Molecular and genetic studies of suicide

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Jul. 27, 07

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

ACKNOWLEDGMENTS:

I would like to sincerely acknowledge the support of several groups of people who have contributed to this project in many ways, both from a scientific and a more personal perspective.

First, I would like to thank my supervisor Gustavo Turecki, for always finding a way to come up with new challenges for me and for giving me the freedom to explore new avenues and to develop my ideas and my skills in ways and fields even if they are unorthodox. Also, I would sincerely like to thank present and past members of the lab with whom I've collaborated. The "new students" who always had fundamental questions that forced me to keep my knowledge up to date in order to be able to answer them, for their support and simply for being there for discussions and for the numerous social activities we had. In order of appearance: Nouzha, Alex, Nadia, Caroline, Aleksandra, Yves, Milena, Sherry, Firoza, Alan, Genevieve, Iris, Lilian, Catalina, Noura, Hana, Simon, Tim, Carl, Jaques, Sara-Jane, Gabriel, Marcelo, Sonia, Vesselina and Veronice. Also a special thanks to people with whom I collaborated more closely: Fuad, Aleks, Lilian, Danielle and especially Firoza for her corrections and last minute help.

Most importantly, I want to thank my parents, my family and my friends who were always there with their continual support and encouragements throughout my studies.

STATEMENT OF ORIGINALITY:

This thesis is the work of Adolfo Sequeirà and has been completed solely in fulfilment of the requirements of the degree of Doctor of Philosophy in the Department of Human Genetics, Faculty of Medicine, McGill University. The following elements of the thesis constitute original pieces of work:

- Chapter two is an original review of the literature concerning the usage of the microarray technology for the study of mood disorders and suicide.

- Chapter three concerning the Wolfram syndrome and suicide constitutes the first molecular evidence for a role of the WFS1 gene in suicidal and impulsive behavior.

- Chapter four concerning the alpha 2a receptor gene and suicide was the first investigation of the implication of several genetic variants in this gene in suicide.

- Chapter five constitutes the first large scale gene expression study in brain cortical areas of suicides and revealed for the first time gene expression and genetic variations implicating SSAT in suicide and major depression.

- Chapter 6 is the first analysis of patterns of expression in the limbic system of suicides with and without depression.

- Chapter 7 represents the first neuroanatomical comprehensive gene expression analysis of suicide and clearly links globally synaptic transmission as well as glutamatergic and gabaergic alterations to suicide and depression.

- Chapter 8 is the first study aiming to identify region specific as well as global molecular alterations implicated in suicide and mood disorders.

ABSTRACT

Suicide is a complex social phenomenon and a major public health problem in the world, ranking among the top ten causes of death for individuals of all age groups. In addition to known sociocultural predisposing factors, research in the past decades has pointed to neurobiological factors and more specifically to genetic factors increasing the predisposition to suicide. In this study, we aimed to investigate the influence of genetic variation on suicide using a candidate gene approach and to explore changes in terms of expression at the mRNA level using microarrays. Using the candidate gene approach a possible role for a Wolfram gene polymorphism in the susceptibility to suicide was identified and suggestive evidence was found linking the alpha2A receptor to suicide. Also, using microarrays we identified a gene implicated in the catabolism of polyamines, SSAT, as differentially expressed in the prefrontal cortex of suicides and depressed suicides. Also, a polymorphism in the promoter region of SSAT influencing its expression and associated with suicide was found. Furthermore, gene expression analysis of limbic areas confirmed the implication of polyamines in suicide and depression as well as the implication of neurotransmission and second messenger signaling in the physiopathology of suicide and depression. Finally, in a global gene expression analysis we identified several pathways as being involved in this complex phenomenon and more specifically, an implication of the GABAergic and glutamatergic systems in suicide and depression. In conclusion, suicide is a complex phenomenon involving, at the molecular and genetic level, not only genes but whole systems; thus, underlying the necessity to use genome wide or systematically comprehensive approaches for the study of suicide in the future.

RÉSUMÉ

Le suicide est un phénomène de société complexe et un problème majeur de santé publique. En plus des facteurs socioculturels connus, la recherche au cours des dernières décennies a mis en évidence l'importance de facteurs neurobiologiques et plus spécifiquement génétiques dans la prédisposition au suicide. Dans cette étude, l'influence de facteurs génétiques et des changements au niveau de l'expression des gènes a été investiguée. Dans un premier temps des études d'association ont été conduites sur des gènes candidats, Wolfram et alpha-2A, avec des résultats positifs dans le cas du premier gène le liant à la prédisposition au suicide et des résultats suggestifs dans le cas du second. Des études d'expression génique à l'aide de micro-puces d'ADN nous ont permis d'identifier un gène, SSAT, impliqué dans le catabolisme des polyamines comme facteur de risque chez des suicidés et chez des dépressifs suicidés. Une étude semblable dans le système limbique, nous a permis de constater des changements globaux dans des mécanismes tels la neurotransmission et les systèmes de messagers seconds dans le suicide et la dépression majeure. Finalement, dans une analyse globale d'expression génique, nous avons identifié plusieurs systèmes impliqués dans ce phénomène complexe et plus spécifiquement des altérations de la transmission GABAergique et glutamatergique. En conclusion, le suicide est un phénomène complexe dans le quel sont impliqués au niveau génétique et moléculaire non seulement des gènes mais aussi des systèmes, ce qui met en évidence la nécessité d'utiliser des méthodes globales d'analyse et une approche plus compréhensive pour l'étude du suicide dans le futur.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Suicide is a complex phenomenon of great magnitude in the world causing more deaths than all wars together. According to the World Health Organization (WHO), suicide accounts for almost 2% of the world's deaths every year (WHO, 2000). Furthermore, in most of the developed countries suicide is among the top-ten leading causes of death for individuals of all ages (Mao et al., 1990) and is the leading cause of death for males younger than 40 years of age (Statistics, 1997).

Suicide is a complex behaviour and probably the result of interactions between biological, psychological and social factors. Suicidal behaviours vary from simple suicidal thoughts, to suicide completion passing through suicidal ideation and suicidal plans. Suicide completion is evidently the most dramatic and extreme form of suicidal behaviour resulting with the death of a person. Despite extensive research in the last decades and some progress in the understanding of factors predisposing to suicide, suicide rates have not decreased and efficient treatments reducing the risk of suicide are not yet available.

Suicide is particularly a major health problem in Quebec since it has one of the highest rates for suicide in the world. Several risk factors for suicide have been identified such as psychiatric disorders. However, the majority of psychiatric patients will never commit suicide thereby implying the role of inherent factors

apart from psychiatric diagnosis. Thus, a better understanding of the suicide phenomenon, as well as a better determination of molecular risk factors that makes some individuals more susceptible to commit suicide is of capital importance in suicide prevention.

In this chapter, first, the phenomenon of suicide in Québec will be presented as well as a clinical perspective of this complex trait followed by an introduction to the neurobiology of suicide with a particular focus on post-mortem studies and genetics of suicide. Finally, the specific hypothesis and objectives of this project will be exposed.

PROBLEMATIC OF SUICIDE IN QUÉBEC

In most western countries suicide is the first cause of death for males aged between 15 and 39 years old (Diekstra, 1993). In Canada, Quebec has one of the highest suicide rates just after the Yukon and the North-Western Territories with a suicide rate of 19 per 100 000 (Figure 1), which is one of the highest in western nations (Institut de la statistique du Québec, 2001).

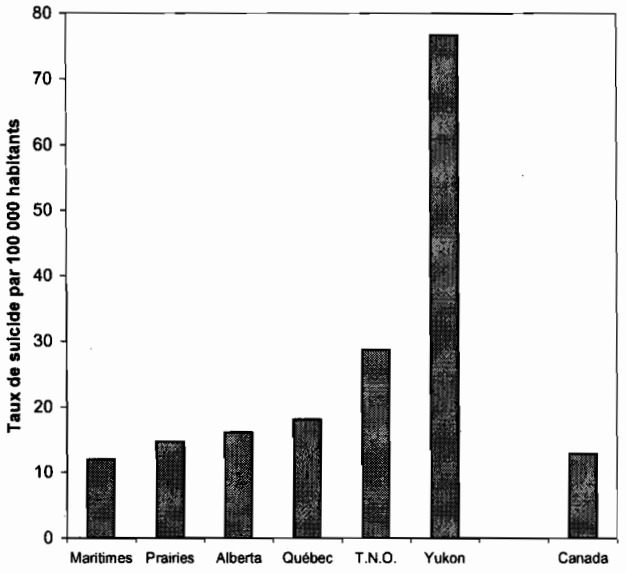

Figure 1 Graphical representation of suicide rates per 100 000 people in some Canadian provinces or territories.

Interestingly, gender appears to influence suicidal outcome, for example in 1995 in Quebec 80% of suicide completers were males with a ratio of 19.5 suicides/100 000 people and suicide was the first cause of death for males aged between 15 and 29 years old, a group with an alarming suicide ratio of 45 suicides/100 000 people (Gouvernement du Québec, 1998). Among females, suicide rates are relatively low compared to male ratios (7.9 suicides/100 000 people) even though females attempt suicide far more often than males (Gouvernement du Québec, 1998). Around 5% of females attempt suicide during their lifetime in comparison to only

3% of males. The main reason is that males utilize more violent and drastic methods to commit suicide and are thus more successful. For instance, females are three times more likely to attempt suicide through poisoning while males, on the other hand, are three times more likely to attempt suicide through a self-inflicted gunshot (Gouvernement du Québec, 1998).

CLINICAL PERSPECTIVE OF SUICIDE

Clinical evidence collected during the past four decades underscores the important role played by psychiatric disorders in suicidal behaviours. The main approach used for investigating the link between psychiatric disorders and suicide is the psychological autopsy method (Kelly and Mann, 1996). The psychological autopsy consists of using informants to reconstitute the life style and personality of the deceased with details on the circumstances, behaviours and events that led to the death of the individual.

Psychological autopsy studies among suicide victims have consistently found a high prevalence of mental disorders among people who have committed suicide (Robins et al., 1959;Barraclough et al., 1974;Lesage et al., 1994). For instance up to 90% of suicide victims suffered from a psychiatric condition at the time of death, with mood disorders, particularly major depression, being the most

common (Barraclough et al., 1974;Goldstein et al., 1991;Miles, 1977;Lesage et al., 1994).

Mood disorders and suicide

Mood disorders (anxiety, major depression and bipolar disorder) are characterized by extreme and prolonged alterations of the mood and constitute important risk factors for suicide (Robins et al., 1959;Barraclough et al., 1974;Lesage et al., 1994). Many studies based on the psychological autopsy method have demonstrated a direct link between mood disorders and suicide. In Quebec, for instance, a study conducted among young men between the ages of 18 to 35 showed that suicide victims had depression rates significantly higher (40%) compared to control subjects (5%) (Lesage et al., 1994). Elsewhere, elevated rates of depression among suicides were also reported (Robins et al., 1959;Barraclough et al., 1974) with around 50% of suicides having major depression prior to death. Moreover, life suicide risk for depressed patients varies from 15% (Jamison, 1986;Guze and Robins, 1970) to 19% (Goodwin and Jamison, 1990) illustrating the importance of major depression as a risk factor for suicide.

Despite the development of new therapeutic approaches such as the introduction of selective serotonin reuptake inhibitors for the treatment of major depression, suicide rates are still high albeit they have been quite stable in the past decade. In

that sense, Oquendo et al showed in 1999 (Oquendo et al., 1999) that the advent of this new class of antidepressants had no effect on suicide prevention and that one of the problems with this disorder is that there is no valid, reliable and easy way to diagnose and identify persons at risk to develop major depression and suicidal behaviours. Thus, Oquendo et al. observed that the majority of depressed patients with a history of suicide attempts, who are at higher risk for future suicide, are pharmacologically undertreated.

Bipolar disorder, a disorder characterized by episodes of depression and mania alternating at certain intervals, is also often associated with suicidal behaviours and suicide completion. Lifetime risk associated with bipolar disorder is similar to that associated with major depression, between 15% (Jamison, 1986;Guze and Robins, 1970) to 19% (Goodwin and Jamison, 1990) and epidemiological studies have demonstrated that 29% of bipolar patients have tried to kill themselves (Chen and Dilsaver, 1996) . Suicide attempts in bipolar subjects seem, however, to be more specifically associated with the depressive episodes (Oquendo and Mann, 2000), reinforcing the link between the depressive state and suicide..

A recent re-assessment of suicide rates in mood disorders and suicide showed that initial studies had overestimated the risk of suicide in mood disorders and revealed that this relative risk is instead around 4-6% in mood disorders (Bostwick and Pankratz, 2000;Inskip et al., 1998). Nonetheless, some patients

with mood disorders are at a higher risk for committing suicide and determining what makes these patients more susceptible to commit suicide is of capital importance for suicide prevention.

NEUROBIOLOGY OF SUICIDE

In this section, a brief summary of the neurobiology of suicide will be presented along with the principal theories and post mortem evidence pointing towards the alteration of several neurotransmitter systems in suicide.

Initial studies using neurochemical approaches after the discovery of serotonin (5-hydroxytryptamine) led to the introduction of the serotonergic hypothesis of mood disorders in the sixties (Coppen et al., 1963) and not too long after that of the monoaminergic hypothesis of suicide (Bourne et al., 1968). Nowadays, still most of the research is focused on serotonergic neurotransmission, but it appears more and more clearly that suicide is a complex phenomenon involving several neurotransmitter systems.

The serotonergic hypothesis of mood disorders was first introduced in the sixties (Coppen *et al.*, 1963), since then numerous evidences have supported the implication of the serotonergic system in both mood disorders ((Veenstra-

VanderWeele et al., 2000) (Eriksson, 2000)) and suicide (Gorwood et al., 2000;Mann et al., 2001;Oquendo and Mann, 2000).

Probably the most well replicated findings in suicide research are studies measuring the levels of 5-hydroxyindolacetic acid (5-HIAA), the metabolite of serotonin, in the cerebrospinal fluid of depressed suicidal patients, first reported by Asberg et al. in 1976 where they observed lower levels of 5-HIAA in violent suicides attempters (Asberg et al., 1976), for a review see (Asberg, 1997). Other seminal papers implicating of the serotonergic system in suicide include the initial work of Stanley, who using tritiated imipramine which binds with high affinity to the serotonin reuptake site, revealed an decrease of reuptake sites in the brain of suicides (Stanley et al., 1982) This initial finding was also confirmed by several (Owen et al., 1983;Crow et al., 1984;Arango et al., 1995) but not all the studies, for a review see Purselle et al. (Purselle and Nemeroff, 2003). Using similar approaches an increase of the 5-HT2 receptor binding has also been consistently observed in the brain of suicide victims (Arango et al., 1990;Hrdina et al., 1993;Owen et al., 1983;Pandey et al., 2002;Stanley and Mann, 1983).

Additionally, studies aiming to measure serotonin directly reported a link between a decrease of brain levels of serotonin and suicidal behaviours (Beskow et al., 1976;Bourne et al., 1968;Shaw Murray et al., 1967). However, other teams failed

to observe altered levels of serotonin or 5-HIAA in the frontal cortex of suicides (Crow et al., 1984;Owen et al., 1983;Stanley et al., 1982).

Another interesting finding linking the serotonergic system to suicide concerns the 5-HT_{1A} autoreceptor which controls the firing rate of serotonergic neurons and is localized in the dorsal raphé nucleus (Sprouse and Aghajanian, 1987). Increased binding for this receptor in the midbrain dorsal raphé was reported in depressed suicide victims (Stockmeier et al., 1998) and some other studies reported either a trend in the same direction or confirmed this increase (Dillon et al., 1991;Arango et al., 1995;Matsubara et al., 1991). This increase was recently shown to be under genetic control as revealed by a functional study that found a polymorphism in the promoter of the 5-HT1A gene (Lemonde et al., 2003).

Another monamine, noradrenaline has also been shown to be linked to suicide and suicidal behaviors. For instance, binding studies in postmortem brains showed an increase in adrenergic α2 receptors density in suicides when compared to controls (Meana and Garcia-Sevilla, 1987;Gonzalez et al., 1994;De_Paermentier et al., 1997). There are three types of α2 receptors in the brain (α2A, α2B, α2C), α2A being the predominant one, particularly in the frontal cortex. Also, an increase in α2A receptor density was observed in the frontal cortex of depressed suicides (Callado et al., 1998) and this increase was confirmed by immunolabelling techniques (Garcia-Sevilla et al., 1999). Coupled to a Gαi protein, the α2A

receptor is expressed in noradrenergic neurons as well as in serotonergic neurons acting as an autoreceptor or heteroreceptor, respectively. The agonist mediated α2A receptor coupling to G proteins was also recently found to be increased in depressed suicides when compared to controls supporting the implication of this receptor in suicide (Gonzalez-Maeso et al., 2002).

