0.262). However, C/G ratio was significantly higher in subjects with lifetime alcohol abuse (t = –2.820; 24 df; p = 0.009) and in subjects with lifetime drug abuse (t = –0.584; 24 df; p = 0.03).

Moreover, comparing the suicide subjects with controls, we did not observe any associations with C/G ratio (p = 0.370), age at death (p = 0.590), and age at onset (p = 0.300); however, there was a significant difference in duration of illness between suicide victims and controls (p = 0.050).

6.3.2 Clinical Analysis of SA and NSA

The demographic clinical data for SA and NSA are summarized in Table 4 - 2. The mean age at the time of the interview was 46.5 years (11.9). The age at onset of psychotic symptoms was 23.8 years (8.0). The duration of illness was 24.2 years (12.9) in the SA group and 20.7 years (8.1) in the NSA group. There were no significant differences in sex ratios, mean age, mean age at onset, Childhood Trauma Questionnaire total score, Stressful Life Events Screening Questionnaire, Beck’s Hopelessness Scale scores, and NEO personality inventory subscales between the SA and NSA groups (Table 4 - 2).

6.3.3 LD Analysis of HTR1B promoter and Association with Suicide Attempts in 80 Unrelated Subjects

We did not find an association between the 5 ′ SNP and suicide attempt history (online suppl. Table 1), but we found several SNP in the promoter in high LD with the C861G SNP; therefore, these variants may influence the allelic ratio between the 2 alleles. The 78 SNP selected were not observed to be significantly associated with suicide attempt when we analyzed suicide attempt as grouping variable. The LD pattern expressed as r^2 (online suppl. Table 2) was not significantly different between SA and NSA ($t = –1.37$, 152 df; $p = 0.171$).

6.3.4 QTDT with Suicide Severity Scores

QTDT analysis of the 78 cis-acting SNP did not reveal preferential transmission for either allele regarding a higher suicidal behavior severity score (online suppl. Table 3). When we tested maternal transmission, we observed a trend for the SNP rs1777755 ($p = 0.050$) with the allele 2 associated with a higher suicide severity score (0.897). When we tested the paternal transmission, we found a slight trend for the rs2504277 with allele 1 transmission distortion towards a higher suicide severity score.

Table 4 - 2. Clinical and demographics of suicide attempters and non-attempters.

	Suicide Attempters	**Non-Attempters**	**P-value**
Participants (N)	38	42	ns-
Age (X, SD)	47 ± 11.13	46 ± 12.68	0.710
Sex **F (N): M (N)**	11:27	16:26	Ns
Age at Onset (X, SD)	22.15 ± 7.45	25.19 ± 8.15	0.088
Duration of Illness (X, SD)	24.21 ± 11.97	20.70 ± 11.98	0.194
CTQ* (X, SD)	40.92 ± 15.10	38.17 ± 13.93	0.399
SLESQ (X, D)**	3.51 ± 2.81	2.75 ± 2.49	0.203
BHS	4.02 ± 3.59	4.29 ± 3.49	0.734
NEO* Neuroticism**	35.08± 7.95	35.85 ± 7.95	0.666
NEO Extraversion	37.60± 7.35	36.60 ± 6.08	0.508
NEO Openness	39.68 ± 6.15	39.73 ± 6.36	0.972
NEO Agreeableness	41.62 ± 3.37	41.24 ± 4.13	0.655
NEO Conscientiousness	43.11 ± 7.81	42.85 ± 6.50	0.871

* Childhood Trauma Questionnaire.

** Stressful Life Events Screening Questionnaire.

*** Neuroticism-Extraversion- Openness

6.4 Discussion

During the past decade, there has been increased interest in possible associations between serotonergic genes and suicidal behavior. However, only a few studies have explored the effect of differential allelic expression of 5-HT genes in suicide brains. Analysis of the expression of rs6296 alleles in suicide victims did not yield significant differences in *HTR1B* C/G allele mRNA ratio levels between suicide victims and non-suicide controls in our postmortem brain samples. Although the cDNA analysis showed no difference in the C/G allele ratio between the suicide and non-suicide group, the expression of the C allele is higher, implying upstream genetic or epigenetic mechanisms that are influencing the differential expression on the 2 chromosomes.

