Involvement of the Polyamine System in Suicide

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ABSTRACT/RÉSUMÉ

The polyamine system plays an essential role in a myriad of cellular functions, and dysregulated polyamine metabolism and functioning has been implicated in a number of pathological conditions, including neurological and psychiatric disorders. In recent years, gene expression and genetic association studies have provided strong evidence implicating the polyamine system in suicide, yet it was unclear how extensively polyamine metabolism is affected across the brains of suicide completers, if similar mechanisms are involved in other psychiatric conditions, nor the molecular events by which these alterations in gene expression arise. The studies comprising this thesis were thus designed to address these issues, as they represent essential considerations both for interpreting previous findings regarding the polyamine system, as well as for evaluating the potential for this system to be used as a therapeutic target for the treatment of suicidal behaviors. To address these issues, a combination of gene expression and genetic association studies were first performed in order to better characterize the extent by which polyamine metabolism is affected across the brains of suicide completers, to determine if similar mechanisms are implicated in other psychiatric conditions, and to assess how the altered expression of these genes is related to the local genetic environment. Following these studies, a series of experiments were performed to investigate the involvement of genetic and epigenetic mechanisms in determining the expression of polyamine genes in the brain, as well as to evaluate the association of these factors with suicide. Collectively, the studies contained herein have not only replicated previous findings implicating the polyamine system in suicide, but have also greatly expanded our knowledge regarding the extent by which this system is affected in suicide, and have extended these findings towards other psychiatric conditions. In addition, several genetic and epigenetic mechanisms were identified which account, in part, for alterations in polyamine gene expression that occur in suicide. Ultimately, these findings have established that the polyamine system represents an important facet of the neurobiological alterations that occur in suicide, and

have provided insight into the means by which this system is involved in the etiology and pathology of suicidal behaviors.

Le système des polyamines joue un rôle essentiel dans une myriade de fonctions cellulaires, et la dysrégulation du métabolisme et du fonctionnement des polyamines ont été impliqué dans quelques conditions pathologiques, incluant les troubles neurologiques et psychiatriques. Durant les dernières années, des études investiguant l'expression des gènes et les associations génétiques ont apporté des preuves convaincantes impliquant le système des polyamines dans le suicide. Ces études, par contre, n'indiquaient pas à quel point le métabolisme des polyamines est affecté dans les cerveaux de suicidés, ni si des mécanismes similaires sont impliqués dans d'autres conditions psychiatriques, et ne traitaient pas des événements moléculaires par lesquels ces altérations dans l'expression des gènes se présentent. Les études comprises dans cette thèse ont donc été conçus pour répondre à ces questions, car ils représentent les considérations essentielles pour interpréter les résultats antérieurs concernant le système des polyamines, ainsi que pour évaluer le potentiel de ce système à être utilisé comme cible thérapeutique pour le traitement des comportements suicidaires. Pour répondre à ces questions, une combinaison d'études examinant l'expression des gènes et d'études d'associations génétiques ont d'abord été réalisées afin de mieux caractériser dans quelle mesure le métabolisme des polyamines est affecté dans les cerveaux de suicidés. De plus, ces études ont permis de déterminer si des mécanismes similaires sont impliqués dans d'autres conditions psychiatriques et d'évaluer comment l'expression de ces gènes est liée à l'environnement local génétique. Suite à ces études, une série d'expériences ont été effectuées pour enquêter sur l'implication des mécanismes génétiques et épigénétiques dans la détermination de l'expression des gènes des polyamines dans le cerveau, ainsi que pour évaluer l'association de ces facteurs avec le suicide. Collectivement, les études contenues dans cette thèse n'ont pas seulement reproduit les résultats antérieurs impliquant le

système des polyamines dans le suicide, mais ont également augmenté considérablement nos connaissances quant à la mesure de l'affectation de ce système dans le suicide, tout en étendant ces résultats à d'autres conditions psychiatriques. En outre, plusieurs mécanismes génétiques et épigénétiques ont été identifiés qui compte, entre autre, des altérations de l'expression génique des polyamines qui se produisent dans le suicide. En fin de compte, ces résultats ont établi que le système des polyamines représente un aspect important des modifications neurobiologiques qui se produisent dans le suicide, et ont permis de mieux comprendre les moyens par lesquels ce système est impliqué dans l'étiologie et la pathologie des comportements suicidaires.

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1.2 Gene Expression Profiling of Suicide Completers

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1.3 Implication of the Polyamine System in Mental Disorders

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3.1 Identification and Characterization of SAT1 Promoter Variants in Suicide Completers

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4.1 Genetic and Epigenetic Influences on Expression of SMS and SMOX in Suicide

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CHAPTER 1: INTRODUCTION

Mental illnesses are common and often chronic conditions which are associated with significant disability and mortality (1). Suicide and suicidal behaviors are among the most devastating consequences of these disorders, and range between ideation, attempts, and completed suicide, which accounts for one million deaths worldwide each year (2). Over the last century, numerous family, twin, and adoption studies have demonstrated the existence of heritable genetic factors which are involved in conferring risk for suicidal behaviors, and while a diagnosis of an Axis I psychiatric disorder is found in the majority of individuals who complete suicide (3), the heritability of these conditions appears to be based upon both shared and distinct genetic factors (4;5). Overall, the heritability rate for suicide ranges between 30-50% (6-8), with the remainder of the risk being due to environmental factors.

While an extensive number of epidemiological and psychological studies have been performed to identify clinical and sociodemographic variables which are associated with suicidal behaviors, it has become increasingly recognized that the etiology and pathology of suicide depends upon the interplay between epidemiological, psychosocial, genetic, and neurobiological factors. In accordance, each of these elements must also be incorporated into the development of effective means to treat and prevent suicidal behaviors. Unfortunately, in many respects the treatment of suicidal behaviors has lagged far behind those of other psychiatric conditions, such that clinicians have focused primarily on addressing co-morbid psychiatric disorders in the hopes that symptoms related to suicidal behaviors will also be diminished. While this approach has not been altogether unsuccessful, likely in part due to shared neurobiological mechanisms, this is clearly not optimal, and it is imperative that better psychological and pharmacological interventions be developed. Clearly, an essential first step in this process is identifying the biological substrates of suicide, as these represent the optimal targets for psychopharmaceutical treatments. While early studies investigating suicide focused primarily on neurobiological pathways

implicated in other psychiatric conditions, it has become increasingly recognized that research must expand its focus beyond studies of candidate genes or pathways in order to identify additional pathological factors. In recent years, studies using large scale profiling techniques have begun to address this issue. Amongst the findings arising from this research, the polyamine system has begun to emerge as an exciting new frontier in the investigation of suicide and other psychiatric conditions. Both expanding our understanding of the nature of the dysregulation of this system in suicidal behaviors, and elucidating mechanisms by which this occurs, comprise the focus of this thesis and the studies described herein.

This introductory chapter first discusses the wide range of genetic and neurobiological findings which have been obtained in studies examining suicidal behaviors to date (Chapter 1.1), then examines more specifically the methodologies and findings obtained through gene expression profiling studies of suicide completers (Chapter 1.2), and finally, summarizes the evidence supporting the involvement of the polyamine system in suicide and other psychiatric disorders and proposes mechanisms by which alterations in components of the polyamine system may ultimately lead to the development of these complex disorders (Chapter 1.3). The final section of this chapter (Chapter 1.4) describes the rationale and objectives for the studies undertaken in this thesis in order to investigate the role of the polyamine system in suicide.

1.1 Genetic and Neurobiological Approaches to Understanding Suicidal Behaviors

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Abstract:

Historically, societal views on suicidal behaviors have largely revolved around the religious and moral implications of ending ones' life. These views have differed greatly across cultures and time, and only recently has it become recognized that suicidal behaviors often reflect underlying mental illnesses and are not simply matters of conscience. This understanding led to the awareness that suicide could be empirically studied: a fundamental idea which has ultimately given rise to the current state of suicide research. This chapter focuses on studies investigating neurobiological processes associated to suicide. We first examine evidence implicating genetic factors in the susceptibility to suicidal behaviors, and subsequently, discuss molecular, neurochemical and other biological approaches used by studies investigating this complex phenotype.

1. Introduction

Over the past century, a myriad of studies have provided recognition and insight into the presence and role of hereditary factors in relation to suicidal behaviors, and have played a crucial role in directing future studies to understanding the development and clinical presentation of these behaviors.

Arising from this, examination of the most basic of inherited factors, the genetic code, became a logical step in uncovering molecular mechanisms involved in suicide. In parallel, neurobiological studies have provided evidence for the presence of specific biological alterations which are functionally associated with suicidal behaviors. Through the integration of genetic and neurobiological findings, the identification of the biological substrates of suicide and the characterization of the mechanisms by which these act and interact will eventually allow us to develop improved methods by which to treat and prevent suicidal behaviors. While historically there has been a separation between epidemiological, psychosocial, genetic, and neurobiological studies, these fields have begun to merge in recent decades as it has become increasingly evident that there is a strong interplay between each of these factors. As such, although the focus of this chapter is on the genetic and neurobiological aspects of suicidal behavior, most of the studies incorporate many additional variables, which may eventually allow us to develop an integrated understanding of the biological and environmental factors involved in the etiology and pathology of suicidal behaviors. This chapter first reviews research into the genetic epidemiology and neurochemistry of suicide, then examines how genetic and molecular studies will be instrumental in gaining important insight into neurobiological factors underlying the suicide process.

2. Genetics

The idea that hereditary factors played a role in suicidal behavior is not new and has been noted by many researchers over time, as early as 1790, when Charles Moore, in his book entitled A *Full Enquiry into the Subject of Suicide,* noted that suicide tended to cluster in families. In the centuries following this publication, case reports of twins concordant for suicide began to emerge, pointing the way towards the understanding that suicidal behaviors may possess what today we interpret as a genetic component. In 1949, Franz Kallman and colleagues noted that, “suicide is likely to be conceded as the least disputable

example of a trait determined largely by heredity, while accidental death is often found to be the favorite choice of a condition attributed to strictly environmental influences" (10).

The idea that suicide possessed a neurobiological basis also began to emerge in the 1900's. In a set of papers published in the 1930's, Nolan Lewis reviewed many of the demographic, social, and medical factors associated with suicide and attempted suicide, and noted the importance of biology, particularly in terms of "adaptation" for suicidal behaviors (11;12). A series of studies followed that progressively helped in the recognition that suicidal behavior had a biological basis. Progressively, researchers became interested in the investigation of the genetic epidemiology of suicide, and more recently, on the study of molecular factors conferring susceptibility to suicidal behavior and underlying the suicide process. This section first examines studies that support the existence of heritable factors in suicidal behavior, then examines methods by which these genetic factors have been studied.

Whereas case-report studies played an important role in first demonstrating the potential for the involvement of heritable factors in suicidality, these types of studies have an inherent risk of representing chance findings, and lack the power to properly assess genetic and environmental effects that may be involved in the transmission of suicidal behavior. In the last few decades, numerous family, twin, and adoption studies have provided the necessary statistical evidence to support the role of genetic factors in the transmission of suicidal behavior and have allowed researchers to identify specific clinical and behavioral components which are associated with the familial transmission of suicidality. In addition, these studies have suggested that genetic factors accounting for the transmission of suicidal behavior are distinct from those influencing the transmission of other psychiatric disorders.

2.1 Family Studies

Family studies allow us to test the hypothesis that a trait aggregates (clusters) in families, as well as to investigate patterns of transmission and other factors, such as co-transmitted conditions, that may help to explain familial aggregation. One of the first studies to quantify the magnitude of familial aggregation of suicide completion was the seminal psychological autopsy study conducted by Eli Robins and colleagues in 1959, which found that between 6 and 31% of 1st and 2nd degree relatives of suicide probands had a history of suicidal behavior, depending on the psychiatric diagnosis of the proband. A series of subsequent studies have supported this observation in different populations (13-16) using primarily a family-history design, i.e., asking the proband or an informant about familial recurrence of suicidal behavior.

Family-history studies are limited by several biases and cannot properly investigate familial aggregation. One of the central questions puzzling researchers investigating familial transmission of suicidal behavior has been whether or not familial aggregation of suicide was explained by familial aggregation of psychiatric disorders, which frequently co-segregate in suicidal families. Properly designed family studies can address this type of question. One of the first studies to suggest that suicidal behavior was transmitted independently from psychiatric illnesses was the family study by Tsuang in 1983, who examined, by means of a combination of direct interviews and medical charts, relatives of patients with schizophrenia, bipolar disorder, and depression, of whom a subset had completed suicide (17). This study identified different rates of risk for suicide in the relatives of suicides, non-suicides, and non-psychiatric controls, suggesting that transmission of psychopathology and suicide were not identical.

Greater proof for the independent transmission of psychiatric disorders and suicide came from the study by Egeland and Sussex, who examined mood disorders and suicide in the Old Order Amish, an isolated religious community in Pennsylvania (18). Using the extensive medical and genealogical records kept by this community over the past century, they identified 26 suicide completers, all

clustered in four families in which mood disorders also displayed familial aggregation. No suicides occurred in other families who also displayed aggregation of similar mood disorders, strongly suggesting that genetic factors which increase the predisposition to suicide completion are distinct from those affecting the predisposition to other psychiatric disorders. However, as no suicides were found among families without mood disorders, it also seemed clear that transmission of suicidal behavior was conditional on the liability for psychopathology.

The first study to properly measure the familial aggregation of suicide, while adjusting for the role of psychopathology, was the study conducted by Brent and colleagues, which focused on adolescent suicide completers. They found higher recurrence in families of suicide completers compared to those of controls, an effect that remained significant after controlling for the increased rates of psychopathology in the probands and their families (4). Specifically, they found adjusted recurrence risks of 5.2 and 2.4, respectively, for 1st and 2nd degree relatives of suicide completers. This study also found that probands with high levels of cluster B traits had higher familial loading of suicide than other probands, an observation that is consistent with the notion that part of the familial aggregation of suicidal behavior could be attributed to cosegregation with impulsive-aggressive traits. Similar results were found in a study conducted by our group in families of adult suicide completers and population controls (19). We found that relatives of suicide completers were over 10 times more likely than relatives of comparison subjects to attempt or complete suicide after controlling for psychopathology. While relatives of suicide completers were not more likely to exhibit suicidal ideation, they had more severe suicidal ideation than relatives of comparison subjects. Similarly to the observations made by Brent et al (1996), we found that familial aggregation was stronger for the suicide completers diagnosed with cluster B personality disorders (19).

As these studies were only able to control statistically for the effects of psychopathology, our group more recently conducted a study using a three-group design. More specifically, we conducted direct and blind assessments of a total of

718 relatives from 51 families of depressed suicide probands, 34 families of living depressed probands without histories of suicidal behavior, and 35 families of psychiatrically normal community controls. We found clear evidence that familial transmission of suicide and major depression are distinct, while partially overlapping. In addition, using this design we could demonstrate that histories of aggression and cluster B personality disorders function as endophenotypes of suicide: in other words, they account for familial recurrence of suicidal behavior (5).

2.2 Twin Studies

Compared to family studies, twin studies can separate environmental from genetic effects, as monozygotic (MZ) twins and dizygotic (DZ) twins share, respectively, 100% and an average of 50% of the genomic variants, while both twin types equally share the environment. The first case report of suicide concordance among MZ twins was made almost two centuries ago, and since that time numerous other case reports and twin studies have emerged, of which the majority have found support for a higher concordance of suicidal behavior among MZ compared to DZ twins (20).

The three largest studies examining suicidal behavior in twins used twin pairs recruited through twin registries, and examined only suicide attempts or suicidal ideation. The largest study was performed by Statham and colleagues using an Australian sample, and found that the history of suicidal behavior in one twin was a predictor for suicidal behavior in MZ but not DZ twins (7). This study also found that genetic factors explained approximately 45% of the variance in these behaviors, and 55% of the heritability for serious suicide attempts. Following this study, Glowinski and colleagues examined a sample of female adolescent twins from the USA, and found higher rates of suicide attempts in MZ twins, with genetic influences explaining 35-75% of the variance in risk (21). Thirdly, using the Vietnam Era Twin Registry from the USA, Fu and colleagues examined suicidal ideation and suicide attempts in men, and found that after

controlling for psychiatric disorders, the heritability for ideation was 36%, and the heritability for suicide attempts was 17% (8).

Given the infrequent nature of suicide completion, studies examining concordance rates for suicide completion have been forced to use much smaller sample sizes. Roy and colleagues examined 176 twin pairs in which at least one twin had committed suicide, and found that the concordance for suicide completion was 11.3% in the MZ twins, and 1.8% in the DZ twins (22). Roy and colleagues also examined rates of suicide attempts in surviving co-twins, and found that 10 of 26 surviving MZ co-twins had attempted suicide compared to 0 of 9 surviving DZ co-twins (23). In order to determine if this elevated risk was due to factors associated with grief, rather than a shared inheritance for suicidality, Segal and Roy examined suicide attempts in surviving co-twins whose twin died by means other than suicide. They found no differences between rates of suicide attempts between MZ and DZ twins, supporting the hypothesis that the higher rates of concordance were specifically due to the inheritance of risk factors for suicide (24).

2.3 Adoption Studies

Similar to twin studies, adoption studies can also differentiate between genetic and environmental factors by comparing the rates of suicidality or other psychiatric disorders between the biological and adoptive relatives of adoptees.

The first study to specifically examine the relationship between suicide completion in adoptees and suicide completion among their adopted and biological families was carried out by Schulsinger and colleagues 30 years ago (25). This study compared rates of familial (biological and adopted) suicides between 57 adoptees who committed suicide and 57 living adoptees, identified using the Danish population registry. Among the 269 biological relatives of suicide completers, 12 had committed suicide, compared to 2 biological relatives of the control adoptees. Additionally, they found no incidents of suicide among

the adoptive families of the suicide completers or controls, highlighting the important role of genetic, rather than environmental influences.

A second adoption study was performed by Wender and colleagues using a larger sample from the Danish population registry, comprised of 71 adoptees with affective disorders and 71 healthy adopted controls (26). They identified increases in both attempted and completed suicides among biological relatives of adoptees with affective disorders compared to the biological relatives of controls. Rates of suicide completions were particularly elevated, and displayed a 15-fold higher prevalence in the biological relatives of the cases. They found no differences in suicide attempts or completion among the adoptive relatives, again demonstrating the importance of heritable factors. Interestingly, results showed only an eight-fold increase in unipolar depression, which provides additional support for the independent heritability for factors related to suicide and other psychiatric disorders. Indeed, they found that affect regulation was a greater indicator than depression for suicidal behavior, and proposed that impulse control may play an important role in suicide.

Third, and most recently, a study using a large sample from the National Swedish Register identified greater rates of suicidal behavior in adoptees compared to non-adopted controls, and observed that psychiatric morbidity in the biological parents accounted for a third of the increased risk (27).

2.4 Identification of Genetic Loci

The results of the family, twin, and adoption studies have made it clear that heritable factors play a role in suicidal behaviors, and in doing so, point towards a role for genetic factors in the etiology and psychopathology of suicide. Below is a summary of studies conducted attempting to map and/or identify molecular genetic factors implicated in suicidal behavior.

2.4.1 Association and Linkage Studies

Linkage studies investigate co-segregation between a phenotype and genetic markers, and have been typically non-hypothesis driven, as they have tested markers spanning the entire genome. Relatively few linkage studies have been conducted with suicidal behavior compared to studies investigating different psychiatric phenotypes. In part this is related to the challenges associated with having access to biological material from affected relatives, some of whom may have died by suicide. As such, only three linkage studies have been conducted investigating loci segregating with suicidal behavior. One study examined suicidality among families with alcohol-dependent probands, and found that suicide attempts and suicidality were linked with regions on chromosomes 1, 2, and 3 (28). Another study using probands with recurrent early-onset major depressive disorder, identified a number of chromosomal regions linked with suicide attempts, including 2p, 5q, 6q, 8p, 11q, and Xq (29). A third study, performed in a large bipolar pedigree, identified regions linked with either suicide completions (2q, 4p, 6q) or suicide attempts (10q) (30). As seen for other phenotypes, there has been a decrease in the level of enthusiasm for linkage studies, at least as conducted until now, as they are seen as not having yielded meaningful and consistent results.

Unlike linkage studies, association studies do not investigate co-segregation, but instead compare genetic variability between all cases and controls. Numerous association studies have been performed to investigate polymorphisms in candidate genes, many of which will be discussed in later sections. Many of these studies also examined the relationship between these polymorphisms and clinical correlates of suicidality, such as co-morbid psychiatric disorders, as well as impulsive-aggression and cluster B personality disorders. Our group maintains a database of these studies, which may be accessed through our webpage (www.douglasrecherche.qc.ca/suicide). As with linkage studies, there has also been a significant decrease in the enthusiasm associated with studies using a candidate gene approach. However, the availability of dense single nucleotide

polymorphism (SNP) panels now allows researchers to instead conduct exploratory, genome-wide studies, investigating large samples of unrelated individuals. These studies, usually referred to as genome-wide association studies (GWAS), are currently being conducted in several psychiatric phenotypes, including suicidal behavior.

To date, however, only one non-candidate gene association study has been performed. This study, conducted by our group, examined the relationship between markers on the X chromosome and suicide completion in a French-Canadian sample (31). This study identified several regions displaying high evidence of association with suicide, as well as loci which were specifically associated with suicide in individuals with co-morbid depressive disorders.

2.4.2 Gene Expression Studies

Gene expression studies provide us with gene functional information. By comparing brain tissue from individuals who died by suicide with tissue from controls, we can obtain valuable information on the functional profile of the brain prior to death. The use of microarray technology allows us to screen genes in parallel and virtually obtain a snapshot of brain gene activity in the moments before death, and as such, gain valuable insight into biological processes underlying suicide. To date, 13 microarray studies have been performed which specifically examined gene expression differences between suicide completers and non-suicide controls. All of these studies have examined postmortem brain tissues, and have mainly focused on the prefrontal cortex (Brodmann areas (BA) 8, 9, 10, 11, 44, 45, 46, and 47) or the limbic area (amygdala, hippocampus, BA 24, and BA 29). Two more recent studies by our group have examined additional brain regions in order to obtain a broader view of gene expression changes occurring in the brain (32;33).

All but one study (34) identified multiple genes that displayed differential expression in suicide completers. As these studies generate large amounts of data, and the reporting of significant findings is often at the discretion of the researcher,

the tendency to focus upon genes within known and potentially relevant pathways can make it difficult to identify trends in "non-traditional" genes which are highly differentially expressed but were not reported. Nonetheless, several pathways have been consistently implicated in these studies, including the glutamatergic and γ-amino-butyric acid (GABA)-ergic neurotransmitter systems, as well as the polyamine system, which will be discussed in later sections of this chapter. Although these large data sets have the potential to generate many false positive results, the consistency of findings in these pathways provides strong evidence to support the notion that differential gene expression is an important factor in suicidality.

Interestingly, microarray studies have only produced minimal evidence for dysregulation of genes involved in the more traditional neurotransmitter pathways. This is in contradiction to the results of many studies that have specifically implicated these pathways in suicide. The reason for this discrepancy is not clear.

2.4.3 Epigenetics

Epigenetics refer to processes that alter gene expression without changing DNA sequences. Two important epigenetic processes are DNA methylation and post-translational histone modifications. Evidence suggests that environmental factors can influence epigenetic processes (35), and as such, epigenetic factors are excellent mechanisms to investigate in suicide and other psychiatric conditions where the environment is thought to play an important etiological role.

DNA methylation is a covalent modification at the 5' position of cytosine, occurring at CG dinucleotides. This modification can prevent the binding of proteins to DNA, and typically has been associated with gene repression when located in promoter regions (36), although increased gene expression also could occur if methylation prevented the binding of repressive elements. Altered levels of DNA methylation have been observed in the promoter regions of several genes in suicide completers. The first study to examine this was performed by our group

using DNA extracted from the hippocampus of suicide completers with a history of early childhood neglect or abuse, and identified increased methylation in the promoter regions of the ribosomal RNA genes which was associated with decreased gene expression (37). Following this, Poulter and colleagues identified altered levels of DNA methyltransferase activities in several brain regions of suicide completers as well as alterations in DNA methylation in the promoter region of the $GABA_A$ α1 subunit which was associated with levels of a particular isoform of DNA methyltransferase (38). Another study examined the role of allele-specific methylation of the serotonin 2A receptor (HTR2A) and found that methylation of the C allele of the T102C polymorphism was significantly different in white blood cells obtained from suicide attempters diagnosed with schizophrenia, but not in attempters diagnosed with bipolar disorder, nor was it differentially methylated in brain tissues of suicide completers (39). Two other studies conducted by our group examining methylation patterns of gene promoters should be mentioned. The first study examined the promoter region of the tropomyosin-related kinase B T1 variant (TrkB.T1) in brains of suicide completers and found that methylation at specific sites was correlated with gene expression, which was associated with the decreased brain expression of this gene in a subset of suicide completers (32). The other study focused on the hippocampus glucocorticoid receptor and translated results to humans that had previously been found in animal models of early environmental influences (40). This study highlighted the importance of early environmental adversity on the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, a system that is important in stress response.

Histones are the core proteins involved in the packaging of DNA into nucleosomes, and can be covalently modified at specific residues by acetylation, methylation, phosphorylation, SUMOylation, and ubiquitinylation (41). The effect of these modifications on gene expression depends upon the histone protein (H2A, H2B, H3, H4), amino acid residue, modification, and positioning within the gene. Alterations in histone modifications have been examined in a number of psychiatric disorders, including suicide (42-44). Our group examined the tri-

methyl modification of lysine 27 in histone 3 (H3K27me3) and found increased levels of this modification in the promoter region of TrkB.T1, which were associated with decreased expression of this gene in BA 10 (44). On the other hand, we conducted another study examining the H3K27me3 modification in the promoter region of two polyamine genes, spermine synthase (SMS) and spermine oxidase (SMOX), and found no association between levels of modified H3K27 and suicide completion or expression of these genes in BA 8/9.

2.4.4 Proteomics

Although alterations in mRNA levels are often indicators for alterations in protein expression, this is not always the case. As the levels of the various proteins comprising the proteome theoretically represent the endpoint for gene expression changes, measurement of their levels should conceivably reveal a more accurate picture of the mechanisms involved in suicidal behavior. However, the field of proteomics has lagged behind that of high throughput genetic research, largely due to a lack of suitable experimental techniques. To date, only two studies have been performed investigating proteome alterations in suicidal behavior. The first study was performed by Brunner and colleagues who investigated the cerebrospinal fluid (CSF) of unmedicated, depressed suicide attempters and non-attempter controls (45). Using 2-dimensional gel electrophoresis, they identified one protein which was found at much lower concentrations in the suicide attempters. However they were unable to determine the identity of this protein due to the limited amount of material. The second proteomic study was performed by Schlicht and colleagues using prefrontal cortex tissues obtained from suicide completers and healthy controls (46). Using 2-dimensional gel electrophoresis and mass spectrometry, they identified five proteins which differed in intensity between the two groups, and were able to identify three of these: crystallin chain B, glial fibrillary acidic protein (GFAP), and manganese superoxide dismutase.

3. Neurotransmitter Systems

Synaptic transmission in the central nervous system (CNS) underlies much of what makes us who we are. It is also the main site of the action of the psychopharmaceutical agents currently in use, and it is therefore not surprising that the role of the neurotransmitter systems represents one of the most commonly studied facets of suicidal behaviors. Classically, these pathways have been examined through measurements of the levels of the neurotransmitters and their metabolites, as well as the binding properties of their receptors, in the blood, urine, CSF, or brains of suicide completers. This early work has now been complemented with numerous molecular studies to examine many aspects of gene transcription, as well as more detailed analyses of the metabolic and downstream signalling pathways. This section will focus on the main neurotransmitter systems investigated in suicide to date, summarizing results from neurochemical studies, as well as those using complementary approaches.

3.1 Serotonin

The serotonin (5-HT) system is the most studied neurotransmitter in suicide research and studies focusing on this system have a long history, dating back a few decades. 5-HT neurons originate in the dorsal raphe nucleus of the brainstem, where 5-HT is synthesized from tryptophan by the actions of tryptophan hydroxylase (TPH) and aromatic L-amino acid decarboxylase. Metabolism occurs mainly by monoamine oxidase (MAO) and aldehyde dehydrogenase to produce 5-hydroxyindoleacetic acid (5-HIAA). At least 14 mammalian 5-HT receptors are known, and all are G-protein coupled receptors (GPCR) except HTR3, a ligand-gated ion channel (47).

Alterations in serotonergic function related to suicidal behavior have been found using techniques aimed at assessing various markers of serotonin in living humans and in postmortem brain tissue. The findings from these studies point to a net reduction in serotonergic neurotransmission as the neurobiological alteration

associated with suicidality. However, not all studies have obtained these results, and it has been recognized that these compounds are highly influenced by postmortem and antemortem factors not associated with suicide (48).

Numerous studies also have examined the expression and function of genes involved in 5-HT neurotransmission, of which the most well-characterized are the 5-HT transporter (SERT, SLC6A4), and the receptors HTR1A and HTR2A. Many studies have examined the levels of SERT in the frontal cortex by measuring the binding of ligands such as [^{3}H]-paroxetine or [^{3}H]-imipramine. As this protein is found on axons of 5-HT neurons, these measurements are interpreted to indicate the integrity of 5-HT innervation. Overall, results have been mixed and although the general trend appears to be for decreased binding in suicide completers, studies have also found increases, or no changes (49). HTR1A also has been studied in suicide completers, however postmortem binding studies have been fairly inconsistent. Several studies have found decreases in HTR1A binding in the brainstem (50;51), and increases have been seen in the prefrontal cortex of suicide completers in some (52;53), but not all studies (54;55). Interestingly, alterations in downstream signalling pathways following HTR1A activation have also been observed in suicide completers (56). Greater support has been found for the involvement of HTR2A than HTR1A. Several studies have found increased binding in postmortem samples using either [^{3}H]-ketanserin or [^{125}I]-lysergic acid diethylamide (57-60), and increased protein and mRNA expression have also been observed (58;61). However, as with SERT and HTR1A, there have been inconsistent findings (55;62-64). Interestingly, imaging studies of suicide attempters have found decreases in HTR2A binding in anxious and depressed individuals, which was negatively correlated with personality measures of hopelessness (65;66). As with HTR1A, alterations in downstream signalling pathways involved in HTR2A signalling have been observed in some brain regions of suicide completers (67).

Expression studies have assessed the role of genes involved in 5-HT metabolism, particularly the TPH genes (1 and 2) and MAO genes (A and B). Several studies have examined the expression of TPH2 in the dorsal raphe nucleus

of suicide completers and have found elevated levels of mRNA, protein, and TPH immunoreactivity in suicide completers (68-70). As this finding is counter to the idea that low 5-HT transmission is involved in suicide, it was suggested that these increases may reflect a compensatory mechanism in the brainstem. MAO-A and MAO-B are involved in the metabolism of 5-HT as well as the catecholamines, and have also been implicated in suicidal behavior. However, most studies have focused on genetic polymorphisms rather than expression or function of these genes. There is some evidence for increased expression of MAO-A, although this was found only in the hypothalamus of suicide completers (71). Although results for the involvement of MAO-B have been typically negative, a recent study found increased density of MAO-B in the frontal cortex of suicide completers (72).

Numerous genetic association studies have been performed in serotonergic genes, including SERT, HTR1A, HTR1B, HTR2A, MAO-A, TPH1, and TPH2 (73-75). The most convincing association has been with a functional promoter insertion/deletion in SERT. A recent meta-analysis found that the short allele, which is associated with decreased gene expression, was overrepresented in those with suicide attempts and violent suicides (75). Although positive associations have been seen with the majority of other serotonergic genes examined, many conflicting results have also been obtained, and there has been no overwhelming evidence to support the involvement of genetic polymorphisms in any of these genes in suicidal behavior.

3.2. Catecholamines

The catecholamines comprise dopamine (DA), norepinephrine (NE), and epinephrine (Epi). The role of DA in psychiatry has been best studied in schizophrenia, where it is the main target of the antipsychotics. NE plays many roles in the sympathetic nervous system, while Epi is a hormone released by the adrenal gland into the bloodstream (76). DA is synthesized from tyrosine through the sequential actions of tyrosine hydroxylase and aromatic L-amino acid decarboxylase. DA is then converted to NE by dopamine-β-hydroxylase, and NE

is acted upon by phenylethanolamine N-methyltransferase to produce Epi. Catecholamine metabolism occurs through the actions of MAO and catechol-O-methyltransferase (COMT). There are five DA receptors, which are all GPCRs, and are classified as D1-like (DRD1 and DRD5), and D2-like (DRD2, DRD3, and DRD4), which differ in their localization and downstream signalling effects (77). Both NE and Epi activate two forms of GPCRs, the α- and β-adrenoceptors, each of which have several subtypes and are associated with specific second messenger systems (78).

Studies examining DA and its metabolites have focused largely on levels of its metabolites, homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC), in the CSF or urine of suicide attempters. Although several studies identified decreased levels of metabolites in suicide attempters (79-83) and completers (84), this has not been found in all studies (85-87). Inconsistent results have also been obtained when assessing the relationship between CSF HVA levels and behavioral correlates of suicidal behavior (88;89). Similar conflicting findings have been found by researchers measuring NE and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in urine or CSF samples from suicide attempters, as well as in postmortem tissues of suicide completers. Some studies have found elevated levels of NE and MHPG (85;90;91), whereas others have found decreased levels (92), or no differences (86;93-95).

Receptor binding studies have assessed the properties of DRD1 and DRD2 in suicide completers. One study examined the basal ganglia of depressed suicide completers and found no differences in numbers or affinities of DRD1 and DRD2 in antidepressant-free suicides, but increased numbers and decreased affinity of DRD2, and increased DRD1 in antidepressant-treated suicides, suggesting that differences were related to treatment rather than suicide (96). Another study investigating DRD2 in the caudate of depressed suicide completers also found no significant differences in number or affinity (97). However, several studies by Pitchot and colleagues assessing downstream effects of DRD2 activation found significant differences between suicide attempters and controls, with suicide attempters demonstrating reduced growth hormone responses after treatment with

a DRD2 agonist (98-100). Two studies examined DA uptake sites in several brain regions of depressed suicide completers and found no significant differences in either numbers or affinities (101;102).

The adrenoceptors also have been examined in suicide completers. Arango and colleagues examined α1 and α2 receptors in the prefrontal and temporal cortices of suicide completers, and found an increase in α1 binding sites in the prefrontal cortex, with no alterations in the temporal cortex, and no differences in α2 receptors in either region (91). The opposite trend was observed by three studies which found decreased α1 binding in the prefrontal cortex, temporal cortex, and caudate of suicide completers (103), the hippocampus of anti-depressant treated suicide completers (104), and the prefrontal cortex of alcoholic suicide completers (105). Another study found no differences in numbers of α1 receptors in suicide completers, but increased α2 binding in the temporal cortex, and decreased numbers of α2 receptors in the occipital cortex, hippocampus, caudate, and amygdala (106). Several other groups have investigated α2 binding and protein levels and have found increased amounts of α2 receptors in the high affinity state (107-109), increased α2 numbers in the hippocampus and frontal cortex (110), and elevated α2 protein levels and mRNA expression in the prefrontal cortex (61;111). However, one study found decreased α2 binding in the frontal cortex of alcoholic suicide completers (105), and a recent microarray study found decreased mRNA expression of α2A receptors in depressed suicide completers (64). Many researchers also have investigated the β receptors in suicide completers, with differing results. Several groups found increased β receptor binding in the frontal cortex (60) and temporal cortex (59) of suicide completers, whereas others have found decreases in these same regions (112;113). In the same vein, although one study found an increased risk for suicide in individuals taking β-blocking medication for cardiovascular diseases (114), this was not confirmed in another study (115).

Several association studies have investigated catecholamine receptors and their metabolism in suicidal behavior. Two studies investigated an exon III polymorphism in DRD4 and found no significant differences between suicide

attempters and controls (116;117). Two studies have found positive associations with suicide attempts and polymorphisms in DRD2 (118;119). Several association studies have also examined polymorphisms in the α2A receptor, and while one study from our group found a weak association with the functional Asn251Lys polymorphism in male French-Canadian suicide completers (120), this was not found in other populations (121;122). Interestingly, another study found an association of a promoter polymorphism with suicide completion in Japanese females, but not males (122), which was also not significantly associated in the all-male sample used in our study (120). Recently, another α2A polymorphism was identified to be associated with increased suicidal ideation during antidepressant treatment (123). The relationship between COMT and suicidal behavior has been extensively studied, in particular the Val158Met polymorphism. The Met allele of this enzyme is associated with decreased activity (124), and a recent meta-analysis of association studies supported its involvement in suicidal behavior (125).

Overall, the results for the catecholamine systems are highly diverse, but several trends have emerged. Measurements in postmortem and peripheral samples points towards a decrease in levels of DA and its metabolites, as well as decreased activity of DRD2 receptors. There also appears to be an increase in NE and its metabolites, as well as increased expression of the α2 and β2 receptors in the brains of suicide completers (126). However, it is clear that additional studies will be required in order to gain a consensus on the role of these systems in suicidal behavior.

3.3 Other Systems

3.3.1 Glutamate and GABA

Glutamate and GABA are the primary excitatory and inhibitory neurotransmitters in the brain, respectively. Glutamate is synthesized either from glucose obtained from the tricarboxylic acid (TCA) cycle, or from glutamine,

which is synthesized by glial cells and taken up by neurons (127). Glutamate transmission is terminated by reuptake into neurons or astrocytes, which then convert it to glutamine to be resynthesized into glutamate by glutaminase (GLS) (127). The metabolism of GABA is intricately tied to that of glutamate, which is the precursor for GABA synthesis by glutamic acid decarboxylase (GAD). Additionally, following its release into synapses, GABA is transported into astrocytes and converted to glutamine (128).

Glutamate acts on four classes of receptors: the ionotropic α-amino-3-hydroxy-5-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) receptors, as well as the metabotropic glutamate receptors (129;130). Several protein studies have assessed the ionotropic receptors in suicide. Two studies examining NMDA receptors in the brain found no differences in antagonist binding between suicide completers and controls (131;132). However, another study found a decrease in high affinity, glycine displaceable binding in suicide completers, although no changes to the potency or efficacy of glycine to inhibit this binding, as well as no differences in antagonist binding (133). Another study examined modulatory sites of the NMDA receptor and found no alterations in the frontal and parietal cortices of suicide completers (134), but significant differences in the hippocampus, suggesting region-specific alterations in NMDA receptor functioning (135). Two studies examining AMPA receptors found increased binding in the caudate (132;136), although no differences in the putamen or nucleus accumbens (132). Another study found no differences in kainate receptor binding in the striatum or nucleus accumbens (132).

Microarray studies have identified alterations in the levels of several glutamate receptors, including subunits for NMDA, AMPA and kainite receptors, as well as the metabotropic glutamate receptor 3 (33;64;137). Additionally, downregulated expression has been observed for GLS, glutamate-ammonia ligase (glutamine synthetase) (GLUL), and glial high-affinity glutamate transporters SLC1A2 and SLC1A3 (33;64;138). The involvement of AMPA receptor 3 (GRIA3) is particularly interesting as it has been associated with a number of

psychiatric conditions, including bipolar disorder, schizophrenia, and citalopram treatment-emergent suicidal ideation (139-142). Treatment-emergent suicidal ideation has also been associated with polymorphisms in kainite receptor 2 (GRIK2) (141;143). Interestingly, several of these glutamatergic genes are localized to glia, which lends support for the involvement of dysregulated astroglial functioning in psychiatric disorders.

The relationship of GABA levels to suicide was assessed by Korpi and colleagues who measured GABA concentrations in several brain regions of suicide completers and controls, and found no significant differences in any region, although there was a trend for increased levels in the hypothalamus (144). Another study measured GABA concentrations in the CSF of suicide attempters with personality disorders, and found increased levels which were highly related to impulsivity in these individuals (145).

GABA acts upon two classes of receptors: ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. Measurements of benzodiazepine binding sites, reflecting quantities of the $GABA_A$ receptors, have been performed by several groups. Manchon and colleagues reported an increased number of type I binding sites in the hippocampus with a slightly increased binding affinity in suicide completers (146). A later study by the same group found no differences in receptor density but increased affinity of $GABA_A$ receptors in the hippocampus of violent suicide completers (147). Pandey and colleagues found significantly increased receptor density in the prefrontal cortex, particularly in violent suicide completers, although no alterations in ligand affinity (148). Other studies have found an increased number of sites in the frontal cortex of suicide completers, with no differences in the temporal cortex, amygdala, hippocampus, or locus coeruleus (149-151). Only two receptor binding studies have examined $GABA_B$ receptors in brains of suicide completers, and have been mainly negative, with the exception of a slight increase in binding affinity in the temporal cortex of drug-free suicide completers (152;153). Other protein studies examining proteins such as GABA aminotransferase (71), GAD (149), and GABA transporter-1 (154), have also been negative. Finally, a recent neuroanatomical study investigating

GAD-immunoreactivity found significantly increased reactivity in the hippocampus of suicide completers (155), which may indicate alterations in the levels of GABAergic neurons in suicide completers.

In spite of the conflicting results from protein studies, expression studies have now observed dysregulated expression of many GABAergic genes in suicide completers. Altered expression of numerous $GABA_A$ and $GABA_B$ receptor subunits was observed across prefrontal and limbic brain regions, as well as $GABA_A$ receptor-associated protein like 1, and a GABA transporter (SLC6A1) (33;64;138;156-158). Moreover, a study from our group found that 16 and 36% of the probesets annotated to be involved in GABAergic signalling were significantly differentially expressed in BA 44 and 46, respectively (64). There also is evidence for the involvement of epigenetic processes in the alteration of GABAergic transmission in suicide, as a recent study by Poulter and colleagues found a hypermethylation of the promoter region of $GABA_A$ α1 in the prefrontal cortex of suicide completers, which was associated with decreased expression (38). This effect was not found in the α5 subunit, indicating gene-specificity.

In sum, although studies investigating glutamate and GABA have yielded some conflicting results, the evidence for their involvement in suicide is overwhelming. Although the traditional focus on suicide research has been the 5-HT system, it seems clear that these two systems show considerable promise for investigating the neurobiological basis of suicide as well as being potential sites for pharmaceutical treatments.

3.3.2 Other

Acetylcholine (ACh) is a key neurotransmitter in both the parasympathetic and sympathetic components of the autonomic nervous system, and plays an important role in the control of attention, learning and memory in the CNS (159-161). The two major classes of ACh receptors are the metabotropic GPCR muscarinic receptors (mAChR) and the ionotropic nicotinic receptors (nAChR) (162).

The role of cholinergic neurotransmission in suicide has been examined in several binding studies. Meyerson and colleagues found increased mAChR densities in the cerebral cortex of suicide completers (163), however no differences were observed by two studies in the frontal cortex, hypothalamus, or pons (164;165). Downstream signalling through mAChR was examined by measuring [^{35}S]GTPγS binding following receptor stimulation in the prefrontal cortex, and was not found to be significantly different in suicide completers (166). Interestingly, a more recent study investigating M1 and M4 mAChR found higher binding in the anterior cingulate cortex of suicide completers, particularly in those with schizophrenia (167), which may indicate a role for specific receptor types in suicide.

On another note, suicide by ingestion of organophosphate pesticides, which irreversibly inhibit the breakdown of ACh in the synapse, is an increasingly-recognized problem in developing countries. And while this reflects the availability of a highly effective means of committing suicide, it has also been suggested that long-term exposure to organophosphates may play a causal role in suicidal behavior (168;169). It also has been recently proposed that ACh may be involved in suicidality through its effects on other neurotransmitter systems (170). Overall there is some evidence for increased AChR or ACh signalling in suicide; however, more studies will be needed to confirm these findings and to investigate the possible relationship between organophosphates and suicide.

Endogenous opioids are a class of short peptides including the enkephalins, dynorphins, endorphins, endomorphins, and nociceptin, which have important roles in mood and nociception. Opioids bind with differing affinities to the four major types of opioid receptors, which are all GPCRs, and display different anatomical distributions (171). Although the effects of opioids on mood are well-known and opioids have been suggested as candidates for new antidepressants (172), relatively few studies have examined the opioid system in suicide. Three studies have examined μ-opioid receptor density and affinity in brains of suicide completers, although the results have not been consistent. Two studies observed elevated receptor density in several brain regions of suicide completers, with no

significant changes in receptor affinity (173;174), while another study found no alterations in receptor density but higher affinity ligand-binding in suicide completers (175). Another study assessed signalling through μ-opioid receptors and found no significant differences between the groups (166). Finally, one study investigated the mRNA expression of μ-opioid receptors and found it to be upregulated in the prefrontal cortex of suicide completers (61). Overall, there is only minimal and conflicting evidence for the involvement of the opioids in the psychopathology of suicide, with results suggesting increased binding affinities and/or expression of μ-opioid receptors. However, given that this system is still not fully understood, further studies are warranted to confirm these findings and to investigate the role of other opioid receptors.

4. Stress Systems

One of the most-widely used models to conceptualize risk for mental disorders, which has also been adopted in suicide research, is based on the notion of a stress-diathesis interaction. In the case of suicidal behavior, this model assumes that suicide results from the combination of stressors and predisposing factors. The systems discussed in this section may be involved in both stress and predisposition, as they represent sites at which the first effects resulting from stress may be apparent. Any factors influencing their capacities to respond to these stressors can have numerous long-term consequences. The two traditional stress systems in humans include the sympathetic nervous system, in which the catecholamines described above play essential roles, and the HPA axis system. A third stress response system whose role in psychiatric disorders has been less studied, is the polyamine system.

4.1 Hypothalamic-Pituitary-Adrenal Axis

The HPA axis plays an essential role in adaptation to biological and behavioral homeostatic stressors. Activation of the HPA system involves the

release of corticotrophin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), resulting in the release of glucocorticoids from the adrenal gland (176). These hormones travel systemically and act to increase the expression of genes involved in metabolism and inflammatory responses, and produce numerous effects in the CNS.

Dysregulation of the HPA system has been proposed for several psychiatric conditions, including depressive disorders (177), psychotic disorders (178), and post-traumatic stress disorder (179). One of the earliest studies to implicate the HPA axis in suicide was by Bunney and Fawcett in 1965, with the identification of high levels of 17-hydroxycorticosteroids in depressed suicidal patients (180). Following this, studies identified elevated levels of CRH in the CSF (181) and frontal cortex (182) of suicide completers, indicative of increased activation of the HPA axis in suicide. One of the most common methods to examine the functioning of the HPA axis is the dexamethasone suppression test, with dexamethasone non-suppression being indicative of elevated HPA activity. Early studies identified increased non-suppression among suicidal adolescents (183), and suicide attempters who later died by suicide demonstrated a greater rate of non-suppression compared to suicide attempters and non-attempters who did not go on to commit suicide (184). Similar results continue to be seen, supporting good predictive power of dexamethasone non-suppression for suicidal behavior (185;186). Gene expression differences have also been found in suicide completers, including a decreased expression of the glucocorticoid receptor in the hippocampus of suicide completers with a history of childhood abuse (40), and elevated expression of the CRH1 receptor (CRHR1) in the prefrontal cortex of suicide completers (158).

Relatively few genetic association studies have been performed for HPA axis genes. Of note are the studies focusing on the CRHR1, which was found to be associated with suicide attempts, but not in individuals exposed to high levels of lifetime stress (187;188). Another study found that genotypes in CRHR1 and the CRH binding protein (CRHBP) influenced suicide attempts and severity of

suicidal behaviors in individuals with schizophrenia (189), and the same group found a small association between suicidal behaviors and CRH receptor 2 (CRHR2) in those with bipolar disorder (190). Although there is only minor evidence regarding the role of heritable factors in HPA dysfunction, recent findings have indicated that first-degree relatives of suicide completers also display blunted cortisol responses under stress, indicating that alterations in HPA functioning are partially due to heritable factors.

Overall, there is strong support for a disturbance in HPA axis functioning in suicidal behavior, although the biological mechanisms linking the two are not yet known. One popular theory regarding the role of the HPA system in depression postulates that long-term activation of the HPA axis, and the resulting elevated glucocorticoid levels, have toxic effects on neurons in the hippocampus (191). As hippocampal neurogenesis is an important mechanism involved in antidepressant and stress responses (192), this chronic over-activity may also be relevant to suicidal behavior.

4.2 Polyamines

The polyamines are ubiquitous aliphatic molecules comprising putrescine, spermidine, and spermine, which contain two, three, and four amino groups, respectively, and which are positively charged at physiological pH. In addition, the guanidino-amine agmatine, whose presence in mammalian brains was discovered much more recently than the other polyamines (193), may also be considered among this group (194). The polyamine system has been identified in all organisms, and plays an important role in numerous essential cellular functions, including growth, division, and signalling cascades (195-198). In addition, evidence supports its involvement in stress responses at both cellular and behavioral levels (197;199). Interestingly, the polyamine stress response (PSR) appears to be developmentally regulated, and the emergence of the adult PSR is correlated with the cessation of the hyporesponsive period of the HPA system (200).