Recent reports have also proposed the implication other neurotransmitters in suicide such as the amino acid neurotransmitters GABA and glutamate. This has been shown for instance through results from binding studies where increased binding of benzodiazepine (BZ), which specifically binds to GABA receptors, in the cerebral cortex of depressed suicides (Cheetham et al., 1988) and in the hippocampus of violent suicides (Rochet et al., 1992), two areas also associated with depression and involved in the control of emotions (Mann, 2003). GABAergic neurotransmission was also shown to be altered in mood disorder patients (Brambilla et al., 2003;Cryan and Kaupmann, 2005), yet again demonstrating the existence of a relationship between suicide and mood disorders. Finally, gene expression studies looking at mRNA levels have also shown altered expression of GABAergic and glutamatergic genes in the brain associating these systems with depression and suicide (Choudary et al., 2005;Merali et al., 2004).

Suicide and mood disorders (major depression, bipolar disorder) are complex psychiatric manifestations resulting from interactions between genetic, socio-

economic, cultural and environmental factors. In the last decades, classical binding and biochemical approaches in post-mortem brains have pointed towards a large number of possible biological markers for mood disorders and suicide, for a review see Gross-Isseroff et al. (Gross-Isseroff et al., 1998), as well as contributed to considerable progress in the understanding of the neurobiology of mood disorders (Manji et al., 2001;Nestler et al., 2002;Manji and Lenox, 2000). However, these studies have also underlined that suicide is accompanied by a complex pattern of neurochemical changes involving several neurotransmitter systems and the necessity to examine mood disorders and suicide in a more globally comprehensive way, focusing not on single genes but rather on systems or biological pathways of genes interacting together.

GENETICS OF SUICIDE.

It has become clear in the last decades that there exists a genetic susceptibility to suicide, as suggested by evidences from family, twin and adoption studies (Turecki, 2001).

Family, twin and adoption studies

The first evidence of the importance of genetics in suicide was shown by family studies. In the middle of the 80's, Egeland and Sussex (Egeland and Sussex, 1985a) ascertained suicides that occurred among the Old Order Amish over a 100-year period and observed that the 26 suicides that occurred during this period of time were clustered in only four families. Also, this familial clustering of suicides seemed independent of the familial clustering of affective disorders also observed in some of those families, thereby, supporting the idea that the genetic predisposition to suicide and the genetic predisposition to affective disorders are independent. Since then the familial aggregation of suicide and suicidal behaviors has been clearly demonstrated in several studies (Brent et al., 1996b;Johnson et al., 1998;Egeland and Sussex, 1985b). Furthermore, this familial transmission of suicidal behaviors appeared to be independent of the familial aggregation of psychiatric disorders often associated with suicide in the following studies (Brent et al., 1996b;Mann, 1998;Qin et al., 2002;Mann, 1998).

Supporting evidence of a genetic component in suicidal behavior comes also from twin studies that have shown higher concordance for suicide in monozygotic twins compared to dizygotic twins (Roy, 1993;Roy et al., 1995;Roy and Segal, 2001b). In 2001 for instance, Roy and Segal found concordance rates of 18.5% in monozygote twins compared to 0.7% in dizygotic twins (Roy and Segal, 2001a). Additionally, a recent twin study showed that genetic factors account for 45% of the variance observed in suicidal behavior in a sample of 5 995 male and female

twins (Statham et al., 1998). Another study assessing 3401 female twins estimated that suicide attempt liability was familial, with genetic and shared environmental influences together accounting for 35% to 75% of the variance in risk (Glowinski et al., 2001a) clearly underlying the importance of genetics in the suicide phenotype.

These results are also consistent with data from adoption studies that also support the important role of genetic factors in suicide (Schulsinger et al., 1979). Adoption studies are important because they provide information pertaining to specific genetic and environmental factors involved in the trait one is studying. In an important adoption study aiming to explore the specific role of genetics and environment in mood disorders and suicide carried out by Wender et al., they observed a 15-fold increase in suicide among biological relatives of the index cases depressed patients (Wender et al., 1986).

Association studies

Association studies using a candidate gene approach is a very useful and powerful design to investigate genetic risk factors for complex disorders. In this type of studies unrelated individuals grouped as cases or controls are screened for genetic variants in genes hypothesized to be implicated in the aetiology of the studied phenotype (Lander and Schork, 1994).

Because of the nature of the suicide phenotype, family-based mapping studies have been difficult to carry out. As a result, the focus has been on case-control association designs using a candidate gene approach. In recent years, a growing number of molecular studies have been carried out to investigate candidate genes that may be involved in suicidal behaviour (Anguelova et al., 2003).

However, this type of design has produced conflicting results and replication of the initial findings, in general, is rarely observed. Several factors can explain the non replication of candidate gene association studies, such as misclassification due to an ill-defined phenotype, the presence of subpopulations in the studied sample leading to admixture and random associations or genetic heterogeneity causing non replication when studying ethnically diverse populations (Lander and Schork, 1994). Nonetheless, by studying an extreme phenotype such as suicide completion instead of suicidal behaviours and using a homogenous population with a known founder effect such as the population from Québec (Scriver, 2001), it is possible to minimize those confounders.

Given the known neurobiological factors involved in suicide, most association studies have focused on genes implicated in monoaminergic and, especially, serotonergic neurotransmission (Mann et al., 2001;Anguelova et al., 2003). To date, however, despite the tremendous effort and the number of studies

undertaken in many different populations, few genes have consistently been replicated apart from the genes coding for the serotonin transporter and tryptophan hydroxylase which are particularly interesting due to their implication in serotonergic neurotransmission.

One of the most studied candidate gene is probably the gene coding for the serotonin transporter (5-HTT). This transporter is responsible for the reuptake and termination of the effect of serotonin in the synaptic cleft and is considered a good index of serotonergic neurotransmission. Located on chromosome 17, the 5-HTT gene has a 44bp insertion/deletion polymorphism leading to either a short (S-allele) or long (L-allele) form of the transporter (Lesch et al., 1996). However, analysis of this polymorphism has produced some conflicting results regarding its involvement in suicide (Bondy et al., 2000;Fitch et al., 2001;Mann et al., 2000), for a review see (Bondy et al., 2006). However, a meta-analysis including 12 studies revealed that the promoter polymorphism is significantly associated with suicide, thus suggesting a role for the 5-HTT gene in the predisposition to suicide.

Another gene that has shown a level of consistency in its association with suicidal behaviour is that coding for tryptophan hydroxylase 1 (TPH1), more specifically the A218C single nucleotide polymorphism, despite the fact that this gene is not expressed in the brain at significant levels. Even though this gene is not expressed in the brain it has been hypothesized that its dysfunction lead to a global

serotonergic alterations (Abbar et al., 2001;Bellivier et al., 2004;Lalovic and Turecki, 2002;Rujescu et al., 2003;Tsai et al., 1999;Turecki et al., 2001;Zalsman et al., 2001). A new brain specific TPH gene was discovered recently (TPH2)(Walther et al., 2003)and the studies published until now have found significant associations between polymorphisms and/or haplotypes and suicide (Zill et al., 2004;De, V et al., 2006;Lopez et al., 2006) suggesting an implication of TPH2 in the susceptibility to suicide, but this has to be confirmed in larger samples and different populations.

The results amassed so far for associations between genetic variation and predisposition for suicide are promising. However, association designs with candidate genes in this area of research are plagued with methodological limitations and problems. The advent of whole genome studies will prove useful in this regard in allowing for the simultaneous investigation of many genes at once contrary to single genes with minimal numbers of SNPs, as has been the case thus far. In addition, new strategies looking at genetic polymorphisms going beyond the investigation of coding variants are needed.

OBJECTIVES

The main purpose of this project was to identify new genetic risk factors and biological markers for suicide completion.

Specific objectives

1. Use a candidate gene approach and a case-control design, to carry out genetic variation studies in genes implicated in serotonergic and noradrenergic neurotransmission as well as other new possible candidate genes.

2. Build a brain gene expression profile of cortical and subcortical (hippocampus, limbic system) regions from suicides and controls by means of microarray technology in order to identify new candidate genes for suicide.

3. To confirm the implication of selective genes shown to be differentially expressed in suicide.

4. To explore the relationship between changes in gene expression and genetics in suicide.

Hypotheses

1) Variation and expression at genes that code for components of the serotonergic and noradrenergic pathways predispose to suicide, probably but not necessarily, through the modulation of traits associated to suicide.

2) Suicide victims have different brain expression profiles than normal controls.

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CHAPTER 2

GENOME WIDE GENE EXPRESSION STUDIES IN MOOD DISORDERS

This work was accepteded as:
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CONTRIBUTION BY AUTHOR:

Sequeira: Review of the literature and writing of the paper.

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PREFACE

Microarrays were first introduced in 1995-96 for the investigation in parallel of a high number of gene's expression. Commercially available and highly reproducible microarrays were introduced at the end of the 90's making possible high throughput screening of gene expression changes in complex multifactorial traits. This technology is particularly useful for the study of complex traits because of their multifactorial character. Several methodological and statistical challenges were also circumvent making this technology mature enough for the study of complex traits.

Numerous studies have been published in the last ten years taking advantage of the microarray technology to study complex traits and more particularly mood disorders. Because of the high frequency of mood disorders among suicide victims, many of these studies included suicide victims to explore patterns of expression in the brain of mood disorders. This review paper explores the platforms utilized as well as design characteristics of the many studies conducted to date as well as the strategies developed in order to find new target genes or pathways implicated in mood disorders and suicide.

ABSTRACT

Microarrays offer the possibility of screening in parallel virtually all genes expressed in a given tissue or to study the molecular signature associated with available treatments. As such, this technology has been increasingly used to investigate multifactorial and polygenic complex traits such as psychiatric disorders, in particular, schizophrenia and mood disorders. This review focuses on microarray studies investigating mood disorders. Study designs, methodologic approaches and limitations, subsequent follow-up strategies, and confirmation of results are discussed. Despite the apparent disparate and not always concordant results, it appears evident that this technology is a powerful and inevitable approach for the study of mood disorders, especially when phenotype-specific confounders are properly accounted for. Thus, alterations of mitochondrial, oligodendrocyte, and myelin related genes in bipolar disorder, of signaling and olidendroglial related genes in depression, and of GABA-glutamate related genes in depression and suicide have been observed and have confirmed new avenues for the study and the treatment of these complex disorders.

INTRODUCTION

Mood disorders, also known as affective disorders, are common conditions characterized by mood dysregulation and neurovegetative dysfunction (Akiskal, 1995), the most severe variants of which are bipolar disorder and major depressive disorder. Major depressive disorder may be recurrent (also known as unipolar depression) or not. Bipolar disorder, classically referred to as manic-depressive illness, differs from major depression by presentation of periods of mania during which mood is elated, in addition to depressive episodes. One-year prevalence estimates are approximately 1.3% for bipolar disorder and range between 6.4% and 10.1% for major depression. As a consequence, mood disorders are among the most significant causes of disability. The greatest loss for society, however, is the mortality associated with suicide, a complication of mood disorders. Between 50 and 70% of suicide completers die during an episode of major depression (Arsenault-Lapierre et al., 2004;Cavanagh et al., 2003) and prospective follow-up studies in major depression suggest that between 7 and 15% of patients will die by suicide (Angst et al., 2002;Angst et al., 1999;Angst et al., 1992;Blair-West et al., 1999).

The investigation of the neurobiological basis of mood disorders goes back to the late 1950's with the advent of the first efficient pharmacological interventions. Since then, there have been increasing efforts to understand the biological basis of these conditions. Bipolar disorder and major depression are intrinsically related,

as the depressive episode of a bipolar patient is phenomenologically similar to the depressive episode of a non-bipolar patient. Therefore, neurobiological findings in mood disorders have not been specific to one diagnostic category, but rather apply both to the clinical phase, and to depression. Initial studies suggested that mood disorders were a consequence of neurochemical imbalance, particularly at the neurotransmitter level (Schildkraut et al., 1983). More recent work has suggested the possible role of neuropeptides (Garlow et al., 1999;Nemeroff, 2002), as well as downstream alterations, particularly in signaling pathways, as etiologically relevant to mood disorders[21-25]. Development in imaging technology has also suggested the implication of certain anatomical circuits in both depression and bipolar disorder (Drevets, 2001;Drevets, 2000;Soares, 2003). From the imaging data of subcortical regions, the hypothalamus and amygdala seem to be implicated, while in cortical areas, the orbital and ventrolateral prefrontal cortices appear to be involved (Drevets, 2001;Drevets, 2000;Soares, 2003). However, in spite of tremendous effort, the precise neurological circuits, neuroplastic and neurochemical alterations related to mood disorders remain elusive.

Most neurobiological findings in mood disorders have been based on studies whose hypotheses were driven by the known effects of therapeutic agents. While useful, these approaches are limited. The development of microarray gene expression screening technologies has allowed us to move beyond these limitations by using whole genome strategies (Schena et al., 1995;Lockhart et al., 1996). In that sense, this type of approach has facilitated the shift from candidate-

target approaches to more comprehensive, whole-genome approaches, where thousands of gene transcripts are concomitantly investigated. In this review we briefly overview the current applications of microarray technologies in the investigation of mood disorders and their most severe outcome, suicide. In addition, we will discuss the methodological challenges and bioinformatics strategies available to separate actual biological variation from random differences (background noise).

BRIEF OVERVIEW OF THE TWO MAIN MICROARRAY TECHNOLOGIES

There are two major types of microarrays used and commercialized nowadays, the short oligonucleotide (Lockhart et al., 1996) and the long cDNA types of arrays (Schena et al., 1995).

Oligonucleotides are short sequences of nucleotides, the basic components of DNA and RNA. Oligonucleotide arrays are based on a technique first introduced in 1991 by Stephen Fodor et al. (Fodor et al., 1991) from the Affymax Research Institute. Using a combination of solid-phase chemistry, photolabile protecting groups and photolithography, this group developed a light-directed parallel synthesis of short oligonucleotides used nowadays by Affymetrix to produce oligonucleotide arrays. These are referred to as "probe sets" and target virtually all genes in the genome. These single strands of nucleotides are specifically

designed to be complementary to the target gene, and thus have binding affinity to this specific genetic sequence. In this manner, Affymetrix arrays are designed to tease apart true variation from non-specific hybridization or "background noise" by using "probe sets" constituted of 11 target specific 25mers probes (perfect match, PM) and 11 probes differing from the PM probes by one nucleotide at position 13 (mismatch, MM) (Lipshutz et al., 1999).

Complementary DNA (cDNA) microarrays consist of longer (~80-2000bp) PCR amplified DNA sequences placed on a glass surface by printing or mechanical deposition by a robotic arrayer (Duggan et al., 1999). The relative abundance of two mRNA samples is measured by hybridizing their cDNA, reverse transcribed and labeled with two different fluorescent dyes (usually Cy3 (green) and Cy5 (red)), onto the same array. The ratio of the respective signal for each spot indicates the relative abundance of the target gene in the two mRNA samples.

CONFOUNDING VARIABLES

Many well studied parameters may influence and increase the variance in global gene expression, making it more difficult to find biological differences related to a given phenotype in addition to increasing the chances of false positive results. Such variables include demographic factors, cause of death, agonal factors, post-mortem delay, tissue pH, post-autopsy tissue handling, dissection procedures and tissue storage conditions (Tomita et al., 2004;Li et al., 2004;Bahn et al.,

2001;Albrecht and Yanagihara, 1979;Barton et al., 1993;Harrison et al., 1995;Cummings et al., 2001;Burke et al., 1991;Johnson et al., 1986;Leonard et al., 1993;Kobayashi et al., 1990;Perry et al., 1982;Preece and Cairns, 2003;Wester et al., 1985;Perrett et al., 1988;Yasojima et al., 2001). Although not all studies agree, of these variables, agonal factors are thought to more directly influence RNA integrity through a large influence on tissue pH (Tomita et al., 2004;Albrecht and Yanagihara, 1979;Harrison et al., 1995).

Traditionally the integrity and quality of RNA, of critical importance particularly in post-mortem brain expression studies, was originally assessed by visual inspection of 28S/18S bands after gel electrophoresis and UV spectroscopy to obtain A260/A280 ratios, but these measures were proved to be unreliable or highly sample consuming (Imbeaud et al., 2005). Nowadays the gold standard for RNA quality and integrity is based on microcapillary electrophoresis and commercialized by Agilent as the 2100 Bioanalyser (Agilent Technologies, USA). This method requires small amounts of RNA sample, is relatively unaffected by contaminants and generates an RNA integrity number (RIN) that can be used as a standardized measure of quality and an objective threshold for acceptable RNA samples (Imbeaud et al., 2005;Schroeder et al., 2006).

GENE SELECTION

One of the most controversial aspects of microarray analysis is establishing selection criteria to determine whether a given gene is differentially expressed in a given phenotype. Proposed approaches include Fold-change analysis (FC), parametric test (t-test, ANOVA)(Cui and Churchill, 2003), SAM t-test (Tusher et al., 2001) as well as graphical methods combining FC and P-values from the parametric tests (Welle et al., 2002;Allison et al., 2006;Cui and Churchill, 2003).

In psychiatry, the most controversial issue is establishing valid thresholds thereby minimize false positives, while still allowing the discovery of, not only genes, but also pathways implicated in complex and highly variable disorders. Because several thousands of transcripts are investigated in parallel, the chances of finding significant results just by chance is relatively high. One way to circumvent the multiplicity problem and reduce the chance of false positives, without diminishing the chances of finding true positives, is to select, prior to the analysis, the genes or transcripts of interest based on the presence criteria (Seo et al., 2004).