There were no significant differences between paternal and maternal transmission ratios in SA, and there was no association between maternal or paternal transmissions and suicide severity scores.

Studies have attempted to investigate a POE effect for serotonin genes in suicide; however, this is the first study to our knowledge that has examined *HTR1B* gene. Previous studies have failed to find other serotonin alleles that are significantly overtransmitted in SA, and our study consistently does not show POE in suicidal behavior.

In the gene expression analysis, we found that the C/G allele mRNA ratio was significantly associated with a history of alcohol and drug abuse. Similar findings have been reported by Lappalainen et al. (1998) who demonstrated positive linkage and association between the *HTR1B* variant G861C and antisocial alcoholism. Furthermore, G861C was also

found to be associated with substance abuse disorder and major depression (Huang et al., 2003). Recent evidence, however, has suggested that there is no association between suicide and polymorphisms in specific serotonergic genes (including *HTR1B*) and alcohol-related suicide (Zupanc et al., 2010).

The lack of association of rs6296 alleles in suicide victims and non-suicide controls appears to be consistent with previous genetic research in SA. A study by Rujescu et al. (2003) showed no relationship between the *HTR1B* polymorphism G861C and suicidal behavior. Similarly, a lack of association between *HTR1B* and suicide was further supported by Turecki et al. (2003), who observed no significant interaction between completed suicide and 7 serotonin receptor genes. This finding has remained consistent over time as earlier reports by Huang et al. (1999) and Nishiguchi et al. (2001) also demonstrated a lack of evidence for a role of *HTR1B* in suicide or psychopathological conditions. Conversely, Wang et al. (2009) performed a haplotype association study, which suggested that the rs11568817-rs130058 haplotype of the *HTR1B* gene is significantly associated with suicidal ideation in major depression.

Several associations and some gene expression studies have investigated whether a relationship between variation in the *HTR1B* gene and suicidal behavior exists, but it is still unclear whether *HTR1B* is involved in increased propensity to engage in suicidal behavior.

Despite our findings showing a lack of association between *HTR1B* and suicide, the series of experiments that we used to analyze this 5-HT receptor may be applied to other genes that have been linked to suicidal behavior, in particular, the genes involved in stress responses, such as those in the hypothalamic-pituitary-adrenal axis. The C861G allelic imbalance was not analyzed before, and, therefore, we cannot compare our analytic technique with other methods.

However, for serotonin receptor 2A (*HTR2A*), it was proven that TaqMan is superior to restriction enzyme analysis (Polesskaya et al., 2006). Future studies should analyze the allelic imbalance of this receptor in different tissues using cutting-edge technologies such as RNA sequencing.

Limitations of this study include sample size, drug treatment effects, inability to examine >1 coding SNP, and the phenotypic heterogeneity of the sample sets (Stanley Neuropathology and Stanley Array). It is difficult to compare tissue from the 2 frontal cortical areas (Brodmann areas 9 and 46) due to potential environmental influences of differently sourced samples. Ideally, future studies should attempt to obtain different tissues from the same subject to address this limitation. Allele-specific expression can also be affected by multiple cis-regulatory elements; however, in our study, we did not find any difference in LD patterns between SA and NSA. Several studies that have reported positive associations between candidate genes and suicidal behavior have failed to be replicated. A number of factors may contribute to the inconsistency in these results, including the heterogeneous phenotype of suicidal behavior and potential methodological shortcomings.