Alterations of the polyamine system have been observed in several pathological conditions including cancer (196), ischemia (201), Alzheimer's disease (202), and mental disorders (203). Although the involvement of the polyamine system in schizophrenia, depression, and anxiety disorders has been studied for over two decades, its role in suicide had not been suspected prior to a study by our group which found consistent decreases in levels of spermidine/spermine N-1 acetyltransferase (SAT1) across several brain regions obtained from French-Canadian suicide completers both with and without major depression. Further, our study identified a SNP (rs6526342) in the promoter region, which was significantly associated with suicide completion (204). Since this initial study, decreased expression of SAT1 has been observed in other brain regions and populations (64;157;205;206). Dysregulated expression of additional polyamine related genes, including SMS, SMOX, and ornithine aminotransferase-like 1 (OATL1), has also been identified (64;157).

We recently characterized the haplotype structure of the promoter region of SAT1, and identified three polymorphisms, including rs6526342, which played a role in determining the expression of SAT1 in the brain and *in vitro* (207). The most interesting results were found with a variable-length tandem adenine repeat in the promoter region, whose length was sufficient to determine the expression of reporter gene constructs, and which was found to be significantly associated with suicide completion among individuals with depressive disorders (208). Additional studies by our group have identified elevated levels of putrescine and spermidine in the brains of suicide completers, which may be a consequence of the decreased activity of SAT1 (209).

Although the mechanism by which altered polyamine levels can influence risk for suicide or other psychiatric disorders is not yet clear, several mechanisms have been proposed. Firstly, the polyamines have been shown to influence transmission through several neurotransmitter systems, including the catecholamines (210-213), glutamate (214), GABA (215-217), and nitric oxide (218), each of which may be involved in psychiatric disorders. Agmatine itself is believed to act as a neurotransmitter through imidazoline receptors, α2-

adrenoceptors, nAChR, and HTR3 receptors: this theory is supported by its storage in synaptic vesicles and capacity to be released upon depolarization (219). Secondly, polyamines interact with several transmembrane channels, including inward-rectifying K^+ channels, ionotropic glutamate receptors, and L-type Ca^{2+} channels, and thus can influence the properties of excitable cells (214;220). Finally, both agmatine and putrescine demonstrate anxiolytic and antidepressant effects in animal studies (221-227), which could be particularly relevant for suicide completers with co-morbid mood or anxiety disorders.

5. Cell Signalling

The main function of cell signalling pathways is to transduce messages from outside the cell to produce specific alterations within the cell. This section will focus specifically on signal transduction pathways that result in changes in gene expression. The two mechanisms by which these effects occur are through receptors located at the cell surface, whose effects on gene transcription occur through second messenger systems, and nuclear receptors, whose inactive forms reside primarily in the cytoplasm and which become activated when bound by their membrane-permeable ligands. These receptors then pass through the nuclear pore to the nucleus where they act directly upon the DNA by binding to specific recognition sites within gene promoter regions.

Cell surface receptors typically possess extracellular, transmembrane, and intracellular domains. Following binding of a ligand to the extracellular domain, conformational changes are induced which can alter its ability to interact with intracellular molecules or activate the enzymatic activity of the receptor itself. Based upon the type of receptor and the nature of the ligand being bound, different intracellular signalling pathways are activated or repressed.

Many of the neurotransmitter receptors are GPCRs, for which ligand-binding allows the intracellular domain to interact with various G-proteins to activate second messenger signalling pathways, mainly those involving cyclic AMP (cAMP) or 1,4,5-inositoltriphosphate (IP3). As discussed above, many of these

receptors have already been implicated in suicidal behavior, and alterations in downstream signalling pathways have also been observed in suicide completers. Although the trend has been for impaired signalling through various components of the cAMP and IP3 pathways in postmortem brain tissues of suicide completers (56;228-232), some studies have either found no change, or evidence for increased signalling (233;234). These differences may be due to differing regulation of second messenger signalling across the brain (235), use of antidepressants (234), or the presence of other psychiatric disorders (236).

Growth factor receptors are another important class of cell surface receptors, and following binding of their ligands, the receptors dimerize and activate the tyrosine kinase activity of their intracellular domain, resulting in autophosphorylation that allows the intracellular domains to interact with proteins involved in various second messenger systems (237). The neurotrophins are a class of peptide growth factors secreted by specific cells to increase the growth and survival of neurons, and include nerve growth factor (NGF), neurotrophin 3 (NT-3), neurotrophin 4 (NT-4), and brain-derived neurotrophic factor (BDNF). Among this group, only signalling by BDNF, and its receptor TrkB, have been implicated in suicide. While microarray studies have failed to identify differences in the expression of BDNF itself, decreased protein levels of BDNF and expression of TrkB have been observed in several postmortem studies of suicide completers (32;238;239), supporting a role for a downregulation of BDNF/TrkB signalling. One of these studies determined that downregulated TrkB expression was due to the decreased expression of one specific TrkB isoform, TrkB.T1, which is expressed exclusively in astrocytes (32). Many association studies have examined the Val66Met polymorphism of BDNF in suicidal behavior, although results have been mixed, and associations generally appear not to be with suicide completion, but rather with other aspects of suicidal behavior (240-243). Although the mechanism by which decreased BDNF/TrkB signalling plays a role in suicide is not yet known, several studies have implicated this pathway in depression. Antidepressant use has been shown to increase the expression of BDNF in both the periphery (244) and the brain (245), which has been associated

with improvement of depressive symptoms (246). Interestingly, a recent study found that SNPs in BDNF and the neurotrophin tyrosine kinase receptor 2 (NTRK2) were associated with antidepressant treatment-emergent suicidal ideation (123).

Fibroblast growth factor (FGF) is another growth factor which has been implicated in suicide. This growth factor is involved in cell proliferation and differentiation in development, and has essential roles in neuronal signal transduction in adults (247). Several microarray studies have identified downregulated expression of proteins involved in FGF signalling in brains of suicide completers (138;248). Similar to BDNF, FGF has also been shown to be upregulated following antidepressant treatment (249).

As discussed earlier, there is some evidence for dysregulated function of the glucocorticoid receptor in suicide, but there have been few studies on the role of other nuclear receptors in suicidal behavior. Estrogen has been implicated in depression and schizophrenia (250), and several studies have examined polymorphisms in estrogen receptors in suicidal behavior, none of which were found to be significantly associated (251-253). Low levels of testosterone, which acts at the androgen receptor, have been observed in suicide attempters, although the relationship with violence of the attempts has not been consistent (254;255). Whether there are defects in androgen receptor functioning in suicidal behavior remains unknown.

6. Cholesterol and Lipid Metabolism

Lipids and their metabolites are involved in numerous biological processes including energy metabolism, immune function, and second messenger signalling, as well as being essential components of cellular membranes. Cholesterol metabolism appears to be particularly important in psychiatric disorders, and beyond its essential role in determining membrane fluidity and localization of proteins in lipid bilayers, is required for the synthesis of steroid hormones and fat-soluble vitamins.

The interest in cholesterol as a possible biological correlate of suicide and related behaviors dates as far back as 1979, when Virkkunen demonstrated that male subjects with antisocial personality disorder — a high risk group prone to violence and suicide — had lower levels of serum cholesterol than the control group of male patients with other personality disorders (256). It was only after concerns were raised over the safety of using cholesterol-lowering medications that particular attention was paid to the seeming relationship between cholesterol and violent behavior. In particular, two large randomized, double-blind lipid-lowering trials revealed that although decreasing serum cholesterol with lipid-lowering medications was effective in reducing mortality from coronary heart disease, the overall mortality rate was not significantly different (257;258). The reduction in cardiac-related deaths appeared to be offset by an increase in violence-related deaths including suicide, accidents, and homicide. To examine the issue more globally, Muldoon and colleagues combined the results of primary prevention trials using meta-analytic techniques and concluded that there was a significant increase in non-illness-related mortality (i.e., deaths from accidents, suicide or violence) in groups receiving treatment to lower cholesterol concentrations compared with controls (259). Although nonsignificant results have been observed among more recent meta-analyses, this may reflect the modification of inclusion criteria in these trials to exclude individuals with histories of psychiatric illnesses and substance abuse (260). Support for a relationship between cholesterol metabolism and suicide has also been found in large epidemiological studies in which low serum cholesterol levels have been frequently associated with suicidal behavior (261-265). Although gender-specific effects have been observed (261;266), this has not been the case in all studies (263). Although a relationship between low serum cholesterol levels and suicide has not been described in all studies (267), the overall evidence strongly supports the involvement of cholesterol in suicidal behavior.

There also has been some evidence supporting a relationship between suicide attempts and low serum levels of cholesterol, as well as other lipids, from studies in psychiatric populations (268-274). Clinical studies, however, have not been as

consistent as epidemiological studies. Nevertheless, intriguingly, many studies have identified low cholesterol levels to be a specific marker for violent suicide methods, such that lower serum cholesterol levels have been observed in violent compared to non-violent suicide attempters (275-279), as well as in the brain tissues of violent suicide completers (280). As with the epidemiological studies, gender-specific differences have sometimes been observed, with greater effects being found in male compared to female suicide attempters (271;272). The relationship between suicidal behavior and the levels of other lipids, such as fatty acids and triglycerides, has been difficult to interpret, as some studies have described decreased levels in the serum of suicide attempters (268;281;282), which has not been observed in the brains of suicide completers (283;284). As the relationship between peripheral and brain levels of cholesterol and other lipids remains unclear (285), these discrepant results could suggest differing roles for lipid metabolism in suicidal behavior between the two systems, or alternatively, alterations in lipid metabolism may be specific to suicide attempters.

In addition to altered levels of the lipids themselves, evidence for a dysregulation in lipid metabolism in suicidal behavior also has emerged from gene expression studies. Recent microarray studies have identified altered expression of several lipid-related genes in the brains of suicide completers, including the low density lipoprotein receptor (LDL-R) (138), chemokine-like factor superfamily 5 (CKLFSF5), erbb2 interacting protein (ERBB2IP), fatty acid desaturase 1 (FADS1), gremlin 1, cysteine knot superfamily (GREM1), interferon gamma-inducible protein 16 (IFI16), leptin receptor (LEPR), myotubularin related protein 10 (MTMR10), Notch homolog 1 (NOTCH1), phosphoinositide-3-kinase (class 2 alpha; PIK3C2A), phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1 (PREX1), S100 calcium binding protein beta (S100B), and stearoyl-CoA desaturase (SCD) (286). The ability of long-term alterations in the expression of cholesterol metabolic genes to confer risk for suicidal behavior has been observed in carriers of a mutation involved in Smith-Lemli-Opitz (SLO) syndrome, an autosomal recessive disorder resulting from mutations in the gene coding for 7-dehydrocholesterol reductase (DHCR7), an enzyme involved in

cholesterol biosynthesis. While carriers of this mutation are phenotypically normal, they display low levels of cholesterol and display suicidal behavior (287).

Several mechanisms have been proposed to explain the role of altered lipid and cholesterol metabolism in suicide and other psychiatric disorders. Many of these theories propose a relationship between cholesterol and 5-HT neurotransmission resulting from alterations in membrane fluidity (288;289). Although unlikely, this mechanism is partly supported by experimental studies confirming a relationship between cholesterol and 5-HT in humans (290). Secondly, altered immune responses, in which lipids play an essential role, have been implicated in other psychiatric disorders (291) and some studies have suggested that the role of lipid metabolism in suicide may be due to a relationship between increased levels of pro-inflammatory cytokines and the levels of cholesterol and fatty acids (292;293). A more recent explanation is associated with the role cholesterol plays in the development and function of neural synapses (294;295). Regardless of the molecular mechanisms that may mediate this relationship, a possible behavioral link may be through impulsive-aggressive traits. Accordingly, it seems reasonable to posit that a relationship should exist between our dietary needs and our behaviors. As such, when in need of dietary intake, our behavior should naturally become more aggressive, which in turn, may increase suicide risk in individuals at risk.

7. Other Systems

As research into the neurobiological basis of suicidal behavior continues, new biological pathways continue to emerge as potential sites harbouring factors involved in this complex trait. While a discussion of all known pathways which have been implicated in suicide is beyond the scope of this chapter, several of the newer findings will be discussed in this section.

As well as being involved in intracellular signalling pathways through its conversion to cAMP, adenosine triphosphate (ATP) is an essential component of cellular energy metabolism. Altered expression of several ATP-related genes has

been observed in two microarray studies. One study observed downregulated expression of the Na+/K+-ATPase alpha 3 subunit (ATP1A3) in the prefrontal cortex of suicide completers with depression, bipolar disorder, and schizophrenia (248). Another study found that TCA cycle and ATP-related genes were significantly associated with gene expression differences between depressed and non-depressed suicide completers, and suggested that these defects may be markers for suicidal behavior in the context of depression (64). Mitochondria are the organelles in which much of the cellular energy metabolism occurs, and it is of interest that altered expression of genes encoded by mitochondrial DNA has also been observed in suicide and other psychiatric disorders (296;297).

Nitric oxide (NO) is a gaseous signalling molecule with important roles in cardiovascular, muscular, immune, and nervous system functions. NO is synthesized from L-arginine by several NO synthases (NOS), which differ in both their tissue and cellular distributions. NO has been implicated in several psychiatric conditions including depression, and appears to play an important role in learning and memory (298). Several studies have now investigated the role of NO function in suicide. Two studies found elevated plasma levels of NO in depressed suicide attempters (299;300). Two studies have also examined polymorphisms in NOS genes, and while one study found that genotypes within NOS1 and NOS3 were associated with aspects of suicidal behavior (301), another group found no association between NOS3 polymorphisms and suicide attempts (302). Clearly additional work will be required to clarify the role of NO in suicidal behavior, although there is some evidence that its involvement may be through increasing impulsive behaviors (303).

In addition to many of the factors described in previous sections, synaptic transmission is also highly regulated by presynaptic factors controlling the vesicle-mediated release of neurotransmitters. Considerable evidence is now emerging implicating altered function of this system in suicide. One study identified a decreased ratio of synapsin/synaptophysin in suicide completers (304), while another group observed alterations in syntaxin and synaptosomal-associated protein (25 kDa) (SNAP25) levels in the SNARE complex (305).

Alterations in the expression of numerous other related genes have been found in microarray studies of suicide completers, including vesicle-associated membrane protein 3 (VAMP3), synaptotagmin XIII (SYT13), synaptophysin-like protein (SYPL), synapsin II (SYN2), synaptosomal-associated protein (23 kDa) SNAP23, SNAP25, and synaptosomal-associated protein (29 kDa) (SNAP29) (33;64). Finally, variants in vesicle-associated membrane protein 4 (VAMP4) were associated with suicide attempts in one study (306).

8. Future Studies

Although the understanding of the role of neurobiological factors in suicide has vastly increased within the last few decades, we are far from developing an integrated picture of how specific alterations at the levels of DNA, mRNA, protein, or neuroactive molecules interact to confer risk for suicidal behaviors. Additionally, we remain far from understanding how this knowledge can be used to chemically treat these behaviors. The majority of studies performed to date have focused on genes and proteins of interest, and while these studies continue to be important, they are not sufficient to address these issues. Microarrays have played an essential role in highlighting new molecular pathways involved in suicidal behavior, and in the future more high throughput methods should be used to study this complex trait.

GWAS have become a popular tool to investigate complex diseases, and have been performed in several psychiatric disorders including depression, bipolar disorder, schizophrenia, and attention deficit hyperactivity disorder. A GWAS has not yet been performed in suicide completers, although efforts to do so are currently underway. Along with identifying SNPs associated with suicide, these studies can be used to examine copy number variations (CNV), large segments of chromosomal deletions and amplifications, which have been observed in other psychiatric conditions, most notably bipolar disorder, schizophrenia, and autism (307).

The majority of microarray studies in suicide performed to date have used 3' expression arrays, which typically probe only the 3' end of mRNAs. Newer arrays have now been developed which can specifically probe the expression of each exon within a gene. Importantly, this will allow for the identification of proteins which show specific splicing differences among suicide completers, and which may improve our understanding of how these genes function in suicide.

While gene expression studies have typically focused on mRNA, the importance of other classes of RNA molecules has become increasingly recognized. MicroRNAs are short, single-stranded RNA molecules which bind to specific mRNA molecules and target them for degradation (308). Recent studies have examined the role of altered microRNA levels and functions in other psychiatric conditions (309-311), and microRNAs are currently under investigation for their role in suicide.

Finally, the epigenetic studies of suicide to date have only focused on genes of interest. Examination of alterations in CpG methylation at a larger scale is now possible using comparative hybridization arrays (312), and very soon through large scale, genome-based deep sequencing. Histone modifications can also be examined at a higher throughput level using ChIP-based methods. These large scale studies will open the door to the systematic investigation of epigenetic effects that may be involved in suicide risk. As epigenetic markings are potentially reversed, this is an exciting area of investigation that creates the opportunity for eventual intervention and future avenues for risk modification.

1.2 Gene Expression Profiling of Suicide Completers

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Abstract

Despite strong evidence for a role of biological factors in the etiology and pathology of suicide, the study of traditional neurotransmitter systems has been able to explain only a small proportion of the neurobiology of what is now recognized as a complex genetic trait. The use of microarrays to simultaneously examine the expression levels of thousands of gene transcripts has vastly expanded our capacity to detect the involvement of additional genes and pathways in suicidality, and has opened many new avenues for the discovery of the biological underpinnings of suicide completion. This review examines microarray studies which have been used to identify genes displaying altered expression in suicide completers, and highlights some of the important methodological considerations and metabolic pathways which have emerged from these analyses.

Introduction

Suicide is one of the leading causes of death worldwide, and the importance of both biological and environmental factors in the etiology and pathology of suicidal behavior has been well established. As with other psychiatric conditions, suicidal behavior is a complex phenotype, and efforts to identify the precise molecular mechanisms involved have been hampered by the large heterogeneity found within groups of suicide completers. Traditional candidate gene studies of

suicide focused largely on the serotonergic, noradrenergic, and dopaminergic systems due to their strong affiliations with other psychiatric disorders. However, while many interesting findings have been generated regarding the roles of these systems in suicidality, genetic and epigenetic variability within these genes has explained only a fraction of the known heritability and environmental influences. To identify additional contributing genetic factors, large-scale studies have been completed, and other studies are in the process of being conducted, to pinpoint genetic loci that may be putatively involved in the etiology of suicide. However, as with all studies of genetic variation, once a promising region has been identified there still remains the need of determining which variant within the associated haplotype block is etiologically implicated, the mechanism by which it exerts its effects, where exactly in the brain and at what cellular level these effects occur, and as importantly, at what stage in development they take place and how they interact with the environment. The use of microarray technology to examine gene expression in suicide completers represents a useful shortcut to most of these challenges and provides us with a functional snapshot of cellular processes in the brain of individuals who died by suicide. When compared to similar information of control samples, we can obtain valuable insight into molecular processes underlying suicide.

This review will focus on studies which have used microarray technology as a screening tool to identify genes displaying altered postmortem brain expression levels in suicide completers. These are shown in Table 1. Although many additional genome wide expression studies have included subjects who died by suicide (reviewed in (296;314;315)), this list comprises only those which 1) used microarrays to measure differences in mRNA levels, 2) specifically assessed differences between suicide completers and non-suicide controls, and 3) used the data as a screening tool rather than to study specific pre-selected genes or pathways.

This review will first discuss the methodological similarities and differences between these studies, then will highlight several of the metabolic pathways which have emerged from these studies. Finally, future directions of gene

expression studies in identifying biological mechanisms involved in suicidality will be examined.

Methodology

This section will discuss methodological issues such as sample characteristics, brain regions, and microarray technologies. Additional considerations such as normalization methods, false discovery correction, and statistical tools to specifically examine confounding factors are beyond the scope of this review.

Sample Characteristics and Confounding Factors

The power to detect significantly differentially expressed genes, and what this information represents in terms of relevance for suicidality, depends both upon the number of samples within each group, and the method by which groups are defined.

Owing to the cost of microarray studies and difficulties in subject recruitment, sample sizes have typically been small, ranging from 6 (316) to 90 (138) subjects overall, with generally between 10 to 15 subjects within each experimental group. Psychopathology is an important confounder in expression studies of suicide. Although familial transmission of suicidality suggest that suicide segregates independently from psychopathology (317), biological factors associated with the latter need to be carefully controlled for, a process that is methodologically and operationally challenging. Three Axis I diagnoses that are frequently associated with suicide, major depression (MD), bipolar disorder (BD), and schizophrenia (SCZ), have been commonly included in the studies performed to date, and several methods have been used to attempt to separate their effect from that of suicide. One method has been to group together all suicide completers regardless of underlying psychopathology and compare this suicide group to a healthy control group (34;137;316;318;319). A second strategy has

been to group suicide completers with each Axis I disorder separately (ie. suicide completers with MD, suicide completers with BD, suicide completers with SCZ) then to compare each different suicide group to the healthy control population (33;64;156;157;204;248). Finally, one study compared, within psychopathological groups, individuals who died by suicide with those who died of other causes (138). The latter strategy is probably the best one to properly control for psychopathology. However, it is operationally challenging to collect a control group affected by psychopathology that is comparable to the suicide group in a number of other variables that could correlate with brain gene expression, including demographic, sudden death, and treatment factors, and that in addition were actively ill at the moment of death.

The potential effects of other confounding factors, such as substances and medication have been less well addressed. Two studies (64;157) identified multiple probesets whose expression was significantly altered in users of alcohol or cocaine, one study (138) included drug or alcohol use in the statistical models, and one study examined the effects of alcohol consumption on specific probesets in another population and in an animal study (33). However, the majority of studies have either excluded subjects based upon toxicology reports, or ignored the issue altogether. These measures are clearly suboptimal. For instance, toxicological reports provide only one level of information about substance use. It is not unusual that individuals with significant patterns of abuse of certain drugs are not intoxicated at the moment of death. More frequently, however, is the opposite. In other words, it is not infrequent that suicide cases were intoxicated at the moment of death with drugs on which no pattern of dependence/abuse existed prior to death. To more appropriately control for the effect of substance, toxicological, six month and lifetime diagnoses of substance disorders should be considered.

Gender is also an important factor to be considered in gene expression studies in general, and particularly so in studies of psychiatric disorders due to known gender differences in their rates and clinical presentations. The rate of suicide completion in males is significantly higher than in females. Unfortunately,

this gender difference makes it also operationally more difficult to recruit large samples from female suicides, and researchers have been more likely to focus exclusively on male samples to avoid gender-specific effects, or to use mixed samples in which the numbers of female subjects are insufficient to specifically examine the influence of gender.

Brain Regions

All microarray studies of suicide completers to date have examined gene expression differences in postmortem brain tissues and have largely focused on tissues obtained from the prefrontal cortex (Brodmann areas (BA) 8, 9, 10, 11, 44, 45, 46, and 47) or the limbic area (amygdala, hippocampus, BA 24, and BA 29). Two more recent studies have examined additional brain regions in order to obtain a broader view of gene expression changes occurring in the brain (32;33).

Microarray Technology and Validation of Findings

The majority of studies have used Affymetrix microarray chips, which are one-color arrays printed with hundreds of thousands of short oligonucleotides. RNA that has been extracted from brain tissues is converted to cDNA, which is then converted to cRNA for hybridization to the chips. Each generation of microarray chips differs slightly, with the most important difference being in the number of sequences that are included. The Illumina BeadChip used by Thalmeier and colleagues (137) utilizes different labeling and hybridization methods, but yields comparable results.

Validation of significant findings is generally required for the publication of microarray studies, although the choice of genes to validate is at the discretion of the researcher and is generally based upon the statistical significance of the findings, potential relevance of the genes to psychiatric conditions, and their involvement in pathways of specific interest to the researcher. Quantitative real time polymerase chain reaction (RT-PCR) is considered to be the "gold-standard"

method of validation, although other methods such as semi-quantitative RT-PCR, Western blotting, and immunohistochemistry have also been used. Two of the studies in Table 1 (204;316) further investigated the involvement of selected genes by performing genetic association studies using polymorphisms found within those genes in larger groups of suicide completers and controls

Dysregulated Genes and Pathways

As mentioned above, the validation and reporting of significant findings is often at the discretion of the reviewer, and the tendency to focus upon genes within known and potentially relevant pathways can make it difficult to identify trends in "non-traditional" genes which are highly differentially expressed but were not reported. Nonetheless, several pathways have been consistently implicated in these studies, including the glutamatergic and γ-amino-butyric acid (GABA)-ergic neurotransmitter systems, as well as the polyamine system.

GABA

GABA is the primary inhibitory neurotransmitter in the brain, and altered expression of genes involved in GABAergic neurotransmission has been observed in several neuropsychiatric conditions (315). Several microarray studies have now observed dysregulated expression of GABAergic genes in suicide completers. Altered expression of numerous GABA receptor subunits was observed across prefrontal and limbic brain regions, including $GABA_A$, α1, (GABRA1), $GABA_A$, α4 (GABRA4), $GABA_A$, α5 (GABRA5), $GABA_A$, β1 (GABRB1), $GABA_A$, β3 (GABRB3), $GABA_A$, δ (GABRD), $GABA_A$, γ-1 and γ-2 (GABRG1 and GABRG2), $GABA_B$, β2 (GABBR2), and $GABA_C$, ρ1 (GABRR1), as well as $GABA_A$ receptor-associated protein like 1 (GABARAPL1), and a GABA transporter (SLC6A1) (33;64;138;156;157) Moreover, one study found that 16 and 36% of the probesets annotated to be involved in GABAergic signalling were significantly differentially expressed in BA 44 and 46, respectively (64).

Glutamate

In opposition to GABA, glutamate is the primary excitatory neurotransmitter in the brain, however it too has been implicated in psychiatric disorders, and these two neurotransmitters share an important metabolic connection through the activity of glutamate decarboxylase (GAD), an enzyme which converts glutamate to GABA, and which has also been implicated in psychiatric conditions (315). Alterations in the levels of several glutamate receptors were observed, including glutamate receptor, ionotropic, N-methyl-D-aspartate (NMDA)-like 1A (GRINL1A), glutamate receptor, ionotropic, NMDA 2A (GRIN2A), glutamate receptor, ionotropic, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors 1-4 (GRIA1, GRIA2, GRIA3, and GRIA4), glutamate receptor, metabotropic 3 (GRM3), and glutamate receptor, ionotropic, kainite 1 (GRIK1) (33;64;137). Additionally, downregulated expression was observed for glutamate-ammonia ligase (glutamine synthetase) (GLUL), the enzyme responsible for removing glutamate from synapses, glutaminase (GLS), which produces glutamate from glutamine, and glial high-affinity glutamate transporters SLC1A2 and SLC1A3 (33;64;138).

Interestingly, several of these genes are localized to glia, which lends support for the involvement of dysregulated astroglial functioning in psychiatric disorders. Choudary and colleagues identified a number of glutamatergic genes which were altered in mood disorders independently of suicide (156), while additional glial-associated genes, including quaking homolog, KH domain RNA binding (mouse) (QKI), glycoprotein M6B (GPM6B), S100 calcium binding protein B (S100B), and a truncated form of the tropomyosin-related kinase B receptor (TrkB.T1), appear to play a specific role in suicide (32;64).

Polyamines

The polyamine system has been implicated in other psychiatric disorders, including SCZ, MD, and anxiety, and the polyamines play important roles in

multiple neurotransmitter systems, including modulating transmission through both GABA and glutamate receptors (203). Unlike the GABA and glutamatergic systems however, the involvement of the polyamine system in suicide had not been suspected prior to the study by Sequeira and colleagues who observed consistent decreases in levels of spermidine/spermine N-1 acetyltransferase (SAT1) across several brain regions (204). Since this initial study, decreased expression of SAT1 was observed in other brain regions and populations (64;157;205;206). Dysregulated expression of additional polyamine related genes, including spermine synthase (SMS), spermine oxidase (SMOX), and ornithine aminotransferase-like 1 (OATL1), has also been identified (64;157).

Future Steps

In the future, newer technologies, including arrays designed to measure transcription of individual exons, microRNAs, and proteins, can be used to examine gene expression differences at a finer level, while single nucleotide polymorphism (SNP) and DNA methylation arrays can be used to identify genetic and epigenetic mechanisms to account for these alterations. In addition, continued recruitment of subjects will increase sample sizes to allow the effects of confounders such as substance use and gender to be more thoroughly examined.

Conclusions

Microarray technology has greatly improved our understanding of the complexity of gene expression changes that occur in the brains of suicide completers, and has expanded our knowledge of genes and pathways which are involved in this complex trait. Combination of these findings with those that can be obtained from more detailed analyses of implicated loci has the potential to clarify the molecular mechanisms involved in suicidality, and which can eventually lead to the identification of pharmacological targets that may one day be used in suicide prevention.

Table 1: Microarray studies examining gene expression in post-mortem brain tissues from suicide completers.

Study	Platform	Tissue (s)	Diagnostic Groups	Gender (s)
Sibille et al., 2004 (34)	HG-U133A	BA 9, 47	MD-S, C	M/F
Yanagi et al., 2005 (316)	HG-U95A	Amygdala	S, C	M
Gwadry et al., 2005 (318)	HG-U133A/B	BA 11	MD-S, C	M
Choudary et al., 2005 (156)	HG-U95Av2	BA 9/46, 24	MD-S, BD-S, C	M/F
Sequeira et al., 2006 (204)	HG-U133A/B	BA 4, 8/9, 11	MD-S, S, C	M
Sequeira et al., 2007 (157)	HG-U133A/B	Amygdala, hippocampus, BA 24, BA 29	MD-S, S, C	M
Kim et al., 2007 (138)	HG-U133A	BA 46/10	BD-S, BS-NS, SCZ-S, SCZ-NS	M/F
Thalmeier et al., 2008 (137)	HumanRef-8 BeadChip	BA 11	S (violent), C	M/F
Tochigi et al., 2008 (248)	HG-U95Av2	BA 10	MD-S, SCZ-S, BD-S, C	M/F
Ernst et al., 2008 (319)	HG-U133A/B	BA 8/9, 11	MD-S, C	M
Klempan et al., 2009 (64)	HG-U133A/B	BA 44, 45, 46, 47	MD-S, S, C	M
Ernst et al., 2009 (32)	HG-U133 plus 2	BA 4, 6, 8/9, 10, 11, 45, 46, 47, cerebellum	S, C	M
Sequeira et al., 2009 (33)	HG-U133A/B	BA 4, 6, 8/9, 10, 11, 20, 21, 38, 24, 29, 44, 45, 46, 47, amygdala, hippocampus, nucleus accumbens	MD-S, S, C	M

BA – Brodmann area; BD – bipolar disorder; C – healthy control; MD - major depressive disorder; S – suicide completer; SCZ - schizophrenia

1.3 Implication of the Polyamine System in Mental Disorders

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Abstract

The polyamine pathway has an essential role in many cellular functions and has been implicated in several pathological conditions. Accumulating evidence suggests that the polyamine system also plays a role in the etiology and pathology of mental disorders. Alterations in the expression and activity of polyamine metabolic enzymes, as well as changes in the levels of the individual polyamines, have been observed in multiple conditions including schizophrenia, mood disorders, anxiety, and suicidal behavior. Additionally, these components have been found to be altered by a variety of psychiatric treatments. Furthermore, the polyamines and their precursors have demonstrated both antidepressant and anxiolytic effects. Overall, findings to date suggest that the polyamine pathway represents an important frontier for the development of neuropharmacological treatments.

Introduction

Mostly due to the elucidation of the molecular targets of effective psychopharmaceutical agents, much of the neurochemical work in mental disorders to date has focused on the role of the monoaminergic system. In spite of the success of monoamine-related pharmacological treatments, they are not effective in many individuals, indicating that these systems are not the sole factors involved in these conditions. An alternative pathway which has been gaining support for its role in mental illness is the polyamine system. Although better known for their role in modulation of the cell cycle, and consequently their relevance to cancer, research in the last few decades has shown the importance of the polyamines in numerous neurodegenerative conditions, and substantial evidence is emerging which supports their role in the pathophysiology of psychiatric disorders. Accordingly, the polyamines represent an important system for understanding the causes of mental illnesses, and in addition, provide a new pharmacological target for their treatment.

This review will focus on evidence pertaining to altered levels of the polyamines and their metabolic enzymes in psychiatry, as well as the possible role the polyamine system plays in the etiology of these disorders and mechanisms by which its effects may occur. Firstly, however, the basic properties of the polyamines, as well as their metabolism, localization in the central nervous system (CNS), and relevant cellular functions will be discussed.

1. Properties of the Polyamine System

The polyamines are ubiquitous aliphatic molecules comprising putrescine, spermidine, and spermine, which contain two, three, and four amino groups, respectively. In addition, the guanidino-amine agmatine, whose presence in mammalian brains was discovered much more recently than the other polyamines (193), may also be considered among this group (194). Due to their essential roles in many cellular functions, their homeostasis is highly regulated through their

biosynthesis, degradation, and transport, as well as the interconversion between individual polyamines.

1.1 Metabolism and Accumulation

Both the polyamine synthesis and interconversion pathways have been extensively studied, and the major reactions are depicted in Figure 1.

Due to their vital roles, the polyamine metabolic pathways are highly regulated. The major rate-limiting enzymes are ornithine decarboxylase (ODC), S-adenosylmethionine (SAM) decarboxylase (AMD1), and spermidine/spermine N^1-acetyltransferase (SAT1), whose activities are controlled at multiple levels by numerous mechanisms including feedback control by the polyamines themselves (196). The activities of spermidine synthase (SRM) and spermine synthase (SMS) are generally constant although there may be induction under certain conditions (320). Polyamine oxidase (PAO) activity appears to be regulated by substrate availability (321).

Multiple transport systems have been identified, and have been found in a variety of cell types including hepatocytes, synaptosomes, synaptic vesicles, and glial cells (322-324).

1.2 CNS Localization

The polyamines and their biosynthetic enzymes are found throughout the body, including the CNS, where they display specific regional distributions. Many methods have been used to assess these distributions in the CNS, which have been reviewed by Bernstein and Müller (325). Both agmatine and its precursor arginine have been shown to cross the blood brain barrier, allowing both the concentration and localization of agmatine in the brain to be determined by peripheral agmatine and arginine levels as well as through endogenous synthesis by the inducible enzyme arginine decarboxylase (193;326). Putrescine, spermidine and spermine possess only a limited capacity to cross the blood brain barrier (327) and as such,

their localization in the healthy CNS largely represents those which have been endogenously synthesized. Concentrations in brain tissues are typically in the nM range (328). The localization and concentrations of each of the metabolic enzymes and polyamines are not identical for brain region or cell type (325;329-336), indicating that synthesis and storage may not occur in identical locations.

1.3 Cellular effects

The polyamines have numerous roles and are involved in many aspects of cellular function. Due to their cationic nature, they interact well with nucleic acids, and not surprisingly, are involved in many aspects of gene expression (196). In addition, polyamines influence the properties of proteins and membranes (196;337), and function as antioxidants and scavengers of reactive oxygen species (338;339).

The polyamines have an important role in cell proliferation, and demonstrate both pro- and anti-apoptotic effects (198). Additionally, the polyamines are involved in many signalling pathways through their effects on G-proteins, protein kinases, nucleotide cyclases, receptors, as well as by regulating the expression of proteins involved in these processes (196-198). Owing to their interactions with several transmembrane channels, they also influence the electrical properties of excitable cells (214). Agmatine is believed to act as a neurotransmitter by its actions through several receptors, and this theory is supported by its storage in synaptic vesicles and capacity to be released upon depolarization (219). Spermine has also been shown to be released from synaptic vesicles upon depolarization, indicating that the polyamines may function as neuromodulators (323). Additionally, polyamines influence the properties of several neurotransmitter pathways known to be involved in mental disorders, including the catecholamines (210-213;340), gamma amino-butyric acid (215-217;341), nitric oxide (NO) (218), and glutamate (221;227;342-344).

2. Possible Implication in Mental Disorders

2.1 Schizophrenia

The role of the polyamine system in the pathology of schizophrenia and other psychotic disorders was first proposed by Richardson-Andrews who noted that the structures of certain neuroleptics and antimalarials both contain a spermidine moiety and are associated with extrapyramidal symptoms and psychosis (345;346). Since this time, alterations of many aspects of the polyamine system have been observed in both human schizophrenic patients as well as in animal models. Furthermore, certain treatments for schizophrenia have been shown to alter both polyamine levels as well as the activities of polyamine-related enzymes, supporting the role of the polyamine system in the pathophysiology of this disorder. A summary of relevant studies performed in human subjects is found in Table 1.

2.1.1 Polyamine Levels

Increased blood levels of all polyamines have been observed in schizophrenic patients (347-351). Levels appear to be related to neuroleptic treatment, as increased concentrations were observed in treated patients compared to untreated patients and control subjects (352). This effect may be related to treatment response as no changes in polyamine levels were found after clozapine treatment of neuroleptic-resistant schizophrenics (350).

Unlike the periphery, a study of human brains found no differences in polyamine levels in the frontal cortex or hippocampus of schizophrenic patients compared to controls (353). However, as levels of the polyamines and some of their metabolic enzymes are known to vary with post-mortem interval (329;330;332;353) which could not be fully controlled for in this experiment, further studies are warranted to confirm these findings.

2.1.2 Enzyme Activities

Studies examining serum from schizophrenic patients have shown increased levels of polyamine oxidative enzymes (354;355), which were normalized in patients who showed improvement in clinical symptoms after electroconvulsive shock therapy (ECT) (354;355). Early studies of the relationship between plasma amine oxidase and schizophrenia demonstrated a trend towards decreased activity, which may have been associated with familial transmission of the disorder (356-358). Although plasma amine oxidase is not specific for the polyamines, decreases in activity combined with increases in polyamine concentrations might be expected to alter its substrate profile.

The role of ODC is less clear. Studies in schizophrenic patients found no differences in ODC levels or activity in the frontal cortex, hippocampus, or entorhinal cortex (353;359). However, increased activity was observed in cortical neurons from a rat model of schizophrenia (360). Although these results may indicate that the animal model does not properly represent the neurobiology of schizophrenia, it may be that differences in ODC activity are found only in specific CNS regions which have not yet been clearly identified in humans. Interestingly, ECT has been shown to increase ODC activity in multiple regions of rat brains (361;362).

Regardless of the findings with ODC, there is support for alterations in ornithine metabolism in schizophrenia as the activities of ornithine aminotransferase (OAT), antizyme inhibitor (AZIN1), and ornithine cyclodeaminase (OCD), were shown to be decreased in the prefrontal cortex of both treated and untreated patients (363).

Information on the activities of other enzymes is lacking, although in addition to no evidence of altered polyamines or ODC activity, Gilad *et.al.* were unable to demonstrate changes in AMD1 or SAT1 activities (353).

2.1.3 Potential Mechanisms

The complexity of this system makes it unlikely that a single mechanism is responsible and hence a simple explanation is impossible. One possibility is that the increased peripheral polyamine concentrations are a result of decreased plasma amine oxidase activity, and that the increased PAO activity is a compensatory mechanism to decrease these levels. It would be of interest to determine if the normalization of PAO activity in clinically-improved patients is also associated with normalization of polyamine levels. Additionally, oxidative deamination by both plasma amine oxidase and PAO yields compounds capable of causing cell damage (198), and as such, alterations in their activities could reflect either a causative role or compensatory mechanisms to reduce this damage.

The mechanism in the brain is even less clear and further studies are necessary in order to provide a consensus on the actual levels of the polyamines in each brain region, as well as the activities and relationships between each enzyme. The decreased expression of OAT and OCD in the prefrontal cortex should theoretically provide increased ornithine for polyamine production but decreased expression of AZIN1 would allow for increased inhibition of ODC. It was proposed that these results may reflect either a mechanism to compensate for increased polyamine levels, or possibly an attempt to downregulate the entire pathway (363).

As mentioned above, the polyamines act on the dopamine pathway. As this system is strongly associated with the pathology of schizophrenia, its modulation by the polyamines could be of great relevance to both the etiology of this illness as well as in influencing the clinical outcome of antidopaminergic treatments.

Polyamines alter the functioning of *N*-methyl-D-aspartate receptors (NMDAR) (214), and the increased polyamine levels in schizophrenia patients has therefore been proposed to be related to the implication of hypofunctional NMDAR signalling in schizophrenia (352). In this case, increased polyamines should be associated with increased glutamate signalling, with increases representing a compensatory mechanism. Alternatively, as excessive glutamate

signalling can produce excitotoxicity (364), polyamines may instead be destructive rather than beneficial. However, if polyamine levels are confirmed to be unchanged in the brain, these mechanisms may not be applicable.

It seems clear that the neuroleptics are capable of influencing polyamine metabolism, however the mechanisms involved are not yet apparent. Although polyamines were higher in treated patients (352), their lack of change in neuroleptic-resistant patients (350), suggests that the effects of neuroleptics on the polyamine system occur further downstream and may mediate responses rather than determining if a response to treatment will occur.

Obviously significant work remains in order to determine the precise role of the polyamine system in schizophrenia, and although it seems clear that dysregulation of the system is associated with this illness, it is not yet certain if these alterations are etiologically related or represent compensatory mechanisms.

2.2 Mood Disorders and Suicide

As with schizophrenia, the ability of antimalarials to produce depressive symptoms was proposed as an indication of a role of the polyamines in depression (346). Although there have been fewer studies in humans examining the polyamine system in mood disorders, evidence also exists to implicate this system in their pathology. In addition, emerging evidence points to a role of the polyamine system in suicidal behavior. A summary of studies examining the polyamine system in mood disorders in human subjects is found in Table 2, while relevant animal studies are shown in Tables 3 to 5.

2.2.1 Polyamine Levels

Although Gilad *et.al.* found no differences in polyamine levels in the hippocampus and frontal cortex of depressed patients (353), a rat model displayed decreased hippocampal putrescine, spermidine and spermine, as well as decreased putrescine in the nucleus accumbens septi (365). In addition, plasma agmatine

was significantly elevated in depressed patients, and was normalized by antidepressant treatment (366). Agmatine produces both antidepressant and anxiolytic effects in animals through mechanisms involving multiple receptor systems (221-226). The antidepressant effects of putrescine also appear to involve NMDAR (227), and the possibility that at least some of the roles of polyamines in depression is due to modulation of NMDAR is supported by the mechanism of the antidepressant eliprodil which acts as an antagonist at polyamine-binding sites (367). SAM also produces antidepressant effects in humans (368). The exact mechanism remains uncertain, but animal studies have indicated that antidepressant doses of SAM could normalize putrescine and partially restore spermine and spermidine levels (365). However, as SAM is also required for synthesis of dopamine, norepinephrine, and serotonin, and is essential for folate and vitamin B12 metabolism, each of which are implicated in mood disorders (369), its antidepressant effects may not necessarily be mediated through the polyamine system.

2.2.2 Enzyme Activities

As with schizophrenic patients, high levels of plasma PAO activity were observed in depressed patients, and these were normalized by ECT (355). Also similar, no differences were observed in ODC levels in the entorhinal cortex of depressed patients (359), or in the activities of ODC, AMD1, or SAT1 in the hippocampus or frontal cortex of patients who were depressed or committed suicide (353). However, studies performed by our group using both depressed and non-depressed suicide completers demonstrated a downregulation of SAT1 in several brain regions (157;204). SAT1 expression was more profoundly decreased in depressed suicide completers (204) and was lower in the posterior cingulate gyrus of depressed compared to non-depressed suicide completers (157), suggesting an important role in depression. Additional studies performed by our group have identified other polyamine-related genes which are dysregulated in the

limbic system of suicide completers (157), providing further support for an involvement of the polyamine pathway in depression and suicide.

2.2.3 Potential Mechanisms

The antidepressant effects of agmatine, putrescine, and SAM lend support for the role of the polyamine system in depression and perhaps other mood disorders. Similar to schizophrenia however, the relationship between polyamine concentrations and activities of the associated enzymes cannot be formulated into a simple explanation. In order to gain a better understanding of the roles of the polyamines in depression and suicidal behavior, it is essential to determine the actual levels of each of the polyamines in the CNS. As with schizophrenia, further studies are required to assess whether dysregulation of the polyamine system should be considered a cause or a consequence of these disorders. However, as discussed below, evidence suggests that dysregulation of the system may precede development of mood disorders.

2.3 Polyamine Stress Response

Of considerable interest to the role of the polyamine system in the morbidity and etiology of psychiatric disorders is the polyamine stress response (PSR). This phenomenon has been reviewed by Gilad and Gilad (197) and is implicated in both the detrimental effects of stress and anxiety, as well as their role in the development of other psychiatric disorders. Studies assessing the PSR as well as animal models of anxiety are summarized in Table 4.

Unlike the peripheral system where acute stressors activate the PSR to increase the concentrations of all polyamines (370), acute stressors in the CNS result in only the elevation of ODC activity and putrescine and agmatine levels (197;371). The PSR can be induced by multiple forms of stress, and its magnitude appears to be related to the intensity of the stressor (372;373). Consistent with this are findings that anxiolytic pretreatment can diminish or eliminate stress-induced

alterations of the polyamine system (374;375). Chronic stress increases ODC activity and putrescine levels after each application, whereas spermidine and spermine concentrations increase only after several treatments, which is suggestive of an adaptive response (376).

In support of a role of the PSR in behavioral responses to stress, increases in polyamine levels and ODC activity were found to be larger in strains of rats exhibiting greater behavioral and physiological responsiveness to stress (197;377). Furthermore, chronic administration of α-difluoromethylornithine (α-DFMO), an irreversible inhibitor of ODC, yielded rats which displayed distinctive behavioral changes when exposed to stressors (376). Interestingly, memory impairments have been observed in both ODC- and SAT1-overexpressing mice, which possess substantially increased putrescine levels (378;379). Additionally, SAT1-overexpressing mice are hypomotoric and display decreased aggressiveness (379). It has been proposed that partial blockade of NMDAR by putrescine may be involved in these effects (380).

The PSR appears to be developmentally regulated, and may be associated with the development of mood disorders. Early post-natal stressors have been shown to alter putrescine concentrations and ODC activity (381), and yield altered behavioral reactivity and an attenuated PSR in adults (382). Furthermore, the emergence of the characteristic adult PSR is correlated with the cessation of the hyporesponsive period of the hypothalamic-pituitary-adrenocortical (HPA) axis system (200). As this developmental stage in rats is equivalent to a period in humans associated with a high incidence of affective disorders, it was proposed that the PSR might therefore be involved in development of these conditions (200). The HPA axis is implicated in depression and effects are believed to be associated with dysregulation of the glucocorticoid system (383). Consequently, the occurrence of a characteristic PSR after glucocorticoid treatment adds further weight to the theory that the PSR and the polyamines are involved in the development of affective disorders (376). Additionally, the combination of treatment with α-DFMO and a polyamine-deficient diet reduced polyamine, catecholamine, and corticosteroid concentrations (210). Overall, these results

suggest that the PSR, through modulation of the HPA axis, may be directly involved in the pathogenesis of depression, and shed some light on the relevance of environmental influences in the etiology of this disorder.

Lithium is commonly used in the treatment of bipolar disorder, and although many cellular effects have been proposed, the precise mechanisms by which it exerts its therapeutic effects have not been fully determined. Considerable work has been performed investigating the influence of lithium on the PSR. Specifically, chronic lithium treatment prevents stress-induced ODC activity in rat brains, thereby decreasing the intensity of the PSR (372;384). Decreased ODC activity is not due to a direct interaction with lithium (385) and was proposed to be a result of interference with a signal required for induction of ODC (197). *In vitro* experiments demonstrated that these decreases may be associated with altered glial cell properties (386).

Conclusions

Several lines of evidence support a possible role for the polyamine system in the neurobiology of major psychiatric disorders and suicide. The significant number of metabolic enzymes which show altered expression in these disorders, the findings of altered levels and ratios of each polyamine, and the effects of psychiatric treatments on many aspects of the polyamine system, each add support for the idea that modulation of this system may represent a possible pharmacological target in the treatment of these disorders. As the precise mechanisms involved have not yet been fully elucidated, it seems clear that the study of this system remains a crucial frontier for understanding the pathophysiology of several mental disorders, including schizophrenia, mood disorders, and suicide.