For instance, Affymetrix HG-133AB GeneChips® contain around 44 000 probe sets, but many of the probe sets are not really expressed at biologically significant or detectable levels in the brain. For these transcripts, any significant variation in signal most likely represents noise. Accordingly, Jongeneel et al. estimated that between 10 to 15 thousand transcripts are actually expressed in human cell lines (Jongeneel et al., 2003). Thus, probe sets can be efficiently filtered to ensure that they are "Present" (according to MAS 5.0) in a certain proportion of subjects,

significantly reducing the total number of analyzed probe sets without resulting in a notable decrease in the number of truly positive genes (McClintick and Edenberg, 2006). When using cDNA (intensity of expression) arrays, a similar approach using intensities achieves the same goal with similar benefits (Altar et al., 2005).

ONTOLOGICAL PROFILING

One of the difficulties when performing genome wide studies, and especially gene expression studies, is to make sense of the massive amount of data that ensues. This issue is intensified by looking at multiple brain regions at a time. Gene ontologies provide a useful tool to explore the overall functional profile of the genes associated with a phenotype (Ashburner et al., 2000a).

Despite the many ontological tools proposed in the recent years (www.geneontology.org/go.tools.shtml), the majority of these tools present several methodological biases and intrinsic drawbacks (Khatri and Draghici, 2005).

A new tool for functional ontological profiling of expression changes was recently implemented in the ErmineJ software (version 2.1.8, Columbia University, NY)(Lee et al., 2005): Gene Score Resampling (GSR). In this type of analysis, the probe set scores (t-test or *P*-values) are not used to determine an

arbitrary threshold (0.05 vs 0.01), but rather to compute an aggregate score for genes in a given category (e.g. the geometric mean of *P*-values). The significance of this score is then determined by random sampling of the data.

Other methods, such as Gene set enrichment analysis (GSEA)(Subramanian et al., 2005) and its variants, parametric analysis of gene set enrichment (PAGE)(Kim and Volsky, 2005), utilize a similar approach. In other words, they do not restrain the analysis to a given subset of genes using an arbitrary threshold. This makes the method more suitable to find processes and pathways distributed across the transcriptome, while allowing for subtle changes at the level of individual genes(Kim and Volsky, 2005).

THE MULTIPLICITY PROBLEM

There has been considerable debate concerning the multiplicity problem when studying brain gene expression in humans. Multiplicity correction procedures, such as Bonferroni or FDR that control for multiple comparisons, usually assume the independence of the hypotheses being tested (Bland and Altman, 1995). In the context of gene expression in brain tissue, this is far from reality. For instance, the probe-sets of genes belonging to a common pathway, or that perform a common molecular function, are expected to vary in concert. In such situations, multicollinearity is the necessary consequence of the relevant genes being regulated in a coordinated manner. In support of such a proposition, many

researchers have not found specific genes, but rather families of genes or pathways to be implicated in psychiatric disorders (Iwamoto et al., 2005;Sequeira et al., 2006;Iwamoto et al., 2004;Konradi, 2005;Benes et al., 2004;Konradi et al., 2004;Vawter et al., 2006b;Choudary et al., 2005;Mimmack et al., 2002;Mirnics et al., 2000;Benes et al., 2006).

Given that the purpose of gene expression studies in psychiatry is to find global gene expression changes in a complex tissue in the context of a complex disorder, Benes and colleagues have suggested placing more emphasis on bio-pathway analysis and using less stringent criteria (P=0.25). This allows for a more inclusive understanding of the disorder' complexities, ranging from aspects such as transduction, signaling and metabolism that may be altered in psychiatric disorders (Benes et al., 2006;Benes et al., 2004).

MICROARRAYS IN THE STUDY OF MOOD DISORDERS

While most initial studies using microarray technology in psychiatry focused on schizophrenia (Mirnics et al., 2000) and addiction (Lewohl et al., 2001), there is a growing number of studies investigating mood disorders and their most severe outcome, suicide (Lewohl et al., 2000). Table 1 lists a summary of global microarray studies investigating bipolar disorder, major depression and suicide published to date.

Initial studies had to deal with the impressive amount of data generated and no means to regroup the findings in a more comprehensive way. It was thus difficult to extract the most informative and biologically meaningful differentially expressed genes (Bezchlibnyk et al., 2001;Tkachev et al., 2003). Subsequently, tools using gene ontology information allowed researchers to perform more comprehensive analyses and undercover systems and pathways that were differentially expressed.

The first high throughput gene expression study in human brain tissue examining mood disorders utilized cDNA technology from Clontech. This study screen approximately 1200 genes in the prefrontal cortex of a mixed suicide/nonsuicide as well as mixed gender sample of individuals with bipolar disorder and normal controls (Bezchlibnyk et al., 2001)(Houston et al., 2001). The authors observed significant changes in 24 genes. More specifically, they observed a down-regulation of TGF-beta 1, leading the authors to hypothesize the involvement of neurotoxic insults in bipolar disorder.

In 2003, Tkachev et al. (Tkachev et al., 2003) performed the first gene expression study of mood disorders using Affymetrix oligonucleotide technology (HG-U133A, MAS5). They examined gene expression in BA9 samples of white and grey matter blocks. The sample consisted of mixed suicide/non-suicide schizophrenics (N=15), bipolar subjects (N=15) and matched controls (N=15). The authors observed a high degree of correlation as relates to expression changes

between bipolar disorder and schizophrenia and an overall reduction in both groups of oligodendrocyte and myelin related genes. However, the specific localization of these changes, white vs. grey matter, was not established.

More recently, Aston et al. conducted a study with 12 major depressive patients and 14 properly matched controls using the Affymetrix HGU95A chip (MAS 4) (Aston et al., 2004). The sample was almost exclusively Caucasian (one single African American), but included both suicides/non suicides. Their significant probe set selection criteria included 1.4 expression fold change (FC), $P<0.05$ (t-test), "presence" in at least 20% of subjects, and average expression intensity of greater than 150 in at least one of the groups. Further functional analysis was performed using EASE (Dennis, Jr. et al., 2003), and lead to the observation of significant alterations in major depressive patients of families of genes implicated in neurodevelopment, signal transduction, cell communication and myelination.

Konradi et al (Konradi et al., 2004) performed a gene expression study in the hippocampi of bipolar disorder (N=9), schizophrenic (N=8) and healthy control (N=10) subjects matched for age and post mortem interval (PMI) using the HG-U95Av2 array (dChip normalized). Using GenMAPP for ontological profiling, the authors observed a significant decrease in the mRNA levels of mitochondrial genes specific to the bipolar phenotype. More specifically, genes regulating oxidative phosphorylation and the adenosine triphosphate-dependent process of proteome degradation exhibited lower levels of mRNA. According to the authors,

this might imply a deregulation of mitochondrial energy metabolism and downstream deficits of adenosine triphosphate-dependent process in bipolar disorder. However, the sample included several controls and cases that died of prolonged chronic diseases, such as renal failure and cancer. These could result in expression changes specifically related to agonal factors, as shown by Tomita et al. (Tomita et al., 2004).

Also in 2004, Iwamoto et al. (Iwamoto et al., 2004) conducted a gene expression assay in the frontal cortex (BA10) of bipolar disorder, major depression, schizophrenia and control subjects using the HU95A chips. The authors performed the statistical analysis only on genes that remained after filtering for (1) the percentage "presence" in each group, (2) the absence of correlation with age, (3) PMI and (4) gender. Significance criteria included 1.3 FC and $P<0.05$ in the t-tests. The authors tried to control for a series of other confounding variables, including medication and suicide status by performing a series of group comparisons based on these variables (t-tests). This, however, did not address the possible interaction of these variables with gene expression and increases the multiplicity problem. In this paper, the authors focused mainly on results obtained for the bipolar disorder versus controls comparison, even though the most significant genes were observed for the major depression vs. controls and schizophrenics vs. controls comparisons. Overall, they noted that the psychiatric conditions did not tend to share expression changes. Further, there was a tendency for down-regulation of genes coding for receptors, channels and transporters and

an up-regulation of genes coding for stress response proteins or molecular chaperons in bipolar disorder. Most notably, the authors observed expression alterations in genes coding for glutamatergic (GRM1, GRK1) and serotonergic (HTR2C) receptors, that have been also found to be altered in mood disorders by our group (Sequeira et al. in preparation) and others (Choudary et al., 2005).

Sibille et al. (Sibille et al., 2004) performed a microarray analysis comparing expression patterns in BA9 and BA47 of depressed suicides versus psychiatrically normal controls matched on the basis of sex, age, PMI, and race using the U133A GeneChip. This chip contains approximately half of the probe sets (22,000) of the U133 chip set. Unlike other studies in the field, however, they observed no evidence for molecular differences that correlated with major depression and/or suicide. As the design relied on group comparison and there is evidence in the prefrontal cortex that gene expression has a strong sex-related component (Galfalvy et al., 2003), the mixed gender sample could have influenced this result. Functional analysis included pattern discovery using Genes@Work (Califano et al., 2000) and a procedure called functional class scoring analysis (Pavlidis et al., 2002), whereby the authors found evidence of epidermal growth factor and enzyme-linked receptor protein signalling pathway implication in suicide and depression.

Following their paper in 2004, Iwamoto et al. (Iwamoto et al., 2005) performed a similar gene expression study in the prefrontal cortex (BA10) of a mixed sample

in terms of gender and cause of death (suicides, non suicides) of bipolar disorder, major depression, schizophrenia and control subjects using the HG-U133A chip (MAS 5.0, RMA). The authors specifically targeted mitochondrial related genes, selected using NetAffx (Cheng et al., 2004) or Gene Ontology (Ashburner et al., 2000b) information. Using this methodology, they observed an overall down-regulation of mitochondrial genes in both bipolar and schizophrenic patients. This may have been due to the effects of medication, however, as the authors note that the opposite effect was observed in medication free patients. Nevertheless, this study shows a possible implication of mitochondrial dysfunction in both bipolar disorder and schizophrenia.

Choudary et al., performed a gene expression study on bipolar, major depression and control subjects (Choudary et al., 2005) using the HG-U95Av2 chip (RMA) in brain cortical areas (BA24, BA9-46) and a mixed cohort of males and females. Comparing results from the cingulate (BA24) and the prefrontal cortex (BA9-46) the authors identified GABA and glutamate related genes dysregulated in both areas or specifically altered in at least one of the areas and related to major depression. Also some GABA receptors were related to suicide regardless of the type of disorder and underlining the presence of a molecular signature of suicide independently of the psychiatric diagnosis. Validation in this study was performed by an alternative technology, the BeadChip Arrays from Illumina (Illumina, San Diego) and *in situ* hybridization.

Finally, using the HG-U133AB chipset and a design including a male only sample of non depressed and depressed suicides, as well as controls, our group (Sequeira et al., 2006) identified SSAT (spermine/spermidine N1-acetyltransferase gene), a gene involved in polyamine metabolism, as being differentially expressed in three cortical brain areas (BA4, BA8,9 and BA11) of suicides with and without major depression. These results were validated and further explored using RT-PCR, immunohistochemistry and Western-Blots in adjacent samples. These findings suggest that polyamines play a role in the neurobiology of suicide and depression. Additionally, a variant located in the SSAT polyamine responsive element regulatory region (SSAT342A/C) revealed a significant effect of genotype on SSAT brain expression levels and further investigation of this variant in an independent sample of male suicides and controls showed a higher frequency of the SSAT342C allele among suicide cases suggesting that this allele may increase predisposition to major depression and suicide.

In conclusion, despite the limited number of studies available in this quickly evolving field, several trends and common themes can be identified in the brains of patients who suffered of mood disorders. In bipolar disorder a global reduction of mitochondrial, oligodendrocyte and myelin related genes seems to be present in several areas of the brain. In depressed subjects signaling, olidendroglial and GABA-glutamate related genes appear to be altered in several postmortem brain areas.

FUTURE DIRECTIONS

Many new usages of microarrays have been developed and implemented in recent years widening the possible applications of this technology for the study of mood disorders. For instance, comparative genomic hybridization analysis (CHG) using microarrays allows the direct comparison of the hybridization patterns between affected and normal tissue in order to detect differences in terms of numbers of copies of genes or DNA sequences at a genomic scale (Pinkel et al., 1998;Pollack et al., 1999). For this type of approach both the affected and normal DNA are co-hybridized onto the same array allowing the detection of gene dosage variations at a genome-wide level (Pinkel and Albertson, 2005). Additionally, with new microarray SNP chips able to interrogate in parallel between around 300K to 500K SNPs, it is now possible to study the effect of common variations on complex phenotypes (Wang et al., 2005) as well as on expression levels (Vawter et al., 2006a). Other applications currently available and applicable to mood disorders are resequencing of specific genomic DNA areas, finding protein-DNA interactions and proteomics using protein chips currently under development.

CONCLUSION

Recently, some concerns have been raised on the quality, the reproducibility and validity of the data produced by microarrays (Ioannidis, 2005). However, many of

these concerns are partly related to the different methodologies employed for the scanning, normalizing and statistical analysis of microarray results, as well as with the proper control of possible confounding variables (laterality, gender, RNA quality, death cause) as highlighted in this review. So far, many of the microarray studies conducted have been undermined by samples of insufficient size to overcome the effect of confounding variables. When these issues are experimentally or statistically addressed, microarrays will likely be of great help in the identification of new molecular targets or new functional pathways implicated in mood disorders. For instance interesting results implicating alterations of mitochondrial, oligodendrocyte and myelin related genes in bipolar disorder and of signaling, olidendroglial and GABA-glutamate related genes in depression or suicide have been observed and confirmed.

Finally, given the amount of data already available, it would be interesting to cross validate the findings in order to identify the most consistent and therefore relevant targets or systems implicated in this family of disorders while correcting for confounding factors.

Acknowledgements: We would like to thank Alexander McGirr for his technical help in the preparation of this manuscript.

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Table 1: Microarray studies measuring gene expression in postmortem brains of subjects with mood disorders.

Study	Platform-chip	Regions	Sample	Suicides	Gender	Confirmation	Functional profilling
Bezchlibnyk, 2001	cDNA (Clontech)	PFC	BD, controls	Yes	M/F	RT-PCR	
Tkachev et al., 2003	HG-U133A	BA9	BD, SZ, controls	Yes	M/F	RT-PCR	
Aston et al., 2004	HG-U95A	BA21	MDD, controls	Yes	M/F	RT-PCR	
Konradi et al., 2004	HG-U95Av2	Hippocampus	BD, SZ, controls	No	M/F	RT-PCR	GenMAPP, MAPPfinder
Iwamoto et al., 2004	HG-U95A	BA10	BD, MDD, SZ, controls	Yes	M/F	RT-PCR	
Sibille et al., 2004	HG-U133A	BA9, BA47	Depressed suicides, controls	Yes	M/F	RT-PCR	Genes@Work, FCS
Iwamoto et al., 2005	HG-U133A	BA46	BD, SZ, controls	Yes	M/F	RT-PCR	
Evans et al., 2004	HG-U133A	BA24, 46	BD, MDD, controls	Yes	M/F	RT-PCR, ISH	
Jurata et al. 2004	HG-U133A, Agilent cDNA	BA46, 10	BD, controls		M/F	RT-PCR	Ease
Choudary et al., 2005	HG-U95Av2	BA9, 24, 46	BD, MDD, controls	Yes	M/F	ISH	
Altar et al., 2005	cDNA (Agilent)	Hippocampus	BD, MDD, SZ, controls		M/F	RT-PCR	Ease
Sequeira et al., 2006	HG-U133AB	BA4, 11, 8-9	Suicides, depressed suicides, controls	Yes	M	RT-PCR, Western blot	NetAffx, DAVID

CHAPTER 3

WOLFRAM SYNDROME AND SUICIDE: EVIDENCE FOR A ROLE OF WFS1 IN SUICIDAL AND IMPULSIVE BEHAVIOR

This work was published as:
Sequeira A, Kim C, Seguin M, Lesage A, Chawky N, Desautels A, Tousignant M, Vanier C, Lipp O, Benkelfat C, Rouleau G, Turecki G (2003) Wolfram syndrome and suicide: Evidence for a role of WFS1 in suicidal and impulsive behavior. Am J Med Genet 119B: 108-113.

CONTRIBUTION BY AUTHOR:

Sequeira: performed the experiments, did the analysis and wrote the paper.

Kim, Seguin, Lesage, Chawky, Tousignant, Vanier, Lipp, Benkelfat: helped in the design of the study and collecting the data.

Desautels and Rouleau: analysis of the data and interpretation of results.

Turecki: writing of the paper and supervision.

PREFACE

Complex traits are thought to be caused by multiple factors having small but significant effects. Known physiopathology can be used in order to identify candidate genes possibly predisposing certain individuals to suicide and suicidal behaviors. A very useful and powerful approach to investigate genetic risk factors for complex disorders is the case-control association study using a candidate gene approach.

In this type of studies unrelated individuals regrouped as cases or controls are screened for genetic variants in genes hypothesized to be implicated in the etiology of the studied phenotype. This is the first of a series of association studies we performed in a Quebec population sample. This population is known to have a founder effect and being more genetically homogeneous.