A major impediment to genetic research of suicidal behavior is the lack of consistency in how “suicidal behavior” is defined. As many psychiatric traits, suicidal behavior falls on a continuous spectrum. Suicidal behavior phenotypes may range from suicidal ideation to suicide attempt and completed suicide. Many studies chose to limit their focus to a certain suicide phenotype, such as attempted suicide or suicidal ideation. As such, it is difficult to draw appropriate comparisons between study results. In our study, we have tried to compensate for this shortcoming by analyzing ideation, attempt, and completed suicide across 3 different

samples, connecting the allelic expression with specific genetic tests that can explain the regulatory effects of the cis-acting variants of *HTR1B*.

In summary, our study does not provide evidence for a major role of *HTR1B* in the predisposition to suicide. Further studies in larger samples may be helpful to investigate a more subtle effect of this gene in this serious phenotype. Overall, our results suggest that differential allele-specific expression of the rs6296 SNP is not involved in suicide. Further analysis of allele-specific expression combined with cis-acting variant analysis of the serotonergic system may help to uncover the mechanism by which serotonergic dysfunction is implicated in suicidal behavior across different psychiatric disorders.

Chapter 7
Conclusion and Discussion

7 Discussion

7.1 Analysis of CpG SNPs in 34 genes: association test with suicide attempt in schizophrenia

In our first analysis (Chapter 3), we attempted to assess the relationship between candidate genes associated with the neurobiology of schizophrenia and suicide attempt in a sample of psychotic individuals. In addition, we aimed to assess whether SNPs within CpG sites played a significant role in predicting a history of suicide attempt in schizophrenia. We found no association between the 384 SNPs selected in our initial panel of candidate genes and suicide attempt history. Furthermore, none of the CpG SNPs we tested were significantly associated with a history of suicide attempt in this sample. Interestingly, we did detect a nominal significant effect for *RGS4* rs2661319, though this finding did not survive statistical multiple testing corrections. We found no evidence of CpG SNPs being significantly associated with a history of suicide attempt in our sample of patients with schizophrenia with or without a history of suicide attempt. While we failed to detect any significant association, our analysis was limited for a variety of reasons. Notably, because of the small sample size, it is difficult to achieve enough statistical power to detect more meaningful results. Nevertheless, the CpG SNP analysis represents one of the first of its kind. We propose here to calculate the potential methylation levels from the polymorphisms that affect the presence of CpG sites in key genes related to schizophrenia.

Therefore, rather than analyzing for SNPs in which the function is not yet determined, we propose a mechanistic approach for identifying potential regions in the genome where higher or lower potential methylation associated with suicide attempt in schizophrenia. As a result, future studies may benefit from incorporating our analysis to achieve a deeper investigation to understand the genetic variants associated with suicide risk.

7.2 Interaction between methylation and CpG single-nucleotide polymorphisms in the HTR2A gene: association analysis with suicide attempt in schizophrenia

In the second study (Chapter 4), we assessed whether DNA methylation and CpG SNPs in the *HTR2A* gene are associated with suicide attempt in schizophrenia patients. We identified 25 CpG SNPs within the *HTR2A* gene and assessed potential methylation as well as direct methylation of CpG sites in exon 1. Our rationale for selecting *HTR2A* for this analysis is due to the reported importance of this gene in the neurobiology of both schizophrenia and suicide. The novelty of this study is in how we selected the SNPs. Historically, tag SNPs were chosen in this gene for genetic association studies. However, here we specifically chose to look at SNPs that directly affect CpG sites in *HTR2A*. In the direct methylation analysis of 32 suicide attempters versus 35 non-attempters, we showed that the polymorphic CpG site nearby the SNP rs6313 was significantly hypermethylated in suicide attempters. Furthermore, in the potential methylation analysis, we did find a significant difference for this polymorphic CpG site between suicide attempters and non-attempters. While these findings are indeed positive, when correcting for SNP effect, this CpG site methylation was no longer significant. Here we define potential methylation as the availability of CpG sites, which is a requisite for the addition of a methyl group onto the cytosine residue. Therefore, the C/T polymorphism is the factor determining the potential for methylation to occur at this site due to the creation of a CpG site when the cytosine allele is present. The strong correlation between overall direct and potential methylation in HTR2A suggests that the methylation process is influenced by the SNPs directly affecting CpG sites. Unfortunately, we are still constrained by sample size and as with any study with low statistical power, it is likely that our results represent a high-degree of false-negative findings. In

conclusion, we did not find strong evidence for epigenetic changes in HTR2A associated with suicide attempt.