Acknowledgements

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Figures

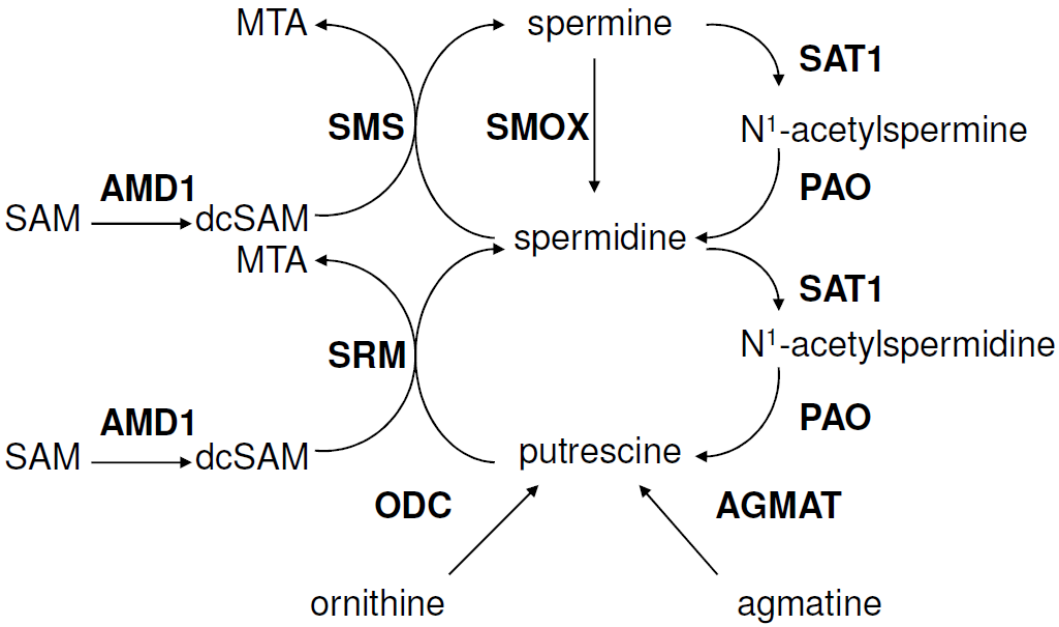


Figure 1: Polyamine synthesis and interconversion pathways. AGMAT agmatinase, AMD1 S-adenosylmethionine (SAM) decarboxylase, dcSAM decarboxylated SAM, MTA 5' methylthioadenosine, ODC ornithine decarboxylase, PAO polyamine oxidase, SAT1 spermidine/spermine N1-acetyltransferase, SMOX spermine oxidase, SMS spermine synthase, SRM spermidine synthase.

Tables

Table 1: Summary of findings from studies analyzing the polyamine system in schizophrenia. AMD S-adenosylmethionine decarboxylase, AZIN1 antizyme inhibitor, ECT electroconvulsive shock therapy, OAT ornithine aminotransferase, OCD ornithine cyclodeaminase, ODC ornithine decarboxylase, PAO polyamine oxidase, SAT spermidine/spermine N1-acetyltransferase

Study	Findings	System
(356)	Plasma amine oxidase activity lower in schizophrenics, only significant for acute cases	Plasma
(387)	Plasma amine oxidase activity unaltered after 3 month neuroleptic treatment	Plasma
(357)	Reduced plasma amine oxidase activity associated with schizophrenia spectrum disorders within families	Plasma
(358)	Small, non-significant, reduction in plasma amine oxidase activity Familial transmission may occur in families where probands have extremely low activity	Plasma
(388)	Plasma amine oxidase activity unrelated to subtype, diagnostic criteria, prognosis, or age at onset	Plasma
(347)	Increased spermidine	Serum
(354)	Increased spermidine oxidase activity, unrelated to gender, age, or treatment	Serum
(349)	Increased polyamines, no correlation with gender or treatment Increased PAO activity	Whole blood, plasma
(350)	Increased polyamines in neuroleptic-resistant patients compared to healthy controls, unchanged after 6 month clozapine treatment	Blood
(351)	Increased polyamines in fibroblasts Increased spermine in culture medium	Skin fibroblasts
(353)	No alterations in polyamines, ODC, AMD1, or SAT1	Frontal cortex, hippocampus
(359)	No alterations in ODC immunoreactivity	Entorhinal cortex
(352)	Increased total polyamines in neuroleptic-treated schizophrenics compared to controls and untreated patients	Skin fibroblasts
(355)	Increased PAO activity, normalized in patients improved after ECT	Serum
(363)	Decreased activities of OAT, AZIN1, and OCD, no relationship to treatment	Prefrontal cortex

Table 2: Summary of polyamine-related findings in human mood disorder studies. AMD S-adenosylmethionine decarboxylase, ECT electroconvulsive shock therapy, OATL ornithine aminotransferase-like, ODC ornithine decarboxylase, PAO polyamine oxidase, SAT spermidine/spermine N1-acetyltransferase

Study	Findings	System
(353)	No alterations in levels of polyamines, ODC, AMD1, or SAT1 in depressed patients or suicide completers	Frontal cortex, hippocampus
(359)	No alterations in ODC immunoreactivity in depressed patients	Entorhinal cortex
(366)	Increased agmatine in depressed patients, normalized after 8 week bupropion treatment	Plasma
(355)	Increased PAO activity in severely depressed patients, normalized in patients showing improvement after ECT	Serum
(204)	Decreased SAT1 expression in depressed and non-depressed suicide completers, results more profound in depressed suicide completers	Orbital cortex, motor cortex, dorsolateral prefrontal cortex
(157)	Increased hippocampal expression of SAT2 and OATL1 in non-depressed suicide completers, increased SMS in both depressed and non-depressed suicide completers Decreased SAT1 expression in depressed compared to non-depressed suicide completers in the posterior cingulate gyrus	Amygdala, hippocampus, anterior cingulate gyrus, posterior cingulate gyrus

Table 3: Summary of findings from rodent studies examining the relationship between depression and the polyamine system. 5-HT 5-hydroxytryptamine, NMDAR N-methyl-D-aspartate receptor, NO nitric oxide, SAM S-adenosylmethionine

Study	Findings
(365)	Depression model displayed decreased putrescine, spermidine and spermine in hippocampus, decreased putrescine in nucleus accumbens septi, no changes in frontal cortex SAM normalized putrescine in nucleus accumbens, partially restored hippocampal spermine and spermidine
(221)	Agmatine antidepressant effects involve NMDAR, L-arginine-NO pathway and α2-adrenoceptors
(344)	Agmatine antidepressant effects involve NMDAR
(389)	Agmatine demonstrated antidepressant effects
(223)	Agmatine antidepressant effects involve 5-HT1A/1B and 5-HT2 receptors
(224)	Agmatine antidepressant effects involve δ- and μ-opioid receptors
(227)	Putrescine antidepressant effects involve NMDAR
(226)	Agmatine antidepressant effects involve imidazoline I1 and I2 receptors

Table 4: Summary of findings from rodent studies examining the relationship between stress, anxiety, and the polyamine system. AMD S-adenosylmethionine decarboxylase, α-DFMO α-difluoromethylornithine, NMDAR N-methyl-D-aspartate receptor, NOS nitric oxide synthase, ODC ornithine decarboxylase, PSR polyamine stress response, SAT spermidine/spermine N1-acetyltransferase

Study	Findings
(381)	Infant cerebellar putrescine and brain ODC activity decreased after maternal separation
(372)	Only chronic lithium treatment prevented glucocorticoid-induced increases in ODC, AMD1, and SAT1 activity in hippocampus and frontal cortex
(384)	Hippocampal ODC activity increased after each episode of chronic intermittent stress, first episode reduced hippocampal AMD1 activity and increased liver ODC activity, but both remained constant after all subsequent treatments Chronic lithium treatment only prevented increases in hippocampal ODC activity
(200)	Decreased hippocampal ODC and AMD1 activities after acute stress at day 5, no change in striatal ODC activity Adult PSR pattern in hippocampus and striatum apparent at day 30 Increased behavioral responses and attenuated increases in ODC activity when stressed at day 7 then rechallenged as adults
(382)	Adult behavioral responses reduced in animals subjected to mild intermittent postnatal stress and increased in animals which received acute postnatal stress No differences in stress-induced ODC activity between adults which received mild or acute postnatal stress Enhanced increase in liver ODC activity when subjected to acute postnatal stressors then rechallenged as adults Adult polyamine concentrations unaffected by postnatal stressors
(373)	ODC activity and putrescine increased in liver and decreased in thymus after acute stress at all ages, only increased in hippocampus of adults Basal ODC activity after adrenalectomy increased in hippocampus and thymus and decreased in liver, increased basal putrescine and spermine in hippocampus, and basal putrescine in thymus Adrenalectomy enhanced stress-induced changes in ODC activity in hippocampus, liver, and thymus, and putrescine changes in liver and thymus
(376)	Putrescine increased in hippocampus after each stress episode, increases in spermidine and spermine delayed and transient α-DFMO combined with stress depleted putrescine and decreased spermidine and spermine in hippocampus, produced behavioral changes
(377)	Acute stress increased putrescine in frontal cortex and hippocampus Chronic stress did not alter putrescine, spermidine or spermine Putrescine concentrations differ between rat strains
(197)	Greater induced changes in ODC and polyamines in rat strain more reactive to stress

(222)	Agmatine anxiolytic effects occur either through NOS, NMDAR or α2-adrenoceptors
(389)	Agmatine demonstrated anxiolytic effects
(371)	Increased agmatine in plasma and frontal cortex during stress, no changes in hypothalamus, medulla, cerebellum, or hippocampus
(374)	Stress increased putrescine and decreased spermine in frontal cortex and hypothalamus, no effects in plasma Anxiolytic pretreatment prevented putrescine increases
(225)	Agmatine demonstrated anxiolytic effects
(390)	Agmatine increased hippocampal neurogenesis in chronically stressed mice
(375)	Stress increased brain putrescine, prevented by anxiolytic pretreatment No alterations in spermine, spermidine, or acetylated products

Table 5: Additional animal studies examining the relationship between the polyamine system and mental disorders. α-DFMO α-difluoromethylornithine, ECT electroconvulsive shock therapy, NMDAR N-methyl-D-aspartate receptor, ODC ornithine decarboxylase

Study	Findings
(211)	Spermidine and spermine acted selectively on mesolimbic, but not striatal, dopamine system
(361)	Transiently increased ODC activity in adrenals, hippocampus, brain stem, frontal cortex, and cerebellum after ECT Dose response for shock intensity and ODC activity in hippocampus and brain stem
(213)	Spermine produced dose-dependent cortical synchronization and sedation Spermidine produced cortical synchronization at low doses and cortical desychronization and behavioral arousal at higher doses Spermine and spermidine inhibited methamphetamine-induced behavior
(362)	Hippocampal ODC increased after repeated ECT
(391)	Increased cerebral ODC after single ECT, effects partially attenuated using an NMDAR antagonist
(210)	α-DFMO treatment with polyamine-deficient diet reduced adrenal polyamines and catecholamines, and plasma corticosterone and aldosterone

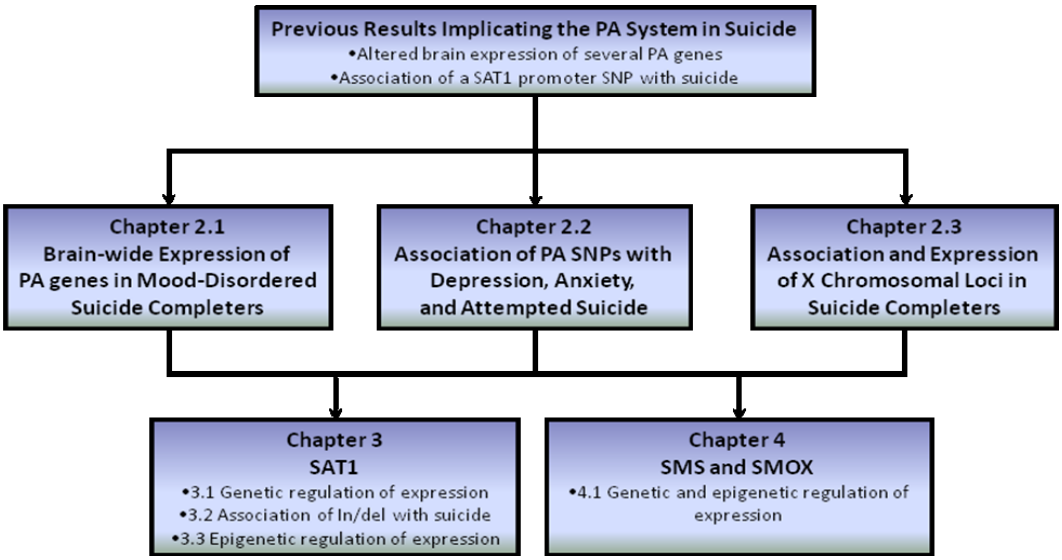
1.4 Rationale and Objectives

Rationale

The initial studies by our group identified four polyamine-related genes, SAT1, SMS, SMOX, and OATL1, which displayed altered expression in the brains of suicide completers, and found a strong association with a promoter variant in SAT1 and suicide. While these studies, as well as results emerging from other groups, strongly support the involvement of the polyamine system in suicide, investigation into the extent by which this system is affected and the molecular mechanisms by which this occurs are essential steps towards gaining a greater understanding of the neurobiology of suicide.

Objectives

- To characterize the extent by which the polyamine system and related metabolic pathways are altered across the brains of suicide completers
- To investigate the polyamine system in other psychiatric conditions
- To identify molecular mechanisms regulating the expression of polyamine genes, and determine how they are involved in the pathology of suicide



Overview of the studies designed to investigate the polyamine system in suicide.

CHAPTER 2: LARGE-SCALE STUDIES INVESTIGATING THE POLYAMINE SYSTEM IN SUICIDE

Our previous studies demonstrated differential expression of several genes involved in polyamine metabolism (SAT1, SMS, SMOX, and OATL1) in the brains of suicide completers, yet little was known regarding how altered expression of these genes influences the functioning of the polyamine pathway as a whole, how polyamine metabolism is affected globally across the brain, if similar mechanisms are implicated in other psychiatric conditions, or how expression of these genes in suicide is related to the local chromosomal environment. The three studies included in this chapter were designed to address these questions, as they represent essential considerations when interpreting our previous findings regarding the polyamine system, as well as for evaluating the potential for this system to be used as a target for the development of pharmacological treatments for suicidal behaviors.

Chapter 2.1 describes a study which addresses questions regarding the expression of genes related, both directly and more distally, to polyamine metabolism, and examines patterns of dysregulated expression across the brains of suicide completers. This study used a larger sample set than our previous brain expression studies, and extended this group to include suicide completers with diagnoses of either major depressive disorder or bipolar disorder. Moreover, where our previous studies examined only a few brain regions in isolation, this study assessed polyamine-related gene expression across twenty-two brain regions, and identified genes and regions displaying patterns of either synchronous or asynchronous expression. As with our previous studies, downregulation of SAT1 remained the most consistent and widespread alteration in suicide, and the differential expression of SMS and SMOX was also replicated. Moreover, we identified eleven additional genes, including several with important roles in polyamine biosynthesis, as well as four involved in cellular energy metabolism, which were differentially expressed in suicide completers. Overall, this study served to expand our knowledge regarding which components of

polyamine metabolism are involved in the neurobiology of suicide, as well as provided a clearer picture of the interplay between polyamine metabolism and genes involved in other closely connected metabolic pathways.

The second study, described in Chapter 2.2, examines the association between genetic variants in SAT1, SMS, SMOX, and OATL1, and additional clinical measures of psychopathology, including suicide attempts, mood disorders, and anxiety disorders. These analyses were performed in a large French-Canadian cohort which has been followed for over 20 years, thereby allowing the relationship between these polyamine genes and numerous mediating and moderating influences to be examined. The results of this study demonstrated that each of the main outcomes were significantly associated with polyamine variants, and that each polyamine gene contained polymorphisms which were significantly associated with at least one of the main outcomes. Particularly interesting among these findings were that while SMS, SMOX and OATL1 were each implicated only in one condition, several SAT1 polymorphisms were associated with multiple disorders, and moreover, that the risk alleles differed across suicide attempts, mood, and anxiety disorders. This study also highlighted the involvement of gene-gene interactions in conferring risk for anxiety disorders, as well as demonstrated that parental psychopathology was an important risk factor for each condition. These results provided greater evidence for a role for polyamine-related genetic factors in suicidal behaviors, as well as demonstrated that not only does dysregulation of the polyamine system represent an important risk factor for completed suicide, it is also implicated in suicide attempts as well as mood and anxiety disorders. Furthermore, these studies suggest that genetic factors may be involved mechanistically in the altered expression of the polyamine system in the brains of suicide completers.

Thirdly, the study described in Chapter 2.3 moves beyond the polyamine system to examine the association of X chromosomal loci with suicide in males, and to identify genes within associated chromosomal regions which are differentially expressed in the brains of suicide completers. This study was motivated, in part, by the fact that three of the differentially expressed polyamine

genes, SAT1, SMS, and OATL1, are located on the X chromosome, which may thus harbor essential cis- and trans-factors involved in regulating the expression of these genes. Moreover, as males are hemizygous for genes on the X chromosome, we expected that regions containing risk or protective factors for suicide would be more apparent, and that there would be a clearer and more direct relationship between genetic loci and gene expression differences than would be observed in a female or mixed gender sample. This study identified two distinct chromosomal regions displaying strong associations to suicide: Xq24-Xq27.3 and Xp22.2-Xp11.4, which contains both SAT1 and SMS. Genes within these regions were analyzed further by examining their expression in the prefrontal cortex, and six of these were found to be differentially expressed in suicide completers, including SAT1. Thus, not only did this study confirm the significance of X chromosomal factors in the etiology of suicide, it also further affirmed the role of the polyamine system in suicide.

Collectively, the studies within this chapter served to replicate our previous findings implicating the involvement of the polyamine system in suicide, as well as expanded our understanding of how this system relates to closely-related metabolic pathways, nearby genetic loci, and other psychiatric conditions.

2.1 Global Gene Expression Profiling of the Polyamine System in Suicide Completers

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Abstract

In recent years, gene expression, genetic association, and metabolic studies have implicated the polyamine system in psychiatric conditions, including suicide. Given the extensive regulation of genes involved in polyamine metabolism, as well as their interconnections with the metabolism of other amino acids, we were interested in further investigating the expression of polyamine-related genes across the brain in order to obtain a more comprehensive view of the dysregulation of this system in suicide. To this end, we examined the expression of genes related to polyamine metabolism across 22 brain regions in a sample of 29 mood-disordered suicide completers and 16 healthy controls, and identified 14 genes displaying differential expression. Among these, altered expression of spermidine/spermine N1-acetyltransferase, spermine oxidase, and spermine synthase, has previously been observed in brains of suicide completers, while the remainder of the genes represent novel findings. In addition to genes with direct involvement in polyamine metabolism, including S-adenosylmethionine

decarboxylase, ornithine decarboxylase antizymes 1 and 2, and arginase II, we identified altered expression of several more distally-related genes, including aldehyde dehydrogenase 3 family, member A2, brain creatine kinase, mitochondrial creatine kinase 1, glycine amidinotransferase, glutamic-oxaloacetic transaminase 1, and arginyl-tRNA synthetase-like. Many of these genes displayed altered expression across several brain regions, strongly implying that dysregulated polyamine metabolism is a widespread phenomenon in the brains of suicide completers. This study provides a broader view of the nature and extent of the dysregulation of the polyamine system in suicide, and highlights the importance of this system in the neurobiology of suicide.

Introduction

Suicide is one of the leading causes of death worldwide (2), and while efforts to identify the neurobiological mechanisms involved in suicidal behaviors have been ongoing for decades, we have yet to obtain a comprehensive understanding of the processes involved. Studies initially focused on examining the functioning of monoaminergic metabolism and neurotransmission: however, it has become exceedingly apparent that suicide comprises a far more extensive set of pathophysiological alterations. High-throughput technologies, such as gene expression microarrays, have provided considerable insight into these processes by highlighting molecular pathways which were not previously suspected to be involved in the neurobiology of suicide, including glutamatergic and γ-aminobutyric acid (GABA) neurotransmission, as well as the polyamine system (313).

The first microarray study to implicate the polyamine system in suicide completion identified decreased expression of spermidine/spermine N-1 acetyltransferase (SAT1) across three brain regions in French-Canadian suicide completers (204), a finding which has now been observed in additional brain regions and populations (64;157;205;206). Moreover, several SAT1 promoter variants have been found to be associated with suicide completion (204;208) and appear to play important roles in determining the expression of SAT1 in vitro and in the brain (207). Additional studies by our group have identified several other polyamine-related genes displaying differential expression, including spermine synthase (SMS), spermine oxidase (SMOX), and ornithine aminotransferase-like 1 (64;157). We were unable to identify genetic or epigenetic factors involved in determining the expression of SMS or SMOX in the prefrontal cortex, suggesting the involvement of other regulatory elements or proteins in producing the differential expression (392). We recently measured the levels of putrescine and spermidine in several brain regions and found elevated levels of both polyamines in suicide completers (209).

Polyamines are low molecular weight cationic molecules, including agmatine, putrescine, spermidine and spermine. In addition to their numerous intracellular functions (393), the polyamines also play essential roles in neurotransmission, in particular at glutamatergic receptors (214). Furthermore, polyamines have been shown to influence GABA receptors (215;216) and dopaminergic pathways in the brain (211;213). Agmatine, which itself is believed to be a neurotransmitter, interacts with imidazoline receptors, α2-adrenoceptors, nicotinic acetylcholine receptors, and 5-HT3 receptors (219), and can influence nitric oxide (NO) transmission through effects on NO synthase (218). The levels of the polyamines are highly controlled through extensive regulation of factors involved in their metabolism, accumulation, and transport. The two rate-limiting enzymes involved in polyamine biosynthesis are ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AMD1), while SAT1 is the rate-limiting enzyme in polyamine catabolism. The expression of each of these enzymes is influenced by numerous factors which affect their levels of transcription, translation, post-translational processing, and degradation (393). Putrescine, spermidine and spermine possess a limited capacity to cross the blood brain barrier (394), and as such, their levels in the CNS is largely due to endogenous synthesis. However, arginine, as well as both agmatine and ornithine, can be transported into the brain (395), and as such, factors affecting the metabolism of related amino acids can influence the polyamine system.

Given the importance of polyamine homeostasis, and the substantial evidence supporting a role for the dysregulation of the polyamine system in suicide and other psychiatric conditions, we were thus interested in obtaining a global view of the expression of genes directly and indirectly related to polyamine metabolism, across the brain of suicide completers. By examining the expression of these genes in a sample of mood-disordered suicide completers and healthy controls, we were able to both identify differentially expressed genes, as well as observe the patterns of dysregulated expression across the brain, thus allowing us to develop a greater understanding of the genes and compensatory mechanisms mediating the effects of the polyamine system in the neurobiology of suicide.

Methods

Subjects:

We analyzed brain tissues from 29 male suicide completers with a history of mood disorders (major depressive disorder and bipolar disorders) and 16 male controls with no history of suicidal behavior. This sample includes several of the subjects analyzed in some of our previous studies using a different microarray platform, testing a limited number of brain regions and addressing different hypotheses (204). All subjects were characterized by the psychological autopsy method using structured clinical interviews eliciting Axis I diagnoses according to DSM-IV criteria, as detailed elsewhere (396). The average age, post-mortem interval (PMI), and pH of all subjects was 39.8±14, 27±11 hours, and 6.6±0.3, respectively. There were no group differences in any of these variables as assessed using both the Welch's t-test and the Wilcoxon rank sum test with continuity correction (Supplementary Table 1).

Brain samples were obtained from the Quebec Suicide Brain Bank (QSBB) (www.douglasrecherche.qc.ca/suicide), where tissues were processed and dissected at 4°C, then snap-frozen in liquid nitrogen before storage at -80°C, following standard procedures (397). All subjects collected by the QSBB died suddenly without a prolonged agonal period. Brain tissues were dissected in accordance with standard neuroanatomical definitions (398). We analyzed a total of 22 brain regions: Brodmann areas (BA) 4, 6, 8/9, 10, 11, 20, 21, 24, 29, 38, 44, 45, 46, 47, as well as the amygdala, cerebellum, hippocampus, hypothalamus, nucleus accumbens, anterior thalamus, dorsomedial thalamus, and lateral thalamus. This study was approved by our local institutional review board.

Microarray Analysis:

All RNA samples used in this study had minimum A260/A280 > 1.9 and 28S/18S rRNA peak height ratios > 1.6, as assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Sample preparation and processing were performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA) in collaboration with Gene Logic Inc. (Gaithersburg, MD). Samples were analyzed using the Human Genome U133 Plus 2.0 array.

GeneChip signal analysis was performed with Reference Robust Multiarray Average (refRMA) (399). Arrays were pre-filtered prior to statistical analysis by assessing several quality indicators, including β-actin 5'/3' ratio, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'/3' ratio, RawQ (noise), scale factor and percent of "present" calls per array. Outlier subjects were excluded in regions where they did not pass quality standards, such that different numbers of subjects were included in the final analyses for each brain region (Supplementary Table 4).

Statistical Analysis:

We performed a NetAffx query (www.affymetrix.com) for probesets related to arginine and proline metabolism, then expanded this list to include additional polyamine-related enzymes identified in the literature. We identified 69 genes, which were represented by a total of 147 probesets. Within each brain region, each probeset was included in the analyses if it was considered present in at least 50% of all subjects.

In order to identify covariates to include in statistical models, we performed Pearson correlations between the $\log_2$ expression levels of all probesets across all regions (1703 tests) and each of the continuous variables for age, PMI, pH, and two RNA quality measures (5'/3' ratios of β-actin and GAPDH). The involvement of two dichotomous variables: toxicological findings of alcohol, or findings of other drugs, were assessed by first dichotomizing the covariates (independently

for each covariate and also combining the two) and then performing t-tests comparing the transformed expression levels between the dichotomized groups. We then performed Shapiro-Wilk normality tests on the Pearson correlation values to determine if the distribution of correlations was normal and centered at zero. These results are shown in Supplementary Table 2. Variables which yielded P-values ≤ 0.05 were considered to be correlated with expression and used to construct the six statistical models shown in Supplementary Table 3.

To identify probesets displaying differential expression in suicide completers, ANOVAs were performed using *maanova* (400), a package for the R statistical environment, using the $\log_2$ transformed expression values of each included probeset while controlling for the covariates included in each statistical model. Differential expression between the suicide and control groups was tested using the F_S test (401). This is an F test where the estimate of the variance in the denominator is obtained by shrinking the probeset-specific variance estimate toward the mean of the variance estimates for all 147 probesets using the James-Stein estimator. The amount of shrinkage depends on the degree of homogeneity of the variance estimates. When variance estimates are homogeneous, individual estimates are completely shrunk to the mean, effectively pooling the estimates. P-values for these tests were obtained using a permutation procedure.

The false discovery rates (FDR) attached to each ANOVA P-value, for all comparisons across all brain regions, were estimated using two methods: Benjamini-Hochsberg (402) and Efron (403) implemented in R using the package *fdrtool*. A probeset was considered significantly differentially expressed if both FDR values were ≤ 0.1 in at least one statistical model in any of the 22 brain regions.

The analysis provided an estimate of the $\log_2$-fold change between the suicide and control groups for each probeset and each brain region. We then performed hierarchical clustering on the data matrix formed by these $\log_2$-fold changes. This analysis must be considered exploratory, as the set of subjects included in the analysis varies between regions. We applied the DIANA (404)

divisive clustering algorithm implemented in the R package using the Euclidian distance.

Validation:

Total RNA for each brain region was extracted from adjacent tissue used for the microarray studies. cDNA synthesis was performed using oligo(dT)-priming (Invitrogen, Carlsbad, CA). Real Time-Polymerase Chain Reaction (RT-PCR) was performed in quadruplicate for each gene using SYBR green on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). β-actin, GAPDH, and β-2-microglobulin (B2M) were used as endogenous controls for relative quantitation. We used three different endogenous controls in order to account for differences in primer efficiencies, which can be an issue using SYBR green chemistry (405). Primer sequences are found in Supplementary Table 5.

Results

Gene expression measurements are known to be influenced by specific post-mortem factors, many of which often cannot be avoided due to the difficulties inherent in recruitment of subjects from specific clinical populations. In this study, our statistical strategy first involved the identification of confounding variables which displayed a direct influence on the expression of polyamine-related genes, then controlled for these variables to identify gene expression changes specific to suicide. Using this methodology, we both avoided unnecessary statistical penalties, as well as identified genes whose expression differences would have otherwise been obscured.

We first assessed the potential confounding effects of several variables commonly associated with post-mortem gene expression studies, including age, PMI, pH, and RNA quality (assessed by the 5'/3' ratios of the housekeeping genes β-actin and GAPDH). To this end, we examined the correlation of each of these

variables with the expression of all polyamine-related probesets, and considered a variable to have a significant effect on expression levels if the combination of all correlation values for that variable were not normally distributed. These results indicated that pH, age, and RNA quality may have confounding effects on gene expression levels (Supplementary Table 2). As the5'/3' ratio of GAPDH was highly correlated with that for β-actin, we retained β-actin 5'/3' ratios as our RNA quality measure. We found no evidence to indicate that expression was influenced by PMI, or the presence of alcohol or drugs (not shown).

ANOVAs were performed to identify probesets which were significantly differentially expressed in the suicide completers compared to the controls, while controlling for each of the variables identified in the first step. The most significant results for each probeset are shown in Table 1. In total, we identified 20 probesets, representing 14 genes, which were significantly differentially expressed in at least one brain region in at least one statistical model. Among these, seven significant findings were retained across all statistical models, including probesets representing ornithine decarboxylase antizyme 1 (OAZ1) and creatine kinase mitochondrial 1A (CKMT1A), as well as five representing SAT1 (not shown).

Overall, 13 of the 22 brain regions contained at least one gene which displayed differential expression in our group. The primary motor cortex, BA 4, displayed the largest number of significant probesets, followed closely by the inferior frontal gyrus, BA 44. Among our statistical models, model 2 (age) yielded the most significant P-values, while model 3 (RNA quality) generated the most significant probesets. However, the probesets yielding significant results were fairly consistent across models 1-3 (not shown), indicating that age and RNA quality have only minimal influence on the expression of our differentially expressed genes.

Altogether, the most significant findings were for SAT1, which showed decreased expression in the suicide completers in 10 of the 22 brain regions examined, which is in agreement with our previous findings (64;157;204;206). Among the SAT1 probesets, the most commonly differentially expressed was

230333_at. Although this probeset targets an intronic region between exons 3 and 4, preliminary studies by our group have found that this does represent a transcribed region of SAT1.Two other genes, SMS and SMOX, have also previously been found to be differentially expressed in suicide completers (64;157), and these two genes displayed similar patterns of altered expression in our current study. Although the downregulation of SAT1 was the most globally observed alteration in the suicide group, half of the differentially expressed genes displayed dysregulated expression in more than one brain region, with all but AMD1 showing a consistent direction across each associated brain region. In addition to demonstrating dysregulated expression across the greatest number of brain regions, calculations of fold changes (FC) between suicide completers and controls showed that SAT1 had the greatest decrease in expression, while ALDH3A2 demonstrated the largest increase in expression.

We performed RT-PCR to validate the most significant findings for each gene, with the exception of creatine kinase, brain (CKB) and CKMT1A, which we were unable to examine in the hypothalamus due to the small quantity of tissue available from this region. The results of this validation are shown in Supplementary Table 6. The majority of the genes showed significant correlations with microarray expression values or significant differences between groups, with the exception of CKMT1A/B and ODC antizyme 2 (OAZ2) in BA 4. However, there was a significant correlation between our RT-PCR results and the expression measured by the CKMT1A probeset 235452_at. As probeset 202712_s_at measures both CKMT1A and CKMT1B, which are different isoforms encoded by the same gene (406), our results suggest that our significant findings in BA 4 may be due to altered expression of the CKMT1B isoform. The results for OAZ2 are less clear, as correlations between RT-PCR results and expression of two other OAZ2 probesets gave both positive and negative correlations.

We next used a hierarchical clustering algorithm in order to gain a better understanding of the relationship amongst the alterations in polyamine-related gene expression across each brain region. Figure 1 depicts a heat map of the $\log_2$ fold changes from statistical model 5 (pH + RNA quality) between the suicide and

control groups for 19 of the 20 probesets with a significant expression difference in at least one region, as well as the dendogram obtained from the hierarchical clustering of the regions and probesets. We initially carried out these analyses using all 20 significant probesets, however the SAT1 probeset 230333_at was removed as its very strong and extensive downregulation obscured our ability to detect relationships between all other components (not shown). Model 5 was chosen for this analysis as it resulted in the greatest range of $\log_2$ fold changes. The clustering clearly separated two groups of genes, with SAT1 (probesets 210592_s_at and 213988_s_at), SMOX and GATM being mostly downregulated across the brain. In addition, probeset 202053_s_at in ALDH3A2 was distinguished from all other included probesets by its extensive overexpression across multiple regions. These expression differences were particularly strong in the cluster of regions formed by BA4, BA21, BA38, BA44, and all regions of the thalamus. Finally, these results demonstrate a distinct pattern of dysregulated expression in the hypothalamus.

Discussion

We investigated the expression of 69 polyamine-, arginine-, and proline-related genes, across 22 brain regions, in a sample of controls and mood-disordered suicide completers, and identified 14 genes displaying altered levels of gene expression, as well as several clusters of genes and brain regions which appear to be particularly important for the involvement of these systems in suicide.

As with our previous gene expression studies in suicide completers, SAT1, the main rate-limiting enzyme in polyamine catabolism and interconversion, demonstrated the most widespread and consistent alterations in expression. Previous studies by our group have also identified upregulated expression of SMS and decreased expression of SMOX in suicide completers (64;157), and these two genes displayed a similar pattern of dysregulated expression in the current study. The consistency of the findings for these three genes, with our use of an expanded

sample set and different microarray platform, reinforces the validity of our previous findings, and highlights their importance in suicide. Altogether, increased polyamine synthesis, combined with decreased polyamine catabolism, would be expected to generate similar, and possibly cumulative effects on cellular and intracellular levels of the polyamines and their metabolites. These alterations are likely related to the increased levels of putrescine and spermidine we recently quantified by GC-MS in the brains of suicide completers (209). Although the direct effects of altered expression of SAT1, SMOX, and SMS on the overall profile of the polyamines and metabolites are unknown, these changes may reflect compensatory mechanisms to reduce the levels of the lower polyamines, or could indicate an increased cellular requirement for spermine. However, as described below, the present study identified dysregulated expression of additional genes with important roles in controlling polyamine levels, and it remains difficult to distinguish between those whose altered expression levels represent compensatory rather than pathological mechanisms.

In this study, our focused approach and expanded sample allowed us to identify additional polyamine-related genes which show differential expression in suicide completers. Whereas our original studies highlighted genes involved in controlling the levels of the higher polyamines, the current study also revealed the importance of enzymes involved in the biosynthesis of the lower polyamines. AMD1 is one of the rate-limiting biosynthetic enzymes, and acts on S-adenosylmethionine (SAM) to produce the aminopropyl donor required for the conversion of putrescine to spermidine, and spermidine to spermine. AMD1 levels are regulated at multiple levels, including transcription, translation, pro-enzyme processing, enzymatic activity, and protein degradation, and several of these processes are known to be influenced by the polyamines (393). In addition to its essential role in polyamine biosynthesis, its effects on the levels of SAM and decarboxylated SAM influences the synthesis of several neurotransmitters, as well as levels of DNA methylation and histone modifications (369;407-411), each of which may play a role in the antidepressant effects of SAM in humans (368). Post-mortem studies have found elevated levels of SAM in BA 9 of psychotic, but

not depressed patients (411), whereas depressed individuals display a downregulation of AMD1 in BA 21 (412). Interestingly, given the important role of putrescine on AMD1 activity, AMD1 is downregulated in BA 4, a region demonstrating elevated putrescine levels in suicide completers (209). Although ODC is the second major rate-limiting enzyme in polyamine biosynthesis, and plays the leading role in the polyamine stress response (197), post-mortem studies in humans have not detected altered expression of this gene in suicide or other psychiatric conditions. However, as this gene is extensively regulated at numerous levels, the apparent lack of alterations in mRNA levels does not necessarily rule out its involvement in suicide. Thus it is of great interest that we identified increased expression of two genes, the antizymes OAZ1 and OAZ2, which directly influence ODC activity and protein levels. The ODC antizymes are a class of three related isoforms which bind ODC and target it for degradation, as well as inhibit the uptake and induce the secretion of polyamines from the cell (413). Interestingly, translation of each antizyme is controlled by a unique frameshift which is induced by elevated polyamine levels (413). Taking into account the presence of increased polyamine levels (209) in conjunction with elevated antizyme mRNA levels, it seems likely that protein levels of antizyme are also increased, with the potential effect of decreasing ODC activity in these brain regions. A recent study found OAZ1 to be downregulated in BA 10 of schizophrenics (414), thus emphasizing the importance of the antizymes in psychiatry.

The production of ornithine by arginases is also an important site for the control of polyamine metabolism as well as other arginine-related pathways. Arginase II (ARG2) is the mitochondrial variant and is found at high levels in the brain where it plays important roles in both polyamine and NO metabolism (415). Arginase induction can result in decreased NO synthesis as well as increased polyamine biosynthesis through the reduction of arginine and elevation in ornithine levels (416). As such, the upregulation of ARG2 is particularly interesting given the relationship of NO with suicidal behaviors (300;303).

Furthermore, altered arginase activity has been observed in plasma of patients with major depressive disorder, schizophrenia, and bipolar disorder (417-419).

In addition to enzymes directly involved in polyamine metabolism, we identified several genes with more distal relationships which may influence the availability of substrates for polyamine biosynthesis. These include aldehyde dehydrogenase 3 family, member A2 (ALDH3A2), CKB, CKMT1A/B, glycine amidinotransferase (GATM), glutamic-oxaloacetic transaminase 1, soluble (GOT1), and arginyl-tRNAsynthetase-like (RARSL). Interestingly, three of these enzymes, CKB, CKMT1A/B, and GATM are involved in creatine metabolism, which plays an essential role in controlling levels of adenosine 5'-triphosphate (ATP) in the cell (420). CKB, CKMT1A, and CKMT1B represent different isoforms of creatine kinase, and are involved in the regeneration of cellular ATP (406). Both CKB and CKMT1A were upregulated in the hypothalamus, which may indicate a higher energy use in this structure, or could reflect a compensatory mechanism to increase low ATP levels. Intriguingly, the hypothalamus displayed a pattern of dysregulated expression which was distinct from those of other brain regions, which is of great interest given the strong support for the involvement of the hypothalamic-pituitary-adrenal (HPA) axis system in suicide (421). In BA 4, we observed a downregulation of GATM, the rate-limiting enzyme in creatine biosynthesis (422), as well as increased expression of CKMT1A and/or CKMT1B, which is in agreement with our previous findings of significant alterations in ATP production in the prefrontal cortex of suicide completers (64). Finally, alterations in CKB have previously been observed in several brain regions of patients with schizophrenia (423;424), and mutations in GATM appear to have behavioral and cognitive effects (425), emphasizing the important neurobiological consequences resulting from dysregulated creatine metabolism.

The relationship between the other differentially expressed genes and the polyamine system is not straightforward. ALDH3A2 is a microsomal enzyme involved in the metabolism of aldehydes, including those produced during putrescine catabolism. Deficiency of this gene is associated with Sjögren-Larsson syndrome, which is characterized by physical and neurological defects resulting

from the accumulation of fatty alcohols (426). Upregulation may reflect increased polyamine catabolism, or may be a consequence its involvement in lipid metabolism, which has also been implicated in suicidal behaviors (421). Indeed, our multivariate analyses demonstrated a distinct pattern of dysregulated expression for this gene, which may indicate that its involvement in suicide is not related to polyamine metabolism. Along the same line, GOT1 catalyzes the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate, which is an essential reaction in several metabolic pathways, thereby making it difficult to determine if its upregulation is directly related to polyamine metabolism. Finally, RARSL uses arginine to produce arginyl-tRNA, which is essential for protein synthesis, and plays a role in protein degradation (416).

There are several limitations to this study. As with all studies of this nature, the effects of post-mortem factors as well as other confounding clinical variables may influence the interpretation of results. However, we used statistical methods to identify and control for confounding variables, and by statistically assessing the effects of these and other variables on overall expression, we were able to identify the most relevant factors in terms of quality (β-actin 5'/3' ratio), post-mortem (pH), and clinical (age) characteristics. Given that our results were fairly consistent across the models, our expression results appear relatively robust to the effects of these variables. Nonetheless, we cannot rule out the possibility that additional confounding factors may have been present which were not controlled for. By expanding our sample to include individuals with depression and bipolar disorders, we may have obscured our ability to detect suicide-specific alterations by introducing disease-specific effects. Additionally, as we did not have a suicide group without mood disorders, we are unable to completely dissociate the processes related to suicide from those associated with mood disorders. Nonetheless, in doing so we may have generated results which can be better generalized to other populations. Finally, it is possible that additional quality or post-mortem variables were present which were not accounted for in our statistical models.

Microarray studies generate large amounts of data, potentially leading to false positive findings when inadequate statistical methods are used to control for multiple testing. Conversely, the use of overly stringent correction methods can prevent the identification of biologically relevant alterations in expression. By pre-selecting probesets to include only those which may be relevant for polyamine metabolism, we have greatly decreased the amount of statistical correction required for multiple testing, while at the same time, we have limited false positive results by the use of two separate FDR methods.

In conclusion, we have performed a global analysis of the expression of polyamine-related genes across many brain regions of suicide completers, and have identified several genes displaying significantly altered expression. This study has allowed us to obtain a broader view of the polyamine-related processes associated with suicide, and has highlighted the importance of the polyamine system in the psychopathology of suicide.

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Figures

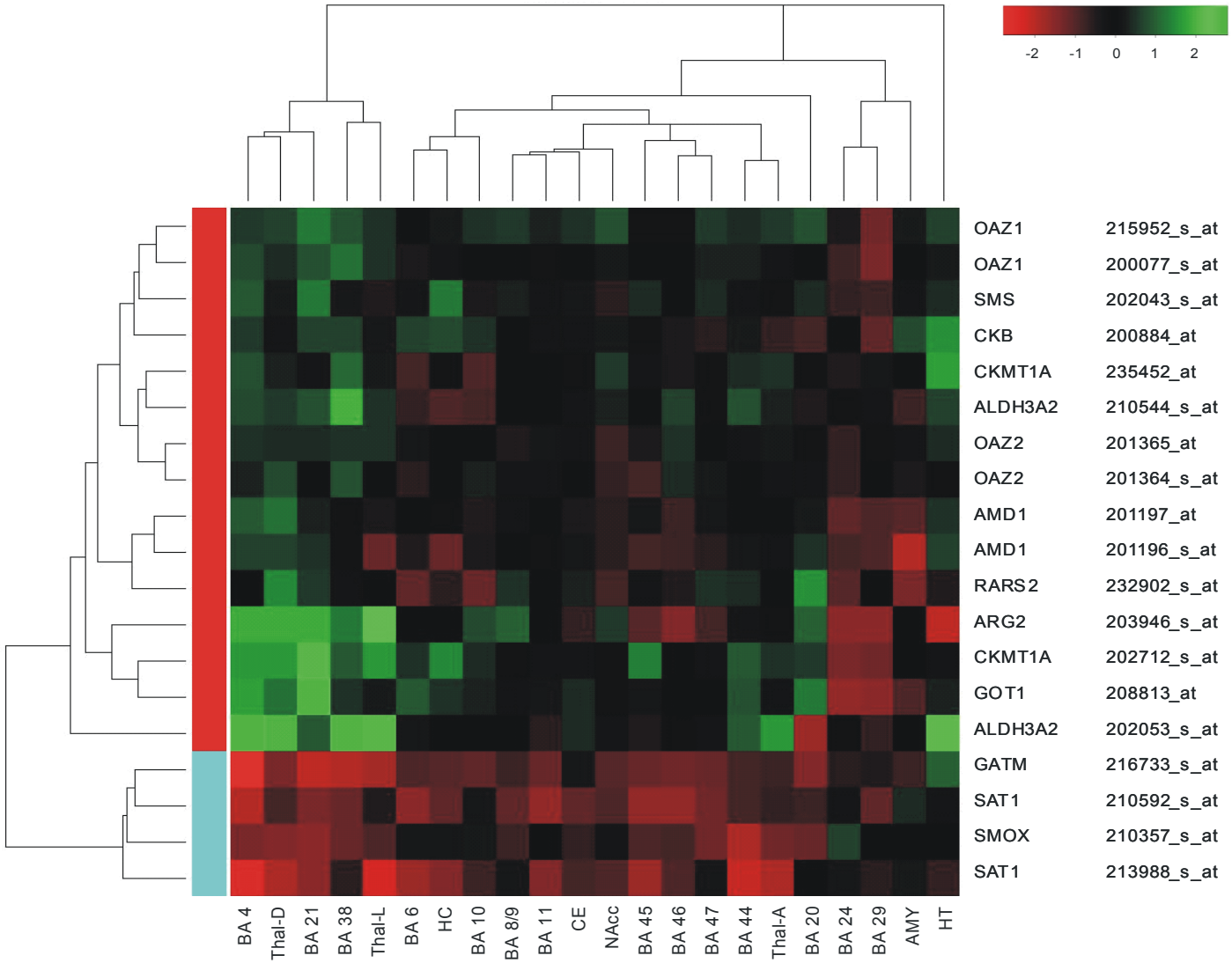


Figure 1: Heat map generated using the DIANA algorithm with the $\log_2$ fold changes from 19 probesets between suicide completers and controls in model 5 (β-actin and pH). AMY: amygdala; BA: Brodmann area; HC: hippocampus; HT: hypothalamus; Thal-A: anterior thalamus; Thal-D: dorsomedial thalamus; Thal-L: lateral thalamus.

Tables

Table 1: Differentially expressed probesets in suicide completers with mood disorders compared to controls. The lowest Efron false discovery rate (FDR)-corrected P-value and the associated statistical model are indicated. Probesets in bold are those which displayed an FDR ≤ 0.1 across all six statistical models. Fold change (FC) indicates expression in suicides relative to controls.

Gene	Probeset	Region	FC	FDR	Model *
ALDH3A2	202053_s_at	BA 4	1.80	0.04	3
	210544_s_at	BA 44	1.29	0.06	2
	202053_s_at	Thal-L	1.72	0.08	6
AMD1	201196_s_at	AMY	0.74	0.08	1
	201196_s_at	BA 4	1.26	0.08	1
	201197_at	BA 4	1.30	0.1	3
ARG2	203946_s_at	BA 4	1.57	0.06	2
CKB	200884_at	HT	1.42	0.04	3
CKMT1A	202712_s_at	BA 4	1.46	0.08	1
CKMT1A, CKMT1B	**235452_at**	**HT**	1.49	**0.03**	**1**
GATM	216733_s_at	BA 4	0.64	0.06	2
GOT1	208813_at	BA 21	1.71	0.08	1
	208813_at	BA 4	1.53	0.06	2
	208813_at	BA 44	1.42	0.06	2
OAZ1	200077_s_at	BA 21	1.28	0.04	1
	215952_s_at	BA 21	1.35	0.07	5
	200077_s_at	**BA 4**	1.27	**0.03**	**2**
	200077_s_at	BA 44	1.20	0.06	2
	215952_s_at	BA 44	1.38	0.06	2
	215952_s_at	BA 47	1.36	0.06	2
OAZ2	201364_s_at	BA 38	1.26	0.1	3
	201365_at	BA 38	1.18	0.1	3
	201365_at	BA 4	1.19	0.08	1
RARSL	232902_s_at	Thal-D	1.41	0.08	1
SAT1	230333_at	AMY	0.43	0.06	6
	230333_at	BA 10	0.47	0.06	2
	230333_at	BA 21	0.46	0.1	3
	210592_s_at	BA 4	0.77	0.04	3
	213988_s_at	**BA 4**	0.72	**0.03**	**1**
	230333_at	**BA 4**	0.39	**0.001**	**4**
	213988_s_at	BA 44	0.76	0.1	3
	230333_at	**BA 44**	0.48	**0.04**	**4**
	230333_at	BA 6	0.49	0.07	5
	230333_at	HC	0.40	0.04	3
	230333_at	Thal-A	0.39	0.04	3
	230333_at	**Thal-D**	0.35	**0.03**	**2**
	230333_at	**Thal-L**	0.23	**0.0004**	**5**
SMOX	210357_s_at	BA 44	0.76	0.06	2
SMS	202043_s_at	BA 4	1.31	0.06	2

[*] Model covariates: 1 (none), 2 (age), 3 (RNA quality), 4 (pH), 5 (RNA quality + pH), 6 (RNA quality + age)
AMY: amygdala; BA: Brodmann area; FC: fold change; HC: hippocampus; HT: hypothalamus; Thal-A: anterior thalamus; Thal-D: dorsomedial thalamus; Thal-L: lateral thalamus

Supplementary Material

Supplementary Table 1: P-values for the comparison of age, post-mortem interval (PMI) and pH between suicide completers and controls.

Test	Age	PMI	pH
Welch Two Sample t-test	0.51	0.16	0.30
Wilcoxon rank sum test with continuity correction	0.56	0.28	0.40

Supplementary Table 2: P-values of Shapiro-Wilk tests performed on the 1703 Pearson correlation values between the expression level and each covariable.

Age	PMI	pH	β-actin	GAPDH
0.006	0.24	0.0002	5.5×10^{-8}	4.4×10^{-5}

Supplementary Table 3: Statistical models used to examine group differences in expression of polyamine-related probesets between suicide completers and controls.