In this project we evaluated the implication of the Wolfram gene in suicide and associated personality traits. The Wolfram syndrome gene is an interesting candidate gene for suicide because homozygous and heterozygous subjects of mutation of this gene are at high risk for being hospitalized for psychiatric disorders and aggressive behaviors and suicidal behaviors.

ABSTRACT

There is evidence suggesting that subjects affected with the Wolfram Syndrome (WFS) and normal carriers present an increased risk of psychiatric disorders, particularly depression and suicidal behavior. We investigated a possible role of the gene involved in WFS (WFS1) in the neurobiology of suicide and the potential modulatory effect on traits associated to suicidal behavior. Genetic variation at WFS1 (H611R, R456H, and I333V) was investigated in 111 suicide victims and 129 normal controls. Possible effects on psychopathology and behavioral traits were investigated in a subsample of suicide cases (N = 31) for whom phenotyping was carried out by means of structured psychiatric interviews and questionnaires adapted for psychological autopsies. We found a significantly higher frequency of the 611R/611R genotype in suicide completers as compared to controls ($\chi^2 = 19.21$, df=2, $P = 0.001$). Suicide completers with this genotype had higher scores on measures of impulsivity (t =-3.15 df = 15.3 $P = 0.006$), novelty seeking (t = -3.35 df = 13.8 $P = 0.005$), and conversely, lower scores of persistence (t = 2.4 df = 16.6 $P = 0.028$). Scores of impulsivity and novelty seeking remained higher in subjects with the associated genotype after adjusting for age, gender and psychopathology. These results suggest a role for WFS1 in the pathophysiology of impulsive suicide, and are consistent with previous clinical reports suggesting an increased risk of suicidal behavior in WFS homozygotes and heterozygotes. However, these findings are preliminary and should be confirmed in independent samples.

INTRODUCTION

Suicide ranks among the top ten causes of death for individuals of all age groups and is the leading cause of death for males younger than 35 years old [Diekstra, 1993]. Although probably determined by a combination of several factors, there is compelling support for a genetic predisposition to suicide, as suggested by family, twin and adoption studies [Turecki, 2001]. This predisposition seems to be complementary, but independent from the liability to major psychiatric disorders commonly observed in suicide cases [Brent et al., 1996; Egeland and Sussex, 1985; Johnson et al., 1998; Statham et al., 1998]. Recently, it has been suggested that subjects who carry mutations predisposing to Wolfram syndrome (WFS) may have an increased vulnerability to some psychiatric disorders, in particular mood disorders and suicidal behaviors [Swift et al., 1998].

Wolfram syndrome (WFS) is a rare autosomal recessive neurodegenerative disorder characterized by the presence of early onset non-immune insulin-dependent diabetes mellitus, diabetes insipidus, progressive optic atrophy and deafness. There is at least one gene implicated in WFS – WFS1 – which maps to chromosome 4p16.1 and has recently been cloned [Inoue et al., 1998; Strom et al., 1998]. The idea that the WFS1 gene may predispose to mood disorders or to suicidal behaviors comes from the observation of significant psychiatric morbidity, particularly mood alterations, suicide attempts, violent and assaultive behavior, as well as delusional ideation in WFS homozygotes [Swift et al., 1991;

Swift et al., 1990]. Furthermore, heterozygous WFS carriers, which may represent 1% of the general population, are reported to be 26-fold more likely to be hospitalized for depression and/or to have increased risk of attempted suicide than non-carriers [Swift et al., 1998].

In this study we investigated the three most common mutations/polymorphisms observed in WFS1 – H611R, R456H and I333V – in a relatively large sample of subjects who committed suicide and report evidence supporting a possible role of H611R in the predisposition to suicide completion. Moreover, our data suggest that its role may involve the modulation of impulsive behavior, a trait that has long been recognized as an important risk factor for suicidal behavior.

MATERIALS AND METHODS

Subjects

Consecutive cases of suicide were collected as part of an ongoing collaboration with the Coroner's Office of the Montreal Central Morgue. Controls in this study were living subjects without a history of suicidal behavior or a major psychiatric diagnosis. All subjects included in this study (or their families in the case of the suicide cases) provided written informed consent, which was approved by the local IRB. A total of 111 suicide completers and 129 normal controls were investigated. All the subjects, cases and controls, were Caucasians from French-

Canadian origin, a homogeneous population with a well-characterized founder effect [Gagnon and Heyer, 2001; Simard et al., 1994]. The mean ages were respectively 32.20 ± 9.38 and 34 ± 10.56 years old for suicide cases and controls.

Phenotypic Assessment

After obtaining a written consent from the next of kin, a blood or tissue sample was collected for DNA extraction. Following an average period of approximately four months, the families were recontacted to proceed with a psychological autopsy. These were carried out as described elsewhere [Seguin et al., in press]. The interviews were carried out with informant versions of the following instruments: impulsive behavior (Barratt's Impulsivity Scale {Patton, Stanford, et al. 1995 861 /id} - BIS), aggressive behavior (Buss-Durkee Hostility Inventory [Buss and Durkee, 1957] – BDHI - assault, indirect and irritability subscales were used), personality variants (Temperament and Character Inventory [Cloninger et al., 1994]– TCI – only temperament measures were used). Estimates of Internal consistency (α) measures for the informant versions used in our data provided the following results: BIS 0.92, BDHI assault 0.86, BDHI indirect 0.66, BDHI irritability 0.76, TCI novelty seeking 0.91, TCI harm avoidance 0.93, TCI reward dependence 0.68, TCI persistence 0.77. Overall, these are satisfactory results and are similar to those reported by others for some of these scales when used by informants [Brent et al., 1994].

WFS1 variants

There are several mutations associated with WFS and most of them (over 80%) are within exon 8 [Inoue et al., 1998; Strom et al., 1998]. The three variants investigated were in exon 8 and lead to an amino acid change in the WFS1 protein. The H611R mutation produces a histidine to arginine change at position 611 (A1832G), the R456H mutation produces an arginine to histidine change at position 456 (G1367A), and finally, the I333V variant results in an isoleucine to valine change at position 333 (A997G).

Genotyping

Genomic DNA was extracted from blood or from frozen brain tissue using standard procedures [Sambrook et al., 1989]. PCR was performed in a total volume of 20 µl consisting in 40-100 ng of DNA, 200 µM each of dATP, dGTP, dCTP, dTTP, 10 pmol of the specific primers and 0.2 units of Taq (Qiagen, Mississauga, Ontario).

For the H611R polymorphism a PCR product of 139 bp was obtained using forward primer: 5' GAGCTCACCAAGATCGCAGT 3' and reverse primer 5' ACACCAGGATGAGCTTGACC 3'. For the R456H polymorphism forward 5' TCATCACCGGCTTCTTTACC 3' and reverse primer 5' CTTCAGGTAGGGCCAATTCA 3' were used to obtain a 170 bp PCR product. For the I333V, since no restriction enzyme could be used to genotype this locus,

two allele-specific PCR reactions were performed for its detection combining one forward primer and two reverse primers [Newton et al., 1989]. A 237 bp PCR product was obtained with forward primer 5' TGAAGGTGGTCAAGTACCCC 3', reverse primer 5' TCGATGGTGAGGTTGCTGAT 3' and reverse mutant primer 5' TCGATGGTGAGGTTGCTGAC 3'. All the primers were from Alpha DNA (Montreal, Quebec, Canada). Each PCR reaction consisted in an initial denaturation at 94°C for 5 min followed by 40 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at an appropriate temperature for each primer and 30 sec of extension at 72°C. A final extension step was performed for 5 min at 72°C. PCR products (7 µl) were digested overnight at 37°C with 0.3 units/µl of *HhaI* (R456H) and *BsrI* (H611R) in a total volume of 10 µl. Digestion products were visualized by ethidium bromide staining after electrophoresis in a 2.5 % agarose gel. For the I333V polymorphism the two PCR products were loaded in the agarose gel for genotyping. All the restriction enzymes were from New England Biolabs, Mississauga, Ontario, Canada.

Statistical Analysis

Genotype and allele frequency distributions were compared between suicide cases and controls using the χ^2 test and odds ratio when applicable. Student t-test or one way analysis of variance (ANOVA) were computed to estimate differences in the scores obtained from the personality trait questionnaires and scales. Adjustment for covariates was carried out by analysis of covariance. Statistical analyses were

performed using the SPSS package (SPSS standard version, release 10.0.5). Corrections were made for multiple testing using the Bonferroni method.

RESULTS

Forty percent of the suicide cases had 6-month and lifetime prevalence rates of psychiatric disorders available. Although psychopathology information was only available for part of the suicide sample, rates were comparable with those previously reported from a different sample of the same population [Lesage et al., 1994], as well as with data from the literature [Barner-Rasmussen et al., 1986; Barraclough et al., 1974; Beautrais et al., 1996; Conwell et al., 1996]. Approximately half of the suicide cases (45.7%) had a major depressive episode within 6 months prior to death and a similar proportion (43%) met criteria for major depressive disorder at least once in their lifetime. Of the remaining major psychiatric disorders, as expected, life- prevalence rates were high for substance abuse or dependence (32.6%), as well as for antisocial (16%) and borderline (26%) personality disorders, or for these two conditions combined (16%). Subjects committed suicide as follows: hanging (57.7%), shooting (10.8%), CO poisoning (9.0%), drug overdose (9.0%), jumping (7.2%), drowning (3%), blunt or penetrating lesion (2.0%), others (1.3%). These figures are similar to available data on suicide method for the Canadian population [Mercier and Saint-Laurent, 1998].

Table 1 shows allelic and genotypic distributions for the three loci investigated. There was a significant difference between suicide cases and controls for genotypes at the H611R locus, with suicide cases having an excess of the GG (611R/611R) genotype ($\chi^2 = 19.21$, df=2, $P <= 0.001$). This difference was still significant even after correcting for multiple testing (6 comparisons, 3 loci, 2 analyses per locus). No differences were found for allelic distribution at this locus ($\chi^2 = 0.754$, df=1, $P = 0.39$) or for genetic variation at the other loci tested. Hardy Weinberg Equilibrium analysis indicated a marginal departure for locus H611R ($\chi^2 = 8.542$, df=2, $P = 0.014$), but this was not statistically significant after correction for multiple testing ($P = 0.084$ – 3 loci, stratifications according to the status).

Table 2 provides mean scores of impulsivity, aggression and temperament according to genotypes in H611R in a subsample of 31 suicide completers for whom complete phenotypic measures (psychiatric diagnoses and personality traits and dimensions) were available. Subjects with the associated genotype – 611R/611R – had significantly higher scores of BIS measures of impulsivity (t =-3.15 df = 15.3 $P = 0.006$). This was in agreement with the observation that subjects with this genotype also had significantly higher scores of TCI novelty seeking (NS), a measure of exploratory behavior (t = -3.35 df = 13.8 $P = 0.005$). Consistently, measures of persistence (P) were significantly lower in subjects with the 611R/611R genotype (t = 2.4 df = 16.6 $P = 0.028$) (figure 1). Because these traits are influenced by demographic factors such as age and gender and correlate

(particularly BIS and NS) with presence of personality disorders (PD), we carried out an analysis of covariance controlling for these factors. Impulsivity (BIS) and novelty seeking scores persisted to be significantly higher in cases with the genotype 611R/611R even after adjusting for age, gender, lifetime history of depression and presence of any axis II diagnosis (BIS: F = 4.92 df = 1,15 P = 0.04; NS: 6.27 df = 1,15 P = 0.02). As expected, these measures were also different between subjects who had and those who did not have a diagnosis of personality disorders (BIS: F = 9.50 df = 1,15 P = 0.008; NS: F = 7.17 P = 0.01). However, no differences were observed in persistence after controlling for these covariates (F = 2.59 df = 1,15 P = 0.12). No behavioral differences were observed for measures of aggression or other temperament dimensions, for the other genotypes investigated.

DISCUSSION

We investigated a sample of suicide completers and psychiatrically normal controls and found a significant association between variation at locus H611R within exon 8 of the WFS1 gene and suicide. Furthermore, a subsample of suicide cases (N=30) for which personality measurements were available indicate that subjects with the associated genotype displayed higher scores of impulsive behavior and novelty seeking. Conversely, these subjects presented lower scores of persistence. The findings from this study are consistent with clinical observations of psychiatric manifestations in subjects affected with Wolfram

syndrome as well as heterozygous carriers, who have been found to have a 26-fold increased probability of being hospitalized for major depression and/or suicide attempts compared to non-carriers. Furthermore, they support the idea that suicidal behavior may be mediated by impulsive and aggressive behaviors.

The study of personality dimensions of suicide completers by means of interviews with informants may raise some issues of concern related to the validity of this procedure. It might be argued that the proper investigation of these traits require self-assessment and that informants may not be able to provide a reliable assessment of subjective states. It would have been interesting to have more than one informant rating personality dimensions per case and then compare the concordance between these ratings. However, similar measures of personality and behavioral trait dimensions showed significant correlation and, more importantly, the adapted questionnaires and scales used in this study showed high internal consistency. This is consistent with findings from other studies using respondent-based behavioral measures [Brent et al., 1994] and suggests that this method provides valid results.

The H611R variant produces an amino acid change from histidine to arginine in position 611 of the protein, a region thought to be inside the cellular membrane [Hardy et al., 1999; Inoue et al., 1998; Middle et al., 2000; Ohtsuki et al., 2000; Strom et al., 1998]. The functional consequence of this amino acid change is not known nor is the function of the protein itself, but it is conceivable that H611R

interferes with the normal functioning of the protein. Although functional assays are needed to formally investigate H611R, the observation that this variant frequently segregated in families with WS [Inoue et al., 1998; Strom et al., 1998] is consistent with this hypothesis (for instance, as a modifier accounting for part of the variation in the disease expression and severity of the phenotype). Supporting this hypothesis is the fact that in the WS families studied while cloning the gene, the R611 allele was proposed as one of the causative mutations in compound heterozygotes for whom no other mutation was found in the chromosome bearing R611 [Hardy et al., 1999].

As it is always the case in association studies, another possibility is that the H611R locus is in linkage disequilibrium with the actual mutation altering the protein function or trafficking. In this regard, a very interesting candidate gene is adjacent to WFS1. This is the gene that codes for the gamma isoform of a B-subunit of protein phosphatase 2A, which is an enzyme highly expressed in brain tissue and that is hypothesized to play a role in neuronal development [Hu et al., 2000].

Even though nonsignificant following corrections, the marginal departure in Hardy Weinberg (HW) equilibrium for H611R, particularly among controls, should be discussed. Departure from HW equilibrium takes place when there are violations of the underlying assumptions used for the analysis of HW distribution. Specifically, (1) the population must be large, (2) there is random mating, (3) there is no selection for a particular allele or genotype, (4) there are no new

mutations and finally, (5) there is no significant migration or isolation. Given that both cases and controls have the same ethnic origin and were drawn from the same population, i.e., they are all Quebecers of French Canadian origin, it is unlikely that that the reasons listed in 1, 2, 4 and 5 could influence HW equilibrium in controls and not in cases. On the other hand, selection could be a possible explanation for some HW departure among controls, as it would be reasonable to assume that if 611R homozygosity is an actual predisposing factor for suicide completion, then the 611R/611R genotype will be less frequently observed among living controls selected according to absence of psychiatric morbidity.

The cDNA sequence of WFS1 predicts an 890 amino acid putative transmembrane protein designated as wolframin with a molecular mass of 100.29 kD [Inoue et al., 1998; Strom et al., 1998; Takeda et al., 2001]. Wolframin seems to be a novel family of transmembrane proteins presenting a central hydrophobic domain with at least nine helical transmembrane segments [Strom et al., 1998]. Although wolframin's cellular function remains to be better characterized, its localization is of particular interest. Indeed, recently, Takeda et al. [Takeda et al., 2001] have shown that wolframin is present in neurons, and predominantly in the hippocampus and locus coeruleus two regions of the brain particularly interesting in the context of suicide and associated disorders. Both regions, the hippocampus and the locus coeruleus have been shown to be involved in the neurobiology of suicide and depression [Bremner et al., 2000; Gonzalez et al., 1994; Klimek et

al., 1997; Ordway et al., 1994; Ordway, 1997; Sapolsky, 2000; Sheline et al., 1996; Zhu et al., 1999].

There is evidence for genetic heterogeneity in Wolfram syndrome as suggested by a linkage study carried out in Jordanian families, some of which provided positive evidence of linkage to loci on 4q22-24 [El Shanti et al., 2000]. Thus far, this second locus (WFS2) has not been cloned. Once WFS2 is identified, it would be interesting to study its possible role in psychiatric disorders and particularly in suicide in the present population.