While our results thus far may seem discouraging, these results are the norm for most studies attempting to find robust genetic predictors for suicidal behavior, particularly in schizophrenia. As a result, an analysis of epigenetic mechanisms has become the next focal point in identifying potential robust biomarkers. Unfortunately, analyzing DNA methylation in mental illnesses remains an ongoing challenge. These disorders primarily affect the brain, but it is difficult to measure brain methylation in living individuals with schizophrenia or other psychiatric diagnoses. As we are currently restricted to extracting saliva or blood from patients, it is difficult to assess whether any methylation changes detected in the blood are indeed representative of what is found in the brain. Unlike genetic studies, assessing DNA sequence that is equivalent in whatever cell type or tissue analyzed, epigenetic studies assess DNA methylation that is highly tissue and cell specific as well as sensitive to environmental factors. This combined with the inaccessibility of brain tissue from living patients makes difficult to draw definitive conclusions for these epigenetic variants. Due to this, we performed another experiment to get a better understanding of how *HTR2A* methylation varies across different tissues.

7.3 Multiple tissue methylation analysis of HTR2A exon I in suicidal behavior

In the third study (Chapter 5), we assessed whether DNA methylation in the HTR2A gene is associated with suicide attempt and completed suicide. We assessed direct methylation at six CpG sites of HTR2A exon I in DNA extracted from both saliva and brain samples. This study did not find any association between this gene and suicide attempt or completed suicide. In addition, we were not able to replicate the results of our findings from Chapter 4 when analyzing direct methylation in 32 suicide attempters versus 35 non-attempters for the polymorphic CpG rs6313 in DNA extracted from saliva. The results of the HTR2A C102T (rs6313) methylation analysis also did not replicate the previous findings by De Luca et al. (2009), which indicated that hypermethylation of C102T may be associated with suicide attempt in SCZ patients. This inconsistency may be because of differences in the molecular techniques that we used in our study. In addition, de Luca et al. (2009) selected only heterozygous C102T in their study, whereas we had both heterozygous and homozygous individuals in our sample. While the association results of our study remain inconclusive, again due to sample size limitations, the results of this study were useful in that we showed that the DNA methylation levels in saliva are strongly and positively correlated to those in blood. According to the previous findings by Ghadirivasfi et al. (2011), which showed that DNA methylation levels in saliva can reflect and be correlated to the methylation levels in the brain, we may use either blood or saliva, which is accessible and less challenging to obtain, to approximate the DNA methylation level in the brain. This represents great news for those of us who are attempting to understand DNA methylation in the context and etiology of mental illnesses.

7.4 Differential Allelic Expression of HTR1B in Suicide Victims: Genetic and Epigenetic Effect of Cis-Acting Variants

In our final analysis (Chapter 6), we attempted to assess the differential allelic expression of *HTR1B* in suicide victims. Only few studies have explored the effect of differential allelic expression of 5-HT genes in suicide brains. The current study did not find any association between *HTR1B* C/G allele mRNA ratio levels and suicide. Despite our findings showed no significant difference in the C/G allele ratio between the suicide victims and controls, the C allele showed higher expression than the G allele, suggesting upstream genetic or epigenetic mechanisms that are influencing the differential expression between the two alleles.