Model	Covariate (s)
1	None
2	Age
3	β-actin
4	pH
5	β-actin + pH
6	β-actin + age

Supplementary Table 4: Numbers of controls and suicide completers used in the analysis of each brain region. BA: Brodmann area.

Region	Controls	Suicides
BA 4	12	18
BA 6	6	15
BA 8/9	6	15
BA 10	7	14
BA 11	10	17
BA 20	8	13
BA 21	10	16
BA 24	9	18
BA 29	10	21
BA 38	6	16
BA 44	11	12
BA 45	6	14
BA 46	5	14
BA 47	9	14
Amygdala	11	13
Cerebellum	11	14
Hippocampus	9	18
Hypothalamus	8	14
Nucleus accumbens	10	18
Thalamus-Anterior	9	14
Thalamus-Dorsomedial	7	15
Thalamus-Lateral	10	12

Supplementary Table 5: Primers used for RT-PCR validation of microarray results.

Gene	Region	Size	Sequence
ALDH3A2	BA4	111	CCTCTGGCTCTTTATGTATTTTCGC
			GAGCGTGAAGTGCATAATGACG
AMD1	BA4	178	GATGGAACTTATTGGACTATTCACATCAC
			CTGTGCGACATTTAGAACTCTGATTAAC
ARG2	BA4	105	TTGCTGAGGAAATACACAATACAGG
			GGTTAGCTGTAGTCTTCGCCTC
CKMT1A/B	BA4	135	ATAGCCGCTTCCCAAAGATCCTG
			CCAGCTCCACCTCTGATTTGC
GATM	BA4	90	TGGATGCCAATGAAGTTCCAATTC
			CCAGGGAATTGGCATTACGAATG
GOT1	BA44	100	GGCATGTTCAGCTTCACTGG
			CACTCACGTTGATTCGACCAC
OAZ1	BA4	106	GACAGCTTTGCAGTTCTCCTGG
			TTCGGAGCAAGGCGGCTC
OAZ2	BA4	130	GCTGATGGGAGCAAAGAAGG
			AGCTGAAGGTCTTCAGGAGTG
SAT1	BA4	179	CAGTGACATACTGCGGCTGATCAAG
			AACCAACAATGCTGTGTCCTTCCG
SMS	BA4	145	TTACACAGGGGAACTGTGTC
			CAAACAGTGTAAAATACCCACAATTCC
SMOX	BA44	193	AGTTCACAGGGAACCCCAAC
			CCGGAAAACAGCACCTGCATG
RARSL	Thal-D	120	CAACCCAGGCATATCGTCAG
			TGAAAAGATGAAGTCTGGCCC
β-actin	Control	93	GCACAGAGCCTCGCCTT
			GTTGTCGACGACGAGCG
B2M	Control	198	CTCACGTCATCCAGCAGAGA
			TCTTTTTCAGTGGGGGTGAA
GAPDH	Control	202	TTGTCAAGCTCATTTCCTGG
			TGTGAGGAGGGGAGATTCAG

Supplementary Table 6: Validation of differentially expressed genes by RT-PCR. P-values from one-tailed Student *t*-tests between controls and suicides, as well as Pearson correlations and associated P-values between microarray expression values and normalized RT-PCR expression values are shown when using β-actin, GAPDH, or B2M as the endogenous control. False discovery rate (FDR) refers to Efron FDR-corrected P-values from the analysis of microarray data.

				β-actin			**GAPDH**			**B2M**		
Gene	**Probeset**	**Region**	**FDR**	***t*-test**	**C**	**P**	***t*-test**	**C**	**P**	***t*-test**	**C**	**P**
ALDH3A2	202053_s_at	BA4	0.04	0.25	0.03	0.87	0.17	0.14	0.46	0.05	0.49	0.01
AMD1	201196_s_at	BA4	0.08	0.40	0.005	0.98	0.12	0.27	0.15	0.04	0.20	0.28
	201197_at	BA4	0.1		0.08	0.67		0.33	0.08		0.23	0.21
ARG2	203946_s_at	BA4	0.06	0.28	0.07	0.73	0.14	0.44	0.02	0.09	0.37	0.05
CKMT1A/B	202712_s_at	BA4	0.08	0.20	-0.22	0.25	0.36	0.20	0.29	0.15	0.07	0.72
CKMT1A	235452_at	BA4	NS		-0.22	0.24		-0.19	0.32		0.39	0.03
GATM	216733_s_at	BA4	0.06	0.24	0.60	0.001	0.37	0.29	0.13	0.28	0.12	0.54
GOT1	208813_at	BA44	0.06	0.35	0.43	0.04	0.43	0.48	0.02	0.05	0.54	0.01
OAZ1	200077_s_at	BA4	0.03	0.37	-0.27	0.15	0.19	0.23	0.23	0.12	0.40	0.03
OAZ2	201365_at	BA4	0.08	0.34	-0.27	0.15	0.24	-0.02	0.92	0.11	0.07	0.72
	201364_s_at	BA4	NS		-0.36	0.05		0.21	0.26		0.03	0.86
	238024_at	BA4	NS		0.47	0.01		-0.33	0.08		-0.40	0.03
RARSL	232902_s_at	Thal-D	0.08	0.003	0.16	0.47	ND	ND	ND	ND	ND	ND
SAT1	213988_s_at	BA4	0.03	0.49	-0.09	0.64	0.27	-0.28	0.13	0.19	-0.23	0.22
	230333_at	BA4	0.001		-0.08	0.66		-0.16	0.41		-0.17	0.36
	210592_s_at	BA4	0.04		0.35	0.06		0.05	0.79		-0.01	0.97
SMOX	210357_s_at	BA44	0.06	0.06	0.52	0.01	0.04	0.53	0.01	0.46	0.25	0.24
SMS	202043_s_at	BA4	0.06	0.29	-0.24	0.20	0.08	0.23	0.22	0.03	0.48	0.01

C: Pearson correlation; ND: not done; NS: non-significant; P: correlation P-value; Thal-D: dorsomedial thalamus

2.2 Association of Polyaminergic Loci with Anxiety, Mood Disorders, and Suicide Attempts

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Abstract

Background: The polyamine system has been implicated in a number of psychiatric conditions, which display both alterations in polyamine levels and altered expression of genes related to polyamine metabolism. Studies have identified associations between genetic variants in spermidine/spermine N1-acetyltransferase (SAT1) and both anxiety and suicide, and several polymorphisms appear to play important roles in determining gene expression.

Methodology/Principle Findings: We genotyped 63 polymorphisms, spread across four polyaminergic genes (SAT1, spermine synthase (SMS), spermine oxidase (SMOX), and ornithine aminotransferase like-1 (OATL1)), in 1255 French-Canadian individuals who have been followed longitudinally for 22 years.

We assessed univariate associations with anxiety, mood disorders, and attempted suicide, as assessed during early adulthood. We also investigated the involvement of gene-environment interactions in terms of childhood abuse, and assessed internalizing and externalizing symptoms as endophenotypes mediating these interactions. Overall, each gene was associated with at least one main outcome: anxiety (SAT1, SMS), mood disorders (SAT1, SMOX), and suicide attempts (SAT1, OATL1). Several SAT1 polymorphisms displayed disease-specific risk alleles, and polymorphisms in this gene were involved in gene-gene interactions with SMS to confer risk for anxiety disorders, as well as gene-environment interactions between childhood physical abuse and mood disorders. Externalizing behaviors demonstrated significant mediation with regards to the association between OATL1 and attempted suicide, however there was no evidence that externalizing or internalizing behaviors were appropriate endophenotypes to explain the associations with mood or anxiety disorders. Finally, childhood sexual abuse did not demonstrate mediating influences on any of our outcomes.

Conclusions/Significance: These results demonstrate that genetic variants in polyaminergic genes are associated with psychiatric conditions, each of which involves a set of separate and distinct risk alleles. As several of these polymorphisms are associated with gene expression, these findings may provide mechanisms to explain the alterations in polyamine metabolism which have been observed in psychiatric disorders.

Introduction

Mood and anxiety disorders represent the two most common forms of mental illness, and are associated with a wide range of behavioural and somatic symptoms as well as substantial disability and decreased quality of life (427). Suicidal behaviors are closely, but not exclusively, associated with both psychiatric disorders. These behaviors, which comprise ideation, attempts, and completed suicide, are amongst the most devastating consequences of psychiatric disorders, and account for over a million deaths worldwide each year (2). Over the years, the importance of genetic factors in psychiatric disorders has become increasingly apparent, with overall heritability rates for depressive disorders, anxiety disorders, and suicide ranging between 30-50% (6-8;428;429), involving both shared and distinct genetic vulnerabilities (5;430). Substantial efforts have been put towards identifying genes and pathways involved in the pathology and etiology of these conditions as these both represent sites involved in conferring risk for their development, as well as act as potential targets for pharmaceutical treatments. Although the substantial evidence emerging from genetic, metabolic, and pharmacological studies investigating these disorders has implicated the involvement of monoaminergic neurotransmission, particularly the serotonin and catecholamine systems, dysregulation of these systems is not sufficient to account for all aspects of the clinical presentations or heritability associated with these disorders, and it has become abundantly clear that additional systems are involved.

The polyamine system represents an important source for neurobiological factors involved in mood disorders, anxiety disorders, and suicide. In recent years, the majority of the focus has been on its involvement in suicidal behaviours, yet considerable research across the last three decades has pointed towards roles for the polyamine system in several psychiatric conditions, including schizophrenia, mood disorders, and anxiety disorders, and particular emphasis has been placed on the importance of this system in the physiological and behavioural responses to stress (203).

The polyamines are ubiquitous aliphatic molecules, comprising spermine, spermidine, putrescine, and agmatine, which are involved in a vast range of cellular functions, including cell cycle modulation, scavenging reactive oxygen species, control of gene expression, and possess important roles in neurotransmission through their modulation of the functioning of cell-surface receptors, involvement in intracellular signalling pathways, as well as their putative roles as neurotransmitters (198;219). Cellular levels of the polyamines are extensively regulated through tight control of their biosynthesis, catabolism, and transport, and much of the evidence for their involvement in psychiatric conditions to date has revolved around variations in the levels of the polyamines, as well as alterations in the expression of genes involved in polyamine metabolism, including spermidine/spermine N1-acetyltransferase (SAT1), spermine oxidase (SMOX), spermine synthase (SMS), and ornithine aminotransferase–like 1 (OATL1) (64;157;204-206;431). Given the extensive molecular functions of the polyamines, their precise roles in the etiology and pathology of psychiatric disorders remain unclear, although evidence from animal studies have suggested that at least some of their antidepressant and anxiolytic effects involve modulation of transmission through N-methyl-D-aspartate receptors, α2-adrenoceptors, imidazoline receptors, and serotonin receptors (203).

In spite of the strong evidence suggesting a role for the polyamine system in depression, anxiety, and suicide, with recent evidence indicating direct polyamine dysregulation in brain tissue from individual who died by suicide (209), only a few studies have investigated polyamine-related genes at the genetic level, all of which have focused exclusively on promoter polymorphisms in SAT1, the main rate-limiting enzyme in polyamine catabolism. Three studies found significant associations: rs6526342 with suicide (204), rs6151267 with suicide in depressed individuals (432), and rs1960264 with anxiety (433). However, another study found no association between either rs6526342 or rs17286006 and suicide (205), nor was rs1960264 found to be associated with schizophrenia (434). Interestingly, these polymorphisms are part of a larger haplotype block which is associated with SAT1 expression in the brain (207), thereby representing a link between genetic

variability and downstream functional consequences. To date, no genetic studies have examined the relationships between polymorphisms in other polyamine-related genes and psychiatric disorders.

The aim of the present study was to expand our understanding of the relationship between polymorphisms in polyamine-related genes and psychiatric disorders, in particular mood disorders, anxiety disorders, and suicidal behaviours, as well as to investigate the potential for epistasis between genetic risk factors, and to identify variables which may influence the effects of genetics on psychopathology. To this end, we genotyped a large number of polymorphisms in several genes involved in polyamine metabolism in a French-Canadian cohort which has been followed for over 20 years. During this time, substantial clinical and epidemiological measures were collected, and a number of these were assessed as potential mediators for our genetic associations. Given the influence of environmental stressors on the polyamine system (197;203) as well as consistent findings implicating early life adversity in the development of psychiatric disorders (435-437), the influence of childhood sexual and physical abuse was assessed. We also investigated several personality measures, comprising externalizing or internalizing behaviors, as endophenotypes for our main outcomes. As our previous research investigating externalizing and internalizing trajectories in young children (ages 6 to 12) did not identify mediating effects with regards to mood disorders or suicide attempts (438;439), in this study these symptoms were investigated at an older age (adolescence).

Overall, we identified several genetic risk factors associated with mood disorders, anxiety disorders, and suicidal behaviours, as well as several clinical variables which mediate these effects.

Methods

Study Participants:

Participants in this study were part of a larger cohort of French-Canadians recruited in 1986-1987, then followed-up for over 20 years. A more detailed description of this cohort as well as the assessment schedules is found in (436;438). In brief, children were recruited from francophone schools in Quebec at age 6, where they were assessed through a variety of demographic, social and behavioral measures in several waves, representing childhood, mid-adolescence, early adulthood, and mid-adulthood. The initial sample comprised 3017 children, of which 2000 were randomly selected and are considered representative of the young French-speaking population. DNA was collected from the 1255 respondents among the initial sample. In the representative sample of 2000 subjects, there were no differences between the respondents and nonrespondents for parental age at birth of first child, maternal socioeconomic status, or proportion living with both biological parents. As both family adversity (described below) and gender were related to attrition in our previous studies in this cohort (438), we used them to construct weights for multivariate analyses. This study was approved by the institutional review boards of the University of Montreal and McGill University. Written informed consent was obtained from all subjects.

Measures:

Genetic Factors:

Single nucleotide polymorphisms (SNPs) were selected from four polyamine-related genes: SAT1 (NM_002970), SMOX (NM_175839), SMS (NM_004595), and OATL1 (NM_001006113). SNPs were located between 5 kb upstream of the transcription start site to 5 kb downstream of the end of the last

exon. Common tag SNPs (minor allele frequencies > 5%) for each gene were selected using HapMap data for the Utah residents with Northern and Western European ancestry (440) and the multi-marker tagging procedure in Tagger (r^2 > 0.8) (441). Additional SNPs in the upstream regions were selected using the NCBI, Pupa, and Ensembl databases. In total, 63 polyamine-related SNPs were genotyped, as shown in Supplementary Table S1. We also included 42 anonymous markers spread in non-coding regions across the genome in order to detect population stratification. Genotyping was performed using a 768-SNP Illumina platform with a custom-designed GoldenGate panel. Following genotyping, several quality control steps were performed as described in (438). Two SNPs (rs1535225 and rs2238958) had call rates of less than 90% and were removed.

Environmental Factors:

Among the overall study group, we defined two subsamples through their exposure to physical (CPA) or sexual (CSA) abuse in childhood (under 18 years of age). Subjects in the CPA group self-reported severe or very severe physical abuse perpetrated by either parent, as assessed in the Conflict Tactics Scales (442;443). Childhood sexual abuse was defined as incidences of sexual violence experienced before the age of 18, and was assessed by self-report as described in (444).

The effects of family adversity were also assessed. As described in (445), we computed a family adversity index based upon maternal reports regarding: (1) family structure (two parent or single), (2) educational level of both parents (or the parent with whom the child was living), (3) occupational status of both parents (or occupation of the parent with whom the child was living) based on the Blishen's occupational prestige scale (446) and, (4) mother's and father's age at birth of the first child. Higher values correspond to higher family adversity levels at the time when the participants were approximately 6 years of age.

Mediators:

Diagnostic Interview Schedule for Children (DIS-C) (447)**:**

Using the DIS-C, self-reports of hyperactivity-impulsivity (8 symptoms), oppositional defiant disorder (9 symptoms), conduct disorder (11 symptoms), generalized anxiety (18 symptoms), panic disorder (13 symptoms), major depression (9 symptoms), and dysthymia (6 symptoms) were assessed. A total externalizing-disruptiveness score was obtained by summing the symptom counts associated with hyperactivity-impulsivity, oppositional defiant disorder, and conduct disorder (Cronbach alpha = 0.77). Two separate internalizing scores were generated: an internalizing-anxiety score was calculated by summing generalized anxiety and panic disorder symptoms (Cronbach alpha = 0.93), while an internalizing-depression score was generated by summing major depression and dysthymia symptoms (Cronbach alpha = 0.94). To equally weight each disorder when calculating the total sum scores, each count variable was transformed to range between 0 and 1 (by dividing the total count by the maximum count after adding 1 as a constant) before calculating the total sum score.

Covariates and Outcomes:

Diagnostic Interview Schedule for Adults (DIS) (448)**:**

This schedule assesses mood (major depression, bipolar disorder and dysthymia), anxiety (generalized anxiety, panic and phobias), disruptive (i.e., antisocial personality), and substance abuse disorders (abuse and/or dependence on drugs, alcohol and nicotine) using DSM-III-R criteria. Mood and anxiety disorders represented outcomes and, in addition, served together with disruptive and substance abuse disorders and suicide attempts (see below) as the covariate "history of psychopathology", following previous research (438). This count variable summarized the number of diagnoses in each individual (see Table 3).

For each of the main outcome variables, the other two outcomes were part of this count variable, in addition to substance and disruptive disorders. By means of controlling for the other disorders, any significant gene-outcome relationship is independent of these potentially confounding effects. The DIS was also used to provide information regarding parental history of suicide attempts, anxiety, and mood disorders. If either parent had a positive history for these control variables, the respective history was coded as '1', otherwise it was coded as '0'.

Suicide attempts:

Suicide attempt status was based on both adolescent and adult assessments. Adolescent history was obtained from parental/adolescent responses to a question from the DIS-C (447): 'Have you already attempted suicide?' Either parental or self-report was sufficient for a person to be classified as an attempter. Adult suicide attempts were assessed with a question from the Suicidal Intent Scale (449): 'Have you already attempted suicide?' A positive attempt status was coded as '1' and negative as '0'.

Statistical Approach:

Population Stratification:

Although the French Canadians descended from a small number of individuals and displays a well-known founder effect (450;451), we nonetheless felt it was necessary to identify population outliers - individuals displaying significantly different allele frequency distributions from the rest of the sample (452). We used the genotype log likelihood test statistic with a cut-off of $P = 0.01$, identifying 12 outliers, which were excluded from subsequent analyses. All SNPs fulfilled Hardy-Weinberg equilibrium.

Univariate analyses:

We first investigated direct effects exerted by SNPs and haplotypes on our main outcomes (suicide attempts, mood disorders, and anxiety disorders). We also postulated that CSA or CPA may create associations of genetic variants with phenotypes only in subjects exposed to these environments and, therefore screened for such moderating or interaction effects by testing for associations of SNPs with the phenotypes in the subsamples of subjects exposed to CSA or CPA.

Firstly, in order to identify redundant SNPs, we used the squared correlations between each SNP to identify all groups of SNPs with $r^2 > 0.99$, then selected only one SNP from within each group of these perfectly correlated SNPs, yielding a set of 43 SNPs which were retained for all further analyses. These are shown in Supplementary Table S1.

χ^2-tests and Fisher's exact tests, in conjunction with a false detection rate (FDR) cutoff of ≤ 0.20, were used to identify significant SNPs under allelic, recessive and dominant genetic models with respect to the minor allele. For X-linked SNPs, males were coded as homozygous when assessing recessive and dominant models. The FDR attached to each P-value for suicide attempts in the total sample as well as all analyses in the CSA and CPA subsamples was estimated using the Efron (403) method implemented in R. The Benjamini-Hochsberg (402) method was used to compute FDRs for analyses of anxiety and depression in the total sample. The Benjamini-Hochsberg method was employed for these analyses as the empirical null distribution was heavily skewed and did not fit a normal distribution, which is required to apply the Efron method. SNPs with FDR corrected P-value ≤ 0.20 were further tested in adjusted multivariate models.

We determined haplotype blocks within each gene in the total sample using *entropy.blocker* (453) implemented in the R statistical environment. Global association tests to haplotypes within each haplotype block were performed for suicide, depression, and anxiety. For SMOX, haplotype analyses were performed using the function *haplo.score* (454) from the package *haplo.stats*, implemented

in R. Analyses for the X chromosome genes were performed using UNPHASED (455). The analysis of SMS haplotypes was more complicated due to the large number of SNPs in the SMS haplotype block. Firstly, we formed ten subgroups of strongly correlated SNPs, then made three different selections of ten SNPs by randomly picking one SNP in each subgroup. These three selections were then analysed using UNPHASED.

Multivariate analyses:

Using a series of regression-based analyses adjusted for psychopathology, we retained all significant SNPs in a given gene ($P < 0.05$), and included them together in a model across genes. We also tested if the results in the final models changed if we applied weights adjusting for the probability of remaining in the sample conditional on the variables related to attrition: gender and family adversity. The expectation-maximization method (EM) was used to impute missing covariate values.

We analyzed two forms of moderating effects: gene-gene interactions, and gene-environment interactions. Gene-gene interactive effects were assessed in order to examine moderation of the effects of polyaminergic loci on the three outcomes by other polyaminergic loci. These interactions were performed between pairs of SNPs, in separate genes, from among those that displayed significant univariate associations to the three main outcomes. To assess these effects, in each regression model, we included two of the significant main effects from the univariate analyses as well as their interaction term. Interactions were only assessed using SNPs which were significant in the univariate analyses, and only between SNPs of different genes. Each set of predictors was tested with a logistic and an additive regression model. We examined allelic–allelic, dominant–dominant, dominant–recessive, recessive–dominant and recessive–recessive model combinations, depending on the significant mode found on the univariate step. Specifically, we conducted ten regressions (suicide attempts: 2, mood disorders: 0, anxiety disorders: 8) and empirically determined whether logistic or

additive link functions yielded a better fit to the data, resulting in two highly correlated sets of regressions and a total of twenty tests. We believe that the low statistical power to detect interaction effects in field studies, as described by McClelland and Judd (456), also applies to psychiatric and genetic studies. We therefore did not employ Type-I Error protection for the gene-gene interaction tests. Following similar procedures, we tested gene-environment interactions to examine the moderation of the effects of childhood abuse on the associations of SNPs with any of the three outcomes. We controlled for confounding effects of passive or evocative gene-environment correlations by adjusting models for parental histories of psychopathology, and by demonstrating that genotypes did not influence the exposure to abuse. Post-hoc tests were used to quantify regression slopes and examine the statistical significance of significant moderating effects (457). Corrections for multiple testing were not performed at this stage in the analyses, as these tests were performed using only SNPs which passed our FDR criteria in the CSA and CPA subsamples prior to the inclusion of covariates. Power analyses for these tests with the mood disorder phenotype are shown in Supplementary Tables S2 and S3. As the prevalence of suicide attempts and anxiety disorders were higher than that of mood disorders in the sample, greater power is expected for these two phenotypes.

In the presence of covariates, count scores of externalizing and internalizing disorders were investigated as endophenotypes mediating the significant main effects identified in the final models of suicide attempts, mood disorders, and anxiety disorders. Specifically, mediation testing was performed to identify variables which accounted for some or all of the associations between our genotypes and main outcomes (458), and the significance of these results was assessed using Sobel and Goodman tests.

Results

Sample:

From the initial 1255 subjects, fourteen subjects with call rates less than 95% were excluded, and 108 subjects were removed as they displayed non-White ethnicity. Assessment for population outliers identified 12 subjects displaying significantly different allele frequencies than the remainder of the population. After exclusions, the total analyzed sample consisted of 1121 (N = 664, 59% female) individuals, as shown in Table 1. Overall, exposure to CSA or CPA was significantly associated with higher rates of suicide attempts, mood disorders, and anxiety disorders.

Univariate analyses:

Our first objective was to determine if genetic variants within these polyamine genes were associated with our main outcomes in order to identify potential risk or protective factors involved in suicide attempts, mood disorders, or anxiety disorders.

Individual SNPs:

We first examined association of the 43 non-redundant SNPs with each of the three adult outcomes (mood disorders, anxiety disorders, and suicide attempts) under allelic, dominant, and recessive models. As shown in Table 2, two SAT1 and five SMS SNPs exhibited recessive modes of inheritance regarding the prediction of anxiety disorders, while three SMS SNPs were significant in the dominant model. These two SAT1 SNPs were also significantly associated with mood disorders, where they exhibited a dominant mode of inheritance. Additionally, six SNPs were found to be significantly associated with mood disorders in the CPA subsample in the dominant mode. Finally, three SNPs,

including one in SAT1 and two in OATL1, were significantly associated with suicide attempts. Additionally, the results of the univariate analyses for the CSA subsample indicated no significant links to any of the adult outcomes, ruling out gene X environment interactions involving childhood sexual abuse.

Haplotypes:

The haplotype block analysis revealed that each gene was contained within a single haplotype block. Overall, association tests between haplotypes from each of the genes and the adult outcomes indicated that common haplotypes did not yield any more information than individual SNPs on the association between the studied genes and the phenotypes (not shown).

Multivariate analyses:

Gene-gene and gene-environment interactions:

Given the extensive regulation of polyamine metabolism, we were next interested in determining if genetic factors found within separate genes (Table 2) may interact in conferring risk for psychiatric disorders. We found no significant interactions for either mood disorders or suicide attempts, as both the interaction terms between the two SNPs in mood disorders, and the three significant SNPs associated with suicide attempts, were not significant.

As there was a high correlation between two of the pairs of SNPs that were significantly associated with anxiety disorders (rs10521911 and rs6654100 in SMS: $r^2 = 0.96$; and rs6526342 and rs3764885 in SAT1: $r^2 = 0.86$), we randomly dropped one of the SNPs of each pair from the interaction analyses. We found a significant interaction in the additive model including the main effects and the interaction term of rs3764885 (SAT1) and rs6654100 (SMS) with respect to anxiety disorders ($\chi^2_{(1)} = 6.25$, P = 0.01). As shown in Figure 1, individuals with homo/heterozyosity for the G alleles in rs3764885 (SAT1) and in rs6654100

(SMS) had an elevated risk for anxiety disorders. No other interaction tests were significant for anxiety disorders.

Next, we tested whether the significant SNPs for mood disorders found in the CPA subsample (Table 2) may yield significant gene-environment interactions in the overall sample. We found a significant interaction in the additive model including the main effects and the interaction term of rs3764885 (SAT1) and CPA as well as parental mood disorder ($\chi^2_{(1)} = 5.51$, P = 0.02). As depicted in Figure 2, individuals with homo/heterozyosity for the G allele in rs3764885 (SAT1) and who were CPA victims had elevated rates of mood disorders. This gene-environment interaction was not confounded by evocative gene-environment correlation as indicated by a nonsignificant correlation between the SNP and CPA (P = 0.75). In terms of passive gene-environment correlations, parental mood disorders were weakly associated with CPA (r^2 = 0.081, P = 0.005). No other gene-environment interactions were significant.

Main/mediating effects:

Our final objective was to identify SNPs that are uniquely associated with our main outcomes, along with clinical variables which mediate these associations. The results of the multivariate analyses of the main effects are depicted in Table 3. Overall, the final logistic models explained approximately 18%, 14%, and 11% of the variance in mood disorders, suicide attempts and anxiety disorders, respectively. Using an equivalent of Cook's statistic for logistic regression, outlier diagnostics indicated no cases of extreme influence except, in the analysis of anxiety, the combination formed by the reference values of all variables. However, the latter represented the largest group of subjects. Multicollinearity was unlikely, as the highest correlation among our variables was r^2 = 10.8%. The final estimates obtained with the EM missing-value imputation method had narrower confidence intervals (CI) but were otherwise similar to unimputed estimates. Weighting for gender and family adversity did not change estimates of significance levels or odds ratios (*OR*) (not shown).

Mood disorders:

As mentioned above, rs3764885 and rs6526342 were highly associated, and were the only SNPs that were significant for the prediction of mood disorders in the overall sample. Table 3 depicts the final additive model that included standardized history of psychopathology in addition to the interaction model described above. We found no evidence for a mediating effect of CSA, although there was a relationship between CSA and mood disorders (r = 0.10, P = 0.0005), after controlling for the covariate effects.

Suicide attempts:

One SNP (OATL1, rs11795513) made a statistically significant contribution to suicide attempts (*OR* = 1.80) independently of parental attempt (*OR* = 3.52) and Axis I diagnoses (*OR* = 2.74). We found evidence for evocative gene-environment correlation for this SNP and CSA (r = 0.10, P = 0.0008) as well as a link between CSA and suicide attempts (r = 0.17, P = 0.0001) after controlling for the covariate effects. After entering CSA in the regression model, the link between the OATL1 SNP and suicide attempt dropped (*OR* = 1.66, CI 1.09-2.51**). A Sobel test indicated significant (partial) mediation (Sobel statistic: z = 2.73, P = 0.0064; Goodman statistic z = 2.77, P = 0.0056). Parental suicide attempts were linked to CSA (r = 0.09, P = 0.0021) and CPA (r = 0.07, P = 0.0129). Although CPA was significantly linked to suicide attempts (r = 0.15, P < 0.0001), it was nonsignificantly linked to the OATL1 SNP (r = -0.03, P = 0.3038), making further analyses unnecessary.

Anxiety disorders:

In addition to the interaction effect of SAT1 (rs3764885) and SMS (rs6654100) described above, one SNP (SMS, rs5951676) made a statistically

significant contribution to anxiety disorders (*OR* = 2.05), independently of the nonsignificant effect of parental anxiety disorders (*OR* = 1.05) and the significant effect of history of psychopathology (*OR* = 1.14). Neither CSA nor CPA demonstrated mediating effects for anxiety disorders.

Candidate endophenotypes:

Externalizing (disruptiveness) symptoms were significantly linked to mood disorders (r = 0.16, P < 0.0001), anxiety disorders (r = 0.09, P = 0.0024), and suicide attempts (r = 0.21, P < 0.0001). Disruptiveness was also linked to the OATL1 SNP rs11795513 (r = 0.09, P = 0.0042) that was linked to suicide attempts (see Table 3). After entering disruptiveness in the regression model predicting suicide attempts, the relationship between the OATL1 SNP and the outcome dropped (*OR* = 1.71, CI 1.13-2.59**). A Sobel test indicated significant (partial) mediation (Sobel statistic: z = 2.25, P = 0.0244; Goodman statistic z = 2.30, P = 0.0215). Disruptiveness did not demonstrate mediating effects with respect to mood disorders or anxiety disorders, although it was significantly linked to rs3764885 (SAT1) (r = -0.09, P = 0.0009) which predicted anxiety disorders and mood disorders.

Neither of the internalizing endophenotypes demonstrated mediating effects with respect to any of the three outcomes.

Discussion

In this study, we examined the relationships between genetic variants in polyamine genes and three main outcomes: suicide attempts, mood disorders, and anxiety disorders. In addition, we assessed the gene-gene and gene-environment interactions as well as investigated the involvement of several potential endophenotypes as mediators for our genetic effects. Overall, not only did each of the four genes examined demonstrate significant associations with at least one of

our main outcomes, but each of these outcomes was in turn associated with polyaminergic variants.

Among the genes examined, SAT1 displayed the greatest range of effects, with polymorphisms demonstrating significant associations with each of the three main outcomes. The SAT1 gene encodes the rate-limiting enzyme in polyamine catabolism, and has provided the most compelling evidence for the involvement of dysregulated polyamine catabolism in psychiatric conditions, particularly in completed suicide where it displays widespread decreases in expression across the brain (64;157;204;206). In the present study, three SAT1 SNPs, rs6526342, rs3764885, and rs1894289, demonstrated significant associations with the main outcomes; interestingly however, our results indicate disease-specificity with respect to the risk alleles at these polymorphisms. Indeed, while rs6526342 and rs3764885 were significantly associated with both mood and anxiety disorders, the major alleles at these SNPs represented the risk alleles in anxiety disorders, whereas the minor alleles conferred susceptibility towards mood disorders. Both these SNPs, as well as two other polymorphisms, rs6151267 and rs1960264, are found on the same haplotype block (207), and greater proportions of the major haplotype at this locus have been associated with both completed suicide and anxiety disorders (204;432;433). Interestingly, the association of rs6151267 with completed suicide was only found when comparing individuals with depressive disorders, indicating that the risk conferred by this polymorphism is specific to suicide performed in the context of depression. The results of the present study may thus help explain this effect, as it could be that the ability of this haplotype to confer susceptibility to suicide may have been more apparent in a sample in which the opposite allele is found at higher proportions. At the molecular level, we recently characterized the role of this haplotype in influencing the expression of SAT1, and found that the more common haplotype was associated with lower gene expression in vitro and in the brain (207). Accordingly, it could be speculated that the relationships between these SNPs and mood and anxiety disorders are mediated by the effects of this haplotype on gene expression. Another SAT1 SNP, rs1894289, also possessed disease-specific risk alleles in

terms of its associations with attempted suicide and mood disorders. Given that this SNP is located approximately 3.5 kb downstream of SAT1, it is unlikely that it possesses a functional role, and rather is tagging the functional variant. Compared to the other SAT1 SNPs examined, the linkage disequilibrium between this SNP and others on the haplotype block is much lower: it is therefore quite possible that this SNP is tagging an adjacent haplotype block, and that the associations of this SNP involve distinct molecular mechanisms.

In this study, SMS, which is involved in spermine biosynthesis, demonstrated strong associations with anxiety disorders through both main genetic effects as well as through a gene-gene interaction with SAT1. Although our previous results had indicated that this gene demonstrates altered expression in the hippocampus of suicide completers (157), this represents the first study indicating that it may also be involved in anxiety. We recently characterized genetic and epigenetic factors in the promoter region of SMS and found no indication that promoter variants, DNA methylation, or chromatin modifications played a role in determining the expression of this gene (392). However, as all of our significant associations in the present study map to introns or regions downstream from SMS, these results may indicate that genetic variants outside of the promoter region could influence the expression of this gene. Alternatively, the functional variants responsible for the associations with anxiety may influence later gene regulatory steps, such as mRNA processing or enzymatic activity. Indeed, several rare mutations in SMS, resulting in altered splicing or enzymatic activity, are responsible for Snyder-Robinson syndrome, a form of X-linked mental retardation which manifests with both intellectual and physical symptoms including alterations in brain morphology (459-462). Certainly additional studies will be required to identify the variants which are responsible for our associations as well as the mechanisms by which they exert their effects. In addition to the main genetic effects, we identified a gene-gene interaction between SMS and SAT1 in conferring risk for anxiety disorders. Interestingly, SMS and SAT1 are located in close proximity on the X chromosome, and are at a chromosomal region which was recently found to be associated with suicide (31). This could

indicate the involvement of shared regulatory mechanisms, which may be involved in the interactive effects between SNPs in these two genes.

Unlike the other genes included in this study, the significant findings regarding SMOX and mood disorders were only apparent in the subset of individuals who had experienced physical abuse in childhood. Within this subgroup, three SNPs, two within the third intron and one located 3 kb downstream, demonstrated significant associations with mood disorders. Along with SMS, we recently examined genetic and epigenetic elements in the SMOX promoter, and found little indication that expression of this gene was influenced through these mechanisms (392). Aside from our previous findings regarding altered expression of this gene in suicide completers (64), little is known concerning the involvement of this gene in psychiatric conditions. As such, the mechanisms by which these SNPs may act to confer susceptibility to mood disorders is unknown.

OATL1 yielded the strongest univariate associations in this study, with two SNPs being significantly associated with suicide attempts. Compared to the other genes in this study, the relationship of OATL1 to polyamine metabolism is more distant, and its physiological function or role in polyamine metabolism has not been well characterized. This gene was selected for this study due to its potential involvement in suicide, as it displays altered expression in the hippocampus of suicide completers (157). While the current study appears to support its involvement in suicidal behaviors, the mechanism by which it exerts its pathological effects remains unclear. Indeed, the two SNPs associated with attempted suicide map outside of the gene, with rs11795513 located approximately 2 kb upstream and rs2249583 approximately 13 kb downstream. These SNPs are part of a larger haplotype block on the X chromosome which encompasses several genes (440;463), and as such, it is also possible that these associations may be due to genes located nearby rather than OATL1.

In addition to main genetic effects and gene-gene interactions, this study examined the role of gene-environment interactions in conferring risk towards each of the three main outcomes, as well as controlled for possible confounding

effects of gene-environment correlations. Our results demonstrated that exposure to either physical or sexual abuse was associated with elevated risk for suicide attempts, mood disorders, and anxiety disorders, however our results indicated that sexual abuse did not moderate the genetic associations of the polyamine genes with these outcomes. Our results identified a significant moderating effect of physical abuse on the association between the SAT1 SNP rs3764885 and mood disorders. Particularly interesting is that while higher levels of the minor allele were associated with mood disorders in the univariate analyses, the effects of physical abuse were to increase the risk for mood disorders in individuals carrying the major allele. Finally, we identified several evocative gene-environment correlations with regards to CSA and its involvement in mood disorders and suicide attempts, however the mechanisms involved in these effects are not clear.

Along with analyses investigating the relationship between genetics and the environment, a growing trend in psychiatric research has been to investigate endophenotypes as a means to disentangle the relationships between genetic variables and psychiatric disorders. Whereas our previous findings demonstrated that externalizing and internalizing trajectories measured in childhood were not suitable endophenotypes for mood disorders or suicidal behaviors (438;439), the present study demonstrates that measurement of these symptoms at an older age may be more appropriate for describing suicidal behaviors. The ability of externalizing behaviors to act as an endophenotype to explain the relationship between our genetic findings and suicide attempts agrees well with the strong association between impulsive aggression and suicidal behaviors (464). In addition, these results agree with our previous findings that trajectories based upon disruptiveness demonstrate a stronger relationship with suicide attempts than those derived from measures of anxiousness (439). We found no evidence that our externalizing or internalizing endophenotypes mediate the relationships between our genetic findings and mood or anxiety disorders. This may reflect a lack of statistical power in our sample to detect these effects, or alternatively, these relationships may be mediated by other personality measures.

There are several potential limitations to this study. Although we removed perfectly correlated SNPs from our analyses in order to reduce statistical corrections, it is clear that many of the remaining SNPs were highly correlated. As such, we likely overcorrected for multiple testing in using the Benjamini–Hochsberg procedure and may have missed additional weaker associations. Moreover, as with all studies of this nature, the presence of linkage disequilibrium prevents us from conclusively identifying the functional variants nor the mechanisms by which they exert their effects. Additionally, our statistical power to characterize genetic effects in the smaller CPA and CSA subsamples was limited and may have prevented us from identifying gene-environment interactions. Also, as CSA and CPA were assessed by self-report, recall bias may have affected these measurements. Finally, as we did not correct our interaction analyses for multiple testing, these findings should thus be interpreted with caution, as they are in need of replication.

In conclusion, this study identified a number of genetic and environmental variables associated with attempted suicide, mood disorders, and anxiety disorders. While the precise biological mechanisms by which these genetic variants confer risk to these disorders remains to be determined, these studies have greatly extended our knowledge regarding the involvement of dysregulated polyamine metabolism in the etiology and pathology of psychiatric conditions.

Acknowledgements

We would like to thank the families for their participation in this study. This work was supported by the Canadian Institute of Health Research (CIHR) MOPs 79253 and 53321 as well as an AFSP award to GT. LMF received a scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC).

Figures

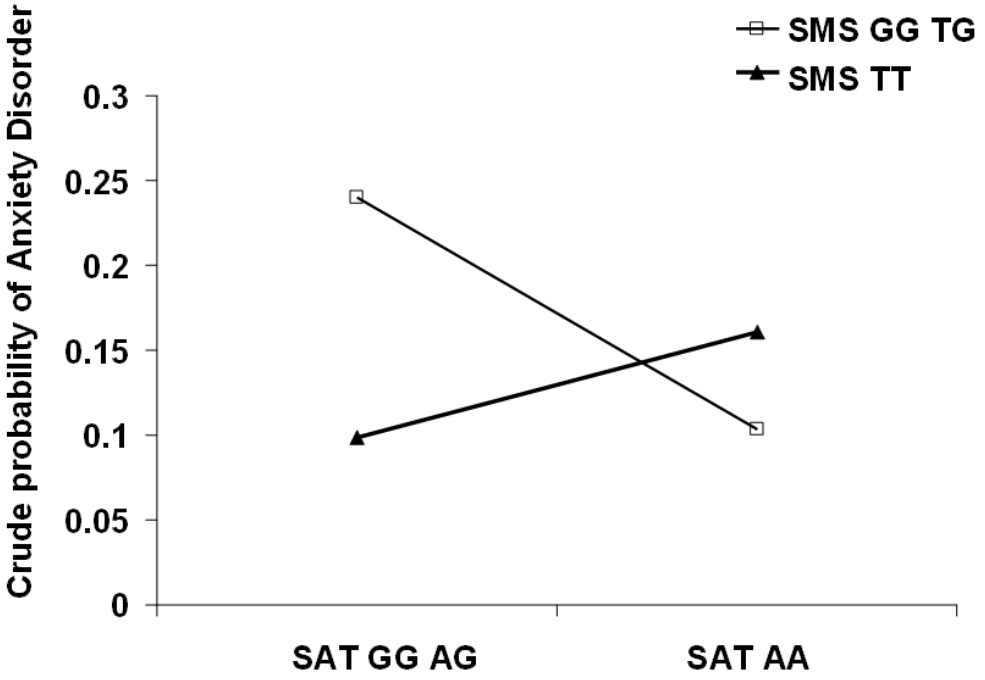


Figure 1: Significant gene-gene interaction for anxiety disorders between SAT1 (rs3764885) and SMS (rs6654100).

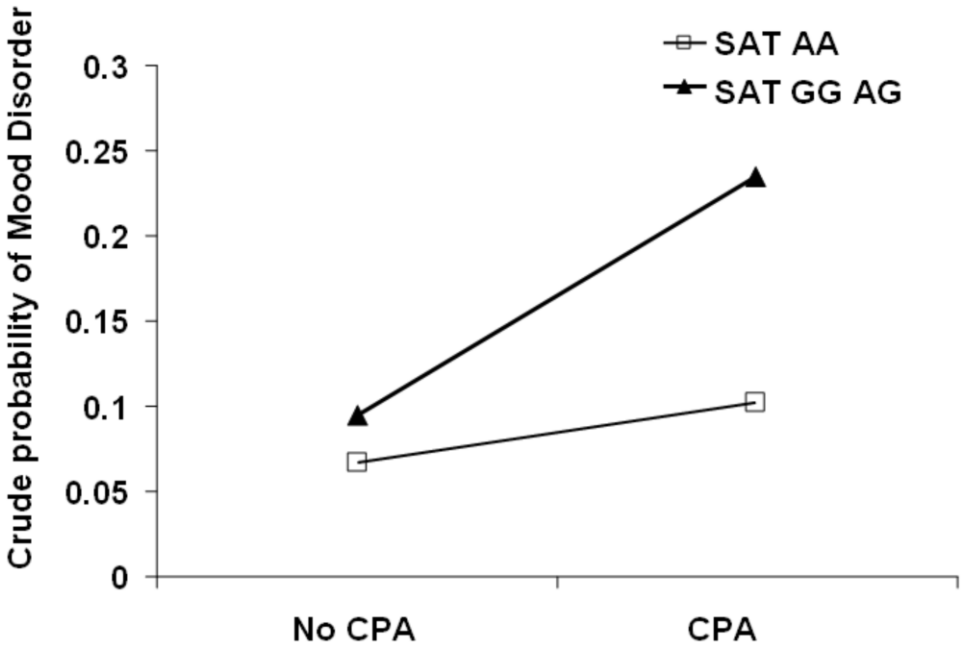


Figure 2: Significant gene-environment interaction between rs3764885 (SAT1) and childhood physical abuse (CPA) for mood disorders.

Tables

Table 1: Characteristics of the total sample and the childhood sexual abuse (CSA) and childhood physical abuse (CPA) subsamples with respect to the main outcomes.

Sample	N	Suicide Attempts	Mood Disorders	Anxiety Disorders
Total	1121	117 (10%)	107 (10%)	239 (21%)
CSA	230	53 (23%) $\chi^2_{(1)} = 48.9$ $P < 0.001$	44 (19%) $\chi^2_{(1)} = 28.1$ $P < 0.001$	77 (33%) $\chi^2_{(1)} = 25.5$ $P < 0.001$
CPA	316	55 (17%) $\chi^2_{(1)} = 22.9$ $P < 0.001$	47 (15%) $\chi^2_{(1)} = 12.9$ $P < 0.001$	88 (28%) $\chi^2_{(1)} = 11.2$ $P < 0.001$

N = number; Percentages refer to the respective sample.

Table 2: Significant univariate associations between polyamine genes and adult outcomes in the total and childhood physical abuse (CPA) samples. False discovery rate (FDR) corrected P-values are shown for each association, and the mode under which the significant effect is observed is indicated. The percentage of affected and unaffected subjects carrying the minor allele is indicated.

Gene	SNP	FDR	Mode	Affected	Unaffected	Sample
Anxiety Disorders						
SAT1 **	rs6526342	0.14	recessive	0.221	0.234	Total
SAT1 **	rs3764885	0.14	recessive	0.217	0.220	Total
SMS *	rs5951672	0.19	dominant	0.071	0.050	Total
SMS *	rs2040357	0.19	recessive	0.333	0.344	Total
SMS *	rs732946	0.19	dominant	0.064	0.042	Total
SMS **	rs5904598	0.14	recessive	0.262	0.310	Total
SMS **	rs10521911	0.16	recessive	0.171	0.214	Total
SMS **	rs5951676	0.14	dominant	0.071	0.046	Total
SMS *	rs5951678	0.19	recessive	0.243	0.260	Total
SMS **	rs6654100	0.14	recessive	0.167	0.210	Total
Mood Disorders						
SAT1 **	rs6526342	0.19	dominant	0.283	0.224	Total
SAT1 **	rs3764885	0.19	dominant	0.262	0.214	Total
SAT1 A**	rs6526342	0.02	dominant	0.305	0.211	CPA
SAT1 A**	rs3764885	0.02	dominant	0.305	0.202	CPA
SAT1 *	rs1894289	0.04	dominant	0.451	0.366	CPA
SMOX A*	rs1622950	0.04	dominant	0.596	0.459	CPA
SMOX A*	rs1765017	0.04	dominant	0.564	0.447	CPA
SMOX A*	rs6084657	0.14	dominant	0.596	0.489	CPA
Suicide Attempts						
OATL1 A**	rs11795513	0.007	dominant	0.400	0.330	Total
OATL1 *	rs2249583	0.03	dominant	0.460	0.411	Total
SAT1 A*	rs1894289	0.07	recessive	0.325	0.406	Total

A allelic test FDR also < 0.20

P-values: *= 0.05-0.01 inclusive,**= 0.0099-0.001 inclusive,*** < 0.001

Table 3: Main and interactive effects for single nucleotide polymorphisms (SNP) and adult outcomes of mood disorders, suicide attempts, and anxiety. Effects and 95% confidence intervals (CI) are shown for each phenotype. Effects for mood disorders are represented by unstandardized regression coefficients, while effects for suicide attempts and anxiety disorders are represented by odds ratios (OR).

Phenotype	Gene	SNP	Effects, CI	Covariates	OR, 95%, CI
Mood disorders					
Model information [D]	SAT1	rs3764885	0.01, -0.02 - 0.05	Parental mood disorder	0.05, 0.01 - 0.10**
Omnibus test: $\chi^2_{(n=1121, 5)} = 87.14^{***}$				History of psychopathology [A]	0.07, 0.05 - 0.10***
Nagelkerke $R^2 = 0.16$				CPA	0.00, -0.03 - 0.04
	SAT1	rs3764885 X CPA	0.10, 0.01 - 0.19*		
Suicide attempts					
Model information	OATL1	rs11795513	1.80, 1.20 - 2.71**	Parental suicide attempt	3.52, 1.84 - 6.72***
Omnibus test: $\chi^2_{(n=1121, 3)} = 80.68^{***}$				History of psychopathology [B]	2.74, 2.12 - 3.55***
Hosmer-Lemeshow test: $\chi^2_{(3)}=2.15, p= 0.54$					
Nagelkerke $R^2 = 0.14$					
Anxiety disorders					
Model information	SMS	rs5951676	1.96, 1.21-3.17**	Parental anxiety disorder	1.49, 0.91-2.45
Omnibus test: $\chi^2_{(n=1121, 6)} = 81.27^{***}$	SAT1	rs3764885	0.39, 0.29-0.77**	History of psychopathology [C]	2.03, 1.67-2.46***
Hosmer-Lemeshow test: $\chi^2_{(4)}=3.76, p= 0.44$	SMS	rs6654100	0.34, 0.17-0.67**		
Nagelkerke $R^2 = 0.11$	SAT1 X SMS	rs3764885 X rs6654100	5.39, 1.11-26.20*		

[A] Anxiety, disruptive and substance abuse disorders, and suicide attempts.
[B] Anxiety, disruptive, mood and substance abuse disorders.
[C] Mood, disruptive and substance abuse disorders, and suicide attempts.
[D] Additive model
P-values: *= 0.05-0.01 inclusive,**= 0.0099-0.001 inclusive,*** <0.001

Supplemental Information

Supplementary Table 1: Single nucleotide polymorphisms genotyped in each gene. Major and minor alleles are shown for each variant.