Suicide is strongly associated with psychiatric morbidity, particularly mood disorders as well as high levels of comorbidity. The observed association of WSF1 with suicide could then be related to a comorbid phenotype instead of suicidal behavior itself. However, although limited by the small sample, our results remained significant when controlling for the presence of psychopathology. Moreover, a few previous molecular studies have investigated WFS1 gene in psychiatric disorders and no evidence was found supporting its role in these conditions, particularly major depression and bipolar disorder [Evans et al., 2000; Furlong et al., 1999; Middle et al., 2000; Ohtsuki et al., 2000]. If confirmed, our results may be interpreted as indicating that WFS1 predisposes to suicide through a modulatory effect of behavioral traits such as impulsivity. However, in this study we were unable to isolate the effect of WFS1 on impulsive behavior from that of axis II, which in our sample is represented mostly by

borderline and antisocial personality disorder or these two conditions combined. This is not surprising as high scores of impulsivity characterize subjects with these disorders. Further studies in larger samples with extended phenotypic information will be helpful to better characterize these results.

ACKNOWLEDGEMENTS

This study was partially funded by Canadian Institute of Health Research grant MOP-78470 and the American Foundation for Suicide Prevention. GT is a CIHR scholar.

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Table I. Genotypic and Allelic Distribution of Three Missense Polymorphisms in Exon 8 of WFS1 in Completed Suicide and Controls.

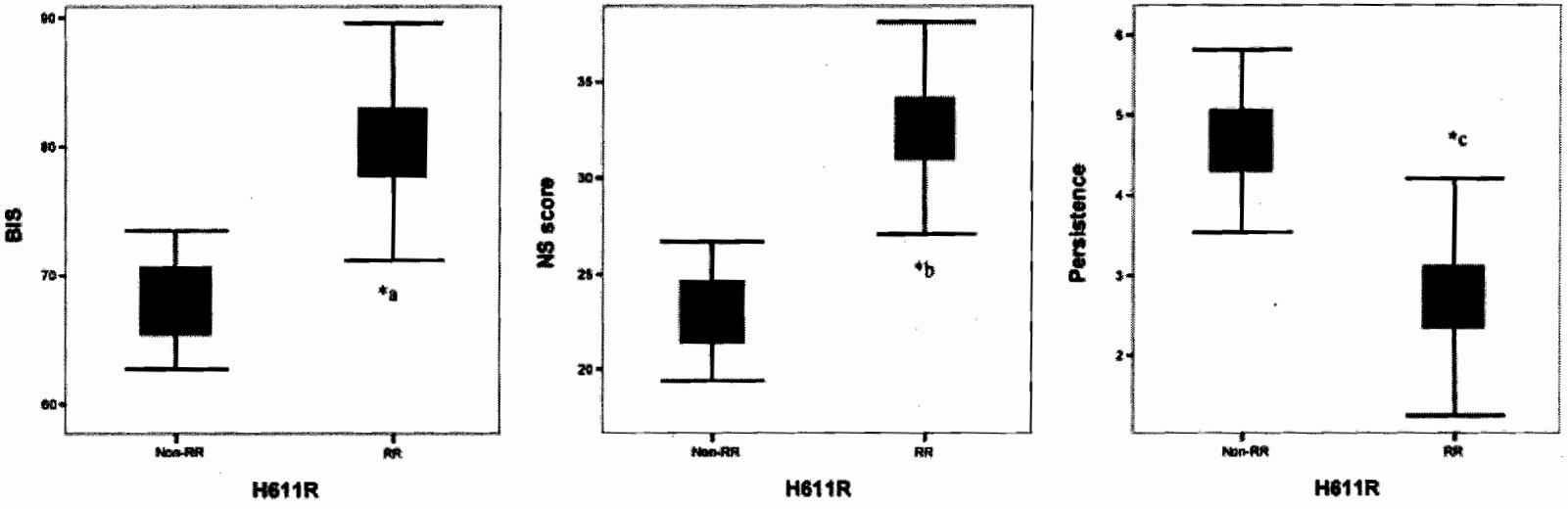
Locus	Allele	Genotype	Status	
			Control	Suicide
H611R	H		139 (0.57)	116 (0.54)
	[a]R		105 (0.43)	100 (0.46)
		HH	24 (0.19)	30 (0.28)
		HR	87 (0.70)	46 (0.42)
		*[b]RR	14 (0.11)	32 (0.30)
R456H	R		20 (0.08)	12 (0.06)
	[c]H		228 (0.92)	204 (0.94)
		RR	1 (0.01)	1 (0.01)
		RH	18 (0.15)	10 (0.09)
		[d]HH	105 (0.85)	97 (0.90)
I333V	I		115 (0.5)	97 (0.48)
	[e]V		115 (0.5)	105 (0.52)
		I I	7 (0.06)	6 (0.06)
		IV	101 (0.88)	85 (0.84)
		[f]VV	7 (0.06)	10 (0.1)

*Indicates statistically significant.
a χ^2=0.754, df=1, *P*=0.39.
b χ^2=9.21, df=2, *P*=0.001.
c χ^2=0.77, df=1, *P*=0.379.
d χ^2=1.51, df=2, *P*=0.471.
e χ^2=0.099, df=1, *P*=0.753.
f χ^2=1.080, df=2, *P*=0.583.

Table II. Mean scores on measures of impulsive and aggressive behavior and related personality variants in subjects who completed suicide according to variation at H611R.

	H611R	N	Mean	SD	t	df	*P*
BIS	Non-RR	22	68.14	12.48	-3.146	15.782	.006
	RR	7	80.43	8.15			
	Total	31	70.06	13.07			
Assault	Non-RR	22	4.14	3.14	-1.536	9.833	.156
	RR	7	6.29	3.25			
	Total	31	4.45	3.23			
Indirect	Non-RR	22	4.95	2.40	-1.564	13.480	.141
	RR	7	6.29	1.80			
		31	5.19	2.27			
Irritability	Non-RR	22	7.50	2.39	-.052	7.960	.960
	RR	7	7.57	3.41			
	Total	31	7.39	2.72			
NS	Non-RR	22	23.05	8.13	-3.348	13.807	.005
	RR	7	32.57	5.97			
	Total	31	24.61	8.90			
HA	Non-RR	22	17.32	8.42	.806	27	.427
	RR	7	13.86	13.89			
	Total	31	16.39	9.54			
RD	Non-RR	22	12.59	3.92	-.683	9.290	.511
	RR	7	13.86	4.38			
	Total	31	13.00	3.88			
Persistence	Non-RR	22	4.68	2.57	2.408	16.644	.028
	RR	7	2.71	1.60			
	Total	31	4.32	2.51			

Figure I. Scores of impulsivity (BIS-Barratt's Impulsivity Scale), novelty seeking (NS) and Persistence according to genotypes in H611R locus in a subsample of 31 suicide completers for whom complete phenotypic measures (psychiatric diagnoses and personality traits and dimensions) were available. *Indicates statistically significant; a(t =-3.15 df = 15.3 $P = 0.006$); b(t = -3.35 df = 13.8 $P = 0.005$); c(t = 2.4 df = 16.6 $P = 0.028$.



CHAPTER 4

ALPHA 2A RECEPTOR GENE AND SUICIDE

This work was published as:
Sequeira A, Mamdani F, Lalovic A, Anguelova M, Lesage A, Seguin M, Chawky N, Desautels A, Turecki G (2004) Alpha 2A adrenergic receptor gene and suicide. Psychiatry Res 125: 87-93.

CONTRIBUTION BY AUTHOR:

Sequeira: performed the experiments, did the analysis and wrote the paper.

Mamdani, Lalovic, Anguelova: performed part of the experiments.

Lesage, Seguin, Chawky,: helped in the design of the study and collecting the data.

Turecki: Writing of the paper and supervision.

PREFACE

With this study, we used the association study approach and a case-control study design to investigate the association between the α2A gene and suicide. We used several genetic markers covering the promoter region but also a SNP in the coding region leading to an amino acid change. The idea behind this approach is to cover most of the gene's genetic variation by testing the most markers in the promoter and coding regions.

Among the first theories concerning the biological basis of mood disorders, the implication of adrenaline and noradrenaline has been documented and confirmed using classical neurochemical approaches and postmortem brain tissue. Advances in molecular genetics revealed recently the existence of genetic variants located in the promoter regions of the α2A adrenergic receptor. Located in cortical and subcortical areas such as the raphé nucleus and the locus coerulus, this receptor is know to be implicated in the control of the release of noradrenaline as well as serotonin. Differences observed in expression levels of this receptor can be due to genetic variants located in the promoter of the gene or in the coding sequence and leading to altered function of expression in suicides. We performed a case-control association study to test this hypothesis in our sample of suicide victims and psychiatrically normal controls from the Quebec population.

ABSTRACT

Suicide is a complex trait resulting from the interaction of several predisposing factors, among which genes seem to play an important role. Alterations in the noradrenergic system have been observed in postmortem brain studies of suicide victims when compared to controls. The purpose of this study was to test the hypothesis that genetic variants of the α2A adrenergic receptor gene are implicated in suicide and/or have a modulatory effect on personality traits that are believed to mediate suicidal behavior. We studied a sample of suicides (N=110) and control subjects (N=130) for genetic variation at four loci, including three in the promoter region (g-1800t, c-1291 g and the g-261a) of the α2A adrenergic receptor gene, and a potentially functional locus, N251K, which leads to an amino acid change (asparagine to lysine). No significant differences were observed at the promoter loci in terms of allelic or genotypic distribution between suicides and controls. However, analysis of the functional polymorphism N251K revealed that the 251 K allele was only present among suicides, though only three suicide cases had this allele, two of which were homozygous. These results are preliminary. If confirmed, they suggest that variation at the α2A adrenergic receptor gene may play a role in a small proportion of suicide cases.

INTRODUCTION

Suicide is an important public health problem that is thought to results from the interaction of different susceptibility factors. Among these, there is increasing evidence suggesting that genetic variability plays a role in susceptibility to suicide (Turecki, 2001). Family studies suggest that the liability to suicide is independently transmitted from the liability to psychiatric disorders often present in suicide victims (Brent et al., 1996). Furthermore, the observation that monozygotic twins have a higher concordance for suicide (Roy et al., 1991) and suicide attempt (Glowinski et al., 2001; Roy and Segal, 2001; Statham et al., 1998) than dizygotic twins supports this evidence.

Alterations in monoamine neurotransmission have been extensively demonstrated in suicidal behaviors. Among these, there is significant evidence suggesting changes in the noradrenergic system of suicide victims. For instance, binding studies in postmortem brains showed an increase in α_2 receptors density in suicides when compared to controls (De_Paermentier et al., 1997; Gonzalez et al., 1994; Meana and Garcia-Sevilla, 1987). There are three types of α_2 receptors in the brain (α_{2A}, α_{2B}, α_{2C}), α_{2A} being the predominant one, particularly in the frontal cortex. Recently, an increase in α_{2A} receptor density was observed in the frontal cortex of depressed suicides (Callado et al., 1998) and this increase was confirmed by immunolabelling techniques (Garcia-Sevilla et al., 1999). Coupled to a $G_{\alpha i}$ protein, the α_{2A} receptor is expressed in noradrenergic neurons as well as

in serotonergic neurons acting as an autoreceptor or heteroreceptor respectively. The agonist mediated α2A receptor coupling to G proteins was also recently found to be increased in depressed suicides when compared to controls supporting the implication of this receptor in suicide (Gonzalez-Maeso et al., 2002).

The altered levels of α_{2A} receptor observed in suicide may be the consequence of genetic variability in the α2A receptor gene, leading to an increased predisposition to suicidal behaviors. Several genetic variants in the α2A receptor gene were identified by Feng el al. in 1998 (Feng et al., 1998). One of them, the N251K polymorphism, located at base 753, leads to a functional change (Small et al., 2000). The possible implication of the promoter variants of this gene have been investigated in panic disorder, mood disorder, temperament factors and antipsychotic response without any conclusive results (Bolonna et al., 2000; Ohara et al., 1998; Ohara et al., 2000; Tsai et al., 2001). Here we report on the investigation of four different α2A receptor gene loci in suicide and associated personality traits.

METHODS

Subjects

Consecutive cases of suicide were collected as part of an ongoing collaboration with the Quebec Coroner's Office and the Montreal Central Morgue. Controls in

this study were living subjects without a history of suicidal behavior or a major psychiatric diagnosis. All subjects included in this study (or their families in the case of the suicides) provided written informed consent, which was approved by the local IRB. A total of 110 suicide completers and 130 normal controls were investigated. All the subjects, cases and controls, were male Caucasians from French-Canadian origin, a homogeneous population with a well-characterized founder effect (Simard et al., 1994). The mean ages were respectively 32.20 ± 9.38 and 34 ± 10.56 years old for suicide cases and controls. Subjects committed suicide as follows: hanging (57.3 %), shooting (10.9 %), CO poisoning (9.1 %), drug overdose (9.1 %), jumping (7.3 %), drowning (2.7 %), blunt or penetrating lesion (1.8 %), others (0.9 %). These figures are similar to available data on suicide method for the Canadian population (Mercier and Saint-Laurent, 1998).

Phenotypic Assessment

After obtaining a written consent from the next of kin, a blood or tissue sample was collected for DNA extraction. A few months later, the families were recontacted to proceed with a psychological autopsy and collect data on related behavioral and personality traits as described elsewhere (Seguin et al.). Briefly, structured interviews were carried out by trained clinicians with an average of two key family members per subject. SCID-I interviews were used to systematically collect information about the mental health history of the deceased. Following the interview, a review of the Coroner's notes and of all relevant medical records was

performed and a case report was written for the purpose of a best-estimate diagnosis. Best consensus DSM-IV axis I and II diagnoses were made by a panel of clinicians based on the diagnosis, interviews and all complementary information available, such as medical charts. In addition to the diagnostic interviews, for 42 random cases of suicide, we had data collected on related behavioral and personality traits. These interviews were carried out with informant versions of the following instruments: impulsive behavior (Barratt's Impulsivity Scale (Patton et al., 1995) - BIS), aggressive behavior (Buss-Durkee Hostility Inventory (Buss and Durkee, 1957) – BDHI - assault, indirect and irritability subscales were used), personality variants (Temperament and Character Inventory (Cloninger et al., 1994)– TCI – only temperament measures were used).

Genetic variants

Genetic variants were chosen based on their reported heterozygosity and their putative functional character. The G-1800T, C-1291G and the G-261A are in the promoter region of the gene and could therefore alter the expression of the gene. The N251K polymorphism, located at base 753, produces an asparagine to lysine change at the third intracellular loop level of the receptor protein. The Lys-251 increases the agonist promoted coupling of the receptor leading to an enhanced inhibition of adenylyl cyclase and an enhanced stimulation of MAP-kinase as compared to the wild type (Small et al., 2000).

Genotyping

Genomic DNA was extracted from blood or from frozen brain tissue using standard procedures (Sambrook et al., 1989). PCR was performed in a total volume of 20 µl consisting in 40-100 ng of DNA, 200 µM each of dATP, dGTP, dCTP, dTTP, 10 pmol of the specific primers and 0.2 units of Taq (Qiagen, Mississauga, Ontario). Each PCR reaction consisted of an initial denaturation at 94°C for 5 min followed by 40 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at an appropriate temperature for each primer and 30 sec of extension at 72°C. A final extension step was performed for 5 min at 72°C. For digestion of PCR products with restriction enzymes, (7 µl) were digested overnight at 37°C with 0.3 units/µl of the enzyme in a total volume of 10 µl. Digestion products were visualized by ethidium bromide staining after electrophoresis in a 2.5 % agarose gel. Restriction enzymes were from New England Biolabs, Mississauga, Ontario, Canada. All the primers were from Alpha DNA (Montreal, Quebec, Canada). For the g-1800t polymorphism, *BmsBI* was used for the digestion of the PCR product obtained with the forward primer (5' AGAAGGCTCTGTTGGCAGAA 3') and the reverse primer (5' ACCTCCGCTTAGGGAAGAAG 3'). For the c-1291g polymorphism *Msp*I was used for digestion after the PCR with the forward primer (5' AGGTTACTTCCCTCGATTTGG 3') and the reverse primer (5' AGACTTAAAGAGGGAGCCCG 3').

For the N251K polymorphism, since no restriction enzyme was available, allele-specific PCR reactions (Newton et al., 1989) were performed combining one forward primer (5' GCCGCGCTGCGAGATCAACGA 3') and two reverse primers (5' TGCGCTCGGGGCCCAGACCG 3' and 5' TGCGCTCGGGGCCCAGACCC 3').

For the g-261a polymorphism we used a SNaPshot PCR and the ABI 3100 Genetic Analyzer from Applied Biosystems for electrophoresis. Genotypes were generated using GeneScan 1.0 and Genotyper 1.0 software. Briefly, genomic DNA was amplified by PCR using one forward primer (5' TCCTTCCAAGTTATCAGGCCA 3') and one reverse primer (5' TTCAGACGCTCTCGGTGGGC 3'). PCR product was then treated with SAP and Exo I to remove the unincorporated dNTPs and primers. The SNaPshot reaction is based on the dideoxy single-base extension of an unlabeled primer (Lindblad-Toh et al., 2000). The added dNTPs were fluorescently marked with four different fluorochromes allowing the genotyping.

Statistical Analysis

Genotype and allele frequency distributions, as well as departure from Hardy-Weinberg equilibrium were compared between suicide cases and controls using the χ^2 test and/or odds ratio, as applicable. Student t-test or one way analysis of variance (ANOVA) were computed to estimate differences in the scores obtained

from the personality trait questionnaires and scales in function of the observed genotypes. Adjustment for covariates was carried out by analysis of covariance. Statistical analyses were performed using the SPSS package (SPSS standard version, release 10.0.5).