Although several studies investigated parent-of-origin effects (POE) of serotonin genes in suicide; this study represents one of the first that has examined POE in *HTR1B* gene. Consistent with the studies attempted to find other serotonin alleles that are significantly overtransmitted in suicide attempt, our results did not show POE in suicidal behavior. We found that the C/G allele expression ratio in *HTR1B* rs6296 was significantly associated with a history of alcohol and drug abuse. However, it has been suggested that there is no association between polymorphisms in *HTR1B* and suicide in alcohol-dependent victims (Zupanc et al., 2010).

The lack of association between rs6296 and suicide has been consistently reported by several studies (Rujescu et al., 2003; Turecki et al., 2003; Huang et al., 1999; and Nishiguchi et al., 2001). However, Wang et al. (2009) performed a haplotype association study, which suggested that the rs11568817-rs130058 haplotype of the HTR1B gene is significantly associated with suicidal ideation in major depression.

Since rs6296 allelic imbalance has not previously been analyzed, we are not able to compare our experimental techniques with other methods. However, for *HTR2A*, it was proven that TaqMan is superior to restriction enzyme analysis (Polesskaya et al., 2006). This study had also several limitations such as sample size, drug treatment effects, inability to examine more than one coding SNP, and the phenotypic heterogeneity of the brain samples. In summary, our results from this study suggest that differential allele-specific expression of rs6296 is not involved in suicidal behavior. Future studies may investigate the allele-specific expression combined with cis-acting variant analysis of the serotonergic system genes to uncover the mechanism by which serotonergic dysfunction is implicated in suicidal behavior.

7.5 Conclusion

A major disadvantage of genetic research on suicidal behavior is the lack of consistency in the definition of suicidality. As many psychiatric traits, suicidal behavior falls on a continuous spectrum. It may range from suicidal ideation to suicide attempt and completed suicide. Many studies have considered only one suicide phenotype, such as suicide attempt or suicidal ideation. Therefore, it is difficult to appropriately compare study results. In one of our studies, we have tried to compensate for this shortcoming by analyzing suicide attempt and completed suicide in three different tissues to investigate the role of genetic and epigenetic changes.

The work described in this thesis does not provide evidence for a major role of 5-HT genes in predisposition to suicidal behavior. Further studies in larger samples may be helpful to investigate a more subtle effect of these genes in this fatal outcome.

Despite our findings showing a lack of association between genetic and epigenetic alterations in serotonin genes, the series of experiments that we used to analyze these 5-HT receptors may be applied to other genes that have been linked to suicidal behavior. Genes involved in stress responses, such as those in the hypothalamic-pituitary-adrenal axis should be investigated in the future.

Chapter 8
Future Direction

8 Future direction

8.1 Future genetic studies of suicidal behavior

Future studies should further examine the involvement of genes in suicidal behavior. In the present work, we examined specific variants within the *HTR1B* and *HTR2A* genes within the serotonergic system in a sample of schizophrenic and bipolar patients. However, suicidal behavior is prevalent across all psychiatric disorders, and therefore in future work, we aim to examine similar markers across multiple psychiatric disorders (i.e. major depression, borderline personality disorder, and post-traumatic stress disorder). This would allow us to increase the generalizability of the findings to find suicide vulnerability markers, independent of psychiatric diagnosis. An example of a study that adopted this strategy of including patients with mood disorders and schizophrenia is the recent meta-analysis conducted by Ottenhof et al. (2018) examining association with *TPH2* polymorphisms. However, in this study, the authors were not able to find a clear association with the suicide phenotype independent of neuropsychiatric diagnosis for *TPH2* variants in coding and non-coding regions.

The association between TNF-α SNPs and serotonergic variants would also be an avenue of investigation in future work. As recent studies have observed an association between TNF-α (de Medeiros Alves et al. 2017; Wang et al. 2018) and serotonergic genes (*5-HTT*) with suicide ideation and completion in a spectrum of psychiatric disorders. Further explorative studies would be indicated to determine whether there is an interaction between the serotonergic receptor genes investigated in our studies (namely *HTR1B, HTR2A, HTR3A, HTR3B,* and *HTR4*) and TNF-α gene in conferring risk for suicide. This interaction may also explain the negative findings in our

work and better elucidate the role of genetic variation in the serotonergic system in conferring risk for suicidal behavior in schizophrenia. Further work is required to illustrate the interaction between 5-HT and other candidate genes in determining predisposition for suicidal behavior.