OATL1	SAT1	SMOX	SMS
rs11795513 (C/T)	rs5925934 (T/C)[B]	rs1741296 (A/G)	rs5951670 (G/A)
rs6608814 (T/C)[B]	rs6526342 (C/A)	rs1741305 (C/T)	rs2238958 (G/A)[A]
rs11795697 (G/A)[B]	rs17286006 (C/T)	rs6076627 (A/T)	rs5951672 (G/A)
rs7880909 (G/A)	rs3764885 (G/A)	rs1741314 (C/T)	rs1007321 (C/G)
rs2293948 (A/G)[B]	rs1894289 (T/G)	rs1741315 (G/A)	rs4824171 (G/A)
rs235834 (T/A)[B]	rs12846646 (G/A)	rs1765014 (A/G)[B]	rs5904497 (T/G)[B]
rs2249583 (G/C)		rs1765016 (G/T)[B]	rs2238962 (G/A)
		rs1741317 (G/A)[B]	rs2238963 (G/A)[B]
		rs1741318 (G/A)	rs2040357 (G/A)
		rs1535225 (G/A)[A]	rs2283723 (A/G)
		rs1622950 (A/G)	rs2238964 (C/T)
		rs1765017 (T/G)	rs12688591 (A/C)
		rs1337281 (G/T)	rs6528074 (G/A)[B]
		rs1741328 (C/T)	rs7059727 (C/T)[B]
		rs6084657 (T/C)	rs3747276 (C/T)
			rs732946 (G/A)
			rs732945 (C/G)[B]
			rs2238966 (C/T)
			rs12013805 (G/T)
			rs7051872 (C/G)[B]
			rs5951490 (A/C)
			rs5951491 (G/A)[B]
			rs5904598 (A/T)
			rs10521911 (C/T)
			rs2239674 (A/G)[B]
			rs1009643 (T/C)
			rs2071136 (T/C)
			rs7887293 (G/T)[B]
			rs7891381 (A/G)[B]
			rs5951676 (T/C)
			rs5904600 (G/A)
			rs5951678 (G/A)
			rs4824217 (T/G)
			rs3891326 (G/A)
			rs6654100 (G/T)

[A] Excluded due to call rate below 90%

[B] Excluded due to perfect (r=1.0) correlation with at least one other SNP in this list.

Supplementary Table 2: Power analyses for association testing with mood disorders in the total sample and the subgroup of individuals exposed to childhood physical abuse (CPA), under the assumption of an interaction between genotype and CPA. Tests were computed under the dominant genetic model by combining carriers (homozygotes and heterozygotes) of the risk allele. Power calculations were assessed for α levels corresponding to false discovery rates (FDR) of 0.2.

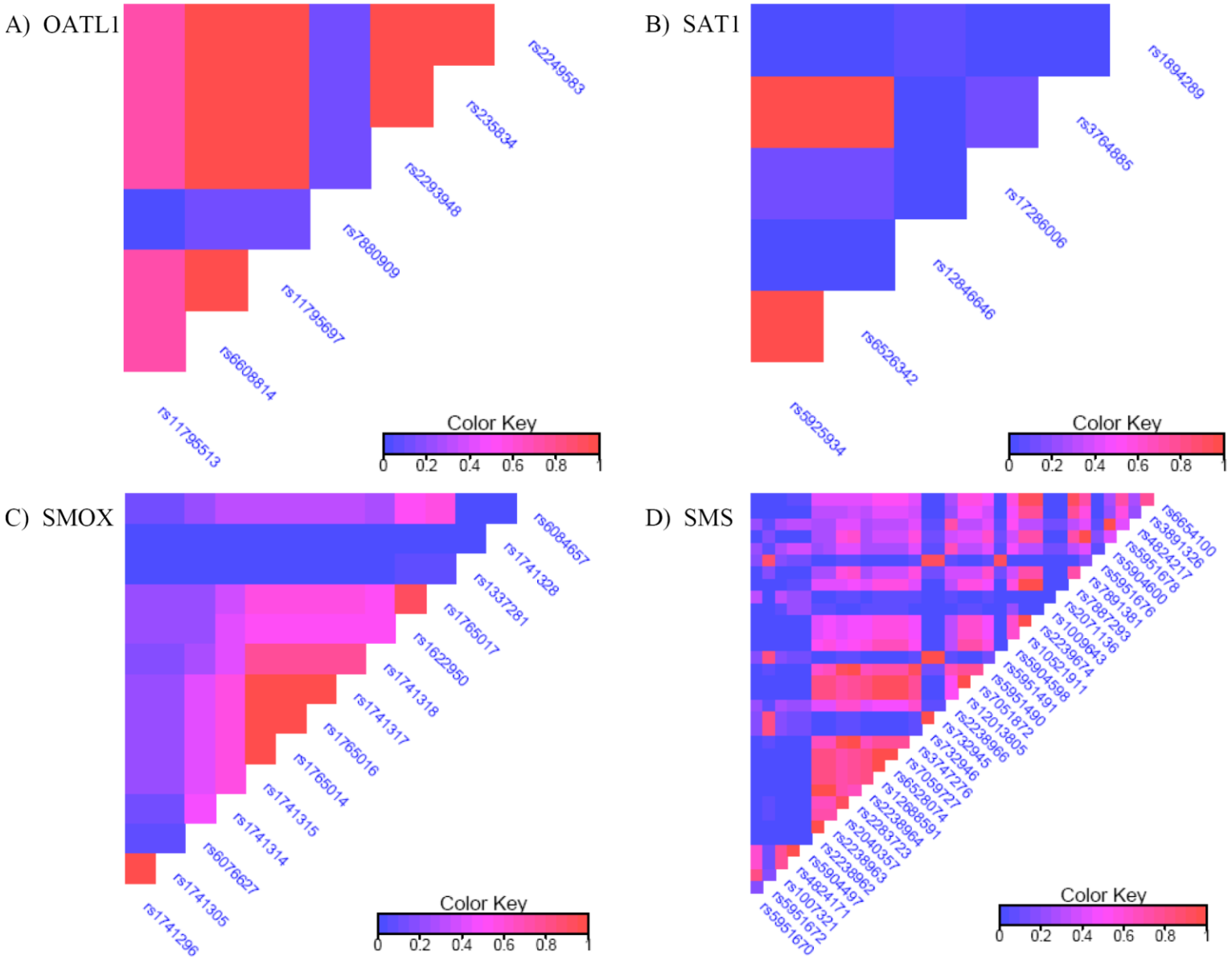
Genotype attributable risk	**Frequency of risk allele (%)**	**Relative risk** *	**Power (%)**	
			CPA (α=0.05)	**Total (α=0.005)**
0.10	0.05	3.84	99	62
	0.10	2.46	86	27
	0.30	1.54	42	5
	0.50	1.37	23	1
0.20	0.10	5.04	100	97
	0.30	2.50	91	57
	0.50	2.02	48	30

* Relative risk of mood disorders between carriers and non-carriers of the risk genotype in the CPA subgroup. The relative risk in the non-CPA subgroup was equal to 1.

Supplementary Table 3: Power analyses for regression analyses of the interaction between a genotype and childhood physical abuse (CPA) on mood disorders. Tests were computed under the dominant genetic model by combining carriers (homozygotes and heterozygotes) of the risk allele. Power calculations were assessed for α = 0.05.

Genotype attributable risk	**Frequency of risk allele (%)**	**Relative risk** *	**Power (%)**	
			Additive	**Logistic**
0.10	0.05	3.84	95	100
	0.10	2.46	76	100
	0.30	1.54	32	83
	0.50	1.37	15	32
0.20	0.10	5.04	100	100
	0.30	2.50	86	100
	0.50	2.02	51	80

* Relative risk of mood disorders between carriers and non-carriers of the risk genotype in the CPA subgroup. The relative risk in the non-CPA subgroup was equal to 1.



Supplementary Figure 1: Linkage disequilibrium (r^2) between polymorphisms within each gene.

2.3 X Chromosome and Suicide

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Abstract

Suicide completion rates are significantly higher in males than females in most societies. While gender differences in suicide rates have been partially explained by environmental and behavioral factors, it is possible that genetic factors, through differential expression between genders, may also help explain gender moderation of suicide risk. This study investigated X-linked genes in suicide completers using a 2-step strategy. We first took advantage of the genetic structure of the French-Canadian population and genotyped 722 unrelated French-Canadian male subjects, of whom 333 were suicide completers and 389 were non-suicide controls, using a panel of 37 microsatellite markers spanning the entire X chromosome. Nine haplotype windows and several individual markers were associated with suicide. Significant results aggregated primarily in two regions, one in the long arm and another in the short arm of chromosome X, limited by markers DXS8051 and DXS8102, and DXS1001 and DXS8106, respectively. The second stage of the study investigated differential brain expression of genes mapping to associated regions in Brodmann areas 8/9, 11, 44, and 46, in an independent sample of suicide completers and controls. Six genes within these regions, ARHGAP6, AP1S2, GPM6B, RPS6KA3, SAT1, and THOC2, were found to be differentially expressed in suicide completers.

Introduction

Suicide is a major public health problem and is one of the leading causes of death in Western countries (465). Although a history of psychiatric disorders is detected in the majority of suicide completers, transmission of psychopathology appears to be independent of suicide (317), supporting the concept that suicidal behavior is a distinct phenotype. There is a significant amount of evidence to support a genetic component to suicidal behavior, as evidenced by twin (466), adoption (25;26) and family studies (317).

A few linkage studies have been conducted investigating loci segregating with suicide completion, suicide attempt, and suicidal ideation. In a sample of alcohol-dependent and control subjects, regions in chromosomes 1, 2, and 3 were linked with suicide attempts (chromosome 2) or suicidality (chromosomes 1 and 3) (28). Using probands with recurrent early-onset major depressive disorder, a number of chromosomal regions were linked with suicide attempts, including 2p, 5q, 6q, 8p, 11q, and Xq (29). In this study, the regions with the two highest LOD scores were 8p22-p21 and Xq25-26.1, in affected relative pairs with depression spectrum disorder, and recurrent early-onset major depressive disorder, respectively. A third study, performed in a large bipolar pedigree, identified regions linked with either suicide completions (2q, 4p, 6q) or suicide attempts (10q) (30).

Suicidal behavior has remarkably different distribution between genders (reviewed in (467)). These differences have been attributed to a number of different factors, including the use of higher lethality methods by male suicide victims, as well as differences in comorbid Axis I and Axis II diagnoses (3;468;469). In addition, both risk and protective factors vary between genders (470). It is possible that genetic factors, through differential expression between the genders, may also help explain gender moderation of suicide risk. Exploring this rationale, candidate gene studies have identified a number of genes with gender-specific effects related to suicidal behaviors, including catechol-O-methyltransferase (COMT) (125;471), the serotonin transporter (5-HTT) (472),

cholecystokinin (CCK) (473), and monoamine oxidase A (MAO-A) (474). MAO-A is particularly interesting as it is located on the X chromosome, and genetic variants have been associated with impulsivity, aggression, and antisocial behavior (475;476), each of which are risk factors for suicide.

As males are hemizygous for genes on the X chromosome, loci that map to this chromosome are an important source of potential variability, and are thus of interest to the investigation of genetic factors that could moderate gender differences in suicidal behavior. In this study, we chose to perform a two-stage case-control study investigating X-linked markers in a sample of suicide completers and controls. The first stage comprised an association study using markers spanning the X-chromosome. This stage took advantage of the genetic structure of the French-Canadian population, which is a young (12 generations) population that remained isolated until very recently because of cultural and religious reasons, leading to a founder effect confirmed by many studies. For instance, among individuals affected by oculopharyngeal muscular dystrophy (OPMD), a disease caused by a gene on chromosome 14, unrelated French Canadian OPMD patients shared 75% of alleles for a 5-cM interval (477;478). Similar results were observed for other diseases (479). As approximately 70% of the gene pool of the present French-Canadian population derives from an initial group of 3380 founders, it is reasonable to hypothesize that there is reduced variability in genetic factors that may be etiologically related to suicide among French-Canadians. Taken together, this means that by testing French-Canadian suicide completers, it is possible to detect ancestral predisposing variants by typing markers within an average interval of 5cM.

The second stage consisted of investigating genes mapping to associated regions by studying their differential pattern of expression in the brain in a sample of suicide completers and controls.

Methods

Subjects:

Genetic Association Study:

We investigated a total of 722 unrelated French-Canadian male subjects, as shown in Table 1A. Cases for this study were 333 individuals who died by suicide (S), as assessed by the Quebec Coroner's office. We also included 389 subjects who did not die by suicide (NS), which comprised both living (311) and deceased (78) individuals. These subjects were recruited from outpatient clinics or from the Quebec general population. Psychiatric diagnoses were assessed using the Structured Clinical Interview for DSM-IV Axis I (SCID-I) (480) or the Diagnostic Interview Schedule using DSM-III-R criteria (481). When possible, interviewees were the subjects themselves; in other cases, the psychological autopsy method was used with the informant best acquainted with the subject. As major depressive disorder is the most common Axis I disorder among suicide completers (3), we selected our non-suicide control group to include a high percentage of subjects with depressive disorders. We classified subjects as being among the depressive disorders group when they had either a present or lifetime diagnosis of major depression, dysthymic disorder, or depressive disorders not otherwise specified (NOS). Informed consent was obtained from either the subject or their next-of-kin, as appropriate.

Gene Expression Study:

An independent sample of French-Canadian male subjects was used to assess gene expression. Brain samples from Brodmann area (BA) 8/9 (dorsolateral prefrontal cortex (DLPFC)), BA 11 (orbital cortex), BA 44 (inferior frontal gyrus), and BA 46 (DLPFC) were obtained from the Quebec Suicide Brain Bank (QSBB) (www.douglasrecherche.qc.ca/suicide). These regions were selected due

to evidence for their involvement in suicide and major depression (482). Brain tissues were processed and dissected at 4°C, and snap-frozen in liquid nitrogen before storage at -80°C, following standard procedures (397). All subjects collected by the QSBB died suddenly without a prolonged agonal period. Brain tissues were dissected in accordance with standard neuroanatomical definitions (398). All subjects were characterized by the psychological autopsy method using structured diagnostic methods eliciting Axis I diagnoses according to DSM-IV criteria, as detailed elsewhere (396). Diagnostic information is shown in Table 1B. The sample consisted of 14 depressed suicide completers who died during an episode of major depression, 8 suicide completers with no history of major depression, and 13 controls with no history of suicidal behavior. These subjects had an average age of 35.6 with an average post-mortem interval of 25 hours. There were no significant differences between the groups for either of these variables. This study was approved by our local institutional review board.

Genotyping:

Genomic DNA was extracted from blood or saliva using standard procedures (483). A total of 37 microsatellite markers were genotyped, with an average inter-marker distance of 5.7 centimorgans (cM) (0.0-11.1 cM). Markers were obtained from the ABI Prism Linkage Mapping Set 2.5 and were analyzed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Genotypes were assessed using GeneMapper 3.7 (Applied Biosystems).

Statistical Analysis:

The likelihood ratio chi-square test statistic was used to identify differences in allele or haplotype frequencies between cases and controls. These association tests were computed using the UNPHASED (version 3.0) suite of software for case-control data (455) and were performed for both single markers as well as marker pairs using a sliding window approach. In order to control for the presence

of depressive disorders, these analyses were also performed with depressive disorders (present, absent, unknown) added as a covariate. As the number of subjects with other Axis I diagnoses was small, their effects were not assessed. Alleles with frequencies less than 0.05 in the entire sample were pooled in order to reduce the effects of rare alleles. All tests of significance were two-sided at the level of $P \leq 0.05$. Corrections for multiple testing were not performed. Linkage disequilibrium (LD) was assessed using the LDMAX algorithm of the Graphical Overview of Linkage Disequilibrium (GOLD) program (484).

Microarray Analysis:

RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA samples used for analysis possessed A260/A280 > 1.9 and 28S/18S rRNA peak height ratios > 1.6. Samples were analysed using the Human Genome U133 A/B set, which comprises 2 GeneChip arrays with 45000 probesets, representing approximately 33000 human genes (http://www.affymetrix.com).

GeneChip analysis was performed with Microarray Analysis Suite version 5.0 (MAS 5), Data Mining Tool 2.0. All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 100. RNA quality indicators used to pre-filter samples prior to analysis included β-actin 5'/3' ratio, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'/3' ratio, RawQ (noise), scale factor and percent of "present" calls per array. Outlier subjects were excluded in regions where they did not pass quality standards, such that different numbers of subjects were included in the final analysis across the four regions (for BA 8/9: 7 C, 5 NDS, and 11 DS; for BA 11: 7 C, 5 NDS, and 11 DS; for BA 44: 12 C, 6 NDS, and 13 DS; and for BA 46: 7 C, 7 NDS, and 12 DS).

Data Analysis:

Gene expression data was analyzed using Genesis (GeneLogic Inc., Gaithersburg, MD) and AVADIS software (Strand Genomics, Redwood City, CA). Gene expression values were log2-transformed, then Student's *t*-tests were performed for each probeset for contrasts of DS vs NDS, DS vs C, and NDS vs C. Genes selected for further analysis had to display, in at least two brain regions, a P-value – fold change (FC) combination of $P \leq 0.01$ and FC of at least ±1.3. Additionally, genes where all significant probesets had a present call of less than 75% were not included.

RT-PCR Validation:

Total RNA for each brain region was extracted from frozen tissue using an RNeasy Lipid Tissue Mini Kit (Qiagen, Canada). RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies), and only samples with an RNA integrity number (RIN) > 5 were included. cDNA synthesis was performed using oligo(dT)-priming (Invitrogen, The Netherlands).

The PCR reactions used 100 ng of cDNA template in a 20 µl reaction volume, and were run on 2% agarose gels. FC and P-values between groups were evaluated using relative quantitation with GAPDH as an endogenous control. All reactions were carried out in triplicate. Semi-quantitative results were analyzed with GeneTools v3.06 (SynGene, Cambridge, England), with automatic computation of gene and control band densitometry. One-tailed Student's *t*-tests were performed (SPSS, v15.0).

Results

A total of 722 subjects were genotyped at thirty-seven microsatellite markers in order to identify loci associated with completed suicide. There was an average genetic distance between contiguous markers of 5.7 cM. Alleles with frequencies

of less than 0.05 were pooled into a single allele. Prior to pooling infrequent alleles, the average marker heterozygosity was 0.71.

Chi-square analyses revealed five markers that were significantly associated with completed suicide. In order to identify haplotypes associated with suicide completion, a sliding window approach was used for each pair of markers. Nine haplotype windows were found to be associated with suicide. Results from both sets of analysis are shown in Figure 1A. For both analyses, the most significant findings were obtained with markers DXS1001 and DXS8106, (P=0.001 and 0.0003, respectively), both of which survive correction for multiple testing. Seven of the marker pairs encompassed single markers which were significant or near-significant, of which the pair DXS1227-DXS8106 survives correction for multiple testing.

Score tests were used to assess the effects of individual alleles for each significant marker and marker pair. These results are depicted in Tables 2A and B. For marker DXS984, the most significant allele represents the pooled alleles. Prior to pooling at this locus, the P-value was 0.034, and the strongest associations were observed with alleles 176 and 188, although these did not reach nominal statistical significance (P-values of 0.078 and 0.057, respectively).

Both protective and susceptibility alleles were observed at the majority of markers and marker pairs. For both DXS1001 and DXS8106, the most significantly associated allele at each locus conferred a susceptibility to suicide. The most strongly protective allele was also found at DXS1001. Among marker pairs, the strongest association was found with DXS1227-DXS8106, with the most significant haplotype being found with a frequency eight times higher among suicide completers.

As risk factors for suicide completion may differ among Axis I disorders, we repeated the analyses while controlling for a history of depressive disorders, the most common diagnostic category associated with suicide (3). When this was done, marker DXS8051, and pairs DXS1106-DXS8088 and DXS8091-DXS8069 lost significance (P=0.070, 0.066, and 0.082, respectively). In order to further characterize this effect, and to identify regions that may be specific to suicide

completions in the context of depressive disorders, these analyses were performed using only depressed suicides and depressed controls (N=302). These results are depicted in Figure 1B and Tables 3A and B. While a number of regions are identical or adjacent (DXS1224 and the pair DXS1060-DXS8051) to those found in the entire sample, an additional region was found with marker DXS8102 and nearby marker pairs DXS1214-DXS8102 and DXS8090-DXS1068. The intervening pair (DXS8102-DXS8090) also displayed a near-significant association (P=0.092). Additionally, a number of markers found to be associated with suicide in the entire sample were no longer significant in the subset of subjects with depressive disorders (DXS8051, DXS1047, and DXS984). The strongest associations were found with DXS1224, DXS8106, and DSX1224-DXS8019, each of which survive corrections for multiple testing.

In order to further characterize the regions showing evidence of involvement in suicide, and to confirm that our use of uncorrected statistical tests identified truly associated regions, we investigated the differential expression of genes mapping to these regions by means of microarray analyses in brain tissues obtained from an independent sample of French-Canadian males who died by suicide as compared to French-Canadian males who died suddenly by other causes. Analyses were performed in four cortical brain regions, BA 8/9, 11, 44, and 46, which are believed to be involved in the neurobiology of suicide and major depressive disorder (482). Based on results from both the entire sample and the subset depressed subjects, we focused on two X-chromosomal regions: between DXS8051 (Xp22.2) and DXS8102 (Xp11.4) (34.1 cM), and between DXS1001 (Xq24) and DXS8106 (Xq27.3) (34.2 cM). These two regions contain 131 and 121 annotated genes, respectively. In order to minimize false positive findings, we focused on genes which were differentially expressed within at least one of the comparisons in at least two brain regions. A total of eight genes fulfilled these criteria and are shown in Table 4.

We subsequently proceeded to the validation of these eight differentially expressed genes. Our group has been working on SAT1 because of results that independently pointed to this gene as important in suicide/major depressive

disorder, and the validation and investigation of its differential expression in these regions has previously been performed by our group (204;206). Of the remaining genes, Rho GTPase activating protein 6 (ARHGAP6), glycoprotein M6B (GPM6B), adaptor-related protein complex 1 sigma 2 subunit (AP1S2), ribosomal protein S6 kinase 90kDa polypeptide 3 (RPS6KA3), and THO complex 2 (THOC2) validated for several of the comparisons that were significant from microarray analysis. The expression of ARHGAP6 in BA44, and AP1S2 in BA8/9 were significantly different for the comparisons of interest, but the FCs were in the opposite direction than those observed from microarray experiments. The reason for this is not clear.

Both stromal antigen 2 (STAG2) and X-linked RNA binding motif protein (RBMX) failed to validate. When examined further, it appears that the RBMX probeset 225310_at is located within an exonic region which has only been observed in a processed transcript (RBMX-004, OTTHUMT00000058510). As the primers we used for validation do not bind to this transcript, this may partially explain the failure of this gene to validate.

Discussion

We conducted a two-stage investigation of X chromosome loci in suicide completion using a sample of male French-Canadian subjects. We first identified a number of chromosomal regions which displayed association to suicide, and subsequently studied patterns of differential expression in the brain for several genes that map within these regions.

This study identified five markers, DXS8051, DXS1001, DXS1047, DXS984, and DXS8106 which were significantly associated with completed suicide in the entire sample. The two strongest associations were obtained with DXS1001 and DXS8106, which are localized to Xq24 and Xq27.3, respectively. Nine marker pairs were also found to be significant, of which the strongest signals were seen in Xp22, Xq24, and Xq27. When suicide was examined in the context of depression, several other markers were found to be significant, producing a

peak spanning a 10MB region from Xp21.2 to Xp11.4. Additionally, several markers which were significant in the entire sample were not significant in the depressed subgroup, which may indicate that these peaks were related to other comorbid conditions. Alternatively, this may simply be a consequence of reduced power in the smaller sample. Overall, markers DXS1001, and DXS8106, as well as pairs DXS1224-DXS8019, DXS1001-DXS8009, DXS1227-DXS8106, and DXS8106-DXS8043, were significant in both analyses.

The identification of DXS1047, found in Xq25, as being associated with suicide is in partial agreement with results from a previous linkage study which showed significant findings for this marker in the context of suicide among families with recurrent, early onset, depressive disorders (29). It is thus interesting that in our study this marker was not significant in the depressed sample. DXS1047 has also been associated with bipolar disorder (485;486) and both this marker and DXS1001 have been associated with autism (487;488). In addition, DXS1001 is closely linked with the glutamate receptor subunit 3 gene (GRIA3) at Xq25, which has been associated with a number of psychiatric conditions including bipolar disorder, schizophrenia, and citalopram treatment-emergent suicidal ideation (139-142).

In the second stage of our study, we focused our efforts on identifying genes within our associated regions that demonstrate differential expression in the brains of suicide completers. Using microarray expression data from BA 8/9, 11, 44, and 46, six genes were identified which were differentially expressed in suicide completers in at least two brain regions.

One of the genes identified in this study, SAT1, was previously identified by our group to be significantly downregulated in a number of different brain regions in suicide completers, and identified a promoter single nucleotide polymorphism (SNP), rs6926342, which was associated with suicide completion and expression of SAT1 in the brain (64;157;204;206). This gene is involved in polyamine catabolism, and the polyamine system has been implicated in a number of psychiatric conditions, including suicide (203).

The remaining five genes also demonstrated decreased expression in suicide completers. Interestingly, RPS6KA3 displayed region-specific alterations in expression, with increased expression in depressed compared to non-depressed suicides in BA 8/9, and decreased expression in BA 44. This gene, which is involved in cognitive function and brain development (489;490), has been implicated in several forms of X-linked mental retardation (XLMR) resulting from both duplications and mutations (490-494). This suggests that dysregulation in the expression of this gene in either direction can have profound effects, and may explain our findings of both increased and decreased levels. AP1S2 has also been implicated in XLMR (495-497), and behavioral effects resulting from mutations are theorized to be due to brain-specific defects in intracellular protein trafficking (497). Dysregulation of ARHGAP6 and GPM6B in suicide completers is also of interest as both of these genes are involved in neuronal function. Indeed, ARHGAP6 has been found to be upregulated during neurogenesis (498), while GPM6B is involved in myelination and is upregulated in the brain during terminal neuronal differentiation and myelination (499-501). Our findings of decreased expression of these two genes may indicate alterations in neuronal functioning or in numbers of the neurons themselves.

One of the aims of this study was to identify genetic factors that may explain the gender moderation of suicide risk. In this light, it is of interest that two of the genes displaying downregulation in our suicide completers, GPM6B and AP1S2, have also been shown to escape X inactivation, and demonstrate increased expression, in females (502-504). This may thus indicate that elevated levels of these genes may be protective against suicide in females. Also, as males are hemizygous for the majority of genes on the X chromosome, they are also more susceptible to the effects of genetic variations, which may alter the function or expression of genes. Indeed, as mentioned above, a promoter variant influencing the expression of SAT1 has already been identified (204), and it could be speculated that heterozygous females possessing the risk allele may be less affected than hemizygous males.

Although the majority of genes on the X chromosome do not escape inactivation in females (502), and therefore, may not be responsible for gender differences in suicide rates, the X chromosome remains of significant interest for the investigation of genes related to psychiatric phenotypes. It has been noted that the X chromosome possesses a disproportionately high number of genes associated with mental function, including intelligence, social-cognition, and emotional regulation (505). Therefore it is not surprising that numerous studies have identified X chromosomal loci linked and/or associated with mental disorders, including bipolar disorder (485;486;506;507), schizophrenia (508;509), major depression (510), autism (511;512), and suicide (29). Our findings of multiple X-linked loci associated with suicide provide additional evidence suggesting that genes mapping to the X chromosome may be important in complex behavioral phenotypes.

Our use of a sample comprised entirely of French-Canadian subjects allowed us to use a genetic strategy to detect suicide-related loci that would not have been possible in samples from admixed populations, where linkage disequilibrium with predisposing genes would have been much lower. The French-Canadian population was established relatively recently and the majority of individuals are descendants of approximately 7000 individuals who immigrated to Quebec from France prior to 1760, and who, until recently, have remained relatively isolated from other populations (479). The existence of a founder effect in this population has been well documented (450;477-479), and the high level of genetic homogeneity has allowed the detection of predisposing ancestral variants by typing markers within relatively small intervals. The power to detect these predisposing variants decreases with increased genetic variability resulting from the introduction of other predisposing variants within a single gene and in other predisposing genes. However, our use of a relatively large sample and a complementary design to follow up on positive findings circumvented these problems.

There are a number of limitations to this study. As this study was performed exclusively in French-Canadians, it is possible that the associations obtained in

this sample may not be found in other populations. Additionally, psychiatric information was not available for all subjects, which limited our ability to examine suicide in the context of other Axis I disorders. Thirdly, we chose not to correct our association tests for multiple testing, which may have increased the likelihood of false positive findings. However, our findings of differentially expressed genes within these chromosomal regions served as a confirmation of the association of these loci with suicide completion in spite of our lack of correction. Finally, it is possible that the genes identified in this study are not those responsible for the association peaks, and that the causal genes remain to be identified. Indeed, it is likely that additional genes are involved as we only validated one gene, THOC2, in the Xq region examined, despite this region displaying markers with stronger associations to suicide.

In summary, we present results demonstrating a number of regions on the X chromosome which are associated with suicide completion, and identified six genes in these regions which display differential expression in suicide completers. These results highlight the importance of the X chromosome in suicidal behavior, and expand our knowledge of pathways involved in this complex trait.

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Figures

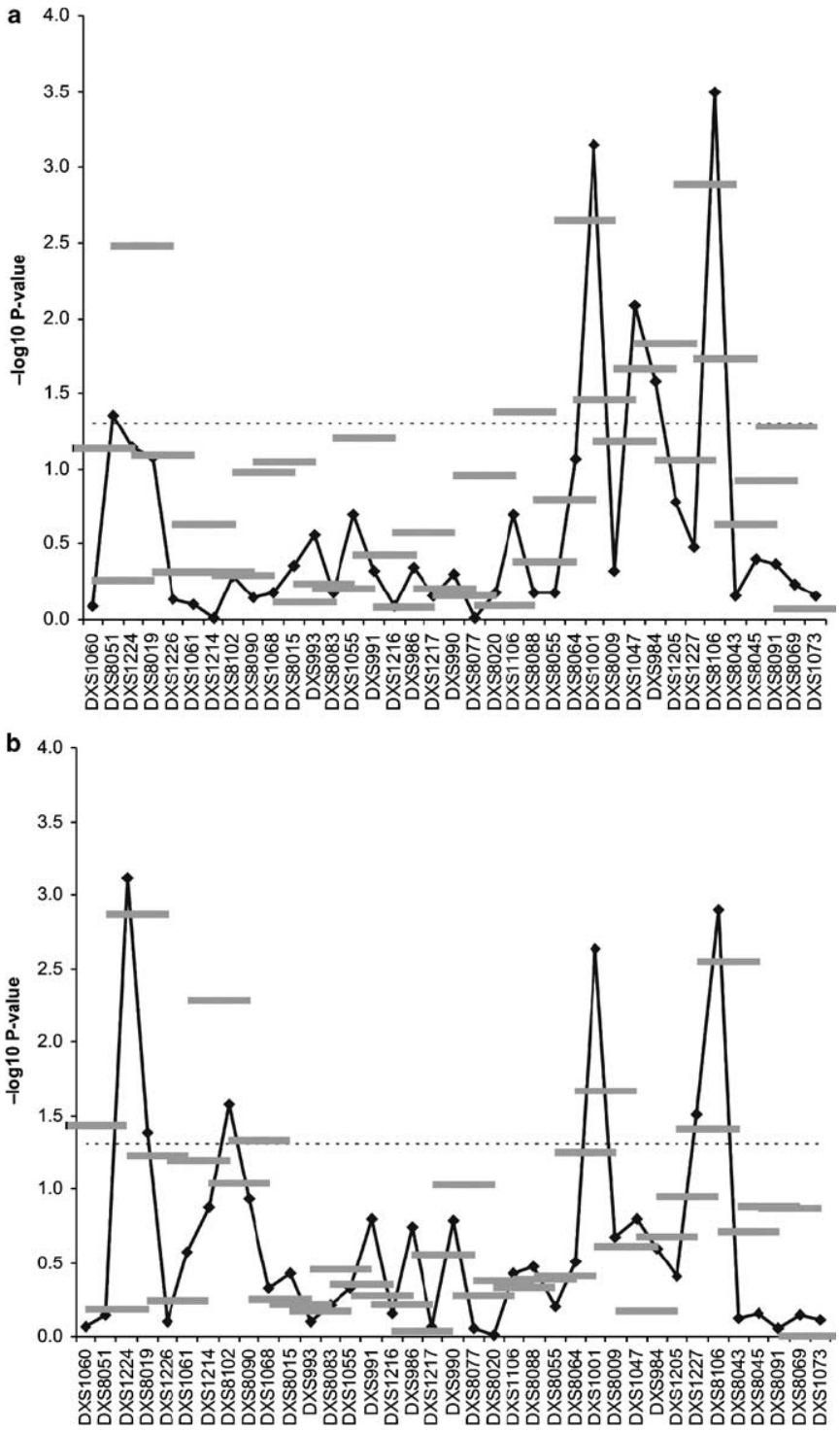


Figure 1: Single and two-marker association tests in (a) suicide and non-suicide subjects in the entire sample, and (b) suicide and non-suicide subjects in the depressed subjects. The results of likelihood ratio chi-square tests are plotted as –log10 P-values. Black lines represent tests for single markers. Grey lines represent the results for marker pairs. The dotted line indicates P=0.05.

Tables

Table 1: Characteristics of case and control subjects used for A) genotyping study and B) gene expression study. Percentages in A) are indicated in brackets; percentages for specific Axis I disorders were calculated within only the assessed individuals. C – control; DS - depressed suicide completers; NDS - non-depressed suicide completers; NS – non-suicide controls; S – suicide completers

A)

	S (N=333)	**NS (N=389)**
Mean age	41.2 ± 13.8	39.3 ± 13.5
Assessed for Axis I disorders	209 (63)	226 (58)
Depressive disorders	121 (58)	181 (80)
Bipolar disorder	20 (10)	2 (1)
Schizophrenia	12 (6)	3 (1)
Panic disorder	9 (4)	14 (6)
Generalized anxiety disorder	5 (2)	14 (6)

B)

	DS (N = 14)	**NDS (N = 8)**	**C (N = 13)**
Mean age	34.9 ± 12.5	34.8 ± 9.3	36.8 ± 10.4
Depressive disorders	14	0	1
Alcohol/substance dependence	6	4	3

Table 2: Significant alleles associated with completed suicide for A) markers and B) two-marker haplotypes. NS – non-suicide; PA – pooled alleles; S – suicide

A)

Marker	P	df	Alleles	S (%)	NS (%)	Score test P
DXS8051	0.044	7	116	19.3	11.9	0.018
			118	7.3	14.6	0.009
DXS1001	0.001	5	197	38.0	23.2	0.0001
			201	14.1	22.9	0.007
DXS1047	0.008	7	158	6.2	13.4	0.008
			160	39.5	30.2	0.024
			168	4.3	9.3	0.030
DXS984	0.026	4	159, 176, 178, 180, 186, 188, 190	20.2	11.7	0.007
			172	44.4	54.5	0.018
DXS8106	0.0003	4	283	10.3	2.2	0.0001

B)

Marker Pair	P	Haplotypes	S (%)	NS (%)	Score test P
DXS1224-DXS8019	0.003	168-163	0.5	5.8	0.002
		170-161	1.4	5.0	0.032
		162-165	14.8	8.6	0.032
		168-167	3.3	0.7	0.033
		PA-161	1.4	0.0	0.045
		170-169	0.0	1.8	0.051
DXS1106-DXS8088	0.042	128-265	5.0	11.2	0.024
		126-265	3.3	0.8	0.048
DXS8064-DXS1001	0.002	217-201	6.1	15.3	0.002
		215-197	5.6	1.4	0.007
		215-203	3.6	0.7	0.019
DXS1001-DXS8009	0.035	197-263	24.4	14.7	0.005
		201-255	0.5	3.5	0.024
		201-263	7.2	13.1	0.033
		197-255	5.7	2.2	0.035
DXS1047-DXS984	0.022	160-PA	11.7	4.0	0.002
		168-172	1.2	7.2	0.004
		158-172	1.8	6.1	0.029
		162-172	2.9	7.2	0.054
DXS984-DXS1205	0.015	172-188	14.4	27.5	0.001
		PA-196	1.5	0.0	0.042
DXS1227-DXS8106	0.001	80-283	8.8	1.1	0.00005
		88-PA	0.9	6.3	0.002
		PA-271	7.9	3.7	0.047
		82-271	1.4	4.5	0.052
DXS8106-DXS8043	0.019	283-159	5.3	0.4	0.001
DXS8091-DXS8069	0.052	86-149	3.5	0.0	0.007

Table 3: Significant alleles associated with completed suicide in depressed individuals for A) markers and B) two-marker haplotypes. NS – non-suicide; PA – pooled alleles; S – suicide

A)

Marker	P	df	Alleles	S (%)	NS (%)	Score test P
DXS1224	0.001	3	152, 158, 160, 164, 166, 168, 172, 176	16.7	2.6	0.0001
DXS8019	0.042	7	165	20.0	10.6	0.045
DXS8102	0.027	4	98	80.4	64.3	0.006
			102	5.4	16.8	0.008
DXS1001	0.002	5	197	40.0	20.7	0.001
			199	9.4	21.3	0.018
DXS1227	0.031	4	80	67.7	54.3	0.031
			88	13.1	24.9	0.021
DXS8106	0.001	4	283	12.8	1.4	0.0003

B)

Marker Pair	P	Haplotypes	S (%)	NS (%)	Score test P
DXS1060-DXS8051	0.038	246-122	0.05	0	0.002
		250-116	0.07	0.02	0.040
DXS1224-DXS8019	0.001	PA-161	0.03	0	0.047
		168-163	0	0.08	0.015
		168-167	0.07	0	0.002
		170-161	0	0.06	0.039
DXS1214-DXS8102	0.005	PA-98	0.06	0.01	0.011
		290-102	0	0.05	0.050
DXS8090-DXS1068	0.047	PA-260	0.09	0.09	0.022
		300-PA	0.05	0.01	0.029
DXS1001-DXS8009	0.022	197-261	0.08	0.02	0.021
		197-263	0.26	0.14	0.026
		201-255	0	0.05	0.050
DXS1227-DXS8106	0.04	80-283	0.11	0.01	0.0003
DXS8106-DXS8043	0.003	PA-147	0	0.08	0.043
		270-163	0	0.10	0.021
		283-147	0.04	0	0.016
		283-159	0.08	0	0.0006

Table 4: Genes differentially expressed in at least two regions in brains of suicide completers in BA 8/9, 11, 44, and 46. BA – Brodmann Area; C – control; DS – depressed suicide completer; FC – fold change; NDS – non-depressed suicide completer; PCR – semi-quantitative PCR; S – suicide completer

				Microarray		PCR	
Gene	**BA**	**Probeset**	**Comparison**	**P**	**FC**	**P[1]**	**FC**
ARHGAP6	11	206167_s_at	DS-C	0.01	-1.30	0.05	-1.06
	44	208085_s_at	DS-NDS	0.01	-1.40	0.005	1.11
GPM6B	8/9	209167_at	DS-C	0.01	-1.80	0.03	-1.18
		209168_at	DS-NDS	0.01	-1.30	0.25	-1.06
	44	209167_at	DS-C	0.01	-1.40	0.0009	-1.40
	46	209169_at	NDS-C	0.00008	1.30	0.21	1.21
		209170_s_at		0.01	1.30		
		209167_at	DS-NDS	0.004	-1.70	0.03	-1.45
		209169_at		0.001	-1.30		
		209170_s_at		0.001	-1.60		
AP1S2	8/9	203300_x_at	NDS-C	0.01	-1.60	0.15	1.05
		243745_at	DS-NDS	0.004	3.70	0.004	-1.15
	11	203300_x_at	NDS-C	0.01	-1.50	0.05	-1.32
	44	243745_at	DS-C	0.01	-2.40	0.05	-1.02
RPS6KA3	8/9	203843_at	DS-NDS	0.01	1.30	0.04	1.02
	44	203843_at	DS-NDS	0.002	-1.40	0.03	-1.10
SAT1	11	210592_s_at	DS-C	0.01	-1.50	0.02 [2]	-1.85 [2]
	44	203455_s_at	DS-C	0.004	-1.40	0.004 [2]	-1.37 [2]
	46	203455_s_at	DS-NDS	0.01	-1.50	0.20 [3]	-1.23 [3]
THOC2	8/9	222122_s_at	NDS-C	0.0004	-1.40	0.09	1.02
		212994_at	DS-NDS	0.01	-1.30	0.05	-1.03
	46	222122_s_at	DS-C	0.001	-1.50	0.03	-1.02
STAG2	8/9	207983_s_at	NDS-C	0.01	-1.60	0.31	1.05
	46	209022_at	NDS-C	0.01	1.30	0.33	-1.05
		209022_at	DS-NDS	0.0002	-1.50	0.12	-1.08
		209023_s_at		0.01	-1.40		
RBMX	8/9	213762_x_at	NDS-C	0.0001	1.40	0.23	-1.05
		213762_x_at	DS-NDS	0.002	-1.50	0.16	-1.06
		225310_at		0.008	-1.60		
	11	225310_at	DS-C	0.01	-1.30	0.39	-1.01

[1] One-tailed

[2] Previously reported in (206)

[3] Previously reported in (64)

Supplemental Information:

Supplementary Table 1: Primers used for validation of microarray results.

Gene	Primer	Sequence
ARHGAP6	ARHGAP6-F2	CTGTCAGATCACCATTCCCA
	ARHGAP6-R4	AGAATCCACTGACATGGCAC
GPM6B	GPM6B-F3	GCCGATGCATCAGTGGAATG
	GPM6B-R3	CGTTTGTGTTGCAGATGTTCTC
AP1S2	AP1S2-F1	CGGCAGTGTCTGTGAACTAG
	AP1S2-R1	TACAGTGTGTGAAATGCCAC
RPS6KA3	RPSKA6-F1	ACCACATCTAGTAAAGGGTGC
	RPSKA6-R1	TCTCTCAGATACGTGCTACC
THOC2	THOC2-F2	GGAGAGAGAAATGGACAAGAAAG
	THOC2-R2	TCTCATTTGGATAAGGAGATTCAC
STAG2	STAG2-F1	GAGAACAGAACTGAAGCCTG
	STAG2-R1	TGCACTTGATCTTGGTAAGC
RBMX	RBMX-F1	CTACTAAAGACAGCTATTCAAGCAG
	RBMX-R2	CTACGTGAGTTACCATAACTCTC



Supplementary Figure 1: Positions of markers significantly associated with suicide completion and genes examined in this study. Lines indicate regions examined for differentially expressed probesets in the gene expression analyses.

CHAPTER 3: MOLECULAR MECHANISMS GOVERNING SAT1 EXPRESSION

The results from both our previous studies as well as the three studies described in Chapter 2 clearly pinpointed SAT1 as playing a central role in the involvement of the polyamine system in suicide as well as other psychiatric disorders. However, while it was clear that both genetic variants and gene expression differences were involved, both the precise mechanisms mediating this relationship, as well as which non-genetic mechanisms may also be involved in regulating SAT1 expression, were unknown. The three studies in this chapter were designed to identify and characterize the molecular mechanisms involved in determining SAT1 expression, as well as to provide direct evidence linking these factors to suicide.

The first study, described in Chapter 3.1, concerned the identification and characterization of genetic variants which were involved in determining SAT1 expression. Promoter regions are known to contain important regulatory sequences, and thus this region was selected as the best site in which to search for polymorphisms influencing SAT1 expression. Genotyping of eighteen promoter polymorphisms was first performed in order to characterize the haplotype structure of the promoter, which was followed by *in vivo* and *in vitro* studies which demonstrated the presence of two major haplotypes which were associated with SAT1 expression. Further experiments found that the differential expression of the two haplotypes was largely due to an insertion/deletion in the promoter region, and two single nucleotide polymorphisms (SNPs) were found to display haplotype-specific effects on expression. This study provided direct evidence for a relationship between genetic variants and gene expression, as well as indicated a potential mechanism through which the genetic variants characterized in Chapter 2.2 may be functionally involved suicide and other psychiatric disorders.

The analyses performed in Chapter 2.2 were based upon SNPs only, and thus the relationship between the promoter insertion/deletion and suicide was not known. As this variant appeared to be the best predictor of SAT1 expression, the

study in Chapter 3.2 was performed in order to determine if this variant was associated with suicide, as well as how the presence of depressive disorders may influence this relationship. To accomplish this, the insertion/deletion was genotyped in a large number of depressed and non-depressed suicide completers and non-suicide controls, and statistical analyses indicated that while this variant was not associated with suicide in the entire population, it was associated with suicide performed in the context of depressive disorders. This study was thus able to provide additional support for the link between SAT1 promoter variants and suicide, as well as indicated that Axis I disorders play an important role in this relationship.

The third study in this chapter, described in Chapter 3.3, was designed to determine if epigenetic factors were also involved in determining SAT1 expression in the brain. Epigenetic modifications have previously been found to be involved in the pathology of suicide, and represent mechanisms by which the environment and other developmental factors may act to influence gene expression. This study assessed two well-known epigenetic modifications, DNA methylation and histone-tail modification, and determined their relationships with suicide and SAT1 expression. Overall, the results indicated that DNA methylation had a strong negative influence on SAT1 expression, and that several polymorphic CpG sites were highly methylated. Accordingly, this study demonstrated both the involvement and the importance of epigenetic factors in the regulation of SAT1 expression.

Altogether, the results of these three studies point to the involvement of specific genetic and epigenetic factors in determining the expression of SAT1, and demonstrate that these factors themselves are altered in suicide completers. Moreover, these results indicate that when investigating SAT1 expression, it is essential that each of these factors, in addition to clinical variables such as age, are taken into consideration.

3.1 Identification and Characterization of SAT1 Promoter Variants in Suicide Completers

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Abstract

Background: We have previously shown that the expression of spermidine/spermine N1-acetyltransferase (SAT1) is decreased in the brain Brodmann areas (BA) 4, 8/9, and 11 of suicide completers, and found an association between rs6526342, a SAT1 promoter SNP, with suicide completion (204).

Methods: We genotyped eighteen promoter polymorphisms in SAT1 in a French-Canadian population. The relationship between haplotypes and gene expression was assessed using microarray analysis of three brain regions, as well as reporter gene assays in three cell lines. Site-directed mutagenesis was used to examine the role of individual polymorphisms in reporter gene expression.

Results: We identified two major and several minor haplotypes in the promoter region of SAT1. Subjects who possessed the haplotype containing the risk allele for rs6526342 demonstrated decreased SAT1 expression in BA 4, 8/9, and 11. This haplotype was also associated with decreased expression in reporter gene assays. Site-directed mutagenesis identified three polymorphisms - an insertion/deletion (rs6151267), and two SNPs (rs6526342 and rs928931) - that were involved in determining reporter gene expression. These polymorphisms do not appear to exert their effects through the polyamine responsive element, as all constructs were induced to a similar extent in the presence of spermine.

Conclusions: Our results indicate that genetic variations in the promoter region of SAT1 are involved in determining levels of gene expression, and may provide a mechanism for the decreased SAT1 expression observed in suicide completers.

Introduction

Polyamines are ubiquitous aliphatic molecules which contain two, three or four amino groups, as well as the guanidino-amine agmatine. Polyamine homeostasis is highly regulated through their biosynthesis, degradation, and transport, and well as the interconversion between individual polyamines. The polyamines have numerous roles and are involved in many aspects of cellular function. Due to their cationic nature, they are well able to interact with nucleic acids, and not surprisingly, they are involved in many aspects of gene expression through their ability to alter DNA structure and stability, as well as their interactions with mRNA, tRNA, and ribosomal subunits (196). Polyamines are involved in many signalling pathways through their effects on G-proteins, protein kinases, nucleotide cyclases, and receptors, as well as through regulating the expression of proteins involved in these processes (196-198). Owing to their interactions with several transmembrane channels, including inward-rectifying K^+ channels, ionotropic glutamate receptors, and L-type Ca^{2+} channels, they also influence the electrical properties of excitable cells (214;220). Agmatine is believed to act as a CNS neurotransmitter by its actions through imidazoline receptors, α2-adrenoceptors, nicotinic acetylcholine receptors, and 5-HT3 receptors, and this theory is supported by its storage in synaptic vesicles and capacity to be released upon depolarization (219). Spermine has also been shown to be released from synaptic vesicles upon depolarization, indicating that the polyamines may function as neuromodulators (323).