RESULTS

Allelic and genotypic frequencies for G-1800T, C-1291G, G-261A and N251K are given in table 1. The genotype distributions at all four *loci* were in Hardy-Weinberg equilibrium, both for cases and controls. No differences were found when comparing allelic or genotypic distribution of cases and controls in *loci* G-1800T, C-1291G or G-261A. The study of locus N251K showed that the only subjects carrying the 251K allele, either in heterozygosity or homozygosity, were suicide cases. The two 251K homozygous were males aged respectively 25 and 53 years and presented a psychiatric disorder at the time of their death. The 25-year-old suicide suffered from major depressive disorder (single episode) and alcohol abuse while the 53 years old had an adjustment disorder with depressed mood. No clinical information was available on the heterozygous suicide.

Personality measurements were available for 42 suicide victims among which the two homozygous 251K/251K. We investigated whether genetic variation at this locus could be associated with personality traits associated with suicide such as impulsive and aggressive behaviors. Table 2 provides mean scores of impulsivity,

aggression and temperament dimensions according to genotypes for *loci* G-1800T, C-1291G, G-261A in a subsample of 42 suicide completers for whom complete phenotypic measures (psychiatric diagnoses and personality traits and dimensions) were available. Analysis of the mean scores obtained from these personality traits in function of the observed genotypes showed no statistically significant differences in terms of impulsive behavior (BIS), aggressive behavior (BDHI) or temperament dimensions (TCI).

DISCUSSION

We studied the genetic variability at three *loci* in the promoter region (G-1800T, C-1291G and the G-261A) and one functional polymorphism (N251K) of the α2A gene in a sample of suicides and control subjects. This is to our knowledge the first study on the implication of the α_{2A} receptor gene in suicide and related behaviors. The genetic variability was not significantly different between the suicide group and the control subjects at the three most polymorphic *loci*. However, the investigation of the N21K polymorphism revealed that the rare 251K allele was only present among suicides, which could, if confirmed, suggest a possible role of this variant in the susceptibility to suicide, accounting for a small proportion of the total genetic variability implicated in this serious phenotype. Since the two 251K homozygous were depressed, the possibility that this variant is associated with depression should be considered in future studies.

The N251K polymorphism, located at base 753, produces a functional asparagine to lysine change in a highly conserved portion of the third intracellular loop level of the receptor protein. The activation of the Lys251 form of the α_{2A} receptor, only observed among suicides, leads to an enhanced inhibition of forskolin stimulated adenylyl cyclase activity and activation of MAP kinase (Small et al., 2000). Interestingly, alterations in several elements of this metabolic pathway have been observed in suicide post-mortem brains when compared to controls, including decreased adenylyl cyclase activity (Cowburn et al., 1994; Lowther et al., 1996). Furthermore, alterations at several levels of this second messenger pathway have also been observed in suicides (Dwivedi et al., 2001; Dwivedi et al., 2002; Odagaki et al., 2001).

It has been suggested that personality traits are at least in part under the control of the noradrenergic system. However, further analysis of the mean scores on measures of impulsive and aggressive behavior and related personality dimensions in subjects who completed suicide according to genotype at the four α_{2A} loci showed no difference. These results do not exclude the possible role of the noradrenergic system on personality traits, as other components of this system may be involved. More importantly, these results should be regarded in the context of the limitations of our study, particularly the small sample size. Nevertheless, these results are consistent with the only other study that to our knowledge has been published investigating the influence of genetic variation at

the α_{2A} gene (the C-1291G promoter polymorphism) on personality traits (Tsai et al., 2001).

In summary, our results suggest that variation at the N251K locus may be implicated in the susceptibility to suicide completion, accounting for a small proportion of the total genetic variability. The 251K allele is a rare variant of the α_{2A} receptor gene and due to this study's relatively small sample and the likelihood of false positives in association designs, independent replication of our results are extremely important in order to assess their validity.

Acknowledgements

This study was partially funded by CIHR grants MOP-38078, MOP-53321 and AFSP.

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Table 1: Allelic and genotypic distribution of four single nucleotide polymorphisms in the α2A receptor gene in a sample of suicides and matched controls subjects.

Locus	Allele	Genotype	Status	
			Suicides	Controls
G-1800T	G		190 (0.92	203 (0.94)
	T		16 (0.08)	13 (0.06)
		GG	90 (0.87)	95 (0.88)
		GT	10 (0.10)	13 (0.12)
		TT	3 (0.03)	0 (0)
C-1291G	C		168 (0.80)	207 (0.82)
	G		44 (0.20)	45 (0.18)
		CC	65 (0.61)	83 (0.66)
		CG	38 (0.36)	41 (0.32)
		GG	3 (0.03)	2 (0.02)
G-261A	G		222 (0.88)	266 (0.90)
	A		30 (0.12)	30 (0.10)
		GG	97 (0.77)	119 (0.80)
		GA	28 (0.22)	28 (0.19)
		AA	1 (0.01)	1 (0.01)
N251K	N		187 (0.97)	248 (1)
	K		5 (0.03)	0 (0)
		NN	93 (0.97)	124 (1)
		NK	1 (0.01)	0 (0)
		KK	2 (0.02)	0 (0)

Fig 1: Graphic representation of the α_{2A} receptor gene with relative position of the tested *loci*.

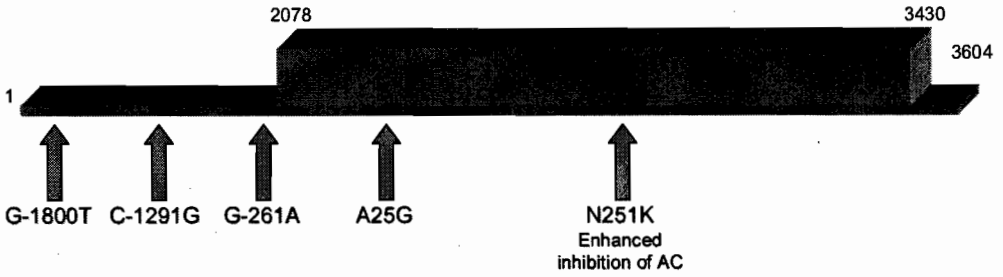

Table 2: Mean scores on measures of impulsive and aggressive behavior and related personality variants in subjects who completed suicide sorted by genotype at four α_{2A} loci*.

	N251K	N	Mean	SD	C-1291G	N	Mean	SD	C-1800T	N	Mean	SD	G-261A	N	Mean	SD
BIS	NN	38	69.89	18.08	CC	26	68.42	19.13	CC	34	69.41	17.42	GG	24	70.50	19.64
	KK	2	66.50	9.19	CG	15	72.07	14.56	CT	5	71.40	19.24	GT	8	70.50	16.41
					GG	1	72.00	.	TT	2	68.00	25.46				
ASSAULT	NN	36	3.97	3.18	CC	24	3.83	3.09	CC	32	4.09	3.14	GG	22	3.73	2.85
	KK	2	3.50	2.12	CG	15	4.40	3.29	CT	5	4.40	3.78	GT	8	4.25	3.69
					GG	1	9.00	.	TT	2	3.00	4.24				
INDIRECT	NN	36	5.17	2.26	CC	24	4.88	2.71	CC	32	5.38	2.35	GG	22	5.55	1.95
	KK	2	4.00	5.66	CG	15	5.53	1.73	CT	5	4.20	3.11	GT	8	5.38	2.77
					GG	1	8.00	.	TT	2	4.50	2.12				
Irritability	NN	36	6.94	2.66	CC	24	7.17	2.91	CC	32	7.13	2.88	GG	22	6.82	2.87
	KK	2	8.00	4.24	CG	15	7.00	2.51	CT	5	6.60	2.30	GT	8	7.25	2.92
					GG	1	10.00	.	TT	2	8.00	1.41				
NS	NN	38	23.97	9.27	CC	26	23.15	9.61	CC	34	24.06	8.97	GG	24	26.08	9.14
	KK	2	18.50	4.95	CG	15	24.20	8.50	CT	5	23.80	8.76	GT	8	22.88	8.24
					GG	1	27.00	.	TT	2	16.50	16.26				
HA	NN	38	15.50	8.68	CC	26	16.85	8.90	CC	34	16.03	8.78	GG	24	13.88	8.89
	KK	2	20.50	13.44	CG	15	14.27	8.53	CT	5	11.20	7.16	GT	8	14.38	7.87
					GG	1	26.00	.	TT	2	26.00	4.24				
RD	NN	38	12.87	4.14	CC	26	12.73	3.48	CC	34	13.26	3.99	GG	24	13.50	3.62
	KK	2	12.00	5.66	CG	15	13.33	4.84	CT	5	11.40	5.03	GT	8	13.25	4.89
					GG	1	7.00	.	TT	2	9.50	0.71				
P	NN	38	4.55	2.58	CC	26	4.69	2.33	CC	34	5.03	2.49	GG	24	4.92	2.45
	KK	2	5.50	3.54	CG	15	4.80	3.17	CT	5	4.40	3.05	GT	8	5.13	2.85
					GG	1	5.00	.	TT	2	1.00	1.41				

BIS: Barrat's impulsivity scale; NS: novelty seeking; HA: harm avoidance; RD: reward dependance; P: persistence.

CHAPTER 5

IMPLICATION OF *SSAT* BY GENE EXPRESSION AND GENETIC VARIATION IN SUICIDE AND MAJOR DEPRESSION

This work was published as:
Sequeira A, Gwadry FG, Ffrench-Mullen JM, Canetti L, Gingras Y, Casero RA, Jr., Rouleau G, Benkelfat C, Turecki G (2006) Implication of SSAT by gene expression and genetic variation in suicide and major depression. Arch Gen Psychiatry 63: 35-48.

CONTRIBUTION BY AUTHOR:

Sequeira: performed the experiments, did the analysis, interpreted the results and wrote the paper.

Gwadry: did part the analysis, interpretation of the results and helped writing the paper.

Canetti and Gingras: performed part of the experiments.

Ffrench-Mullen and Benkelfat: helped in the design of the study and collecting the data.

Casero: provided the anti-SSAT antibody and helped for the discussion of results.

Rouleau: design and interpretation of the results.

Turecki: Design, writing of the paper and supervision.

PREFACE

The use of candidate gene approaches while highly knowledge-driven are limiting for the study of complex traits, which by definition are caused by multiple factors and probably multiple genes each having a small effect. Since the completion of the human genome sequence and the development of high throughput expression screening technologies, whole genome strategies can be developed for study of complex multigenic traits.

In this study, we used a genome wide approach by using microarray expression assays to test in parallel thousand of genes expression levels. We performed a genome wide expression study in three areas of the brain and identified SSAT, a gene involved in polyamine metabolism, as being differentially expressed in three cortical brain areas of suicides with and without major depression, as well as matched controls. These results were validated and further explored using complementary techniques. Genetic variation studies indicated that a variant in the promoter of this gene affects SSAT expression and is associated to suicide in the general population. These findings suggest that polyamines play a role in the neurobiology of suicide and depression.

ABSTRACT

Context: A large body of evidence suggests that predisposition to suicide, an important public health problem, is mediated, to a certain extent, by neurobiological factors.

Objective: To investigate patterns of expression in suicide with and without major depression and to identify new molecular targets that may play a role in the neurobiology of these conditions.

Design: Brain gene expression analysis was performed using the Affymetrix HG-U133 chipset in the orbital cortex (Brodmann area [BA]11), the dorsolateral prefrontal cortex (BA8/9), and motor cortex (BA4). Subsequent studies were carried out in independent samples from adjacent areas to validate positive findings, confirm their relevance at the protein level, and investigate possible effects of genetic variation.

Subjects: We investigated 12 psychiatrically normal controls and 24 suicide victims including 16 suicides with major depression and 8 without major depression in the brain gene expression analysis, validation and protein studies. The genetic studies included 181 suicide completers and 80 psychiatrically normal controls. All subjects investigated were males of French-Canadian origin

Main Outcome Measures: Gene expression measures from microarray, semiquantitative reverse transcription –polymerase chain reaction, immunohistochemistry, and Western blot analyses.

Results: Twenty-six genes were selected because of the consistency of their expression pattern (fold change, >1.3 in either direction [$P \leq 0.01$] in at least 2 regions). The spermine/spermidine N1-acetyltransferase gene (SSAT) was successfully validated by reverse transcription polymerase chain reaction, immunohistochemistry, and Western blot analyses. A variant located in the SSAT polyamine-responsive element regulatory region (SSAT342A/C) demonstrated a significant effect of genotype on SSAT brain expression levels ($F1=5.34$; $P=.02$). Further investigation of this variant in an independent sample of 181 male suicide completers and 80 male controls showed a higher frequency of the SSAT342C

allele among suicide cases (odds ratio, 2.7; 95% confidence interval, 1.4-5.3; P=.005), suggesting that this allele may increase predisposition to suicide. **Conclusions:** These data suggest a role for SSAT, the rate-limiting enzyme in the catabolism of polyamines, in suicide and depression and a role for the SSAT342 locus in the regulation of SSAT gene expression.

INTRODUCTION

Suicide is a major public health problem, and in many countries, it is the leading cause of death for men younger than 35 years of age[1]. Over the last decades it has become increasingly clear that individuals who commit suicide have a certain biological predisposition, part of which is given by genes[2]. Psychopathology, particularly major depressive disorder (MDD), is commonly associated with suicide, but the genetic liability to suicide is likely independent from the liability to psychiatric disorders[3-6]. A growing effort has been in place to identify biological markers for suicide and depression[7], but most of the studies have focused on components of the serotonergic[2] and noradrenergic systems[2]. However, it is clear that additional systems play a role in the neurobiology of these conditions.

This study aimed to identify new molecular targets that may play a role in the neurobiology of suicide with and without major depression. To identify potential risk factors, a gene expression study was initially conducted as a screening strategy. This was conducted in three brain cortical regions: Brodmann areas (BA) BA4, BA8,9 and BA11. Post-mortem and neuroimaging studies over the past years have revealed the implication of BA8,9 (dorsolateral prefrontal cortex) and BA11 (orbital cortex) in suicide and depression[8;9]. Because of the common motor function alterations in depressed patients[10;11], BA4 (motor cortex), primarily a motor region linked to motor deficits[12], was also investigated. The brain gene

expression screening study identified an interesting target – SSAT, the gene that codes for spermine/spermidine N1-acetyltransferase – which, subsequently, was further investigated using a series of complementary strategies. Here we present evidence suggesting that polyamines, and particularly SSAT, may play a role in the predisposition to suicide and major depression.

METHODS

SUBJECTS

Gene expression studies

All subjects were males of French-Canadian origin, a homogeneous population with a founder effect[13]. Cases and controls were matched on the basis of age and post-mortem interval. All subjects died suddenly without a prolonged agonal state or protracted medical illness. Brain samples were obtained from the Quebec Suicide Brain Bank and were collected with post-mortem intervals of less than 36 hours at autopsy. Brodmann area 4 (BA4), BA8,9 and BA11 were sampled at 4 °C and snap-frozen in liquid nitrogen before storage at -80 °C. This study was approved by our local IRB and informed consent was obtained from next of kin.

All subjects were psychiatrically characterized by psychological autopsies, which are validated methods to reconstruct psychiatric history by means of extensive proxy-based interviews, as outlined elsewhere[3]. Briefly, psychological autopsies

were carried out using structured interviews by means of the SCID-I, which was administered by trained clinicians with an average of 2 informants per family. Following the interview, a review of the Coroner's notes and of all relevant medical records was performed and a case report was written for the purpose of a best-estimate diagnosis. Best consensus DSM-IV axis I diagnoses were made by a panel of psychiatrists based on the analysis of the case reports. The sample consisted of depressed-suicide victims (SD; n=16) who died during an episode of major depression; suicide victims (S; n=8) with no lifetime history of major depression; and matched controls (C; n=12) with no history of suicidal behavior or a major psychiatric diagnosis.

Validation and protein studies

Samples from all subjects included in the microarray screening were used for the semi-quantitative RT-PCR and Western Blot experiments. Three subjects per group and per region (n=27) were included in the immunohistochemistry studies. Subjects were selected blind to the microarray results and based on quality of the tissue for immunohistochemistry experiments.

Genetic variation studies

The population-based gene association study was conducted in a larger sample comprising all subjects included in the microarray studies and an independent sample totaling 181 male suicide completers and 80 male controls, all of which were of French-Canadian origin. Suicide completers were consecutively collected

from the Montreal Central Morgue and controls were psychiatrically normal subjects following DIS assessments that were drawn from the Quebec general population and were matched by age, gender and ethnic origin.

MICROARRAY ANALYSIS

RNA extractions utilized in the current study had a minimum A260/A280 ratio of > 1.9. The samples were further checked for evidence of degradation and integrity. Samples had a minimum 28S/18S ratio of > 1.6 (2100-Bioanalyzer, Agilent Technologies). We used the Human Genome U133 Set, which consists of two GeneChip arrays with ~45,000 probesets representing >39,000 transcripts derived from ~33,000 well substantiated human genes (http://www.affymetrix.com).