8.2 Copy number variation analysis in suicidal behavior

Future studies should investigate copy number variants in a sample of schizophrenic patients assessed for suicidal behavior because no studies have tested this specific hypothesis. With respect to suicidal behaviors, only two studies performed CNV analysis (Gross et al. 2015; Perlis et al. 2012). However, these studies included mood disorders subjects in which the effect of CNVs is less evident than in SCZ. Copy number variations (CNVs) are deletions or duplications of a segment of DNA usually larger than one kilobase. Evidence suggests that copy number variants have a robust and reproducible impact on SCZ (Sebat et al. 2007). 22q11.2 Deletion represents one of the greatest known genetic risk factors for development of psychosis. Genes within the 22q11.2 locus have been implicated in the etiology of psychosis (Williams et al. 2007). Given the fact that genetic studies of suicide focused mainly on SNP analysis, we will aim to look at the effect of deletions and duplications in conferring risk for suicide. To date, we have completed a preliminary pilot analysis testing the association of chromosome 22 deletions with lifetime suicide attempt in a SCZ sample, and have observed no direct CNV effect, but there appears to be several confounding and experimental variables that influence deletion events and their detection. A study by Tombacz et al. 2017 examined CNV variants in whole-exome sequencing of suicide victims with major depressive disorder and found no significant association. Nevertheless, in the future, we will aim to investigate CNV variation in suicide attempt and ideation to determine whether the suicide phenotype is influenced by deletions and duplications across the entire gene.

8.3 Future epigenetic studies of suicidal behavior

Brain epigenetics is a distinct field that requires the development of specific analytical tools to address unique experimental challenges. In fact, accumulating evidence indicates that epigenetic processes in the brain compared with other organs present unique properties such as unique pattern of non-CG methylation (He et al., 2015), high levels of hydroxymethylation (Kriaucionis et al., 2009) and higher complexity of non-coding RNAs (Frye et al., 2016). We can speculate that the brain may have evolved as the most sensitive organ to process changes in environmental conditions to improve adaptation to the environment. Accordingly, throughout the evolution, this functional specialization may have required potentially more complex molecular and epigenetic processes mediating the interplay of the environment and the genome. In addition, because epigenetic changes can be long lasting (Nestler et al., 2016), they represent a form of genomic plasticity that could help explain psychiatric phenotypes, such as depressive illness and suicide, that associate with distal environmental stressors (i.e. early life adversities) (Sweatt et al., 2016).

Moreover, in the present investigations, we used patients treated mainly with antipsychotic medications and given the chronicity of schizophrenia; many of the patients included had been on medication for a significant amount of time prior to epigenetic analysis. A recent study by Shi et al. (2017) showed that *HTR2A* methylation level was influenced by treatment with risperidone in Chinese Han schizophrenia patients. Therefore, future epigenetic studies of suicidality must also take into consideration the effects of psychotropic drugs on DNA methylation as it may lead to lasting changes within the epigenetic signature presented on *HTR2A* and other genes.

Furthermore, in the epigenetic analysis of our sample, we used the pyrosequencing technique to investigate DNA methylation of 5-HT genes, measuring few CpG sites. However, Illumina has released a cost-effective and high throughput approach to epigenome-wide association studies (EWAS), the Infinium Human Methylation450 Bead chip (HM450) (Illumina Inc., San Diego, CA, USA). This technology allows for interrogation of >485,000 methylation sites at single nucleotide resolution (Dempster et al., 2013). The CpG sites are distributed in the promoter regions, 5′ UTR, first exon, gene body, and 3′UTR and covering 96% of CpG islands, island shores, and regions flanking them. Recently, Illumina has released the MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling, that covers over 850,000 CpG sites, including >90 % of the CpGs from the HM450 and additional 413,743 CpGs. Future epigenetic studies of suicide may use one of these methods to perform a genome-wide methylation analysis. However, the downside of these new technologies is that the majority of probes on the array are located in CpG-rich promoters, which may not be optimal for studying the complex phenotypic variability observed in suicidal behavior.