Spermidine/spermine N1-acetylatransferase (SAT1) is the rate-limiting enzyme in the interconversion pathway, and is involved in regulating polyamine levels. SAT1 is located on Xp22.1 and contains 6 exons (513), yielding mRNAs of approximately 1.3 and 1.5 kb, depending on the length of the poly-A tail (514). The promoter region of this gene lacks a TATA box (513), however transcription appears to be controlled by the Sp1 element which has been shown to bind a GC-box at -42 to -51 bp (515). Additionally, the upstream region contains sites bound by other transcription factors including NF-κB (516;517), and peroxisome

proliferator-activated proteins (PPAR) (518), and certain cell lines have been shown to constitutively bind NF-E2 related factor (Nrf-2) at the polyamine responsive element (PRE) (519), which is involved in increasing transcription in the presence of polyamines or polyamine analogues through interactions with polyamine-modulated factor-1 (PMF-1) (520). The promoter region also contains potential binding sites for numerous other factors including Ap1, heat shock factor (HSF), GAGA factor, cAMP response element binding protein (CREB), CCAT/enhancer binding protein-β (C/EBPβ), and additional Sp1 sites (513;515). In addition to transcriptional regulation, the levels of SAT1 are influenced by the regulation of mRNA stability, translational efficiency, and stabilization of the SAT1 protein (reviewed in (521)). Through these mechanisms, levels of SAT1 can be influenced by many factors, including polyamines (522), polyamine analogues (523), non-steroidal anti-inflammatory drugs (524), amino acid deprivation (525), and other stressors (526).

Previously, using post-mortem brain tissues collected from French-Canadian suicide completers and healthy control subjects, our group demonstrated decreased expression of SAT1 in Brodmann areas (BA) 4, 8/9, and 11, and identified a single nucleotide polymorphism (SNP), rs6526342, in the promoter region of SAT1 that was significantly associated with suicide completion (204). Decreases in SAT1 expression have now been identified in other populations of suicide completers (205;206) as well as in an animal model of stress and major depression (527). Additionally, another promoter SNP in SAT1, rs1960264, has been reported to be associated with anxiety disorders (433).

To follow up on our previous results, we investigated genetic factors in the promoter region of SAT1 in order to identify potential causes for the downregulation of SAT1 expression in suicide completers. To do so, we examined the influence of promoter polymorphisms on gene expression in BA 4, 8/9, and 11, and used site-directed mutagenesis and reporter gene assays to individually characterize several of these polymorphisms.

Methods and Materials

1.0 Genotyping

1.1 Subjects

Our sample consisted of 96 male subjects of French-Canadian origin. This sample included brain tissues obtained from a group of 40 subjects, including those originally studied in (204), comprised of 14 control subjects, 10 non-depressed suicide completers, and 16 subjects who committed suicide in the context of a major depressive episode. These samples were obtained from the Quebec Suicide Brain Bank (www.douglasrecherche.qc.ca/suicide), and the cause of death was assessed by the Quebec Coroner's office. The remaining 56 subjects were obtained from a cohort of individuals representative of the Quebec general population (described in (435)).

Psychiatric diagnoses of brain tissue donors were obtained using the psychological autopsy method with the Structured Clinical Interview for DSM-IV Axis I (SCID-I) (480), as described elsewhere (528). Individuals from the cohort analyzed in this study were free of psychopathology and history of suicidal behavior as assessed by a large number of instruments and measures (435). Written informed consent was obtained for each subject or next of kin, as applicable. This study was approved by our local institutional review board.

1.2 Analysis of Polymorphisms

We defined the promoter region of SAT1 (NM_002970) as comprising the sequence from 5000 bp upstream from the transcription start site (TSS) to 500-bp into the first intron. Using public databases, we selected seventeen SNPs for analysis. One insertion/deletion (in/del) polymorphism comprising 15 adenine residues (rs6151267) repeat was also examined.

Genomic DNA was extracted from blood, saliva, or frozen brain tissue using standard procedures (483), and SNP genotyping was performed using the SNaPShot method with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Genotypes were determined using GeneMapper, version 3.7 (Applied Biosystems). The in/del, rs928932, rs1960264, and rs17286006 were genotyped by sequencing.

Linkage disequilibrium between polymorphisms was determined using Haploview version 4.1 (529). Prediction of transcription factor binding sites was performed using Transcription Element Search System (TESS) (530).

2.0 Expression

2.1 Correlation of SAT1 Expression with Genotype

The expression of SAT1 has previously been reported and validated for the 40 subjects described above in BA 4 (motor cortex), 8/9 (dorsolateral prefrontal cortex), and 11 (orbital cortex) using the Affymetrix U133A/B chipset (204). In order to determine if haplotypes were related to expression in these brain areas, ANOVAs were performed with $\log_2$ expression values from Affymetrix probesets 203455_s_at and 210592_s_at (www.Affymetrix.com) and haplotypes determined through genotyping.

2.2 Cell Culture

Dual-reporter gene assays were performed in three different cell lines: COS7 (green monkey kidney), HTB15 (human glio/astrocytoma), and CRL2137 (human neuroblastoma) (American Type Culture Collection, Manassas, VA). Cultures were maintained at 37°C with 5% CO_2, and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Canada) supplemented with 10% fetal bovine serum (Gibco),100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Where

indicated, COS7 cells were treated with 100μM spermine tetrahydrochloride (Biochemika, Canada).

2.3 Cloning

A 1.8-kb region, shown in Figure 1A, was cloned into the pGL3 Basic Luciferase Reporter Vector (Promega, Madison, MI), upstream of the firefly luciferase gene. Site-directed mutagenesis was then used to alter each of the polymorphisms in this region. Additional information regarding methods used for cloning is found in the Supplemental Methods. All constructs were confirmed by sequencing.

2.4 Transfection and Dual-Reporter Gene Assays

One day prior to transfection, cells were seeded into 24-well plates and grown for 24 hours. Transfections were performed using either Lipofectamine 2000 (Invitrogen, Canada) or GeneJuice (Novagen, Canada) systems, as appropriate for each cell line. Each well was treated with equivalent amounts of DNA for each of the vectors of interest, as well as with an internal control vector coding for renilla luciferase, pRL (Promega). Cells were grown for an additional 24 hours prior to assaying luciferase activity. Reporter gene expression was measured using the Dual-Luciferase Assay system (Promega) using an Orion II Microluminometer (Berthold, Canada). Each vector was transfected into three wells per experiment, and at least three independent experiments were performed for each condition. For experiments examining the effects of spermine, COS7 cells were seeded in either medium with spermine supplemented to a concentration of 100μM, or in medium prepared with added distilled water. Two hours following transfection, the appropriate media was again added to the cells, which were then incubated for an additional 22 hours prior to measuring luciferase activity. Firefly luciferase expression values were normalized to those

of pRL. Unpaired Student's *t*-tests between haplotypes were performed, with $P \leq 0.05$ considered significant.

Results

Genotyping:

As our previous results had shown an association between a promoter SNP in SAT1 and suicide, we were interested in determining the promoter haplotype structure in order to identify the SNPs which may be in linkage disequilibrium (LD) with this SNP. To this end, we genotyped seventeen SNPs: fourteen located 5' of the TSS, one within exon 1, and two within intron 1, as shown in Figure 1A. Five of these (934, 342, 941, 646, and 885) were genotyped as part of the HapMap project (440). We also analyzed one in/del of 15 adenine residues which is part of a larger tandem adenine repeat.

SAT1 is located on the X chromosome, and as an all-male sample was used in this study, only one allele was observed in each subject. The frequencies of the major haplotypes are shown in Figure 1C. Genotyping revealed one LD block, shown in Figure 1D, which included most SNPs upstream of the TSS as well as both intronic SNPs. Two SNPs, 646 and 006, were found only on the more common haplotype block. SNPs 941 and 952 showed no variation in this sample. The in/del polymorphism and the adjacent SNP 932 were associated with the LD block for 94% of subjects, such that the 25A repeat of the in/del and the G allele of SNP 932 were most frequently observed with the most common haplotype, while the less frequent haplotype displayed the 40 A repeat and the A allele of SNP 932. Of the subjects where the in/del and SNP 932 did not follow this trend, the majority possessed the less common haplotype with a short repeat, and the G allele of SNP 932.

Expression:

As we have previously shown that expression of SAT1 is significantly decreased in suicide completers in a number of different brain regions (64;157;204;206), we were interested in determining if the haplotypes identified in the present study were correlated with SAT1 expression levels observed in BA 4, 8/9, and 11, which were validated in a previous study (204). These analyses are shown in Table 1. We grouped both alleles at SNP 646 (haplotypes 1 and 4), and both combinations at the in/del and SNP 932 (haplotypes 3 and 5) due to small numbers of subjects within these groups. Overall, our results demonstrate that expression is associated with haplotypes in all three regions. Our strongest findings were obtained when only the two major LD blocks were considered (1/2/4 vs 3/5), and post hoc analyses indicated that the most frequent haplotype displayed the lowest expression.

SNP 006 does not appear to be involved in determining expression levels, as genotype at this SNP was not associated with expression either when analyzed as part of the overall haplotype structure (1/4 vs 2), or when assessed alone (not shown).

Dual-Reporter Gene Assays:

To further investigate the functional impact of SAT1 promoter variability on gene expression, we selected one region to be analyzed by reporter gene assays. This region was designed to possess both the core promoter of SAT1 (515), as well as the PRE, as we hypothesized that nearby polymorphisms may influence the ability of polyamines to induce expression through this site. This region is shown in Figure 1B, and contains 162-bp of exon 1 and 1667-bp of upstream sequence. Of the genotyped variations, the in/del, as well as SNPs 342, 264, 931, 932, 646, and 006 are found within this region. Due to the low variability of SNP 646, and the lack of association between SNP 006 and gene expression, only the major alleles for these SNPs (G and C) were examined. This region was

sequenced and cloned in two subjects with each haplotype: CTTDG (haplotype 1) and ACCIA (haplotype 3). These haplotypes correspond to haplotypes 1 and 3 described in Figure 1C. One vector from each haplotype, as well as the empty pGL3 vector, were used to transfect COS7, HTB15, and CRL2137 cells for dual-reporter gene assays. Results are shown in Figure 2.

Both haplotypes demonstrated substantially higher expression than the empty vector. The promoter activity of both haplotypes was different in each cell line such that COS7 cells produced the largest fold change increase compared to pGL3, followed by CRL2137, then HTB15. Additionally, there were differences between the expression of the two different haplotypes, with haplotype 3 showing significantly increased expression compared to haplotype 1 in COS7, HTB15, and CRL2137 cell lines (P=0.0007, 0.000005, and 0.003, respectively).

Site-Directed Mutagenesis:

In order to identify the polymorphisms directly responsible for these differences in expression, we individually altered each polymorphism in these two haplotypes. This yielded twelve different constructs, including the two wildtypes. As this region contains the PRE, which is involved in the induction of SAT1 by spermine and spermidine (519), we performed dual-reporter gene with (Figure 3C) and without (supplementary material) supplemented spermine. As the functional cell assays indicated the haplotype effect on gene expression is similar across the three different cell types, we conducted these experiments only in COS7 cells.

As shown in Figure 3C, alteration of the in/del polymorphism was sufficient to completely reverse the expression levels in both mutants to that of the opposing wildtype haplotype. Changes at SNP 342 reversed the expression when this SNP was altered in the haplotype 3 background (CCCIA), but no changes were observed in the haplotype 1 mutant. For SNP 931, the opposite trend was observed, such that a reversal of the expression phenotype was only seen in the haplotype 1 background (CTCDG). These two trends were observed both with

and without supplemented spermine. For SNPs 932 and 264, all mutants showed increased expression, but the patterns were similar to those of their respective wildtypes (supplementary material).

The addition of spermine increased reporter gene expression, with a mean fold change of 1.38±0.02, relative to cells grown in media without added spermine. There did not appear to be differences in induction between any of the wildtypes or mutants.

Discussion

In this study we followed up on previous results of significantly decreased expression of SAT1 in the brains of suicide completers and the association of the promoter SNP 342 C allele with suicide and decreased brain expression (204). We identified two major promoter haplotypes in SAT1 which showed significant differences in expression in both reporter gene assays as well as in human brain samples. Additionally, one in/del and two promoter SNPs were found to be involved in determining expression under these conditions.

This study revealed one LD block comprising SNPs both upstream of the first exon and within the first intron of the SAT1 gene, which is consistent with results obtained by the HapMap project (440;463). In our sample, two major haplotypes were observed. Two SNPs, 646 and 006, were found only on the more common haplotype block. The in/del polymorphism and SNP 932 were in high, but not complete, LD with this haplotype block. As mentioned above, one of the SNPs that was genotyped within this sample (342) has previously been found to be significantly associated with suicide completion (204). SNP 342 belongs to the LD block we observed, and thus may act as a tag SNP for the other SNPs examined in this study, and to a lesser extent, can predict variation at the in/del polymorphism and SNP 932. These results expand our knowledge of the effects of promoter variants on SAT1 gene expression, and which polymorphisms may confer susceptibility to suicide.

Our analysis found a significant influence of haplotype 1 on SAT1 expression in BA4, 8/9, and 11, and indicated that this common haplotype, which contains the SNP 342 C allele, was associated with lower expression in these brain regions. This gives good support for the presence of a direct relationship between our association results and the findings of decreased SAT1 expression in suicide completers. Interestingly, the T allele of SNP 264 is also found on the more common haplotype 1 (Figures 3A and B), and has been recently associated with anxiety disorders (433). This may indicate that haplotype 3 is protective against both anxiety and suicidal behaviors.

The region examined in the reporter gene assays contained SNP 342 as well as four additional polymorphisms. This region demonstrated increased promoter activity compared to the empty vector in all three cell lines examined. This is consistent with the assumption that the core promoter is located between -82 and +139-bp of the TSS (515), a sequence completely contained within the region. Significant and consistent differences in reporter gene expression between the two haplotypes were observed in each of the three cell lines. The expression of haplotype 3 ranged from 1.3 to 2.1-fold higher than haplotype 1, and this trend is in line with our brain expression results. This consistent increase in gene expression, observed in cell lines of different origins, indicates that similar factors regulating SAT1 expression are present in a variety of cell types, although the differences in the magnitude of the increased expression could indicate that tissue-specific factors may also be involved.

The addition of spermine to the culture medium increased reporter gene transcription by approximately 1.4-fold. The ability of polyamines and polyamine analogues to increase SAT1 transcription is well-established, and is dependent upon the cell type as well as the polyamine or analogue used (for examples, see (522;523;531-533)). As mentioned above, one mechanism for the induction of SAT1 expression by polyamines involves the interaction between PMF-1 and Nrf-2, which is constitutively bound to the PRE in analogue-sensitive cells (519). The PRE is in close proximity to each of the polymorphisms examined by reporter gene assays, and we had speculated that variants in this region may influence the

ability of Nrf-2 to bind to the PRE. However, this did not appear to be the case as the expression of all constructs was induced to a similar extent by the addition of spermine. It has previously been observed that normal intracellular concentrations of polyamines can be sufficient to induce the transcription of SAT1 following transfection, and that higher increases in transcription are observed when cells are depleted of intracellular polyamines prior to transfection (519). In this light, it may be possible that slight differences do exist between the constructs, which could not be observed due to background levels of induction resulting from polyamines already present in the cells.

Due to LD, all SNPs within this haplotype will give the same strength of association to suicide. Site-directed mutagenesis is an important tool for identifying causal variants and assessing potential interactions between polymorphisms. The results of the site-directed mutagenesis experiments indicate that the promoter activity resulting from variations in the region examined is the result of a combination of factors. On its own, the in/del was sufficient to produce the observed promoter activity, as changing the length of this extended adenine repeat completely reversed the expression in the mutants to those of the reciprocal wildtypes. There are a number of mechanisms by which promoter variants can influence transcription, and generally involve alterations of either the binding of transcription factors, or the accessibility of the DNA (534). It may be speculated that the effects of in/del variation may result from its increased AT content, which decreases the melting temperature of the DNA, or by the 15-bp difference in length, which may impact interactions between transcription factors. The results obtained for SNPs 342 and 931 demonstrate that the in/del is not the only polymorphism involved in determining transcription. Both these SNPs appear to exert haplotype-specific effects, as changing their sequence was only sufficient to reverse the phenotype in one haplotype, with no effects on the other. This suggests that there are interactions between these SNPs and other variations present within this region. The precise mechanism for these effects is not yet clear. The sequence surrounding SNP 342 is not predicted to bind any transcription factors, whereas a number of transcription factors are predicted to

bind at SNP 931, several of which appear to demonstrate allele-specificity. The results obtained by Tomitori and colleagues suggest that at least one repressor element may bind in the sequence between -1639 and -777-bp (515), although this has not been confirmed and may not be relevant to the current study. A study analyzing a large number of promoter regions found that the majority of functional variants did not disrupt any known transcription factor binding sites, suggesting that many transcription factor consensus sequences may not yet be known, and that other mechanisms may be involved (535). Clearly, more work is required in order to determine the precise mechanisms involved in determining transcription levels, and future experiments should be designed to identify proteins displaying allele-specific binding to the SAT1 promoter.

There are a number of potential mechanisms by which the decreased expression associated with haplotype 1 of SAT1 may be involved in increasing the susceptibility to suicide. Spermine has been shown to induce neuronal cell death through activation of *N*-methyl-D-aspartate receptors (536), and it could be speculated that long-term decreases in SAT1 activity may cause damage to the CNS. Alternatively, as mentioned above, the polyamines influence numerous receptor systems, and as such, alterations in their relative proportions may alter the functioning of these systems. Finally, both agmatine and putrescine have been shown to produce antidepressant and anxiolytic effects, and their levels have been shown to increase following stressors (197;221-227;371). Decreased SAT1 expression may result in lower levels of these polyamines, and thus may diminish their mood altering effects and prevent normal stress responses. As both mood disorders and stress are associated with suicide (537), downregulation of SAT1 in suicide completers may simply reflect the involvement of these factors in suicide, or alternatively, could indicate that SAT1 mediates their role in the development of suicidal behavior. Future studies are needed in order to differentiate between these effects, and to determine the precise role for SAT1 in suicide.

A recent study by Guipponi and colleagues also demonstrated decreased expression of SAT1 in BA11 of suicide completers, however they were unable to replicate our previous association of SNP 342 with suicide, or find a relationship

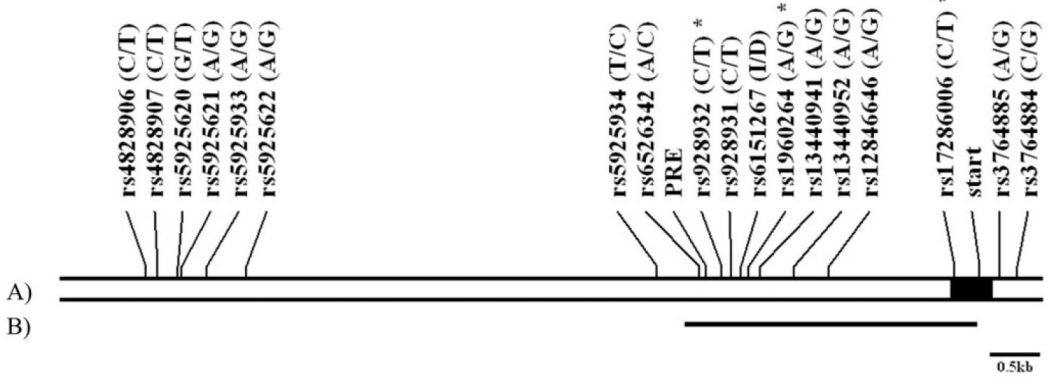
between this SNP and SAT1 expression (205). There are a number of potential causes for these different results. Firstly, the study by Guipponi and colleagues combined males and females when assessing the correlation between genotype and expression, whereas our study was performed exclusively in males. It may be possible that gender-specific differences are involved in regulating SAT1 expression, particularly as this gene is located on the X chromosome, and as the levels of polyamines and their metabolic enzymes have been shown to differ between genders (210;538;539). Additionally, we conducted our studies in a sample composed exclusively of French-Canadians, a population with a well-documented founder effect (450). Accordingly, the reduced genetic heterogeneity in our population may have increased our ability to identify genetic factors involved in suicide. Alternatively, our results may reflect differences in the frequency and properties of haplotypes between populations, which may be especially important for our results with the in/del as it was not in complete LD with SNP 342. As this polymorphism appears to play an important role in determining SAT1 expression, differences in the LD structure at this haplotype block may yield population-specific relationships between expression and genetic variations. Future studies will be required in order to assess the role of SAT1 promoter haplotypes in females, in whom it will also be possible to characterize coding polymorphisms which display differential allelic expression *in vivo*. This would allow for a more detailed understanding of the role of polymorphisms in determining the expression of SAT1.

In summary, we present results indicating that specific promoter variants in SAT1 have an effect on SAT1 gene expression. Considering the association between suicide and the lower-expressing haplotype, one could speculate, given the role of polyamines as a stress system that is quickly activated, that individuals with the lower-expressing haplotype may be less able to deal with the consequences of stress. This proposition, however, remains to be validated and further studied.

Acknowledgements

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Figures



C)

	Haplotype	Frequency (%)
1	TCTAGATCTTDGGGGCGC	41
2	TCTAGATCTTDGGGGTGC	30
3	CTGGAGCACCIAGGGCAG	16
4	TCTAGATCTTDGGGACGC	5
5	CTGGAGCACCDGGGGCAG	5
6	other	3

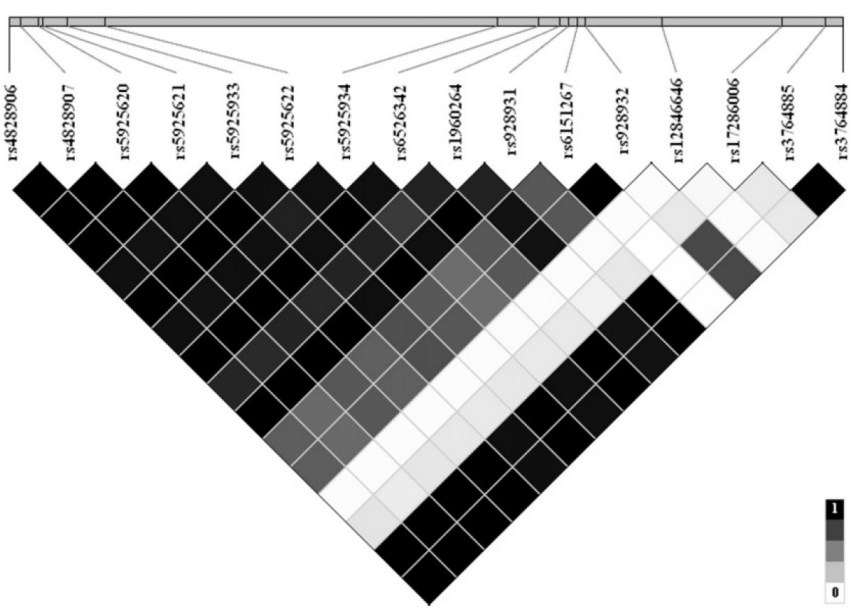


Figure 1: Regions and polymorphisms of the SAT1 promoter analyzed in this study. A) Location and sequences at polymorphic sites. Exon 1 is shaded in black, and the translation start site and polyamine responsive element are indicated. B) Region analyzed for reporter gene assays. SNPs with stars were identified during sequencing of this region. C) Haplotypes and frequencies of genotyped polymorphisms in the promoter region of SAT1. D) Linkage disequilibrium between polymorphisms plotted as r^2. I/D – insertion/deletion; PRE – polyamine responsive element.

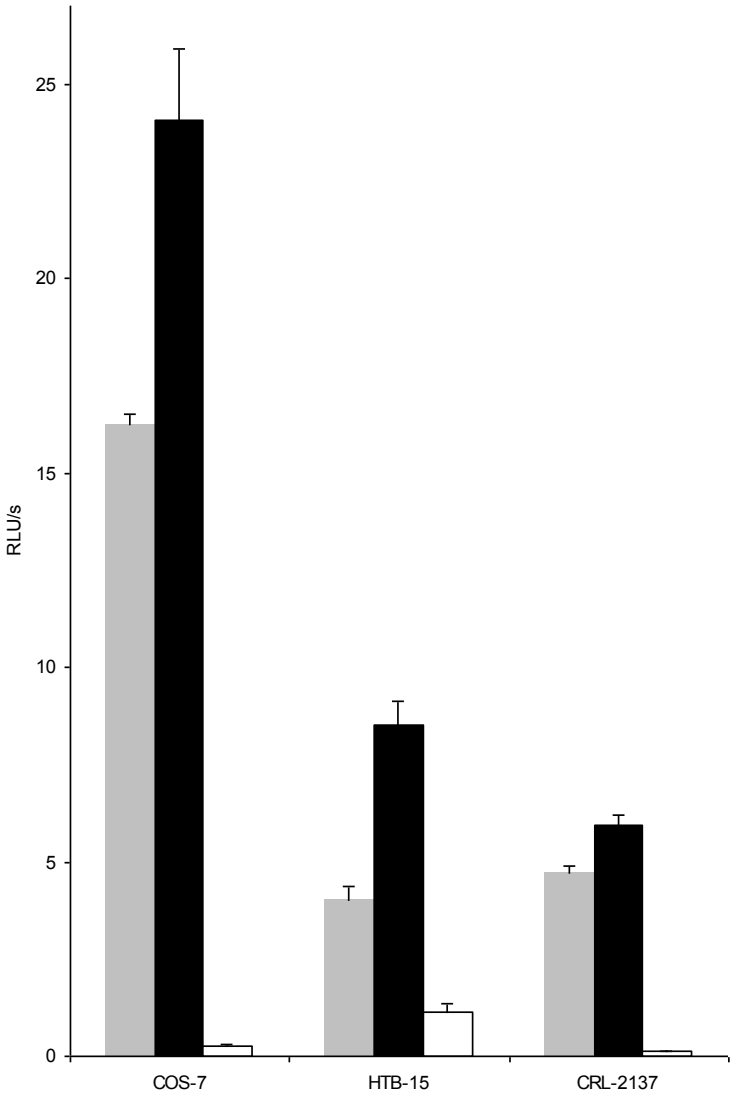
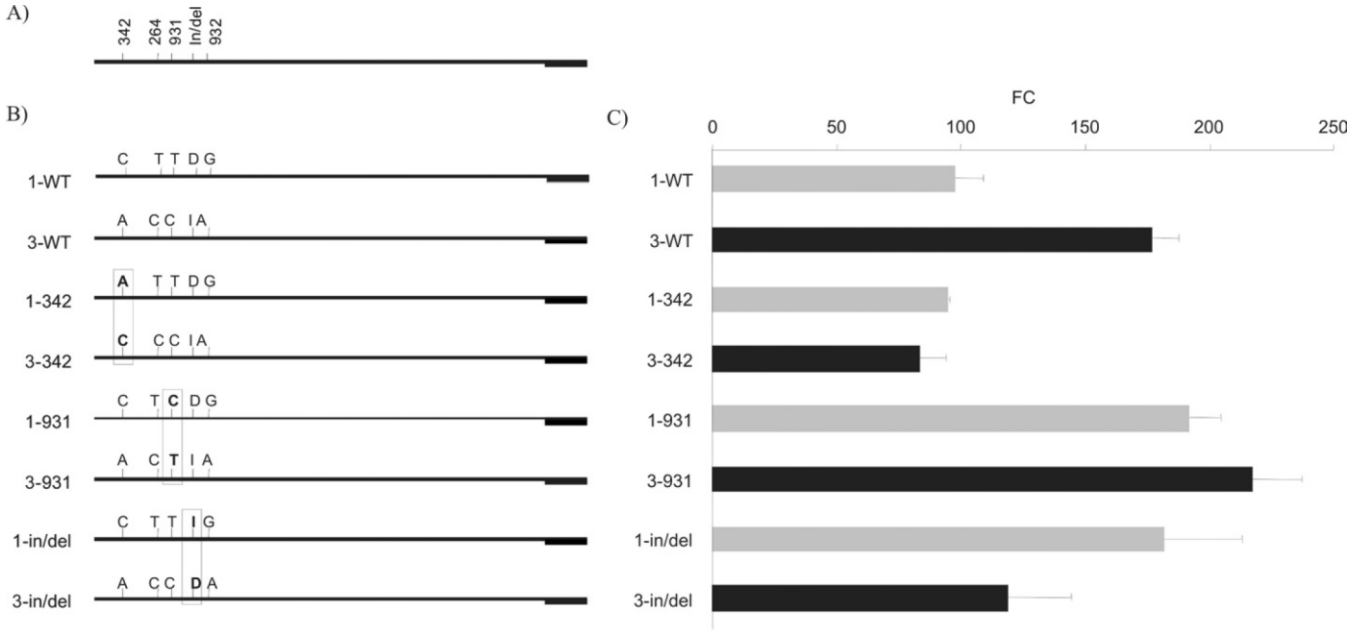


Figure 2: Results from reporter gene studies for haplotype 1 (grey), haplotype 3 (black), and empty pGL3 (white) in COS7, HTB15, and CRL2137 cell lines. Normalized relative light units/second (RLU/s) and standard errors were obtained from triplicate replications of each assay.



D)

	P-value	
Construct	Haplotype 1	Haplotype 3
1-WT		*0.0002*
3-WT	*0.0002*	
1-342	0.895	*0.007*
3-342	0.413	*0.0003*
1-931	*0.0001*	0.366
3-931	*0.0002*	0.077
1-in/del	*0.008*	0.834
3-in/del	0.379	*0.035*

Figure 3: Reporter gene assays for wildtypes and mutant constructs. Constructs are labeled as haplotype-polymorphism. A) and B) Sites and sequences at polymorphisms in reporter gene constructs. Boxes indicate polymorphisms whose sequences have been altered. Exon 1 is indicated as a thick bar. C) Reporter gene expression for haplotype 1 (grey) and 3 (black) wildtypes and mutants in COS7 cells grown with media containing 100μM spermine. Values + standard errors were calculated as fold change (FC) relative to empty pGL3. D) P-values for comparisons of reporter gene expression for each construct with wildtype haplotypes 1 and 3. D – deletion; I – insertion; WT – wildtype.

Tables

Table 1: Relationship between promoter haplotypes and SAT1 gene expression in BA 4, 8/9, and 11. A) Number of subjects included in each region for each haplotype. B) Student *t*-tests for comparisons between different haplotypes.

A)

		Number of Subjects		
Haplotype		**BA 4**	**BA 8/9**	**BA 11**
1	TCTAGATCTTDGGGGCGC	10	8	9
2	TCTAGATCTTDGGGGTGC	3	6	6
3	CTGGAGCACCIAGGGCAG	2	1	2
4	TCTAGATCTTDGGGACGC	1	1	1
5	CTGGAGCACCDGGGGCAG	3	3	2

B)

BA	**Probeset**	**1/4 vs 2**	**1/4 vs 3/5**	**2 vs 3/5**	**1/2/4 vs 3/5**
4	203455_s_at	0.50	0.29	0.24	0.07
	210592_s_at	0.12	0.19	0.86	0.07
8/9	203455_s_at	0.30	*0.01*	*0.02*	*0.009*
	210592_s_at	0.48	*0.03*	*0.02*	*0.01*
11	203455_s_at	0.55	0.45	0.71	*0.03*
	210592_s_at	0.93	0.95	1.00	0.25

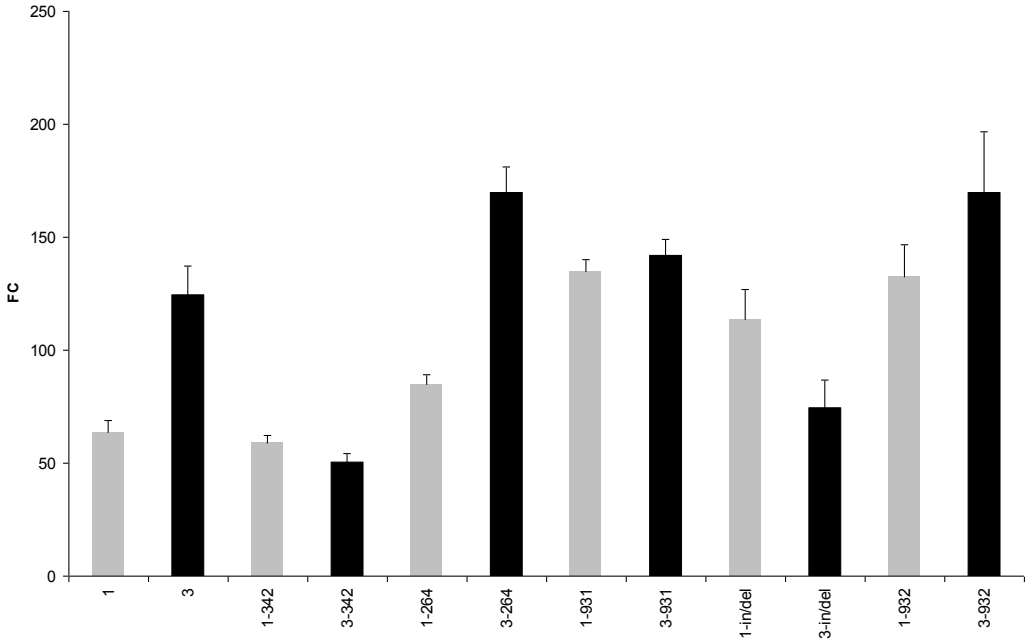
Supplemental Methods

Cloning

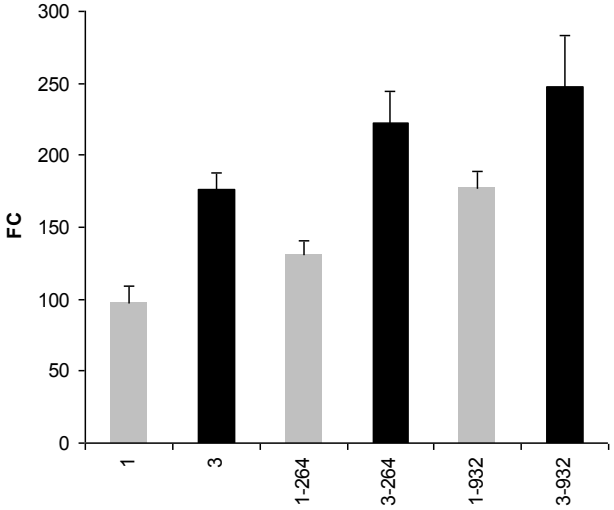
A 1.8-kb region was cloned into the pGL3 Basic Luciferase Reporter Vector (Promega, Madison, MI), upstream of the firefly luciferase gene. Polymerase chain reaction (PCR) was performed using HotStar High Fidelity DNA Polymerase (Qiagen), with primers containing recognition sites for *Kpn*I or *Xho*I. Primer sequences, with restriction sites shown in italics, were SSATf-F-*Kpn*I, 5'-CGG*GGTACC*TTACAGCTACTAGTCTTTCCTGATAGTG and SSATf-R-*Xho*I, 5'- CCG*CTCGAG*CGTCTTTTGCTTTTCTTCCCTTTGCG. PCR products and pGL3 were digested with *Xho*I and *Kpn*I (New England Biolabs (NEB), Canada), then purified with the GenElute PCR Clean-up Kit (Sigma-Aldrich, St Louis, MO). Digested products were ligated together with T4 DNA ligase (NEB), and transformed into competent JM109 cells (Promega) by heat shock. All inserts were sequenced.

Site-directed mutagenesis was used to alter each of the variable SNPs in this region. SNPs were mutated in the wildtype plasmids using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). To generate in/del mutants, SNP 932 mutants from both haplotypes were digested with *Bst*XI and *Msc*I (New England Biolabs) to generate 5581- and 1041-bp products. The 1041-bp products were swapped and ligated with the reciprocal 5581-bp product to generate SNP 931-in/del mutants, then the SNP 931 site was returned to the wildtype sequence by site-directed mutagenesis to create mutants in which only the in/del was altered. Mutations were confirmed by sequencing.

Supplemental Figures



Supplemental Figure 1: Reporter gene expression for wildtypes and mutants. Constructs are labeled as haplotype-polymorphism. Haplotype 1 (grey) and 3 (black) wildtypes and mutants were grown in COS7 cells without supplemented spermine. Values + standard errors were calculated as fold change (FC) relative to empty pGL3.



Supplemental Figure 2: Reporter gene expression for wildtypes and SNPs 264 and 932 mutants. Constructs are labeled as haplotype-polymorphism. Haplotype 1 (grey) and 3 (black) wildtypes and mutants were grown in COS7 cells with media containing 100μM spermine. Values + standard errors were calculated as fold change (FC) relative to empty pGL3.

3.2 Association of the SAT1 In/del Polymorphism with Suicide Completion

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Abstract

Several studies have observed decreased expression of spermidine/spermine N1-acetyltransferase (SAT1) in the brains of suicide completers, and we previously identified a single nucleotide polymorphism in the promoter region of SAT1 which was associated with suicide completion and SAT1 expression in the brain. We recently characterized the haplotype structure of the SAT1 promoter region, and identified an insertion/deletion (in/del) of 15 adenine residues. This variant appears to be a predictor of SAT1 expression, and we were thus interested in determining if the lower-expressing deletion allele was found more frequently among suicide completers. To this end, we genotyped the in/del in a sample of 771 French-Canadian males, comprised of 326 suicide completers and 445 non-suicide controls. We found no significant difference in the frequencies of the two alleles between suicide completers and controls in the entire sample. However, we observed a significantly higher frequency of the deletion in the depressed suicide completers compared to the depressed non-suicides. These results add support for a role of SAT1 in conferring a risk for suicide completion, in particular in the context of depressive disorders.

Suicide is a major public health problem, and considerable effort has been made to identify molecular mechanisms involved in the etiology and pathology of suicidal behavior. Although the majority of studies have focused on the role of monoaminergic neurotransmission, there is growing evidence to support the involvement of other systems underlying suicidality. One molecular system which has been gaining support for a role in suicide and other mental disorders is the polyamine system (203).

The polyamines comprise agmatine, putrescine, spermidine, and spermine, and are involved in many cellular functions, including growth, division, and signalling cascades (196-198). Of particular importance to psychiatric disorders, the polyamines influence the properties of several neurotransmitter pathways, including the catecholamines (210-213;340), gamma amino-butyric acid (215-217;341), nitric oxide (218), and glutamate (221;227;342-344). Numerous animal studies have demonstrated the antidepressant and anxiolytic effects of agmatine and putrescine (221-227;389), and alterations in the levels of the polyamines and their metabolic enzymes have been observed in several psychiatric conditions, including suicide (203).

To date, the polyamine-related gene which has been most strongly implicated in suicidal behavior is spermidine/spermine *N*-1 acetyltransferase (SAT1), the rate-controlling enzyme in polyamine catabolism and interconversion. SAT1 is located on Xp22.1 and contains 6 exons (513), yielding mRNAs of approximately 1.3 and 1.5 kb, depending on the length of the poly-A tail (514). The expression of SAT1 is highly regulated through control of transcription, mRNA stability, translational efficiency, and stabilization of the SAT1 protein (521).

Downregulation of SAT1 has been observed in multiple brain regions in suicide completers (64;157;204-206), as well as in an animal model for stress and depression (527). In addition, two polymorphisms in the promoter region of SAT1 have been shown to be associated with suicide (204) and anxiety (433). One of these variants, rs6926342, was also found to be associated with SAT1 expression in several brain regions. To further investigate SAT1 promoter polymorphisms, we recently characterized the haplotype structure of the promoter region, and

assessed the relationship between haplotypes and expression of SAT1 in several brain regions and cell lines. We found a significant relationship between promoter variants and expression, and identified two single nucleotide polymorphisms (SNPs) (rs6926342 and rs928931), and one insertion/deletion (in/del) of fifteen adenine residues (rs6151267), which were in high linkage disequilibrium (LD) and were directly involved in controlling reporter gene expression *in vitro* (207). Whereas the SNPs appeared to exert haplotype-specific effects on expression, altering the in/del was sufficient to reverse the expression phenotypes in our system, such that the deletion was associated with decreased expression both *in vitro* and in the brain. The in/del thus appears to be a predictor of SAT1 expression, and may represent an additional functional link between decreased SAT1 expression and the association of SNP rs6926342 to suicide. Accordingly, we chose to investigate the association of the in/del polymorphism to suicide completion in a sample of male French Canadian suicide completers and controls.

We investigated a total of 771 unrelated French Canadian male subjects. Cases for this study were 326 individuals who died by suicide, as assessed by the Quebec Coroner's office, with an average age of 40.8±13.5. We also included 445 subjects who did not die by suicide. These subjects had an average age of 40.4±13.5 and were recruited from outpatient clinics or from the Quebec general population. French Canadians represent a relatively young population with a well-documented founder effect (450;477-479), and our use of both cases and controls from this population minimizes the potential for population stratification effects.

Psychiatric diagnoses were assessed for a subset of the sample using the Structured Clinical Interview for DSM-IV Axis I (SCID-I) (480) or the Diagnostic Interview Schedule using DSM-III-R criteria (481). Diagnostic information on suicide completers was obtained by means of the psychological autopsy method, a well-established and validated method to obtain diagnostic information by proxy-based interviews. We conducted interviews with the informant best acquainted with the subject (for detailed information, please consult (540)). Information regarding psychiatric diagnoses is found in the Supplementary Material online. Overall, psychopathology was available for 277 and 213 of the control and suicide

subjects, respectively. In order to investigate the effects of suicide independently from those of depressive disorders, we selected our non-suicide control group to include a high percentage of subjects with depressive disorders. In total, 178 of the non-suicide controls, and 120 of the suicide completers were diagnosed with depressive disorders. Informed consent was obtained from either the subject or their next-of-kin, as appropriate. This study was approved by our local institutional review board.

Genomic DNA was extracted from blood, saliva, or frozen brain tissue using standard procedures (483). We performed a 10μL polymerase chain reaction (PCR) with Taq DNA polymerase (Qiagen) using primers SSAT-TA-F1 (CTTGAGCCCAGGAGTTCGAG) and SSAT-TA-R1 (TGGTGACTAAAAGAATATGTGCTTGTG), to generate products of 161 or 176 bp, depending on the in/del. The entire reaction was analyzed on 3% agarose gels. The in/del was sequenced in twenty of these subjects to verify our results. Our sample also included the ninety-six subjects in which we previously genotyped the in/del by sequencing (207).

As SAT1 is located on the X chromosome, only one allele was observed for each subject. This 15 residue in/del is part of a larger tandem adenine repeat, yielding alleles with either 25 or 40 consecutive adenine residues. Overall, 87% of our subjects possessed the shorter allele, which we previously demonstrated to be in high LD with the C risk allele of rs6526342 in a French Canadian sample (207).

Pearson χ^2 tests were performed to assess association to suicide in both the overall sample, and in the subset of individuals with current or lifetime histories of depressive disorders. The results of these analyses are shown in Table 1. There was no significant difference in the frequencies of the two alleles between suicide completers and controls in the entire sample. However, when we examined the frequencies in the subset of individuals with depressive disorders, there was a significantly higher frequency of the D allele in the depressed suicide completers compared to the depressed non-suicides. These results remained significant when controlling for age. Although false positives are an important concern in

association testing, our results in the depressed subgroup remain significant even after a correction for multiple tests, which suggests that our findings do not simply reflect a spurious association. Although the size of the depressed subgroup and the number of individuals with the I allele are fairly small, additional support for the importance of our findings lies in the relationship between decreased SAT1 expression and the D allele (207), and our findings of widespread decreases in SAT1 expression in the brains of suicide completers (64;157;204;206). Moreover, as the D allele is on the same haplotype as the C risk allele of rs6526342, our results add further weight to the importance of this haplotype in suicide completion. Interestingly, this haplotype also includes the T allele of rs1960264, which has been found to be significantly associated with anxiety disorders (433). Given the high LD among promoter variants, these associations may all reflect the involvement of a single polymorphism in increasing the susceptibility to suicide and anxiety. As our previous results indicated that, among these variants, the in/del had the greatest role in determining SAT1 expression (207), it is not unreasonable to speculate that the in/del is the functional variant being tagged by these association studies.

It should be noted that Guipponi and colleagues failed to replicate our previous findings for an association of rs6526342 with suicidal behavior (205). This may be due to the increased genetic homogeneity in our sample due to our use of exclusively French Canadian subjects, or may reflect differences in the frequency and properties of alleles between populations.

The fact that our results only reached statistical significance in the depressed subgroup may indicate a specific role for SAT1 in depressed suicide victims. This is supported by our previous findings of greater decreases in SAT1 expression in depressed compared to non-depressed suicide completers (64;157;204;206). Indeed, the polyamine system as a whole has been implicated in mood disorders and stress response (203), and SAT1 downregulation has previously been observed in an animal model of stress and depression (527). However, our results suggest that the role of SAT1 in suicidal behavior is not directly mediated by depression, as we found no direct association between the in/del and depression in

our sample (not shown). Our results instead appear to indicate that depression moderates the effect of the in/del on suicide. However, this remains to be validated and further studied. It is also possible that our results may simply reflect the increased homogeneity in the depressed subsample compared to the overall group, which included individuals with multiple other Axis I disorders. Indeed, a previous study found no association for an involvement of rs1960264 in schizophrenia (434), indicating that SAT1 promoter polymorphisms are indeed specific for certain psychiatric conditions.

There are a number of mechanisms by which SAT1 may be involved in the etiology of suicidal behavior. SAT1 is the rate-limiting enzyme in polyamine catabolism and interconversion, and thus plays an important role in controlling the relative and absolute levels of each of the polyamines. As both agmatine and putrescine have been shown to display both antidepressant and anxiolytic effects in animals (221-227), decreased levels of these compounds due to a downregulation in the expression of SAT1 may prevent or diminish these effects. In addition, as mentioned above, the polyamines influence several neurotransmitter systems, which could be affected by alterations in the relative proportions of each of the polyamines.

There are a number of limitations to our study. We conducted this study in a sample composed exclusively of French Canadians, which as mentioned above, is a genetically homogenous population. It is thus possible that the association obtained in this sample may not be found in other populations. Additionally, as this study was performed in only male subjects, the applicability of these results to females is unclear, particularly as SAT1 is found on the X chromosome. Finally, psychopathology was not available for the entire sample. However, the rate of depressive disorders in our suicide group was 56.3%, which is comparable to those previously reported in the French-Canadian population (396;469).

In summary, our results indicate that the in/del variant in the promoter region of SAT1 is implicated in the predisposition towards suicidal behavior in the context of depressive disorders. Given the putative role of this promoter variant in determining brain expression of SAT1, our results lend support for the

involvement of decreased levels of SAT1 expression in suicidal behavior, and highlight the importance of the polyamine system in mental disorders.

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Tables

Table 1: Allelic frequencies of the in/del in suicide completers and controls. Chi-square (χ^2) tests were performed between suicide completers (S) and non-suicide controls (NS) in both the entire sample as well as in the depressive disorder (DD) subgroup.

Subjects	N	D (N, %)	I (N, %)	χ^2	P	OR (95% CI)
NS	445	380 (85.4)	65 (14.6)	1.74	0.19	1.34 (0.87 – 2.06)
S	326	289 (88.7)	37 (11.3)			
NS-DD	178	147 (82.6)	31 (17.4)	6.06	0.01	2.60 (1.19 – 5.69)
S-DD	120	111 (92.5)	9 (7.5)			

Supplemental Information

Supplementary Table 1: Characteristics of case and control subjects. Ages are reported ± standard deviation. Percentages are indicated in brackets; percentages for specific Axis I disorders were calculated within only the assessed individuals. NS – non-suicide; S – suicide completers

	NS (N=445)	**S (N=326)**
Mean age	40.4 ± 13.5	40.8 ± 13.5
Assessed for Axis I disorders	277	213
Depressive disorders	178 (64.3)	120 (56.3)
Bipolar disorder	3 (1.0)	21 (9.9)
Schizophrenia	3 (1.0)	13 (6.1)
Anxiety disorders	33 (11.9)	18 (8.5)

3.3 Epigenetic Regulation of SAT1 in Suicide

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Abstract

Background: We have recently shown that the expression of spermidine/spermine N1-acetyltransferase (SAT1) is downregulated across the brains of suicide completers, and that its expression is influenced by genetic variations in the promoter. Several promoter polymorphisms in SAT1, including rs6526342, have been associated with suicide and other psychiatric disorders, and display haplotype-specific effects on expression. However, these effects cannot explain total variability in SAT1 expression, and other regulatory mechanisms, such as epigenetic factors, may also be at play.

Methods: We assessed the involvement of epigenetic factors in controlling SAT1 expression in the prefrontal cortex of suicide completers by mapping CpG methylation across a 1880-bp region of the SAT1 promoter, and measuring levels of tri-methylated histone-3-lysine 27 (H3K27me3) at the promoter in suicide completers and controls.

Results: CpG methylation was significantly negatively correlated with SAT1 expression. Although we found no direct evidence that overall or site-specific CpG methylation was associated with suicide or SAT1 expression, we observed high levels of methylation at the polymorphic CpG site created by rs6526342, indicating a relationship between promoter haplotypes and methylation. There was no association between H3K27me3 and suicide, nor was this modification associated with SAT1 expression.