GeneChip analysis was performed with Microarray Analysis Suite (MAS) 5.0, Data Mining Tool (DMT) 2.0, and Microarray Database software. All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 100.

Various microarray RNA integrity indicators were used in this study (**Tables 1 and 2**) to filter samples for quality for final analysis. Principal Component Analysis (PCA) was used to quickly identify outlier arrays. Microarray quality control parameters included the following: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, and consistent β-

actin and GAPDH 5'/3' signal ratios. Outlier subjects were excluded on a region basis without any subject being excluded from all the regions. Similar number of subjects were included in the final analysis across the three regions (BA4: C=7, S=5 SD=10; BA8,9: C=6, S=6, SD=7; BA11:C=6, S=5, SD=8).

DATA ANALYSIS

We selected genes for analysis on the basis of "Present Calls" by MAS 5.0. In the current study, for a gene to be included, it had to be "Present" (detectable) in at least 75 % of the subjects in at least one of the three groups in order to reduce the chances of false positives. Expression data was analyzed using Genesis (GeneLogic) and AVADIS software (Strand Genomics). Gene expression values were floored to 1 and then $\log_2$-transformed.

One-way analysis of variance (ANOVA) was performed for each gene to identify statistically significant gene expression changes. To identify differences between depressed-suicides (SD) and suicides (S), statistically significant genes were subjected to a post-hoc test for the contrasts SD versus C, S versus C, and SD versus S. In all, two criteria were used to determine whether a gene was differentially expressed. A gene had to have an ANOVA p-value of less than or equal to 0.01. Second, for a given contrast a gene had to have a fold change\p-value combination of 1.3 fold change in both direction and $P \leq 0.01$.

Cluster analysis was performed using average-linkage hierarchical cluster analysis with a correlation metric. Both expression patterns in individuals and genes were clustered. Principal component analysis (PCA) was performed based on the initial gene sets and on the selected genes (according to our significance criteria). PCA based on the initial gene set did not discriminate the groups. PCA based on the selected genes showed discrimination of the three groups.

SEMI-QUANTITATIVE RT-PCR

Reverse transcription was performed in a total volume of 40µl with 2µg of total mRNA using M-MLV reverse transcriptase (Gibco, Burlington, Ontario) and oligo(dT)16 primers. PCR amplification was carried out using the AmpliTaq Gold from Applied Biosystems (Foster City, California). mRNA-specific primers, were designed using Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) in order to avoid amplification of contaminating genomic DNA. PCR products were visualized using ethidium bromide staining after electrophoresis in a 3% agarose gel. Images were digitalized and analyzed using Gene Tools (Syngene, Cambridge). New samples collected from adjacent tissue from the all the subjects included in the microarray expression studies were used in this analysis (SD=16, S=8, C=12).

IMMUNOHISTOCHEMISTRY AND WESTERN BLOT ANALYSIS

Sections from tissue adjacent to that used for the microarray experiment were used for immunohistochemistry. Three subjects per group and per region (n=27)

were included in the analysis and in average three slides per subject were examined. Immunohistochemical labeling was performed using standard protocols. In brief, frozen samples were sectioned at 10μm, air dried at room temperature, fixed in acetone and conserved at -80°C. Prior to the incubation with the primary antibody, slides were acclimated at room temperature for 15 min., incubated with TBS for 10 min and with normal rabbit antiserum (Santa Cruz Biotechnology, CA) to avoid non specific binding. SSAT antibody was diluted 1:75. For the staining, the Dako LSAB2 System peroxidase (Carpinteria, CA) was used according to the manufacturer's indications. Evaluation of the sections was made by two of the authors (AS, LC) blind to phenotype and brain region. Quantification of immunopositive cells was done with ImageJ from the NIH (version 1.29x).

Western blots were carried out on additional samples adjacent to the previous dissections from all the subjects used in the microarray experiments (SD=16, S=8, C=12). Briefly, 50 μg of total protein were loaded in Tris-Glycine 4-20% pre-cast gels (Invitrogen, Carlsbad, CA) and transferred on to nitrocellulose membranes. Membranes were blocked with 6% milk in TBST, hybridized to the anti-SSAT polyclonal primary antibody (1:1000) overnight at 4 °C and then to a peroxidase conjugated secondary antibody (1:5000, Santa-Cruz Biotechnology, CA). The proteins were visualized by chemiluminescence (Bio-Rad, CA). For standardization and comparisons, the membranes were also hybridized to a

primary anti-β-actin antibody (1:5000, Sigma, St. Louis, MO). Films were digitalized and the bands quantified with Gene Tools (Syngene, Cambridge).

GENOTYPING

Genomic DNA was extracted from blood or from frozen brain tissue using standard procedures[14]. A description of the PCR method used for amplification can be found elsewhere[15]. Genotyping was performed using the SNaPshot[16] procedure and the ABI 3100 Genetic Analyzer from Applied Biosystems (Foster City, CA) following the manufacturer instructions. Genotypes were automatically generated using GeneScan 1.0 and Genotyper 1.0. Four single nucleotide polymorphisms (SNPs) were genotyped, two in the coding sequence (SSAT460 and SSAT495) and two SNPs located a few nucleotides away from the PRE motif and within the regulatory region (SSAT342 and SSAT624). Confirmation of SSAT342A/C genotypes was done by MspI digestion followed visualization by ethidium bromide staining after electrophoresis in a 2.5 % agarose gel. MspI is from New England Biolabs, Mississauga, Ontario, Canada.

RESULTS

Analysis of demographic parameters revealed no significant difference in terms of age and post-mortem interval between the groups (**Table 3**). Consistent with

previous reports[17-19], analysis of post-mortem interval on RNA quality control parameters revealed no significant effect in our sample (**Figure 1**).

As an initial evaluation of the discriminatory ability of the microarray gene expression assay, we compared cortical samples (BA4, BA8,9, BA11) from normal controls to samples obtained from the cerebellar tissue of the same individuals (n=9). PCA was performed based on the ~22,000 genes on the HG-U133A Chip (**Figure 2**). Spatial separation on the first component demonstrate the sensitivity and the power of the microarray experiment design to detect the subtle gene expression pattern changes between the cortical and cerebellar tissue due to their respective functional and neuroanatomical particularities.

To reduce the number of comparisons and the chances of false positives, statistical comparisons were performed on selected genes instead of the total number of genes present in the chipset. The Venn diagrams in **Figure 3** summarize the number of differentially expressed genes observed per comparison using as criteria p-value≤0.01 and fold change (FC) ≥1.3 and the extent of overlap of genes differentially expressed between in each brain region. The common genes differentially expressed in BA4, 8,9 and 11, between the two groups of suicides versus the controls are represented in the intersection of the Venn diagrams. A total of 26 genes were common between 2 or 3 regions and a total of 200 common genes were differentially expressed between groups across brain regions (**Figure 3**). As shown in **Table 4**, the fold changes for the two

comparisons, SD-C and S-C, are highly consistent and going in the same direction for all the genes in all the regions.

PCA performed with differentially expressed genes in each region showed good separation on the first component between the three groups (**Figure 4C** for BA11, **Figures 5C** for BA4 and **Figure 6C** for BA8,9, respectively). The spatial discrimination observed demonstrates that the difference between the groups of subjects is based on genes that are differentially co-regulated between the groups.

In BA11, 375 genes from an initial set of 14,864 selected genes were identified as being differentially expressed (**Figure 3**). The majority of these genes, 275 out of the 375, were misregulated in the depressed suicides when compared to controls as shown by the Venn diagrams (**Figure 3**). Cluster analysis showed mainly four clusters of genes with similar expression as seen in the cluster image map (CIM) in **Figure 4A and B**. Genes in cluster 1 (**Figure 4B**) were on average over-expressed in both suicide groups when compared to controls but showed no differences between the two suicide groups, suggesting that genes in this cluster are co-regulated in the same manner in suicides indistinctly of the diagnosis. Cluster 2 genes were on average down-regulated in the depressed-suicide group versus the suicides and the controls with no major differences between the suicides and the controls, suggesting that these genes may be associated with depression. The spermine/spermidine N1-acetyltransferase (SSAT) gene implicated in stress response was present in this cluster. A graphical

representation of SSAT levels in BA11 is shown in **Figure 7**. Clusters three and four were much smaller and showed an opposite pattern from each other. Gene ontology analysis based on these clusters showed clear differences in terms of the most common gene ontology terms between the clusters (**Figure 4D**).

In BA4, 162 genes were identified as being differentially expressed from initially 14,034 selected genes (**Figure 3**). Clustering analysis of differentially expressed genes in BA4 showed 4 main clusters of genes (**Figure 5A**). The largest cluster was composed of 61 genes on average over-expressed in the two suicide groups upon comparison to controls (**Figure 5A and 5B, cluster 1**). The second cluster in BA4 consisted of 45 genes generally down-regulated among the depressed suicides when compared to the suicides and the controls and mostly implicated in protein metabolism and signal transduction. The SSAT gene was also present in this cluster and a graphic representation of its differential expression is shown in **Figure 7**. Cluster 3 was composed of 37 genes with in average lower expression among suicides when compared to depressed suicides and to the controls. Cluster 4 was the smallest in BA4 with 21 genes mainly having higher expression in suicides versus controls. Gene ontology analysis was also performed in the BA4 cased on the clusters observed and showed clear differences in terms of the most frequent terms between the clusters (**Figure 5D**).

In BA8,9, 282 of an initial set of 14,519 selected genes were identified as being differentially expressed (**Figure 3**). The distribution of differentially expressed

genes shows that the majority of genes were misregulated in suicides in relation to controls (140 or ~50%) and in relation to depressed-suicides (75 or ~25%). Nine genes, five up-regulated and four down-regulated, were common in both depressed-suicides and suicides in relation to controls (**Figure 3, Venn diagram**). Cluster analysis of BA8,9 showed a particular expression pattern with the two major clusters of genes showing, on average, primarily differences between the suicides versus the depressed suicides and the controls, suggesting that this region may be more suicide specific (**Figure 6A**). Cluster 1 was composed of 151 genes in average less expressed among the suicides when compared to the controls. Cluster 2 was the second largest cluster in BA8,9 with 114 genes. Genes in this cluster were also differentially expressed in the suicides when compared to the depressed suicides and the controls with the difference that they were over-expressed in the suicides in this cluster. Cluster 3 was composed of only 17 genes mainly up-regulated in the two suicide groups in comparison with controls. Gene ontology analysis based on the three clusters observed in BA8,9 also showed clear differences confirming the specificity of these clusters (**Figure 6D**).

A total of 26 genes were found to be differentially expressed in at least two out of the three regions investigated as shown by the intersections in the Venn diagram (**Figure 3** and **Table 5**). According to 1-way analysis of variance, one of these genes, SSAT, was differentially expressed according to the ANOVA in BA4 and BA11 at the $P<0.001$ level and at the $P<0.05$ level in BA8,9 (**Figure 7**). In BA4 SSAT was significantly down-regulated in depressed suicides and suicides in

relation to controls with fold-changes of -1.6 (P=0.005) and -1.4 (P=0.016) respectively. In BA11, SSAT was significantly down-regulated in both depressed-suicides and suicides in relation to controls with fold-changes of -1.8 (P=0.002) and -1.4 (P=0.005), respectively. Finally, in BA8,9, SSAT was down-regulated in depressed-suicides in relation to controls with a fold-change of -1.4 (P=0.02).

As indicated in **Table 3**, some of the subjects included in the study had psychopathology other than major depressive disorder. In addition, several subjects had a positive history of dependence/abuse of substance, which may act as important confounders of the gene expression analyses. An analysis of covariance controlling for positive history of dependence/abuse of substance, presence of psychopathology other than major depressive disorder and result of the postmortem toxicological screening exam indicated that the group effect on SSAT expression in BA4 (F=3.89, df=2, p=0.042) and BA11 (F=12.2, df=2, p=0.001) was independent of the effect of these possible confounders.

Differential expression of SSAT in BA4, BA8,9 and BA11 was confirmed by semi-quantitative RT-PCR (**Figure 8A and C**) on independent samples from the same individuals. Lower expression of SSAT was confirmed in BA4 for the suicides (FC = -1.2) and for the depressed suicides (FC= -1.3) when compared to controls (F=3.75; df=2; P=0.035). In BA8,9, SSAT expression was lower among suicides and depressed suicides when compared to controls with fold changes of -1.5 and -1.4, respectively (F=8.08; df=2; P=0.002). Similarly, in BA11 SSAT

expression was 1.5-fold lower in suicides and 1.4-fold lower in suicide with major depression than in controls (F=9.20; df=2; *P*=0.001).

Altered expression at the transcriptional level does not necessarily lead to altered protein expression and SSAT is known to undergo extensive post-transcriptional regulation[20;21]. Therefore, confirmation of the observed changes at the protein level was carried out by immunohistochemistry analysis in tissue sections prepared from the same brain regions using a SSAT polyclonal antibody[22]. **Figure 9A** illustrates the observed changes in SSAT immunoreactivity in BA4, BA8,9 and BA11 of a control, a suicide and a depressed suicide. Quantification of immunopositive cells in a subgroup of subjects (three subjects per group, per region, n=27) showed a lower SSAT protein expression in both suicides with (FC=1.36; df=2; *P*=0.04) and without major depression (FC=1.35; df=2; *P*=0.005) when compared to controls. The changes at the protein level were also confirmed by quantification of SSAT by Western blot analysis in adjacent samples from the same regions from all subjects initially selected for the microarray analysis (SD=16, S=8, C=12). Significant alteration of SSAT protein levels were observed between the groups in BA4 (F=4.47; df=2; *P*=0.020), BA8,9 (F=4.80; df=2; *P*=0.015) and in BA11 (F=4.08; df=2; *P*=0.026), with lower expression among the suicides, particularly the suicides with major depression when compared to the controls (**Figure 9B**). Thus, the microarray evidence, confirmed by the semi quantitative RT-PCR reflect relevant changes at the protein

level and suggest a possible role of SSAT in the pathophysiology of suicide and depression.

It was recently shown that SSAT expression is closely regulated by a *cis* polyamine responsive element (PRE) located in the promoter region of this TATA-less gene [23]. We studied the genetic variation at four loci in the SSAT gene and investigated their influence on SSAT expression. As SSAT is an X-linked locus not located in the pseudoautosomal region, males are hemizygous, and as all brains were from male donors, we could investigate the direct relationship between SSAT allelic variants and the altered expression of SSAT. SSAT342A/C, the only polymorphic locus, and located in the PRE regulatory region, showed a significant effect on SSAT levels in the BA4, BA8,9 and BA11 ($F=5.34$, $df=1$, $P=0.024$) with subjects having the SSAT342A variant showing more expression. Because in suicide brains we observed lower SSAT expression levels, we hypothesized that suicide cases from the general population would have less frequency of SSAT342A. To test this hypothesis, a sample of 181 French-Canadian male suicide completers and 80 French-Canadian male controls from the general population were genotyped. This analysis (**Figure 8B**) showed a protective role of the SSAT342A variant since not having this variant significantly increased the risk of committing suicide (OR=2.6; CI=1.3-5.2; $P=0.006$). Thus, the SSAT342A variant, associated with higher expression of SSAT in the BA4 and in the BA11, is significantly less frequent among suicides when compared to controls suggesting that this locus may play a role in suicide

predisposition possibly through a regulatory influence on SSAT expression in the brain.

COMMENT

Using microarray brain expression analysis as a screening tool in a group of suicides with and without major depression and a group of controls, we have identified SSAT as a gene being differentially expressed in BA4, BA8,9 and BA11. SSAT differential expression was confirmed by semi-quantitative RT-PCR, immunohistochemistry analysis and Western blot. Analysis of genetic variation at the SSAT342 (A/C) locus in the vicinity of the polyamine responsive element (PRE) located in the promoter of the SSAT gene revealed an effect of the genotype on gene expression, with the A allele associated with higher levels of SSAT expression. Conversely, the evaluation of the SSAT342 polymorphism in an independent sample of 183 suicide and 80 control subjects showed a lower frequency of the A allele among the suicides suggesting a protective role against suicide and depression. The main conclusions of our study concerning SSAT are based on the consistency of the significance in different brain regions (BA4, BA8,9 and BA11), the validation of these differences using alternative (RT-PCR) and complementary methods (immunohistochemistry, Western blot), as well as the observation that variation at the promoter region influences levels of expression and may play a role in predisposition to suicide and depression.

SSAT is the rate-limiting enzyme in the catabolism of polyamines[24] (spermidine and spermine) and is implicated in the Polyamine Stress Response (PSR)[25;26]. Polyamines, especially spermine are stored in synaptic vesicles and released by depolarization like neurotransmitters[27]. There is evidence suggesting the implication of polyamines in mood disorders, such as the observation that lithium prevents the stress induced PA response in rats[25;28;29]. In addition, spermidine and spermine block the serotonin transporter transient current in a way similar to fluoxetine and cocaine[30]. Finally, glutamatergic neurotransmission is closely controlled by intracellular levels of polyamines, spermine and spermidine being specific modulators of NMDA and AMPA receptors activity[31]. Consequently, significant down-regulation of SSAT would be expected to disrupt polyamine homeostasis resulting in regional increases in spermine, spermidine or both. Considering the multiple processes in which CNS polyamines have been implicated, changes in polyamines levels may produce profound effects.