Future methylation studies of suicidal behavior should also investigate non-CG methylation. Although DNA methylation is largely restricted to sequences composed of cytosines followed by guanines (CG dinucleotides), recent results have identified a non-canonical form of DNA methylation in non-CG, namely CH contexts (where H stands for A, C or T). High levels of CH methylation (mCH) were first identified in embryonic stem cells; however; recent findings have revealed that the highest levels of mCH across mammalian tissues are in the brain (He et al., 2015). Results also showed that mCH accumulation is much more pronounced in neurons than in glial cells (He et al., 2015; Lister et al., 2013) and that mCH levels measured in the whole tissue (5%) are considerably lower than for the CG context (70–

80%). Nevertheless, a large number of cytosines in CH, compared with CG, contexts has led to estimates that mCH may ultimately account for as much as a quarter of all methylated cytosines (Lister et al., 2013). Similar to mCG, mCH tends to negatively associate with transcriptional activity. Therefore, differences in gene expression associated with suicide might partly result from differential mCH levels, particularly for genes that are dysregulated in cells with high mCH levels (likely neurons rather than glial or other cell types). Importantly, mCH may be particularly relevant to so-called 'sensitive periods', defined as time windows in brain development during which critical processes must take place to achieve proper maturation of essential physiological functions. This concept, which was primarily investigated for sensory-motor functions (Barkat et al., 2011) and more recently in relation to emotional regulation (Makinodan et al., 2012) may partly explain the relationship between early life adversities and suicide (Lutz et al., 2014).

As mentioned above, ELAs are important predictors of suicidal behavior, and their effects are thought to be mediated partly through DNA methylation. Considering that mCH progressively accumulates in neurons during the first few years of life (Lister et al., 2013) it is tempting to speculate that this newly identified epigenetic mark may be particularly sensitive to early life adversities and therefore, highly relevant in determining suicide risk. However, compared with DNA methylation in the canonical CG context, mCH transcription regulation is only starting to be explored (Jin et al., 2016). Nevertheless, mCH should be investigated in the next generation of epigenetic studies of suicide.

8.4 Future imaging studies of suicidal behavior

Suicide prevention research could benefit from neuroimaging investigation. Clinical assessment of individuals who are at risk to engage in suicidal behaviors remains a daunting task for clinicians. Therefore, future studies need to find imaging biomarkers, which can help to better predict individuals that may be at risk for suicidal behavior. Recent neurocognitive and functional/structural neuroimaging findings may be an appropriate resource for clinicians to improve the risk assessment of suicidal behavior. However, a clinical strategy for detection and efficient treatment of suicidality should be based not only on state-like biomarkers, generated from functional MRI and pharmacological imaging but also on trait-dependent imaging biomarkers, such as structural characteristics generate from volumetric and DTI imaging studies.

Functional imaging studies of suicidal brain, using modalities such as fMRI and tasks like the Go-No-Go response inhibition, have found reduced activation in the ACC, left insula and supramarginal gyrus. The dorsolateral and orbitofrontal cortices, which are involved in executive, emotional and reward processing/inhibition (Jollant et al. 2011), are of utmost importance in the context of suicide. Therefore, alterations in serotonergic neurotransmission to these areas may result in the impaired ability to regulate behavior and may consequently lead to a greater propensity to engage in suicidal behavior.

Future neuroimaging efforts could benefit from adding epigenetic biomarkers to fill existing gaps in understanding suicide risk trajectories and intervention responses that can better establish risk and prevention models specific for different psychiatric disorders.

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