Conclusions: Our results indicate that epigenetic factors in the promoter region of SAT1 influence gene expression levels, and may provide a mechanism for both our previous findings of haplotype-specific effects of promoter variations on SAT1 expression, as well as the widespread downregulation of SAT1 expression observed in the brains of suicide completers.

Introduction

Suicide is one of the leading causes of death in Western countries (2), which results from the interaction of social, environmental, and genetic factors. While there is strong evidence for the involvement of the monoaminergic neurotransmitter systems in the neuropathology of suicide, it has become increasingly evident that additional systems are involved in this complex condition. In recent years, evidence has emerged implicating dysregulation of the polyamine system as an important factor in suicide and other psychiatric disorders (203). To date, alterations of spermidine/spermine N1-acetyltransferase (SAT1), the rate-limiting enzyme in polyamine catabolism, have been one of the most robust findings implicating this system in the neurobiology of suicide. Decreased expression of SAT1 has now been observed in a number of brain regions in suicide completers (64;157;204-206), and two promoter polymorphisms, rs6526342 and rs6151267, have been associated with suicide in French Canadians (204;208). Several single nucleotide polymorphisms (SNPs) in the promoter were recently found to be involved in determining expression levels in vitro as well as in the brain, and interestingly two SNPs, rs6526342 and rs928931, produced haplotype-specific effects on expression (207).

In addition to DNA sequence variants, epigenetic regulation is another important determinant of gene expression, and can involve methylation of DNA as well as modification of histones. DNA methylation is a covalent modification at the 5' position of cytosine, occurring at CG dinucleotides (CpG), and is often associated with gene repression when found in promoter regions (541). Histones are the core proteins involved in packaging of DNA into nucleosomes, and can be covalently modified at specific residues by acetylation, methylation, phosphorylation, SUMOylation, and ubiquitinylation (41). The effect of these modifications on gene expression depends upon the histone protein (H2A, H2B, H3, H4), specific amino acid residue, the nature of the modification, and its positioning within the gene.

Interestingly, polyamine metabolism has been shown to influence both DNA methylation and histone modifications (for examples, see (407-410)), and increased S-adenosylmethionine, a precursor in polyamine biosynthesis, has been implicated in the downregulation of several genes in the prefrontal cortex of psychotic patients through altering levels of promoter methylation (411). The expression of several polyamine-related genes has also been shown to be influenced by DNA methylation, including polyamine modulated factor-1 (PMF-1) (542), and methionine adenosyltransferase (MAT1A) (543), although a recent study performed by our group found no relationship between DNA methylation or histone modifications and expression of spermine synthase or spermine oxidase in the brains of suicide completers (392). However, the expression of ornithine aminotransferase and μ-crystallin has been shown to be influenced by histone methylation in the prefrontal cortices of schizophrenia patients (42), suggesting that histone modifications are important modulators for the expression of polyamine genes. Several studies have now identified altered levels of CpG methylation and histone modifications in gene promoter regions in a number of psychiatric disorders, including suicide (32;37-39;42;43;544). Overall, SAT1 appears to be a compelling target for assessing the involvement of epigenetic factors in the regulation of the polyamine system and in conferring risk for suicide.

In this study, we were interested in determining the role of epigenetic modifications in determining the expression of SAT1, and to assess their involvement in downregulating the expression of SAT1 in suicide completers. In addition, we were interested in investigating if epigenetic mechanisms were responsible for the haplotype-specific effects of the functional promoter variants, particularly as rs6526342 and rs928931 create polymorphic CpG sites. Using prefrontal brain tissue from a sample of suicide completers and controls, we focused on promoter CpG methylation and levels of tri-methylated histone-3-lysine 27 (H3K27), epigenetic modifications which are both associated with gene repression (541;545). Both of these modifications have been shown to be relatively unaffected by complications arising in post-mortem tissues such as

altered pH and post-mortem interval (546;547), and are thus well-suited for this type of analysis. Additionally, tri-methylated H3K27 was recently implicated in the downregulation of the TrkB receptor in the prefrontal cortex of suicide completers (44).

Materials and Methods

Subjects:

We examined 20 subjects, comprised of 10 non-suicide controls and 10 suicide completers. Subjects were matched for age, post-mortem interval (PMI), and brain pH. Cause of death was assessed by the Quebec Coroner's office. Samples were obtained from the Quebec Suicide Brain Bank, where they were processed and dissected at 4°C, then snap-frozen in liquid nitrogen before storage at -80°C, following standard procedures (397). All subjects died without a prolonged agonal period. Brodmann area (BA) 8/9 (dorsolateral prefrontal cortex) was dissected in accordance with standard neuroanatomical definitions (398). Psychiatric diagnoses were obtained using the psychological autopsy method with the Structured Clinical Interview for DSM-IV Axis I (SCID-I) (480), as described elsewhere (528). This study was approved by our local institutional review board.

Quantitative Real Time Polymerase Chain Reaction (RT-PCR):

Total RNA was extracted from frozen tissue using an RNeasy Lipid Tissue Mini Kit (Qiagen, Canada). RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA synthesis was performed using oligo(dT)-priming (Invitrogen, Carlsbad, CA). RT-PCR was performed in quadruplicate for SAT1 with β-actin as an endogenous control using Taqman on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA).

CpG Methylation Mapping:

Genomic DNA from BA 8/9 was extracted as above, then treated and purified with the Qiagen EpiTect Bisulfite kit. Bisulfite-treated DNA was amplified by PCR using primers listed in Supplementary Table S1. PCR products were purified with the GenElute PCR Clean-up Kit (Sigma-Aldrich, St Louis, MO), ligated into the pDRIVE vector and transformed into EZ Competent cells (PCR Cloning Plus Kit, Qiagen). Plasmids were extracted and sequenced using the reverse primers for each region. CpG sites are labelled relative to the transcription start site (TSS), with 39 consecutive adenine residues at the tandem adenine repeat at -1274 bp (insertion allele at rs6151267).

Chromatin Immunoprecipitation of Tri-Methylated H3K27 (H3K27me3):

Immunoprecipitated DNA was prepared as described in (548). Briefly, tissue from BA 8/9 was treated with micrococcal nuclease (Sigma-Aldrich), which cleaves DNA between adjacent nucleosomes. Intact nucleosomes were extracted, and a portion of sample was treated with anti-trimethyl-H3-lysine 27 antibody (Upstate Biotechnology, New York, NY), while the remainder was used as an input control. The antibody-treated sample was purified and both input and bound fractions were digested with protease before purifying DNA. Quantitative RT-PCR using SYBR green (Applied Biosystems) was used to measure DNA in each sample, in quadruplicate. Relative quantitation was performed with β-actin as an endogenous control, and ratios of bound:input were calculated for each subject. Primer sequences are found in Supplementary Table S1.

Statistics:

Analyses were performed using SPSS, v15.0. Student's *t*-tests and Pearson correlations were used to analyze differences between groups as well as determine

the relationship between CpG methylation, H3K27me3, and SAT1 expression in BA 8/9.

Results

CpG Methylation:

In order to determine if CpG methylation is involved in the differential expression of SAT1 in suicide completers and the relationship between promoter haplotype and expression, we analyzed methylation in BA 8/9 of 20 subjects (10 suicide completers and 10 controls). We examined the 1880 bp region of the SAT1 promoter shown in Figure 1. This region spans -1751 bp to +129 bp from the transcription start site (TSS) and contains a total of 131 CpG sites, including the site created by rs6526342 (-1566), as well as two additional sites (-1429 bp/rs1960264, and -1371 bp/rs928931) affected by SNPs. These are part of the larger haplotype block extending from 5 kb upstream of the TSS to the end of the first intron, which is associated with SAT1 expression in the brain and in vitro (207). Our sample comprised 16 subjects (9 suicide completers, 7 controls) with the low-expressing haplotype, which contains the C allele at rs6526342, and 4 subjects with the high-expressing haplotype, possessing an A at rs6526342.

The results of the CpG methylation mapping are shown in Figure 2. The overall percentage of methylation at all sites was 10.5%. Although all but five sites were methylated in at least one clone, the majority of sites were only sparsely methylated. However, 18 sites were methylated in at least a third of the clones, including the site created by rs6526342 which was methylated only in the low-expressing haplotype, with an average of 88.6%. There were also high levels of methylation at rs1960264, whereas rs928931 was completely unmethylated. Aside from methylation at these sites, there were no significant differences in total methylation between subjects with the high- or low-expressing haplotypes.

We assessed the expression of SAT1 in BA 8/9 for these subjects and found results consistent with our previous studies in larger samples, indicating a

decrease in SAT1 expression among suicide completers (one-tailed P = 0.07). To assess the relationship between overall promoter CpG methylation and SAT1 expression, we performed Pearson correlations in the sample as a whole (irrespective of phenotype). There was a significant negative correlation between expression and overall percentage methylation (P = 0.008, Pearson = -0.64), which was not affected by age, pH, or PMI. We were next interested in determining if this effect was due to methylation in specific promoter regions, particularly in order to determine if methylation at the region containing rs6526342 played a functional role in the brain. Given that there appeared to be two distinct patterns of methylation, the promoter region was divided into sparsely (+78 bp to -412 bp) and heavily (-433 bp to -1672 bp) methylated regions. Additionally, the sparsely methylated region was further subdivided to examine the role of methylation in exon 1. These results are also shown in Figure 3A and demonstrate that, with the exception of exon 1, methylation in all regions is strongly negatively correlated with SAT1 expression; however, the strength of correlations between expression and methylation in any of the subdivisions does not exceed that between expression and overall methylation. Moreover, we found no evidence that methylation at any individual site was significantly correlated with expression (Supplementary Figure S1B). Overall, these results indicate that CpG methylation of the entire region plays a role in determining SAT1 expression.

We subsequently compared percentages of methylation between suicide completers and controls and found no differences in overall methylation (P = 0.51), as shown in Figure 3B. Interestingly, although there were no significant differences between suicide completers and controls for site-specific methylation (Supplementary Figure S1A), the rs6526342 site showed a trend for increased methylation in suicide completers (94% vs 82% among subjects possessing the C allele, uncorrected P = 0.07).

H3K27 Methylation:

We were next interested in assessing the role of chromatin modifications in determining levels of SAT1 expression in the brain. We focused on the tri-methyl modification of lysine 27 of histone 3 (H3K27me3), as this modification has been shown to be relatively unaffected by post-mortem factors (547). These results are depicted in Figure 4. There was no significant difference between levels of H3K27me3 in controls and suicide completers (P = 0.72), nor was H3K27me3 significantly correlated with SAT1 expression (P = 0.56, Pearson = 0.17) or the overall percentage of CpG methylation (P = 0.33). Interestingly, there was a correlation between the levels of H3K27me3 and the percentage of exonic CpG methylation (P = 0.03, Pearson = 0.57). However, as neither of these factors appear to play a large role in determining SAT1 expression, the relevance of this finding is unclear.

Discussion

In this study, we examined the role of two epigenetic modifications, CpG methylation and histone methylation, in determining the expression of SAT1 in the brain, and assessed their involvement in the decreased expression of SAT1 previously observed in the brains of suicide completers (64;157;204;206).

We found a strong relationship between CpG methylation and SAT1 expression, such that increased overall levels of CpG methylation was correlated with decreased expression. This effect was not limited to sites close to the TSS, and in fact overall promoter methylation showed a stronger correlation with expression than any promoter subdivision. In general, CpG methylation is associated with transcriptional repression, which can occur through several mechanisms including blocking access by transcription factors, binding of repressive transcription factors, and interfering with transcriptional elongation (541). Given the apparent pattern of CpG methylation in the SAT1 promoter, with a paucity of methylation in exon 1 and the first few hundred bases upstream, then

high levels of methylation at specific sites in the more distal promoter regions, it appears likely that multiple mechanisms are involved in repressing SAT1 transcription. The promoter region of SAT1 was characterized by Tomitori and colleagues, who showed that while the promoter region lacks a TATA box, transcription appears to be controlled by the Sp1 element which binds a GC-box at -42 to -51 bp (515). Additional transcription factor binding sites in the proximal promoter region include several sites for NF-κB (516;517), as well as a peroxisome proliferator-activated proteins (PPAR) site (518). The promoter region also contains potential binding sites for numerous other factors including Ap1, heat shock factor (HSF), GAGA factor, cAMP response element binding protein (CREB), CCAT/enhancer binding protein-β (C/EBPβ), and additional Sp1 sites (513;515). Alterations in CpG methylation levels may influence the binding of any of these factors, or may recruit repressive factors such as methyl-CpG-binding proteins (541).

The polyamine responsive element (PRE) is found within the more distal promoter region. This 9 bp sequence, located between -1518 and -1526 bp from the TSS, has been shown to constitutively bind NF-E2 related factor (Nrf-2) in certain cell lines, which is involved in increasing transcription of SAT1 in the presence of polyamines or polyamine analogues through interactions with polyamine-modulated factor-1 (PMF-1) (519;520). It is therefore of interest that it is located immediately between two highly methylated sites at -1529 and -1516 bp. Given the importance of this mechanism in controlling SAT1 expression in the presence of elevated polyamine levels, even small changes in the levels of CpG methylation may alter the efficiency of the interaction between the PRE and Nrf-2, or between Nrf-2 and PMF-1, which may have important consequences for the regulation of polyamine levels. It is also possible that methylation at these sites is involved in the cell-type specific binding of Nrf-2 to the SAT1 promoter, which could result in variable effects of elevated polyamine levels across different cell-types, and possibly even across different brain regions.

We recently characterized the role of polymorphisms in the promoter region of SAT1, and found haplotype-dependent effects of two SNPs, rs6526342 and

rs928931, on SAT1 expression in vitro (207). These two polymorphisms, as well as a third SNP, rs1960264, whose effect on SAT1 expression was less clear, create haplotype-specific CpG sites, such that individuals with the low-expressing haplotype have an additional site at -1566 bp (rs6526342), whereas those with the higher-expressing haplotype have additional sites at -1429 bp (rs1960264) and -1371 bp (rs928931). In this study, rs6526342 was among the highly methylated sites, which could represent a potential mechanism for its haplotype-specific effects on expression. Although methylation at this site was not significantly correlated with SAT1 expression, it is possible that in this study we lacked sufficient power to detect this effect. Although not statistically significant, we observed a trend for increased methylation at rs6526342 in suicide completers. Given that the C allele at this site is more frequent among suicide completers, these results may indicate that methylation at this SNP is involved in the pathology of suicide. Interestingly, while our previous studies indicated that altering the C allele to an A had no effect in the low-expressing haplotype, switching to a C allele in the high-expressing haplotype resulted in a phenotype similar to the low-expressing haplotype (207). These results suggested that the C allele confers lower expression moderated by other components of the haplotype block, such as the insertion/deletion (rs6151267), which was sufficient to generate the expression phenotype on its own. Although the mechanism for these effects remains unclear, examination of this region identified several potential transcription factor binding sites, including those for NF-E2, YY1, and AP-1, and it is possible that the presence of a C allele at rs6526342, with its increased methylation may affect the binding of transcription factors by altering the physical structure of the DNA or accessibility of binding sequences (541). Alternatively, methylation at the C allele could allow the binding of methyl-CpG-binding proteins, which may recruit additional proteins to exert repressive effects on transcription (541). Clearly additional work will be required in order to identify proteins which bind at this site, and the conditions which influence their effects.

We found no association between the H3K27me3 modification and suicide in our sample, nor did this modification appear to be involved in determining

levels of SAT1 expression. Nonetheless, numerous other histone modifications exist, and we cannot discount the possibility that alterations in chromatin structure can influence the expression of SAT1.

A previous study by Guipponi and colleagues did not identify a relationship between CpG methylation of SAT1 in BA 11 and suicide or expression (205). There are several possible explanations for the differing results between studies. The previous study examined only a small region, spanning -70 bp to +32 bp from the TSS which, in our study, showed a much weaker association with expression. The previous study included subjects with a range of Axis I disorders, whereas the majority of our suicide completers died in the context of a major depressive episode. It is thus possible that disorder-specific differences may exist, although we found no evidence for a relationship between depressive disorders and methylation in our sample. Finally, our study examined the dorsolateral prefrontal cortex, whereas Guipponi and colleagues examined the orbital prefrontal cortex, and it may be possible that region-specific differences exist.

There are possible limitations to this study. As this study was performed in only male subjects, the applicability of these results to females is unclear, particularly as SAT1 is found on the X chromosome. Indeed, Guipponi and colleagues observed increased CpG methylation in females, irrespective of psychopathology (205). Secondly, although we examined a large section of the SAT1 promoter, we cannot rule out the possibility that additional important epigenetic factors are found beyond the region examined in this study. In particular, intronic sequences may also harbor important regulatory elements and were not examined in this study. We also cannot exclude the possibility that chromatin modifications other than H3K27me3 are involved in determining SAT1 expression or are altered in suicide, as numerous histone modifications have been described (549), of which several appear to be involved in controlling the expression of polyamine-related genes and to be affected in psychiatric conditions (42). Finally, it is possible that the sample investigated in this study lacked sufficient power to detect effects of discrete magnitude, which may have been of

particular relevance for evaluating the haplotype-specific effects of methylation on expression.

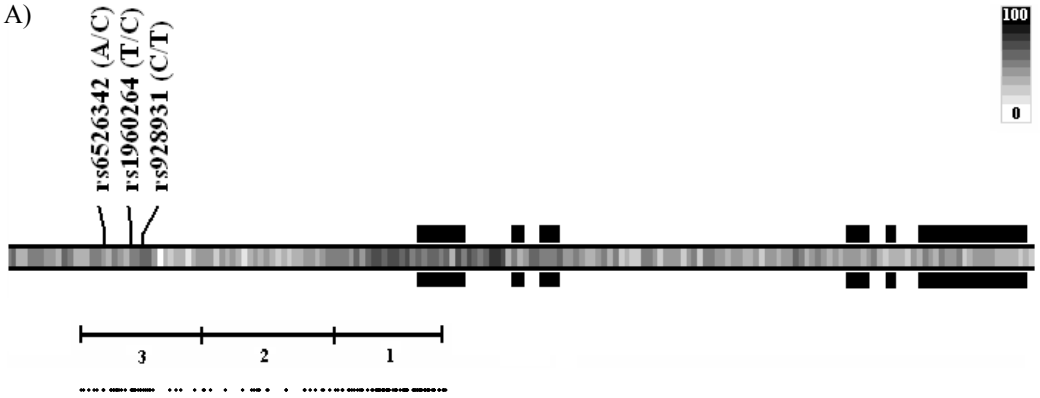
In summary, our results indicate that CpG methylation plays an important role in determining the expression of SAT1 in the brain. Given the widespread downregulation of SAT1 in the brains of suicide completers, this may represent an important epigenetic mechanism involved in conferring risk for suicide. Moreover, the haplotype-specific CpG site created by the rs6526342 polymorphism was found to be highly methylated, which may indicate that methylation at this site could mediate the genetic association between the C allele and suicide.

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Figures

A)



B)

Region	Size (bp)	CpG Sites
1	600	74
2	681	21
3	655	36
Total	1880	131

Figure 1: Promoter region of SAT1 examined by methylation mapping. A) GC content of the SAT1 gene and upstream promoter region. Black boxes represent exons. Black dots denote CG dinucleotides. B) Details of the three subdivisions used to map CpG methylation.

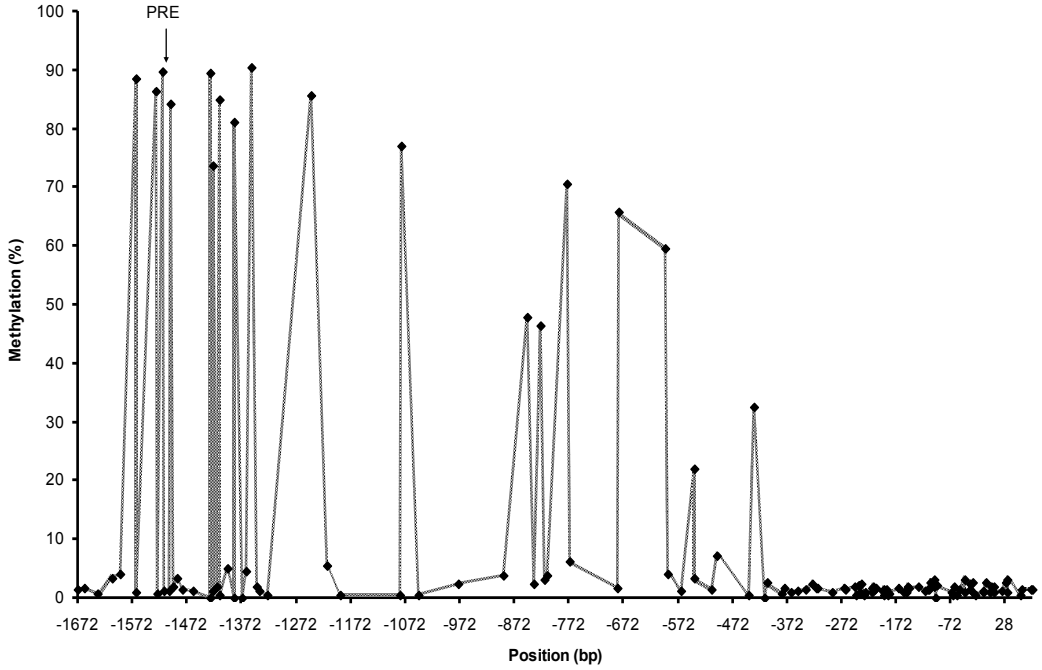


Figure 2: CpG methylation in the promoter region of SAT1 in all subjects. Positions are relative to the transcription start site. Sites affected by polymorphisms are indicated in grey. The calculated percentage methylation at these sites was obtained from individuals possessing the C alleles. The position of the polyamine responsive element (PRE) is indicated.

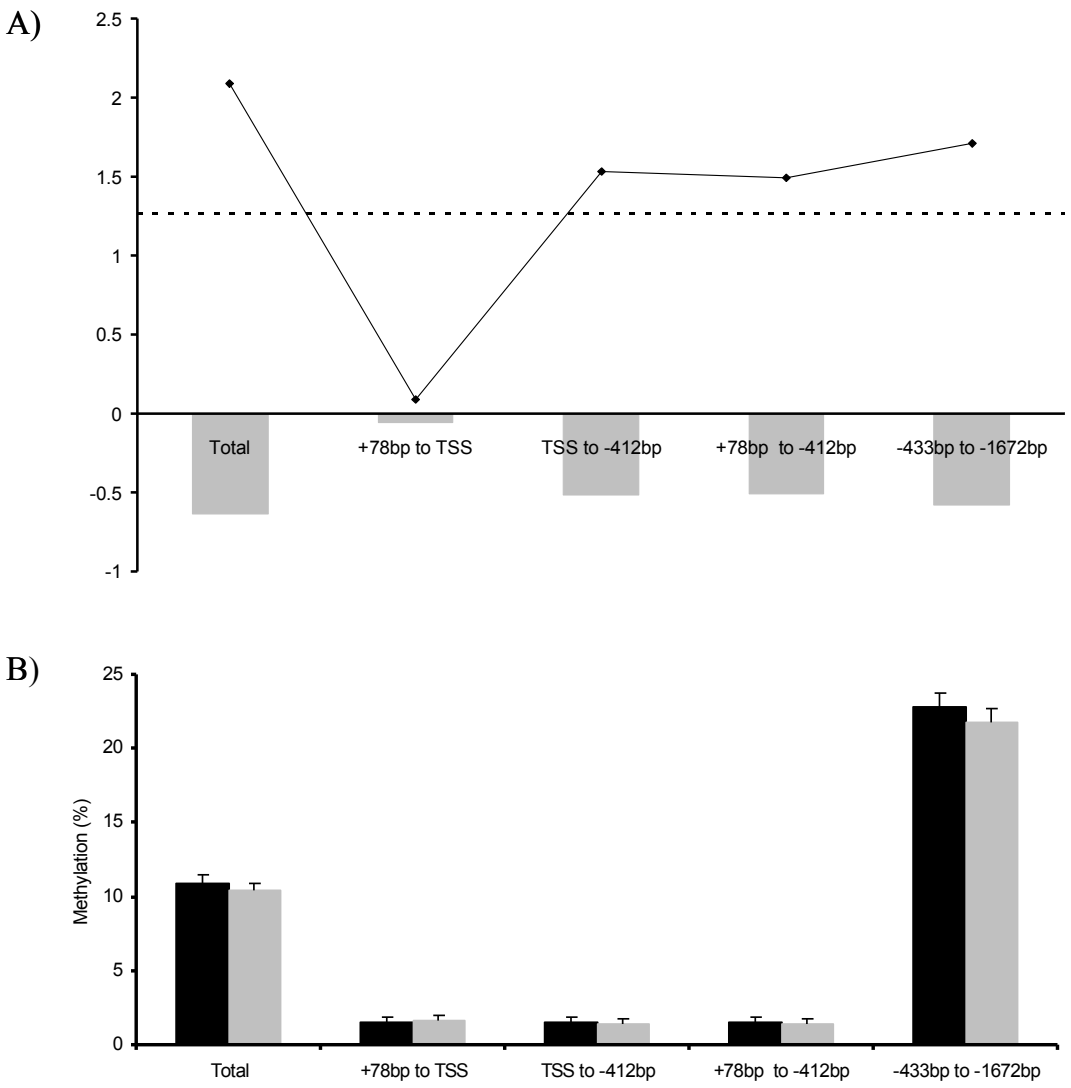


Figure 3: Relationship of CpG methylation to expression and suicide. Results are shown for the entire region as well as sections of the promoter. Sites are shown relative to the transcription start site (TSS). A) Pearson correlation (grey bars) and $-\log_{10}$ P-values (black line) for correlation of percentage methylation with SAT1 expression. The dotted line indicates P = 0.05. B) Percentage methylation + SEM for suicide completers (black) and controls (grey).

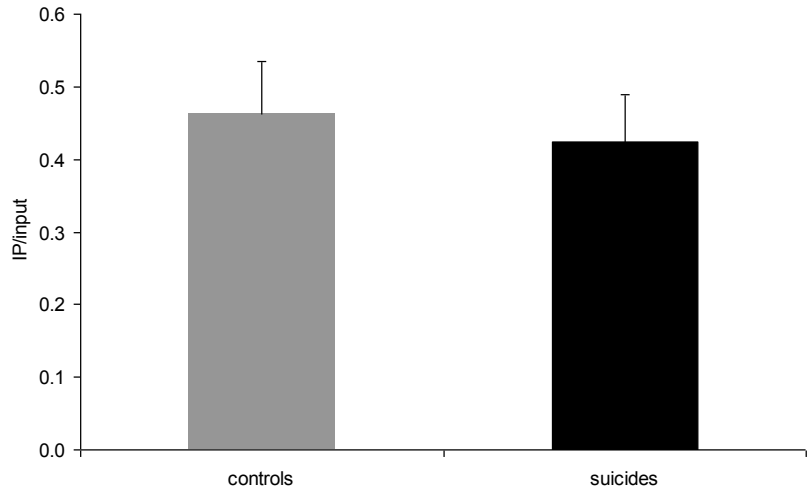
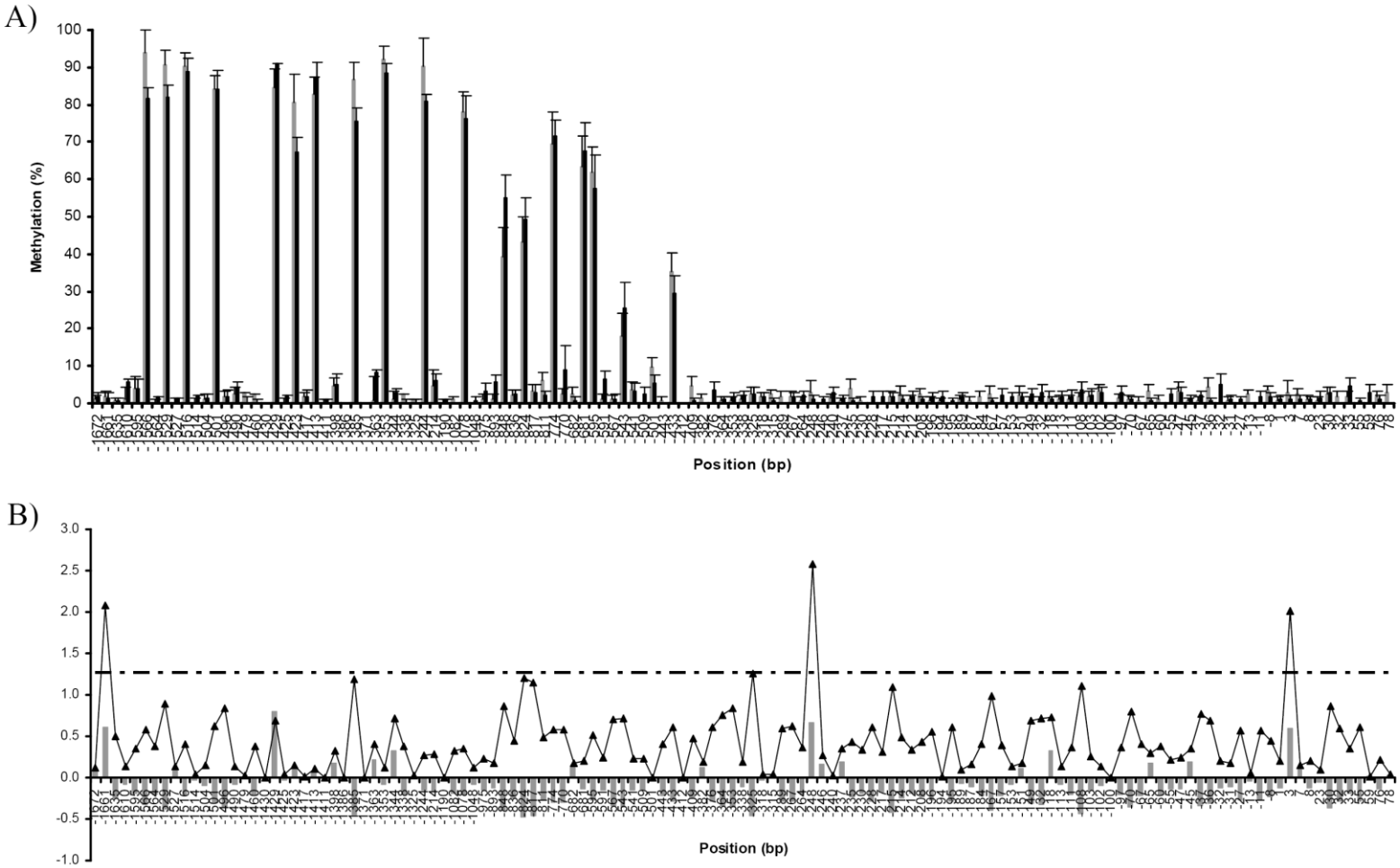


Figure 4: Tri-methylation of H3K27 in the promoter region of SAT1. Methylation + SEM for controls (grey) and suicides (black) are expressed as the ratio of immunoprecipitated (IP) DNA to input DNA.

Supplemental Information

Supplementary Table 1: Primers used in this study.

Study	Primer	Sequence
CpG	SSAT-ep-F1	GAAATTTTAGAAAGGATTTTGGGATTTG
	SSAT-ep-R1	CCCCTATACTTAAACAATAAAAAA
	SSAT-ep3-F1	GGATTTTGAGTTATTTTGGGAGATTATG
	SSAT-ep3-R1	ATTATAATCCCTACTTAATTCATCTTCTAT
	SSAT-ep2-F1	ATAGAAGATGAATTAAGTAGGGATTATAAT
	SSAT-ep2-R1	CAAATCCCAAAATCCTTTCTAAAATTTC
H3K27	SSAT-ChIP-F	TCCTGAGTTTGCTTCCCACT
	SSAT-ChIP-R	GGTGTGTCCCCCAGTAACAT
	BActin-F	AGGATGGCAAGGGACTTCCTGTAA
	BActin-R	AATGTGGCCGAGGACTTTGATTGC



Supplementary Figure 1: Methylation of CpG sites in the promoter region of SAT1. Sites are shown as position relative to the transcription start site. A) Percentage methylation + SEM for suicide completers (black) and controls (grey). B) Pearson correlation (grey bars) and $-\log_{10}$ P-values (solid black line) for correlation of percentage methylation with SAT1 expression. The dotted line indicates uncorrected P = 0.05.

CHAPTER 4: INVESTIGATION OF GENETIC AND EPIGENETIC VARIABILITY ON THE EXPRESSION OF SMS AND SMOX

Similar to SAT1, both our previous studies as well as those described in Chapter 2 provided strong support for the involvement of SMS and SMOX in suicidal behaviors. Given the strong findings in Chapter 3 demonstrating the involvement of numerous genetic and epigenetic mechanisms in determining the expression of SAT1, it was of interest to determine if similar mechanisms were also at play in the regulation of SMS and SMOX expression. Accordingly, the promoter regions of these two genes were examined for genetic variants associated with expression, as well as both DNA methylation and H3K27 methylation. However, unlike SAT1, the study described in Chapter 4.1 failed to identify genetic or epigenetic regulators which played significant roles in determining expression under the conditions examined. It is therefore clear that additional studies will be necessary in order to identify the mechanisms involved in the differential expression of these genes in suicide completers, which may be found elsewhere in the gene, may involve other epigenetic mechanisms not assessed in this study, or may result from the differential expression of other proteins or compounds which regulate the levels of SMS or SMOX transcripts.

4.1 Genetic and Epigenetic Influences on Expression of SMS and SMOX in Suicide

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Abstract

Alterations in the levels of spermine synthase (SMS) and spermine oxidase (SMOX), two enzymes involved in polyamine metabolism, have previously been observed in brains of suicide completers. To characterize the roles played by genetic and epigenetic factors in determining expression levels of these genes, as well as to identify potential mechanisms by which to explain our findings in suicide completers, we 1) assessed the role of promoter polymorphisms in determining expression in the brain and *in vitro*, and 2) examined CpG methylation and levels of methylated histone-3-lysine 27 in the promoter regions of these genes in the prefrontal cortex of suicide completers and healthy controls. We identified several promoter haplotypes in SMS and SMOX, but found no consistent effects of haplotype on expression levels in either the brain or in reporter gene assays performed in three different cell lines. We also found no overall effects of epigenetic factors in determining expression, with the exception of a relationship between CpG methylation at one site in the promoter of SMOX and its expression in Brodmann area 8/9. In conclusion, the genetic and epigenetic factors examined in this study show little influence on the expression levels of SMS and SMOX, and do not appear to be responsible for the dysregulated expression of these genes in suicide completers.

Introduction

The polyamine system has been identified in all organisms and is essential for life (195). The polyamines comprise agmatine, putrescine, spermidine, and spermine, and are involved in a myriad of essential cellular functions, including growth, division, and signalling cascades (196-198). The levels of the polyamines are highly regulated and are controlled through the activities of enzymes in both the biosynthetic and interconversion pathways, which are depicted in Fig 1. Putrescine can be derived from either L-ornithine by the enzyme ornithine decarboxylase (196) or from agmatine by agmatinase (550). Spermidine and spermine are synthesized by spermidine synthase and spermine synthase (SMS), respectively, which transfer aminopropyl groups produced from S-adenosylmethionine (SAM) through SAM decarboxylase (AMD1) (196). The interconversion pathway is responsible for back-conversion of spermine and spermidine to putrescine. Two mechanisms can be used to convert spermine to spermidine of which the better characterized pathway involves acetylation of spermine by spermidine/spermine N1-acetyltransferase (SAT1), followed by oxidative deamination by polyamine oxidase (PAO) to produce spermidine (551). In addition to the SAT1/PAO pathway, spermine can also be converted to spermidine through spermine oxidase (SMOX). This enzyme is specific for unacetylated spermine, and does not work upon spermidine or acetylated spermidine (552). As such, only the first mechanism is used for conversion of spermidine to putrescine.

Alterations of the polyamine system have been observed in several pathological conditions including cancer (196), ischemia (201), Alzheimer's disease (202), and mental disorders (203). The first evidence for the involvement of the polyamine system in suicide was the observation of downregulation of SAT1 expression in brain Brodmann areas (BA) 4, 8/9, and 11, in suicide completers (204). Following this finding, downregulation of SAT1 in suicide completers was observed in several additional brain regions by our group and others (64;157;205;206), and we identified numerous other polyamine-related

genes which were differentially expressed in suicide completers, including SMS, SMOX, ornithine aminotransferase-like 1, AMD1, and ornithine decarboxylase antizymes 1 and 2 (64;157;206). In addition, we recently characterized several polymorphisms in the promoter region of SAT1, and found three to be associated with expression levels in both the brain and *in vitro* (207).

SMS is located on Xp22.1, and encodes 11 exons to produce a 366 amino acid protein. Disruption of the function of this gene can have deleterious effects, and mutations in SMS are responsible for Snyder-Robinson syndrome, a form of X-linked mental retardation which manifests with both intellectual and physical symptoms, including alterations in brain morphology (459;461;462;553). In addition, mouse Gy mutants, which possess a deletion of a section of the X chromosome containing multiple exons of SMS, display neurological abnormalities and reduced lifespans, which has been attributed to the loss of SMS function (554). Elevated levels of SMS appear to produce far fewer effects, as transgenic mice display only slight increases in spermine and decreases in spermidine levels (555). Under normal conditions, the expression of SMS appears to be regulated primarily by substrate availability although there is evidence for induction under certain conditions (320).

SMOX was isolated much more recently than other enzymes involved in polyamine metabolism (552). This gene is found at 20p13 and has at least 9 known exons, with the translation start site being found in the second exon. Numerous splice forms have been identified although their transcription does not appear to be differentially regulated (556-558). Expression of this gene has been shown to be upregulated following exposure to polyamines and their analogues (558). The increased protein levels appear to be due primarily to increased transcription and stabilization of SMOX mRNA, and the area spanning -55 to -1117-bp from the transcription start site is implicated in this response (558).

Based on the strong evidence for a global dysregulation of polyamine metabolism in suicide completers, we chose to further investigate genetic and epigenetic factors which may be involved in the altered expression of SMS and SMOX, two important polyaminergic genes that have been shown to be

differentially expressed in suicides as compared to controls in our previous studies (64;157). To do so, we examined the influence of promoter polymorphisms on brain gene expression as well as in reporter gene assays. Epigenetic differences are another important source of non-coding functional variations, and alterations in the patterns of both CpG methylation and histone modifications in gene promoter regions have been associated with a number of psychiatric disorders, including suicide (32;37-39;42;43). To determine if methylation is involved in determining the levels of expression of SMS and SMOX, we characterized promoter CpG methylation patterns and levels of trimethylated histone-3-lysine 27 (H3K27me3) in the prefrontal cortex from a sample of suicide completers and healthy controls.

Materials and Methods

1.0 Genotyping

1.1 Subjects

Our sample consisted of 96 male French-Canadians. This sample included brain tissues obtained from 40 subjects, comprised of 14 healthy controls, 10 non-depressed, and 16 depressed suicide completers. These samples were obtained from the Quebec Suicide Brain Bank, and the cause of death was assessed by the Quebec Coroner's office. The remaining 56 subjects were obtained from a cohort of individuals representative of the Quebec general population (described in (435)).

Psychiatric diagnoses of brain tissue donors were obtained using the psychological autopsy method with the Structured Clinical Interview for DSM-IV Axis I (SCID-I) (480), as described elsewhere (528). Individuals from the cohort analyzed in this study were free of psychopathology and history of suicidal behavior as assessed by a large number of instruments and measures (435).

Written informed consent was obtained for each subject or next of kin, as applicable. This study was approved by our local institutional review board.

1.2 Analysis of Polymorphisms

We defined the promoter regions of SMS (NM_004595) and SMOX (NM_175842) as comprising the sequence spanning 5000-bp upstream from the transcription start site (TSS) to 500-bp into the first intron. SNPs from these regions were selected from public databases including the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), Ensembl (www.ensembl.org), and HapMap (www.hapmap.org).

Genomic DNA was extracted from blood, saliva, or frozen brain tissue using standard procedures (483), and SNP genotyping was performed using the SNaPShot method with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Genotypes were determined using GeneMapper, version 3.7 (Applied Biosystems). Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium were assessed using Haploview version 4.1 (529).

2.0 Expression

2.1 Correlation of Expression with Genotype

Microarray analyses were performed on the 40 brain tissue donors described above in brain regions BA 8/9, BA 47, and the hippocampus. We focused on these regions as previous work indicates polyaminergic dysregulation in prefrontal tissues and the hippocampus in suicide (64;157). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA samples used for analysis possessed A260/A280 > 1.9 and 28S/18S rRNA peak height ratios > 1.6. Samples were analysed using the HG-U133 A/B set, and GeneChip analysis was performed with Microarray Analysis Suite version 5.0 (MAS 5), Data Mining Tool 2.0. All genes were globally normalized and scaled

to a signal intensity of 100. RNA quality indicators used to filter samples prior to analysis included β-actin 5'/3' ratio, glyceraldehyde-3-phosphate dehydrogenase 5'/3' ratio, RawQ (noise), scale factor and percent of "present" calls per array. Outlier subjects were excluded in regions where they did not pass quality standards.

In order to determine if promoter haplotypes were related to brain expression, ANOVAs were performed with $\log_2$-transformed expression values from probesets 202043_s_at (SMS) and 210357_s_at (SMOX) (www.Affymetrix.com) and haplotypes determined through genotyping, P-values were corrected for 3 tests, with corrected $P \leq 0.05$ considered significant.

2.2 Cell Culture

Dual-reporter gene assays were performed in three different cell lines: COS7 (green monkey kidney), HTB15 (human glio/astrocytoma), and CRL2137 (human neuroblastoma) (American Type Culture Collection, Manassas, VA). All cultures were maintained at 37°C with 5% CO_2, and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Canada) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco).

2.3 Cloning

A 2.1-kb region of SMS and a 1.4-kb region of SMOX were cloned into the pGL3 Basic Luciferase Reporter Vector (Promega, Madison, MI), upstream of the firefly luciferase gene. These regions are depicted in Fig 2B and 3B, and primer sequences are shown in the Supplementary Table S1. All inserts were sequenced.

2.4 Transfection and Dual-Reporter Gene Assays

Cells were seeded into 24-well plates and grown at 37°C for 24 hours to reach 90-95% confluency. Transfections were then performed using either

Lipofectamine 2000 (Invitrogen, Canada) or GeneJuice (Novagen, Canada) systems, as appropriate for each cell line. Each well was treated with equivalent amounts of DNA for each of the vectors of interest, as well as with an internal control vector coding for renilla luciferase, pRL (Promega). Cells were grown for an additional 24 hours at 37°C prior to assaying luciferase activity. Reporter gene expression was measured using the Dual-Luciferase Assay system (Promega) using an Orion II Microluminometer (Berthold, Canada). Each vector was analyzed in triplicate within individual experiments, and three independent experiments were performed for each cell line. Firefly luciferase expression values were normalized to those of the internal control. ANOVAs and unpaired Student's *t*-tests between haplotypes were performed, with $P \leq 0.05$ considered significant.

3.0 Methylation in BA 8/9

3.1 Subjects

A subset of 20 subjects taken from our 40 brain samples was examined, and comprised 10 control subjects and 10 suicide completers. Subjects were matched for age, post-mortem interval, and pH.

3.2 CpG Methylation Mapping

Genomic DNA from BA 8/9 was extracted as above, then treated and purified with the Qiagen EpiTect Bisulfite kit. Bisulfite-treated DNA was amplified by polymerase chain reaction (PCR) using primers shown in the Supplementary Material. PCR products were purified with the GenElute PCR Clean-up Kit (Sigma-Aldrich), ligated into the pDRIVE vector and transformed into EZ Competent cells (PCR Cloning Plus Kit, Qiagen). Plasmids were extracted and sequenced using primers SMS-ep-R2 or SMOX-ep-R3. Using percentages of CpG methylation, unpaired Student's *t*-tests and Pearson

correlations were used to analyze differences between groups as well as determine the relationship between methylation and expression in BA 8/9, and P-values were corrected for the number of methylated CpG sites in each gene.

3.3 Chromatin Immunoprecipitation of H3K27me3

Immunoprecipitated DNA was prepared as described by Matevossian and Akbarian (548). Briefly, tissue from BA 8/9 was treated with micrococcal nuclease (Sigma-Aldrich), which cleaves DNA between adjacent nucleosomes. Intact nucleosomes were extracted, and a portion of sample was treated with anti-trimethyl-H3-lysine 27 antibody (Upstate Biotechnology, New York), while the remainder was used as an input control. The antibody-treated sample was purified and both input and bound fractions were digested with protease before DNA was purified. Quantitative real time PCR (RT-PCR) using SYBR green (Applied Biosystems) was used to measure DNA in each sample, in quadruplicate. Relative quantitation was performed with β-actin as an endogenous control, and ratios of bound:input were calculated for each subject. Student's *t*-tests and Pearson correlations were used to analyze differences between groups and the relationship between methylation and expression in BA 8/9.

Results

We previously observed altered expression of SMS and SMOX in several brain regions of suicide completers (64;157). In order to identify molecular mechanisms that may be involved in determining the expression levels of these transcripts, we examined genetic polymorphisms in the promoter regions of these two genes and their relationship to expression in the brain and *in vitro*, as well as assessed methylation at both the level of DNA and histones.

Genotyping:

Our first objective was to characterize the haplotype structures of SMS and SMOX in order to identify haplotypes which may be involved in determining expression in the brain. To this end, we genotyped six and fourteen SNPs in the promoter regions of SMS and SMOX, respectively.

The results for SMS are shown in Fig 2. SMS is located on the X chromosome, and as an all-male sample was used in this study, only one allele was observed in each subject. All of the SNPs but rs7066893 were genotyped as part of the HapMap project (440). Two SNPs, rs7066303 and rs7062138 showed no variability in this sample. The other four SNPs produced three haplotypes, as shown in Fig 2D. The LD structure is shown in Fig 2E, and reveals that both pairs of non-adjacent SNPs are in LD with each other.

The results for SMOX are depicted in Fig 3. Eight of these SNPs (rs1741289, rs6052399, rs6139327, rs1741291, rs2422915, rs7265946, rs945772, and rs945771) were genotyped in Hap Map populations (440). No variability was observed at rs7265946, and only one subject possessed the alternate allele at rs6139327. Four haplotypes were identified with a frequency of at least 5% in our population, and represented approximately 96% of all alleles. The LD structure is shown in Fig 2E. Two major haplotype blocks were observed, comprising rs1741289 to rs1764998, and rs6052399 to rs945771. Interestingly, rs1631654 is included as part of the second block. Most of the variability at this locus is found in the first LD block, with only two major haplotypes being found in the second.

Brain Expression:

Our next step was to determine if promoter haplotypes were related to expression levels of SMS and SMOX in the brain. We examined brain regions BA 8/9, BA 47, and the hippocampus as these regions are implicated to be involved in psychiatric disorders (482). In order to determine the relationship between promoter haplotypes and expression, ANOVAs were performed between

expression levels and haplotypes of SMS (Table 1) and SMOX (not shown). We found no association between haplotype and expression for either gene in any of the regions, which suggests that promoter haplotypes may not be responsible for the differential expression of SMS or SMOX in suicide completers.

Dual Reporter Gene Assays:

To further investigate the functional impact of promoter variability on gene expression, we performed dual reporter gene assays to examine the relative promoter activities of the major haplotypes from both genes.

We selected a 2.1-kb region of SMS, as shown in Fig 2B. The region contains 158-bp of exon 1, including the translation start site, as well as all four polymorphic SNPs. Two individuals for each haplotype were sequenced in order to identify additional SNPs in LD with the genotyped SNPs. This revealed two additional SNPs, of which one, rs34507903, has been previously identified. Based upon these results, the three haplotypes examined were TCACGA (haplotype 1), TTACAG (haplotype 2), and CTCAAG (haplotype 3), of which underlined SNPs represent those genotyped in our sample. One vector from each haplotype, as well as the empty pGL3 vector, were used to transfect COS7, HTB15, and CRL2137 cells. As shown in Fig 4A, all constructs had promoter activity compared to the empty pGL3 vector, with average fold changes (FC) of 4.1, 1.8, and 11.6 for COS7, HTB15, and CRL2137, respectively. There were significant differences between haplotypes in COS7 cells (ANOVA $P = 0.04$). In this case, haplotype 2 was expressed significantly higher than haplotype 1 (FC = 1.15, $P = 0.007$) and haplotype 3 (FC = 1.18, $P = 0.04$). This partially agrees with our findings in the brain, where haplotype 1 demonstrated the lowest expression levels. There were no significant differences in HTB15 or CRL2137 cell lines ($P = 0.69$ and 0.39), which suggests that the haplotype effects may be cell-type specific. Alternatively, as the FCs were relatively small, differences in the other cell lines may have been masked by the increased variability between replicates in these assays.

We analyzed a 1.4-kb region of SMOX, which contains all of the upstream sequence studied by Wang and colleagues (558). This region is shown in Fig 3B, and contains three of the variable SNPs from the second LD block, giving two haplotypes: CGC (haplotype 1) and GTG (haplotype 4). Sequencing of this region did not reveal any additional SNPs. The results for the two haplotypes are shown in Fig 4B. Both constructs demonstrated promoter activity, with FC of 88.5, 4.0, and 36.7 in COS7, HTB15, and CRL2137 cells, respectively. However, there were no significant differences between the two haplotypes in any of the cell lines examined (P = 0.70, 0.61, and 0.84).

CpG Methylation:

Another possible mechanism for regulation of gene expression is by epigenetic control, which is mediated through different mechanisms, an important one being promoter methylation at CG dinucleotides (559). In order to determine if CpG methylation was involved in the differential expression of SMS and SMOX in suicide, we analyzed CpG methylation in ten suicide completers and ten healthy controls. This analysis was performed in BA 8/9, as previous studies have identified the prefrontal cortex as being an important source of differential patterns of CpG methylation in suicide (32;38;39).

We examined the 741-bp region of SMS shown in Fig 2C. This region has a 62% GC content, and contains all of exon 1 as well as 567-bp of upstream sequence. The results for methylation at individual CpG sites are shown in Fig 5A. Overall, there was a low level of methylation, with an average of 2.4% at all CpG sites. Five sites were completely unmethylated, and the majority of other sites were methylated in less than 5% of all clones. The main exception was the site at -471, which was methylated in 36% of clones. There was no difference in overall methylation between suicide completers and controls (P = 0.57), nor was methylation at any individual sites significant after correcting for multiple testing. We also assessed the relationship between methylation and expression of SMS in

BA 8/9. These results are shown in Fig 5B. We found no relationship between expression and methylation overall (P = 0.57), or at specific sites.

The 566-bp region of SMOX is shown in Fig 3C, and has a 72% GC content. The results for each site are shown in Fig 6A. As with SMS, this region was only sparsely methylated, with an average of 3.1% methylation. We did not identify any relationship between methylation and promoter haplotypes. Similar to SMS, there were no differences in either overall (P = 0.92), or site-specific methylation between suicide completers and control subjects, nor was there a significant correlation between overall methylation and expression of SMOX in BA 8/9 (P = 0.19). As shown in Fig 6B, we found a significant correlation between methylation and expression at only one site (+73-bp, corrected P = 0.035, Pearson = -0.7).

Histone-3-Lysine-27 Methylation:

Histone modifications are another important form of epigenetic regulation. We chose to focus on tri-methylated H3K27, a modification which is associated with gene repression when found in promoter regions (545), and which we recently found to display increased levels in the promoter region of the TrkB receptor in the prefrontal cortex of suicide completers (44). We examined levels of H3K27me3 in the promoter regions of SMS and SMOX in BA 8/9 from the same 20 subjects used for the CpG methylation study. The results of these experiments are depicted in Fig 7. We found no significant differences between levels of H3K27 methylation at the promoter region of SMS (P = 0.80) or SMOX (P = 0.89) in suicide completers compared to controls. In addition, there was no relationship between H3K27 methylation and promoter haplotypes, gene expression, or levels of CpG methylation for either gene (not shown).

Discussion

In this study, we followed up on our previous findings of differential expression of SMS and SMOX in the brains of suicide completers by examining the role of both genetic and epigenetic factors in determining the expression of these two genes.

We identified three major promoter haplotypes in SMS, however we found no relationship between haplotypes and expression in the brain, and differential expression of the haplotypes was only observed in one cell line. We also saw no relationship between brain expression and either of the epigenetic mechanisms we examined. Promoter CpG methylation in SMS has previously been examined in bipolar disorder, although findings were inconclusive, and, in agreement with our results, methylation was not found to be significantly correlated with SMS expression (560).

The overall percentage CpG methylation and levels of H3K27me3 in the promoter region of SMS did not differ between suicide completers and controls, although this is not overly surprising as we saw no differences in expression between these two groups in BA 8/9. We also found no indication that promoter haplotypes were involved in the increased expression of SMS in the hippocampus of depressed suicide completers.

The haplotype structure of SMOX was more complex than that of SMS. We were unable to identify any relationship between various haplotypes and the expression of SMOX in prefrontal brain regions. However the complexity at this locus and the fact that unlike SMS, individuals possessed two alleles at each SNP, may have prevented us from identifying any small magnitude effects. Nonetheless, it does appear unlikely that the second LD block plays a role in determining expression under normal conditions, as there were no significant differences between the two haplotypes in this block in reporter gene assays. However, this region has previously been shown to be responsive to polyamine analogues (558), and it remains possible that the two haplotypes may differ in their ability to induce SMOX expression in the presence of polyamines.

We also examined CpG methylation of the promoter region of SMOX and found that methylation at one CpG site in exon 1 was significantly negatively correlated with expression, which could indicate that this site plays a regulatory role in determining transcription levels. As with SMS, we did not identify any significant differences in CpG or H3K27 methylation in the promoter region of SMOX between suicides and controls. Interestingly, SMOX has been observed to display monoallelic expression in a human cell line (561), which could have obscured the effects of genetic and epigenetic factors involved in determining expression.

Overall, we failed to identify molecular mechanisms with significant effects in determining the levels of SMS and SMOX transcripts, and which could explain the differential expression of these genes in suicide completers. Although we found little evidence for a role for genetic polymorphisms in determining transcript levels, this study focused only on SNPs within the upstream promoter regions of these genes. Introns also play important functions in regulating levels of gene transcripts by providing additional sites for transcriptional regulatory elements, influencing alternative splicing and nonsense mediated mRNA decay, and allowing export of mRNA to the nucleus (562): and each of these mechanisms may be influenced by polymorphisms in these genes. In addition to their regulation at the level of gene transcription, sequences in the processed transcript can affect RNA stability and rates of degradation. Indeed, SMOX mRNA has been found to be stabilized by polyamine analogues (558), indicating the importance of post-transcriptional mechanisms in regulating levels of SMOX transcripts.

Although we found no evidence for a role of H3K27 methylation in determining the expression levels of SMS or SMOX, numerous other histone modifications are known to play a role in regulating various aspects of transcription (549). Indeed, the expression of two polyamine metabolic genes, ornithine aminotransferase and μ-crystallin, has previously been shown to be influenced by elevated methylation at H3-arginine 17 in prefrontal cortices of schizophrenia patients (42). We thus cannot rule out the possibility that

modifications at the chromatin level are involved in regulating the expression of SMS or SMOX.

There are a number of possible limitations that should be considered. Our study was conducted in a sample composed exclusively of French Canadians, a population with a well-documented founder effect (450). It is therefore possible that the frequency and properties of the haplotypes investigated in this study are different in other populations. Similarly, as this study was performed in only male subjects, the applicability of these results to females is unclear, particularly as SMS is found on the X chromosome. Although the subjects used for the epigenetic studies were matched for age, pH and PMI, it is possible that additional factors may have confounded our results. It is equally possible that the sample investigated in this study lacked power to detect methylation effects of discrete magnitude.

In summary, we found little evidence to support roles for either genetic or epigenetic factors in regulating the expression of SMS and SMOX. It is clear that additional mechanisms are involved in determining the levels of SMS and SMOX transcripts in the brain, and further work will be required to identify factors responsible for the dysregulated expression of these genes in suicide completers.

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Figures

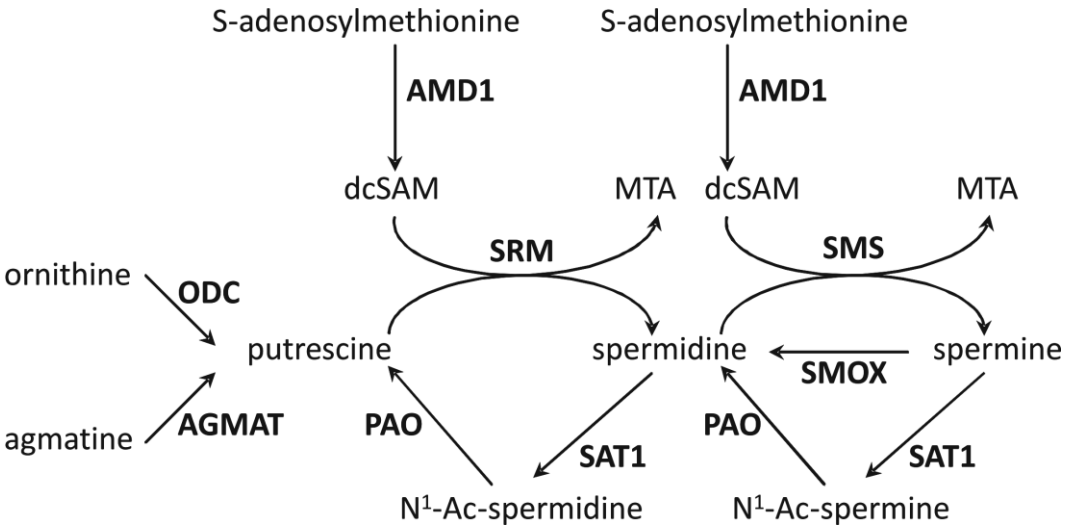


Figure 1: Polyamine synthesis and interconversion pathways. Ac: acetyl, AGMAT: agmatinase, AMD1: S-adenosylmethionine (SAM) decarboxylase, dcSAM: decarboxylated SAM, MTA: 5' methylthioadenosine, ODC: ornithine decarboxylase, PAO: polyamine oxidase, SAT1: spermidine/spermine N^1-acetyltransferase, SMOX: spermine oxidase, SMS: spermine synthase, SRM: spermidine synthase.

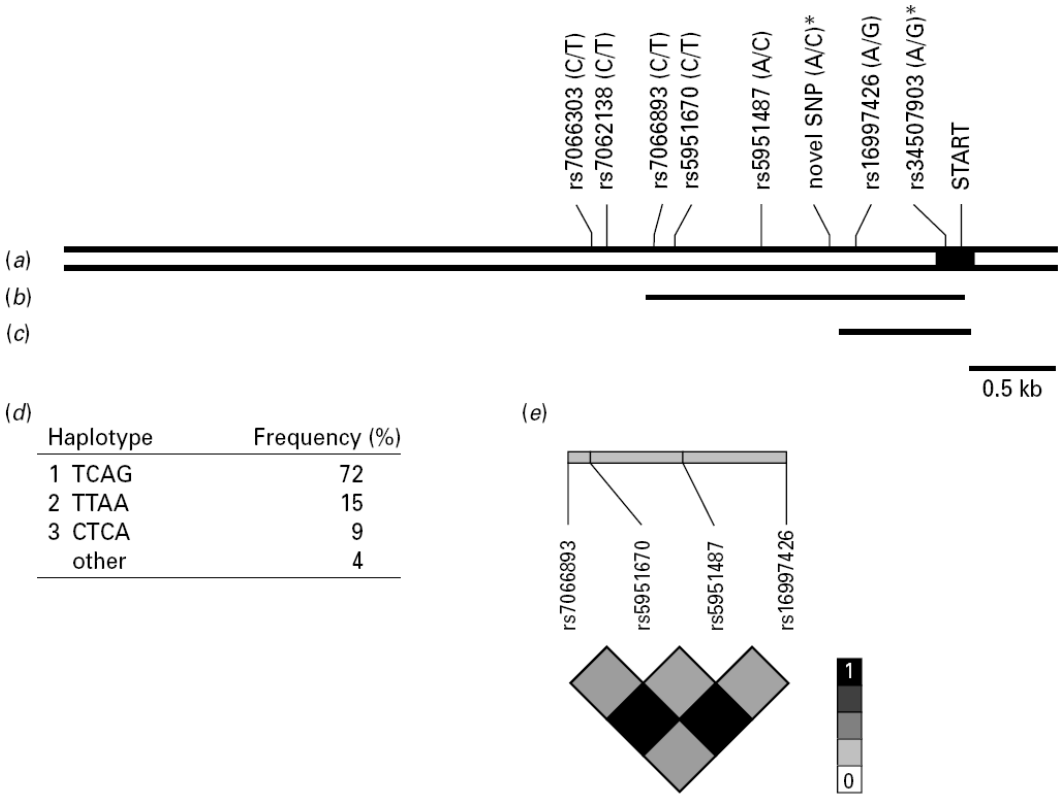


Figure 2: Regions and polymorphisms of the SMS promoter analyzed in this study. (a) Location and sequences at polymorphic sites. Exon 1 is shaded in black, and the translation start site is indicated. (b) Region analyzed for reporter gene assays. SNPs with stars were identified during sequencing of this region. (c) Region analyzed for CpG methylation. (d) Haplotypes and frequencies of genotyped polymorphisms. (e) Linkage disequilibrium between polymorphisms plotted as r^2.

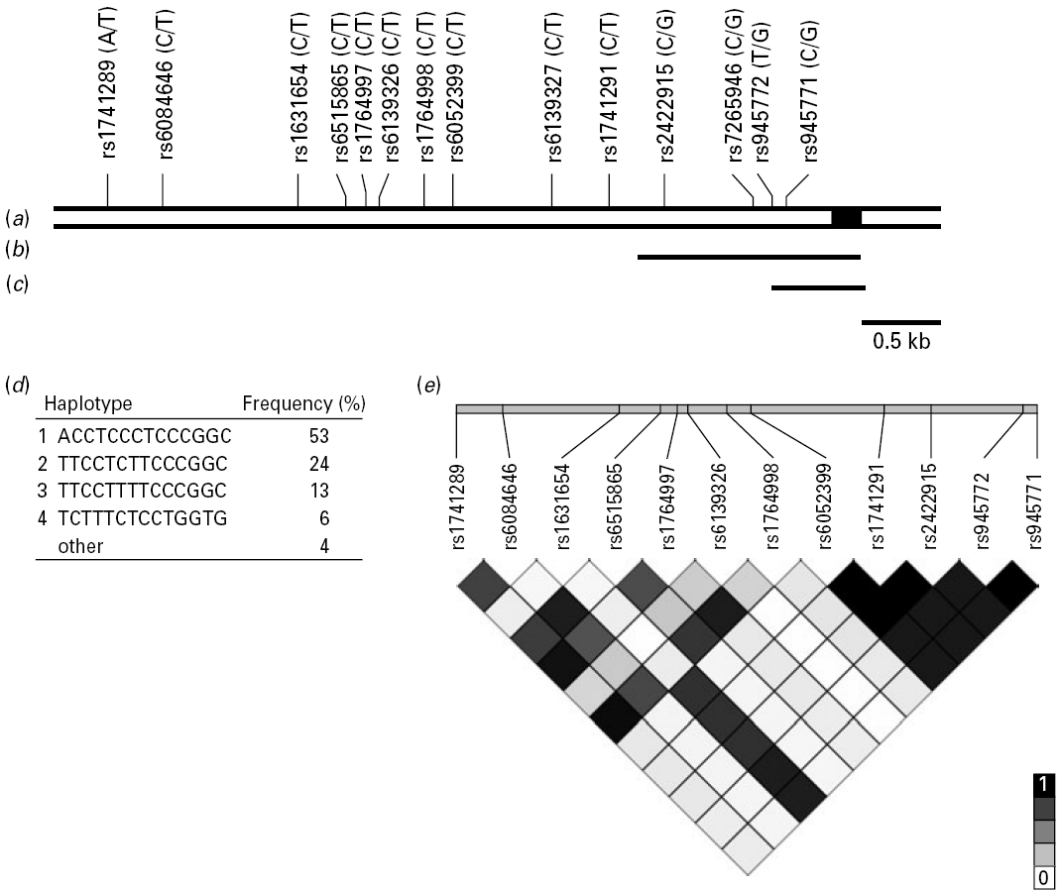


Figure 3: Regions and polymorphisms of the SMOX promoter analyzed in this study. (a) Location and sequences at polymorphic sites. Exon 1 is shaded in black. (b) Region analyzed for reporter gene assays. (c) Region analyzed for CpG methylation. (d) Haplotypes and frequencies of genotyped polymorphisms. (e) Linkage disequilibrium between polymorphisms plotted as r^2.

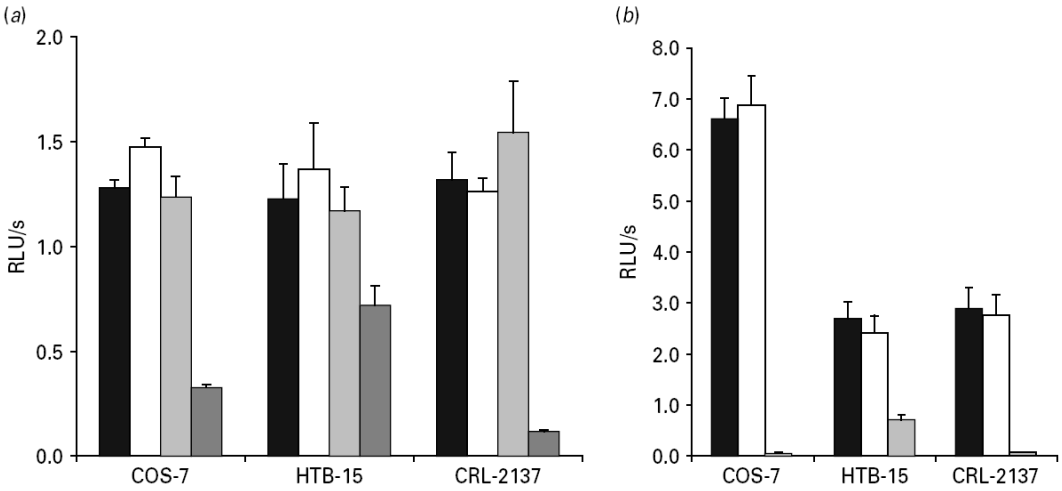


Figure 4: Results from reporter gene studies in COS7, HTB15, and CRL2137 cell lines. Normalized relative light units/second (RLU/s) and standard errors were obtained from triplicate replications of each assay. (a) SMS haplotype 1 (black), haplotype 2 (white), haplotype 3 (grey), and empty pGL3 (striped), and (b) SMOX haplotype 1 (black), haplotype 4 (white), and empty pGL3 (grey).

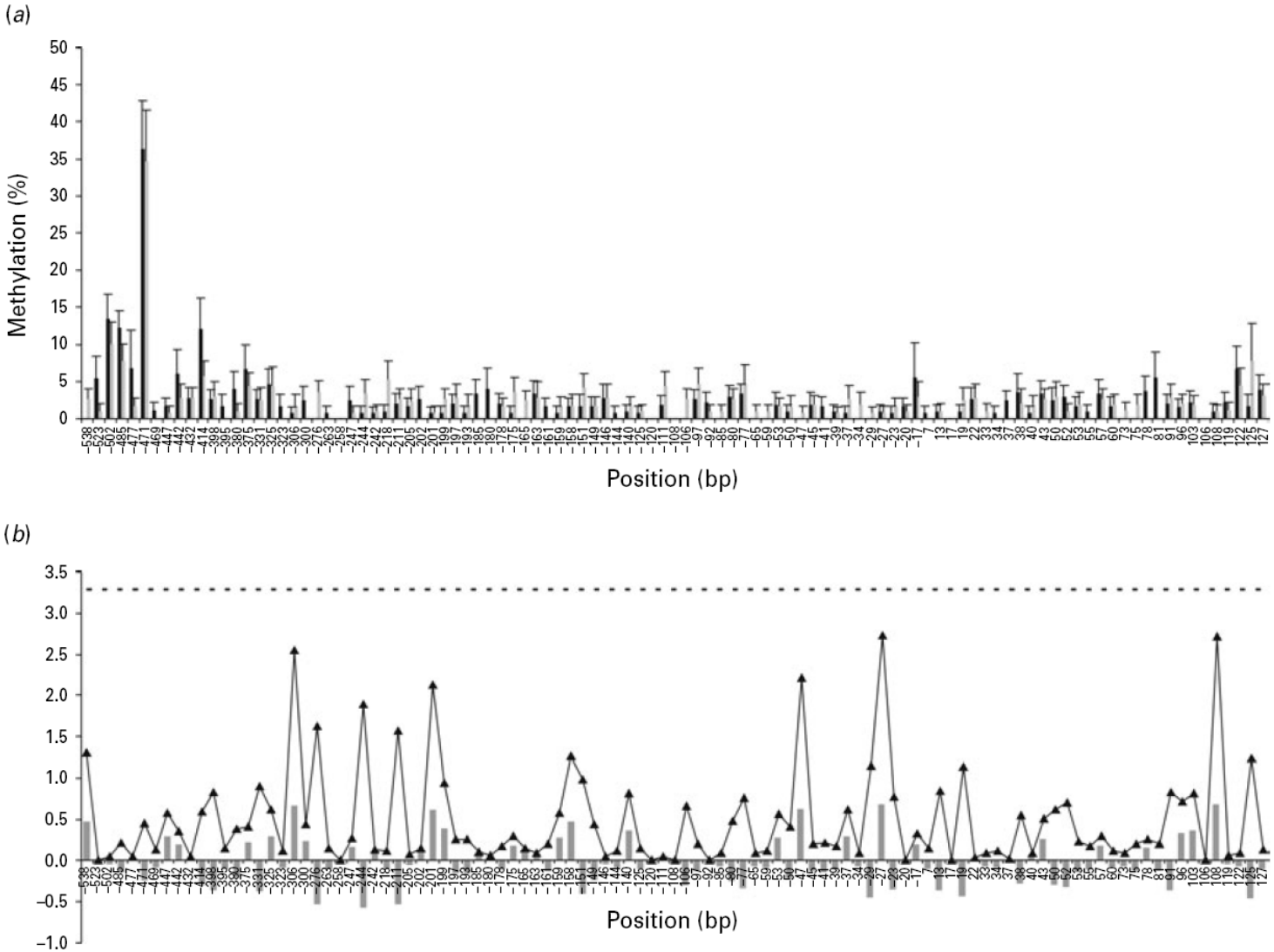


Figure 5: Methylation of CpG sites in the promoter region of SMS. Sites are shown as position relative to the transcription start site. (a) Percentage methylation + SEM for suicide completers (black) and controls (grey). (b) Pearson correlation (grey bars) and $-\log_{10}$ P-values (solid black line) for correlation of percentage methylation with SMS expression. The dotted line indicates corrected P = 0.05.

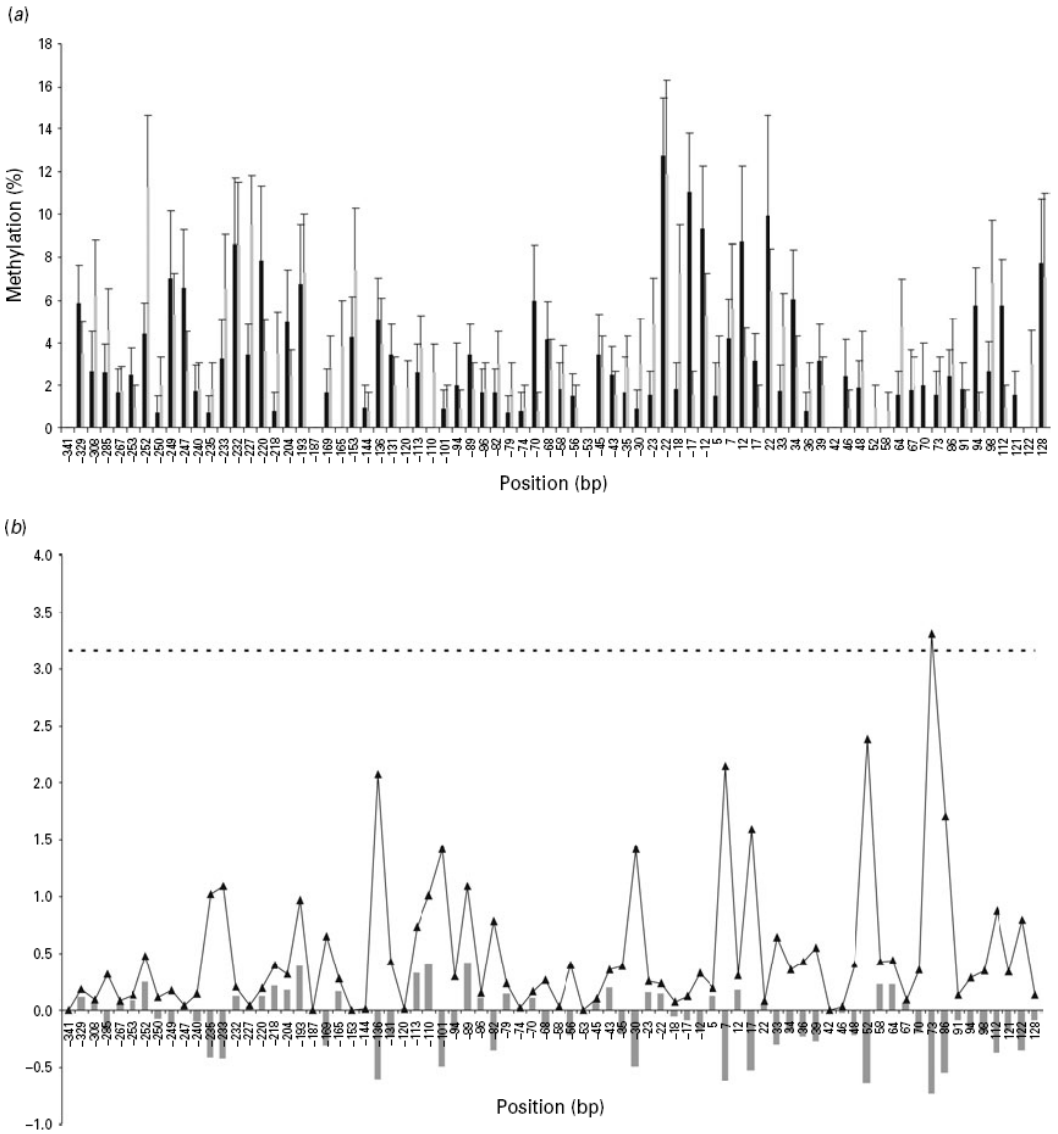


Figure 6: Methylation of CpG sites in the promoter region of SMOX. Sites are shown as position relative to the transcription start site. (a) Percentage methylation + SEM for suicide completers (black) and controls (grey). (b) Pearson correlation (grey bars) and $-\log_{10}$ P-values (solid black line) for correlation of percentage methylation with SMOX expression. The dotted line indicates corrected P = 0.05.

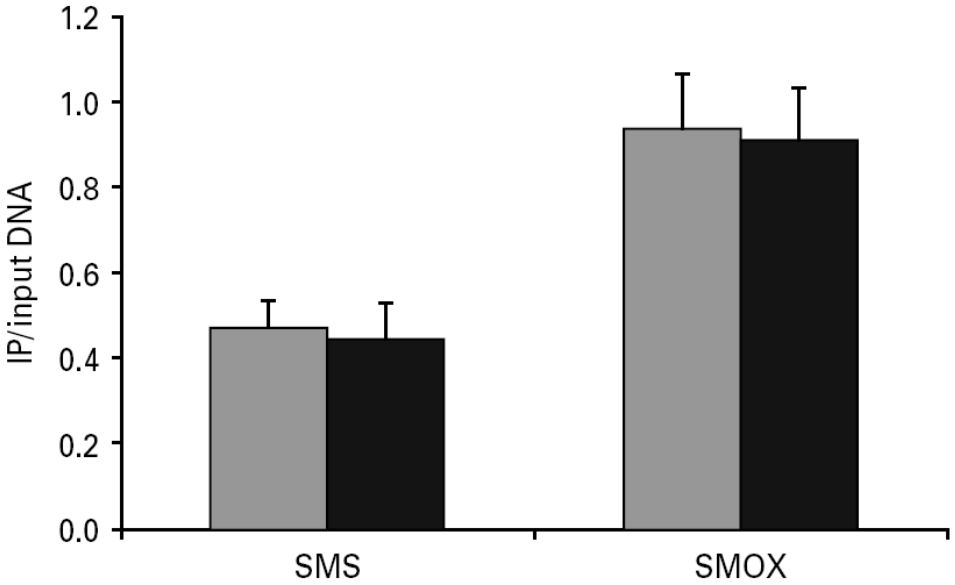


Figure 7: Methylation of H3K27 in the promoter regions of SMS and SMOX. Methylation + SEM for controls (grey) and suicides (black) are expressed as the ratio of immunoprecipitated DNA to input DNA.

Tables

Table 1: Relationship between haplotype and brain expression of SMS. Corrected ANOVA P-values and mean log_2-expression are shown for subjects with each haplotype.

		Haplotype		
Region	**ANOVA**	**1**	**2**	**3**
BA 8/9	0.09	7.42	7.70	7.86
BA 47	0.14	7.29	7.47	7.55
hippocampus	0.85	7.13	6.89	7.24

BA: Brodmann area.

DISCUSSION AND CONCLUSIONS

Overall Findings and Implications:

When the studies contained within this thesis were first initiated, our knowledge regarding the involvement of the polyamine system in suicide was restricted to three gene expression studies demonstrating altered expression of a few polyamine-related genes in the brains of suicide completers (64;157;204), and one study showing a significant association between a polymorphism in the promoter region of one of these genes and completed suicide (204). Given that altered expression of the polyamine gene SAT1 was among the most significant findings arising from our gene expression studies of suicide, and that the involvement of the polyamine system had not been previously suspected in suicide, we were greatly interested in expanding our knowledge regarding the role of this system in the neurobiology of psychiatric disorders. To address this issue, a series of experiments were designed in order to examine concepts related to the extent by which polyamine and related metabolic pathways are affected in the brains of suicide completers, if the polyamine system may also be implicated in other psychiatric conditions, and to identify the molecular mechanisms responsible for the dysregulated expression of polyamine-related genes in suicide.

The study described in Chapter 2.1 was primarily designed to address the first research question in order to characterize the extent by which polyamine and related metabolic pathways display differential gene expression in the brains of suicide completers. While our previous microarray experiments had examined only a few brain regions, and assessed gene expression across the entire transcriptome, this study greatly increased the coverage of the brain, and pre-selected probesets related to polyamine metabolism in order to reduce the amount of required statistical corrections. The overwhelming conclusions arising from this study were that the expression of genes related to polyamine metabolism was extensively altered in suicide completers, and that the downregulated expression of SAT1 remained one of the most pervasive changes observed in brains of

suicide completers. Moreover, we identified altered expression of four genes, AMD1, OAZ1, OAZ2, and ARG2, which play essential roles in the early steps of polyamine biosynthesis. These results were particularly interesting given that among our previously-identified genes, SAT1, SMOX, and SMS all act at higher steps in polyamine metabolism. Thus, not only did these new findings confirm our previous results for SAT1, SMOX, and SMS, but they also demonstrated that alterations in polyamine metabolism are found at all levels of biosynthesis and catabolism. Additionally, this study identified dysregulated expression of several genes related more distally to polyamine metabolism, indicating that altered expression of polyamine metabolism does not occur in isolation. Rather, it comprises one part of a network of arginine- and proline-related metabolic genes which are dysregulated in suicide completers.

While both our previous results and those in Chapter 2.1 examined the involvement of the polyamine system in suicide completers, the studies described in Chapter 1.3 strongly suggested that alterations to the polyamine system may be characteristic of a variety of psychiatric conditions. The experiments described in Chapter 2.2 were thus designed to examine the relationship between genetic variants in polyamine-related genes and a variety of psychiatric conditions - including suicide attempts, mood disorders, and anxiety disorders – and subsequently to characterize how these effects may be mediated by environmental or psychological variables. Each of the four genes examined, SAT1, SMS, SMOX, and OATL1, displayed significant associations to at least one of the main outcomes, and we observed evidence of several gene-environment and gene-gene interactions. Interestingly, the results for SAT1 indicated that the minor allele of rs6526342, which was previously associated with completed suicide, was associated with higher rates of mood disorders yet lower rates of anxiety disorders. As our previous results for this SNP found a lower proportion of the minor allele in suicide completers, these results indicate distinct roles of SAT1 in these conditions. Overall, this study established that alterations in the polyamine system are not constrained to completed suicide, but that they are also involved in conferring risk for attempted suicide, as well as mood and anxiety disorders.

These findings indicate that the involvement of the polyamine system in suicide is complex, and that alterations in polyamine metabolism reflect a combination of factors related to suicidal behaviors and co-morbid psychiatric conditions. However, these findings also offer hope that treatment of polyamine-related perturbations may not only reduce or prevent suicidal behaviors, but may also ameliorate symptoms associated with other psychiatric conditions.

The findings from the first two studies indicated that not only is altered polyamine metabolism part of a larger network of metabolic genes displaying dysregulated expression in suicide, but it also plays a role in a variety of psychiatric conditions. Intriguingly, several of our initially-identified genes, as well as a number of other genes which have previously been associated with suicidal behaviors, are located on the X chromosome. Our next question was therefore to determine if our findings of alterations in the expression of polyamine-related genes could be extended to proximally located genes, as they may be subject to similar factors regulating their expression. Moreover, we hypothesized that the X chromosome was a strong candidate for genes which could be involved in the large gender differences associated with attempted and completed suicide. The study described in Chapter 2.3 was therefore undertaken as a means to identify chromosomal locations harbouring genetic risk factors associated with suicide completion, and to determine if genes found within these regions display differential expression in the brain (31). Not only did this study accomplish both these objectives, but it also demonstrated that two of the polyamine-related genes on the X chromosome, SAT1 and SMS, are found within regions strongly associated with suicide. These results provided even further support for the involvement of these two genes in suicide, and suggested that factors related to their differential expression may lie in nearby chromosomal locations.

Arising from the studies in Chapter 2 was the notion that not only are genes related to polyamine metabolism differentially expressed in the brains of suicide completers, but that these alterations in polyamine metabolism likely result from the effects of specific genetic variations combined with developmental and

environmental factors. Our next objective was to elucidate the mechanisms by which these genetic and environmental factors are involved in conferring risk for suicide and other psychiatric disorders, as these represent important targets for treatment as well as potential biomarkers for these conditions. With this in mind, we undertook the studies described in Chapters 3 and 4 in order to identify and characterize genetic and epigenetic factors which may be involved in influencing the expression of SAT1 (Chapter 3), SMS, and SMOX (Chapter 4).

Given the widespread decreases in SAT1 expression across the brains of suicide completers, as well our previous association study and the genetic findings from Chapters 2.2 and 2.3, this gene appeared to be an ideal candidate to harbour important genetic factors directly influencing its expression. Additionally, given that the effects of a number of SAT1 SNPs appeared to be related to environmental influences on the risk for mood disorders, we were also interested in assessing the effect of epigenetic factors on SAT1. The three studies described in Chapter 3 were thus aimed towards elucidating the genetic and epigenetic factors associated with SAT1 expression and understanding the mechanisms by which they exert their effects. Overall, these studies highlighted the involvement of both genetic and epigenetic mechanisms in regulating SAT1 expression, as well as provided a functional link through which the SNPs associated with psychopathology exert their phenotypic effects. Promoter haplotypes were identified which were directly involved in controlling SAT1 expression, and three specific promoter variants responsible for these effects were uncovered (207). Of particular interest was the finding that the minor allele of rs6526342 was on the higher-expressing haplotype, indicating that the lower percentage of this allele in suicide completers may be involved in the decreased expression of SAT1 in the brain. These results also suggested that similar effects may be at play in individuals with anxiety disorders, whereas the opposite effect could be involved in mood disorders. One of the variants on this haplotype, an insertion/deletion of 15 adenine residues, appeared to play a particularly important role in determining SAT1 expression, and was found to be significantly associated with suicide completion in the context of depressive disorders (208). However, it was not

significant when examined in subjects with a mixture of Axis I disorders. It is quite possible that the effect of this insertion/deletion may be stronger in subjects with mood disorders due to the fact that the opposite allele represents the risk allele for these disorders. Although the insertion/deletion was sufficient to determine SAT1 expression in both haplotypes, two other variants, rs6526342 and rs928931, displayed haplotype-specific effects. It was thus of great interest that rs6526342, rs928931, as well as another SNP on this haplotype, rs1960264, generate sites for CpG methylation. Investigation of epigenetic modifications of the promoter region demonstrated that CpG methylation had a significant negative effect on SAT1 expression in the brain, and that rs6526342 and rs1960264 were both highly methylated. Neither levels of the overall CpG methylation nor the H3K27me3 modification were significantly different in suicide completers, although these results may also be due to insufficient statistical power. Indeed, methylation at several specific CpG sites, including rs6526342, displayed a trend for increased levels in suicide completers, which may be involved in the downregulation of SAT1 expression. Altogether, the studies in Chapter 3 provided considerable insight into the molecular mechanisms regulating expression of SAT1, which in turn resulted in a greater understanding of the means through which genetic factors in SAT1 associated with psychiatric conditions may act to confer these phenotypes.

Given the strong evidence regarding the role of genetic and epigenetic factors in regulating SAT1 expression, the next objective was to determine if these represented common mechanisms for the dysregulation of the polyamine system in suicide completers. As both SMS and SMOX displayed consistent findings across microarray experiments as well as strong associations with mood and anxiety disorders, these two genes were selected for the study described in Chapter 4 (392). Although these experiments did serve to better characterize the genetic and epigenetic landscapes of the promoter regions of these two genes, there appeared to be relatively little influence of promoter haplotypes, CpG methylation, or H3K27me3 on influencing gene expression. In some respects, it is not overly surprising that SMS and SMOX do not display the same strong genetic

and epigenetic regulation as SAT1, given that while SAT1 demonstrates a widespread downregulation across the entire brain of suicide completers, the differential expression of SMS and SMOX is constrained to only a few brain regions. While it remains possible that genetic or epigenetic regulators of SMS or SMOX expression do exist but are located outside of the regions examined in this study, it is equally possible that their differential expression is due to other factors in those brain regions, such as altered expression of various transcription factors, microRNAs, or other compounds such as the polyamines themselves.

In addition to the studies described in this thesis, we recently developed and validated several methods to allow for the accurate quantitation of polyamine levels in post-mortem brain tissues (563;564). Using gas chromatography coupled with mass spectrometry in several brain regions obtained from suicide completers and healthy controls, we found that suicide completers displayed elevated levels of putrescine and spermidine (209), as well as decreased levels of agmatine (Chen et.al., In Preparation). While these methods are not capable of distinguishing between free and bound polyamines, nor can they indicate where in the cell the polyamine levels are altered, they strongly support the concept that dysregulated expression of polyamine-related genes has direct effects on polyamine levels, and that changes in cellular polyamine profiles may have important consequences on polyamine targets.

Our initial results regarding the decreased expression of SAT1 and SMOX, combined with the elevated expression of SMS, had been theorized to result in increased levels of the higher polyamines with a concomitant decrease of lower polyamines such as putrescine and agmatine. This concept became less clear when combined with the newer gene expression results in which elevated expression of AMD1 and ARG2 would have been expected to increase polyamine biosynthesis whereas elevated expression of the ODC antizymes, OAZ1 and OAZ2, should have decreased conversion of ornithine to putrescine. Our measurements of agmatine, putrescine, and spermidine indicate that the picture is even more complicated. Although measurements of spermine and ornithine will be required to gain a more complete profile, it seems clear that while some of the

alterations in gene expression are involved in the etiology of suicide, others likely represent compensatory mechanisms.

Putative Mechanisms:

Given the extensive and diverse cellular functions of the polyamines, it is difficult to pinpoint their precise roles in the etiology and pathology of psychiatric disorders. Moreover, given that the polyamine system has been implicated in a variety of psychiatric conditions, it seems likely that multiple downstream effects of altered polyamine metabolism are relevant, and that these act in conjunction with other systems involved in suicide, schizophrenia, mood disorders, and anxiety disorders to produce each specific phenotype. It is evident that while alterations in the polyamine system likely have direct functional consequences on polyamine-influenced systems, indirect functions, such as those arising from dysregulated expression of genes more distally related to polyamine metabolism, are also relevant. This section discusses several potential mechanisms by which alterations in polyamine metabolism may be involved in the development and neurobiology of suicide and other psychiatric disorders.

Among the direct effects of the polyamines, their involvement in influencing the electrophysiological properties of NMDARs has been among the best-studied neurobiological function of the polyamine system. The role of these receptors in schizophrenia and depression continues to gain support, and dysregulated expression of genes related to glutamatergic neurotransmission has been among the most consistent findings in gene expression studies of suicide completers. Alterations in polyamine concentrations may have an important effect on transmission through NMDARs, and the antidepressant effects of putrescine, agmatine, and NMDAR antagonists which act through the polyamine binding site lend support to the involvement of this mechanism in suicide and other psychiatric conditions. In addition, spermidine has been shown to inhibit the binding of haloperidol, ifenprodil, trifluperidol, and spiperone to NMDARs (342;343), which may be relevant for treatment responses. NMDARs also play

essential functions in learning and memory, and many studies have shown that experimental manipulation of polyamine levels has direct effects on memory tasks through glutamatergic neurotransmission, which appear to be particularly relevant for anxiety-like behaviors (565;566).

GABAergic neurotransmission has also been among the pathways which have been consistently implicated in gene expression studies of suicide, and this system has been shown to be influenced by the polyamines. Functioning of GABA receptors has been shown to be modulated by spermine, possibly through an inhibition of receptor desensitization (217). While the binding of GABA itself does not appear to be altered by polyamines (216), binding of diazepam, a positive allosteric modulator of GABA receptors, is potentiated by putrescine, spermidine, and spermine (215;216). In addition, polyamine catabolism can lead to synthesis of GABA through reactions involving diamine oxidase or monoamine oxidase (341), thus indicating the potential for a direct connection between alterations in polyamine metabolic enzymes and GABAergic functioning.

The catecholamine systems also appear to be influenced by the polyamines. Polyamine depletion decreases adrenal concentrations of dopamine, epinephrine and norepinephrine (210), while polyamine administration produces effects, including behavioral sedation, cortical synchronization, and inhibition of methamphetamine-induced behavior, which are similar to those observed with the classical neuroleptics whose therapeutic effects result primarily from their modulation of dopamine neurotransmission (213). Evidence indicates that the polyamines also act on dopaminergic pathways but through different mechanisms than the neuroleptics, such that polyamine administration produces effects on the mesolimbic dopamine system similar to that of dopamine receptor antagonists, but without the effects on striatal dopamine functions typically seen with neuroleptics (211). Spermine has been shown to inhibit the binding of cocaine to dopamine transporters, with kinetics suggesting a non-competitive interaction with the ligand binding site (212). Finally, some of the antidepressant effects of agmatine involve α2-adrenoceptors (221;222).

There is also evidence supporting a connection between the polyamines and serotonergic neurotransmission, although these effects appear largely constrained to agmatine. Specifically, the antidepressant effects of agmatine involve several serotonin receptors (223), and modulation of imidazoline receptors by agmatine has been implicated in the antidepressant effects of SSRIs (567). While extracellular spermine and spermidine have been shown to block the hyperpolarization-activated transient current of serotonin transporters, this effect was only observed at high polyamine concentrations, suggesting that this effect may not be significant *in vivo* (568). Furthermore, the binding of paroxetine to the serotonin transporter was not affected by spermine, suggesting that the polyamines have little influence on serotonin reuptake (212).

The nitric oxide pathway may also represent an important site for the involvement of dysregulated polyamine metabolism in psychiatric conditions. This pathway has been implicated in several psychiatric disorders, including schizophrenia, depression, panic disorder, and suicide (299;569-571), and is directly related to polyamine metabolism through arginine. Indeed, induction of arginase activity has been shown to decrease nitric oxide synthesis by increasing polyamine biosynthesis (416), and agmatine has been found to inhibit nitric oxide synthase (218). Given the importance of the nitric oxide system on other neurotransmitter systems (570;572;573), modulation of this pathway by polyamines and polyamine metabolism may have wide-reaching neurobiological consequences.

Finally, interactions between the polyamine system and imidazoline binding sites offer a particularly interesting mechanism by which altered polyamine metabolism may be involved in suicide. Although the existence of imidazoline binding sites has been recognized for several decades, efforts to characterize both the substrates and the receptors are ongoing. Nonetheless, it has been well established that these sites are distributed both peripherally and centrally, and are bound by a variety of substrates, including agmatine (193). Additionally, research has shown that imidazoline binding sites are involved in a variety of physiological effects, among which alterations in monoamine synthesis and neurotransmission

are particularly relevant for suicide and other psychiatric disorders (574). Indeed, alterations in imidazoline receptor binding sites have been implicated in depression and anxiety, and their importance in modulating behavioral stress responses has become accepted (575-577). Moreover, some of the antidepressant and anxiolytic effects of agmatine involve imidazoline receptors (226;366;567), strongly suggesting that imidazoline binding sites play meaningful roles in the relationship between polyamines and psychopathology.

Ancillary to their direct actions at specific receptors and other polyamine-binding sites, the polyamines play essential roles in a myriad of additional cellular functions. Considerable research has focused on their involvement in cellular growth, replication, and death, with particular emphasis on their roles in cancer (578). While suicide and other psychiatric conditions do not display such extreme alterations in cell numbers or function, numerous studies have demonstrated a variety of neuroanatomical changes associated with these disorders (482), and the importance of neurogenesis in the development and treatment of psychiatric disorders has been increasingly recognized (192;579). It is therefore of great interest that manipulation of the polyamine system produces significant effects on both central nervous system development and adult brain neurogenesis (328;580), and that variations in polyamine levels are associated with both pro- and anti-apoptotic effects in neuronal cells (581;582). Several studies have found relationships connecting the neurogenic and neuroprotective effects of the polyamines with stress and depression (390;583;584), providing evidence that dysregulation of the polyamine system may act in part through these mechanisms.

While it is clear that there are numerous mechanisms by which alterations in polyamine metabolism may influence psychopathology by affecting the physiological functioning of the polyamines, it may also be proposed that some of the effects of altering their concentrations result from decreasing the availability of substrates for other pathways. The use of SAM in polyamine biosynthesis is of particular distinction given that this molecule is used as a methyl donor for production of several neurotransmitters and other important CNS molecules (369). Of further interest is the relationship between polyamine metabolism and

epigenetic modifications, including DNA methylation and histone tail modifications, which also use SAM as the methyl donor (407-410).

Future Work:

Certainly the studies comprising this thesis have done much to expand our knowledge regarding the involvement of the polyamine system in suicide, as well as to extend these findings to other psychiatric conditions. However in doing so, they have also generated many new questions regarding how each aspect of dysregulated polyaminergic functioning interacts to confer the phenotypes examined in these studies, the precise molecular mechanisms involved and their downstream effects, and as importantly, the developmental sequence of molecular events that ultimately lead to these alterations. While addressing all of these questions represents a substantial undertaking, several of the more imperative issues are described here.

While the studies described above have demonstrated specific differences in gene expression and polyamine levels in suicide completers, we are still unable to clarify neither which among these represent pathological changes nor which reflect compensatory mechanisms. In addition to obtaining a more complete profile of the metabolic alterations occurring in the polyamine system, efforts must be undertaken to identify the location, both within the cell and in which type of cell, where these effects occur. Given the evidence for cell-type specific effects regarding both polyamine metabolism and storage, and the fact that the polyamines exert their functional effects at numerous intracellular and extracellular sites, addressing these questions is imperative for understanding how the polyamine system contributes to psychiatric phenotypes. In order to properly address these issues, experimental procedures which allow for a more precise examination of the polyamine system, such as *in vitro* hybridization, laser capture microdissection, and fluorescence-activated cell sorting, must be employed.

Another fundamental concept which must be addressed regards the trajectories by which specific genetic and environmental effects combine to

generate the alterations in polyamine metabolism observed in suicide completers. Identifying the developmental sequence of molecular events that occur along the route to completed suicide is invaluable, particularly as this knowledge could provide important information for identifying individuals at risk for completed suicide as well as potential avenues for therapeutic interventions. However, to address this issue poses significant experimental challenges as it requires the study of living subjects. One method to overcome this obstacle could be to identify peripheral markers of altered central polyamine metabolism, then to examine how these markers vary over time and in relation to specific clinical measures. Additionally, animal models could be used to identify metabolic alterations correlated with the development of particular psychological symptoms.

To add to these studies, additional investigations must be performed in order to elucidate the molecular mechanisms involved in the dysregulated expression of polyamine genes in suicide completers. While the studies described in this thesis point towards the involvement of several genetic and epigenetic factors in regulating the expression of SAT1, the mechanisms involved in the differential expression of other polyamine-related genes remain elusive. Moreover, additional studies are required to resolve how the specific SAT1 variants result in the differential expression of each haplotype. Examination of a variety of molecular mechanisms, including differential binding of transcription factors and regulation by microRNAs, in addition to further genetic and epigenetic studies, will be required to address these issues. Of further interest, one particularly fascinating finding which has emerged from our gene expression studies regards the significant and pervasive differential expression of the SAT1 probeset 230333_at, which maps to an intronic region between exons 3 and 4 of this gene. We are currently undertaking studies to identify the nature of this transcript and to examine the possible involvement of differential splicing of SAT1 in suicide completers. In addition to examining alterations in gene expression, studies examining the levels and functionality of the proteins themselves are imperative.

Finally, among the most interesting findings arising from the studies described herein has been the apparent disease-specificity regarding the

polyamine system. While addressing this question is likely to involve a large variety of carefully-planned experiments, the studies described above will provide greater information regarding elements which should be both assessed and taken into consideration when investigating these topics.

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