Some of the most important limitations of this study include a possible confounding effect of comorbidity with substance and limited power of the microarray expression study. In our sample, as expected, several subjects had a history of substance dependence/abuse (alcohol and cocaine). This was the case of the two suicide groups, and to a lesser degree, also of the control group (alcohol). However, controlling for the presence of these factors, as well as other relevant potential confounders, the group effect on SSAT expression remained significant

in BA4 and BA11, suggesting that the results observed in the gene expression screening, at least with SSAT, are not a consequence of these other factors.

While the sample used in the microarray study is of limited power and multiple testing may lead to a high rate of false discoveries, the following should be taken into account when interpreting our major findings. 1) We used a number of procedures to avoid false positive results. For instance, we filtered out genes not present in at least 75% of the subjects per group. This procedure reduces significantly the number of comparisons by decreasing probe sets being tested (~15 000 probe sets instead of the ~44 000). In addition, the criteria we used to determine if a gene was differentially expressed combined both fold change and p-value criteria. 2) Our brain expression studies were only used as a first step of a screening procedure to identify potential targets of interest. Several levels of internal consistency were used to select the target of further study. Finally, the findings implicating SSAT are based on different levels of observation: a) consistency between different gene probes signals, b) between brain-region consistency, c) validation using an alternative method (RT-PCR), d) confirmation at the protein level using 2 complementary methods, immunohistochemistry and Western-Blot, e) genetic evidence suggesting that variation at the promoter region may influence levels of expression.

In a recent study Sibille et al. [32] performed a microarray analysis comparing expression patterns in BA9 and BA47 of depressed suicides versus psychiatrically

normal controls matched on the basis of sex, age, post-mortem interval, and race. They observed no evidence of differences in gene expression that correlated with major depression and suicide. Many differences between the two studies could explain the discrepant results obtained. First, in our study we only included males, and as demonstrated by the same group [33], prefrontal cortex gene expression has a strong sex-related component probably increasing the gene expression variability if males and females are combined in the study. Second, all subjects included in this study are of French-Canadian origin, a population with a well-known and well-characterized founder effect [13]. It is possible that by investigating subjects from this young (approximately 12 generations) and isolated population, we may have reduced in our study total variability in gene expression patterns. Finally, another significant difference is that our analysis was performed using the Human Genome U133 Set, which consists of two GeneChip arrays with ~45,000 probe sets while the analysis that was performed in the study by Sibille et al [32] used only the U133A GeneChip, which containing approximately half of the probe sets (22,000 probe sets) of the U133 set.

In this study, we simultaneously screened the expression levels of genes using microarrays analysis in post-mortem cortical regions from subjects who died by suicide with and without major depression versus a group of controls. SSAT was identified as a candidate mediating risk for suicide. This effect appears to be moderated, to a certain extent, by the presence of major depressive disorder. However, our study design does not allow us to completely separate the effect of

suicide from the underlying psychopathology. Such resolution could be obtained by investigation of controls with depression that did not die by suicide. However, collecting such a sample is operationally challenging given the mean age of the suicides. Confirmation of our results and further investigation of the role of SSAT and other polyamine metabolizing enzymes in the neurobiology of suicide and MDD is warranted.

Acknowledgements: We would like to thank the support of the Bureau du Coroner du Québec. We would also like to thank Wen Hua Zheng, Amanda Li, and Allan Chen for their technical support.

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Table 1. Quality control parameters for brain sample microarrays from BA4 (C=6; S=6; SD=7), BA8,9 (C=6; S=6; SD=7) and BA11 (C=6; S=5; SD=8). The lower percent present calls in the B chip compared to the A chip is due to the fact that the B chip contains primary probe sets representing EST clusters. As a result overall signal intensities on the B chip are lower which is reflected by higher scaling factors. Note that RNA-QC (including β-actin and GAPDH signal ratios) are consistent across chips. Values were derived from MAS 5.0 array analysis.

	U133A (mean ± sem)				
	RawQ	**Scale Factor**	**%Present Calls**	**Actin 5'/3' ratio**	**GAPDH 5'/3' ratio**
BA4	2.47+0.06	1.18+0.09	40.83+0.64	0.50+0.03	0.75+0.04
BA8,9	2.51+ 0.09	1.19+0.08	42.21+0.87	0.46+0.03	0.75+0.04
BA11	2.64+0.13	1.34+0.11	41.33+0.98	0.52+0.03	0.74+0.03
	U133B (mean ± sem)				
	RawQ	**Scale Factor**	**%Present Calls**	**Actin 5'/3' ratio**	**GAPDH 5'/3' ratio**
BA4	2.50+0.09	2.73+0.20	28.43+0.68	0.49+0.03	0.67+0.03
BA8,9	2.36+0.08	3.15+0.29	28.14+0.82	0.47+0.03	0.68+0.04
BA11	2.60+0.11	2.87+0.28	27.94+0.83	0.52+0.03	0.67+0.03

Table 2. Summary of the quality control parameters for brain sample microarrays from BA4 (C=6; S=6; SD=7), BA8,9 (C=6; S=6; SD=7) and BA11 (C=6; S=5; SD=8). RNA QC = mean 5'/3' signal ratios of Actin and GapDH across U133A and U133B chips. Values were derived from MAS 5.0 array analysis.

	RNA QC (mean ± sem)	**RSQ (PMI,RNA QC)**
BA4	0.59+0.04	0.062
BA8,9	0.59+0.03	0.025
BA11	0.61+0.03	0.004

Table 3. Demographic characteristics of the subjects included in the microarray expression studies, validation and protein studies.

Group	Age	Sex	pH	PMI	Cause of death	DSM-IV (six months diagnosis)	Toxicology
Control	55	Male	6.75	24	Car accident		
Control	51	Male	6.83	15	Car accident	Alcohol dependence	Alcohol
Control	27	Male	6.55	20.5	Cardiac arrest		
Control	21	Male	6.42	24	Cardiac arrest	Social phobia	
Control	46	Male	6.42	19.5	Myocardial Infarction		
Control	31	Male	6.67	29.5	Car accident		
Control	41	Male	6.00	24	Myocardial Infarction		
Control	28	Male	6.32	27	Car accident		
Control	30	Male	6.37	30	Cardiac arrest		
Control	47	Male	6.49	12	Cardiac arrest	Alcohol abuse	
Control	19	Male	6.55	32	Car accident		
Control	31	Male	5.95	24	Cardiac arrest	Alcohol dependence	
SD	40	Male	6.84	23	Hanging	MDD, Alcohol dependence	
SD	28	Male	6.21	20	Hanging	MDD, Alcohol dependence	Alcohol
SD	24	Male	6.71	20	Hanging	MDD	
SD	39	Male	7.28	19	Overdose	MDD	
SD	18	Male	6.81	27	Hanging	MDD	
SD	40	Male	5.50	22	Hanging	MDD	
SD	26	Male	6.00	34	Hanging	MDD	Cocaine
SD	49	Male	6.96	32	Hanging	MDD, Alcohol abuse	
SD	22	Male	6.67	11.5	Hanging	MDD, Alcohol dependence	Alcohol, cocaine
SD	39	Male	6.57	25.5	Hanging	MDD	
SD	35	Male	6.60	31	Hanging	MDD, Alcohol dependence	
SD	45	Male	6.57	20.5	Self inflicted gun shot	MDD, Pathological gambling	
SD	42	Male	6.40	21	Drowning	MDD	
SD	53	Male	6.30	29	Hanging	MDD, alcohol dependence	
SD	19	Male	6.17	29.5	Hanging	MDD	
SD	26	Male	6.35	21.5	Carbon monoxide	MDD, Alcohol abuse, cocaine dependence	Cocaine
Suicide	42	Male	6.10	27	Carbon monoxide		
Suicide	33	Male	6.68	18	Hanging		
Suicide	51	Male	6.12	21	Self inflicted gun shot	Alcohol dependence	
Suicide	36	Male	6.54	25	Hanging		
Suicide	29	Male	6.15	26.5	Hanging		
Suicide	31	Male	6.27	32.5	Hanging		
Suicide	21	Male	6.59	21	Asphyxiation	OCD, alcohol dependence	Alcohol
Suicide	38	Male	6.00	23	Hanging	Alcohol dependence, cocaine dependence	Alcohol

*PMI: post mortem interval; MDD: major depressive disorder; OCD: obsessive compulsive disorder

Figure 1. RNA quality control parameters. Relationship between RNA-QC and the post-mortem interval (PMI) in BA4, BA11 and BA8,9 showing no effect of the PMI on the quality control measurements. RNA-QC is an average of the 5'/3' ratios for β-actin and GAPDH.

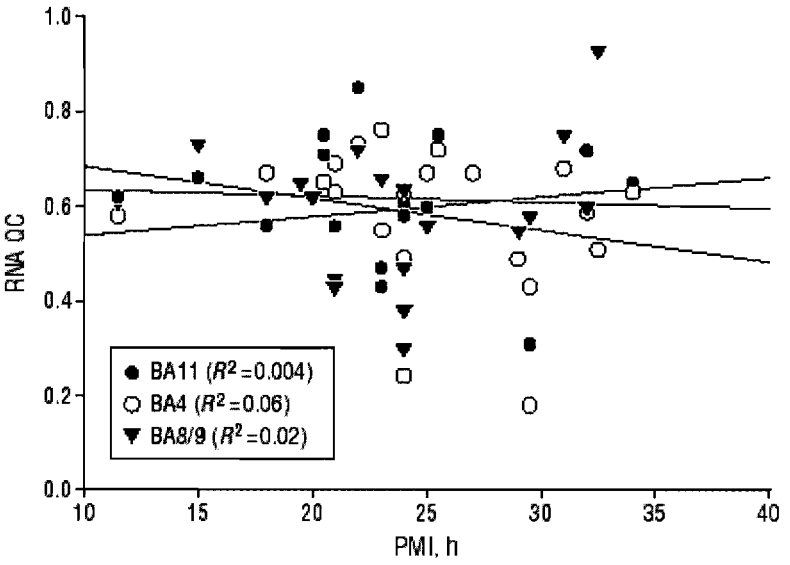


Figure 2. Principal component analysis (PCA). Analysis was based on the ~22,000 genes on the HG-U133A Chip from control samples in three cortical regions (BA11, BA4, BA8,9) and the cerebellum. The first three components accounted for 31.66% of the total variance. Components 1, 2, and 3 accounted for 14.05, 10.65, and 6.96 % of the variances respectively.

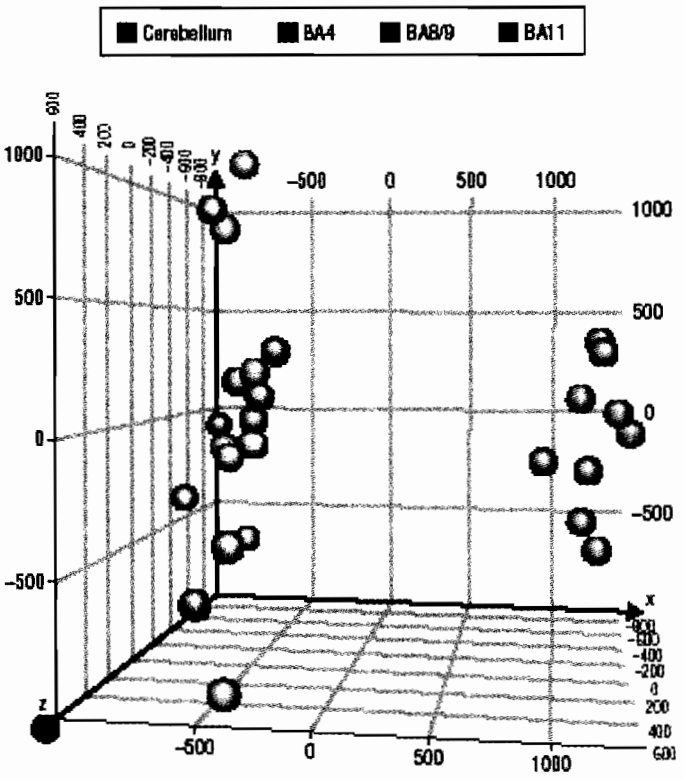


Figure 3. Venn diagrams showing the number of genes identified as differentially expressed and the overlap of genes between the different comparisons in the motor cortex (BA4), the dorsolateral prefrontal cortex (BA8,9) and the orbital cortex (BA11). Each circle represents a single contrast. Up and down arrows indicate the number of up- and down-regulated genes. The numbers of genes that are differentially expressed in a single contrast are shown. The intersections of the circles indicate the number of genes common between contrasts. The between-region Venn diagram shows the differentially expressed genes in BA4, BA8,9 and BA11. The numbers of genes that are differentially expressed in a single brain region are shown. The intersections of the circles indicate the number of genes differentially expressed between brain regions.

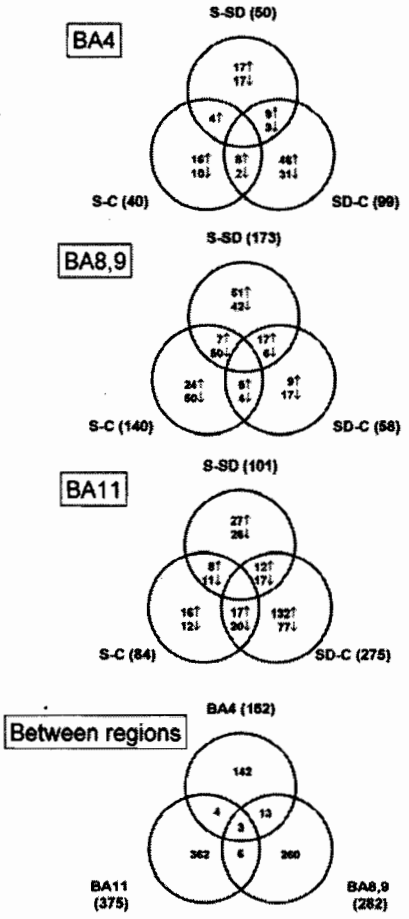


Table 4: Common genes between the depressed suicides versus controls (SD-C) and the suicides versus controls (S-C) comparisons in BA4 A), BA8,9 B) and in BA11 C). The chromosomal location and the fold change per comparison are also shown.

A) BA4

Gene Title	Gene Symbol	Location	Fold Change	
			SD-C	S-C
emilin and multimerin-domain containing protein 1	EMU1	22q12.2	-1.3	-1.6
Homo sapiens cDNA FLJ36689 fis	---	---	-1.5	-1.6
calpain, small subunit 1	CAPNS1	19q13.13	1.5	1.5
myeloid leukemia factor 2	MLF2	12p13	1.4	1.4
leucine rich repeat (in FLII) interacting protein 1	LRRFIP1	2q37.3	1.5	1.5
helicase with zinc finger domain	HELZ	17q24.3	1.3	1.4
nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	NR3C1	5q31	1.4	1.5
trans-golgi network protein 2	TGOLN2	2p11.2	1.4	1.3
hippocalcin-like 1	HPCAL1	2p25.1	1.3	1.5
chromosome 9 open reading frame 5	C9orf5	9q31	1.4	1.5

B) BA8,9

Gene Title	Gene Symbol	Location	Fold Change	
			SD-C	S-C
arrestin, beta 1	ARRB1	11q13	-1.4	-1.3
hypothetical protein DKFZp761G058	DKFZp761G058	4q22.1	-1.7	-1.8
phosphoinositide-3-kinase, class 2, alpha polypeptide	PIK3C2A	11p15.5-p14	-1.7	-2.2
Homo sapiens transcribed sequences	---	---	-2.1	-2.6
GDP dissociation inhibitor 2	GDI2	10p15	1.7	2.1
zinc finger protein 6 (CMPX1)	ZNF6	Xq13-q21.1	1.9	1.8
lysosomal associated protein transmembrane 4 beta	LAPTM4B	8q22.1	1.5	1.5
Hepatoma-derived growth factor, related protein 3	HDGFRP3	15q11.2	1.4	1.5
Protocadherin 19	PCDH19	Xq13.3	1.6	1.6

C) BA11

Gene Title	Gene Symbol	Location	Fold Change	
			SD-C	S-C
Homo sapiens, clone IMAGE:4812754, mRNA	---	---	-1.7	-1.5
Homo sapiens transcribed sequences	---	---	-1.3	-1.4
ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e	ATP6V0E	5q35.2	-1.5	-1.3
complement component 4A	C4A	6p21.3	-1.6	-1.7
CDC42 effector protein (Rho GTPase binding) 4	CDC42EP4	17q24-q25	-1.6	-1.5
Centromere protein B, 80kDa	CENPB	20p13	-1.5	-1.6
Chemokine-like factor super family 6	CKLFSF6	3p22.3	-1.4	-1.5
Cathepsin H	CTSH	15q24-q25	-1.9	-1.7
dentin sialophosphoprotein /// dentin sialophosphoprotein	DSPP	4q21.3	-1.9	-1.9
hypothetical protein FLJ22672	FLJ22672	1q23.1	-1.9	-1.4
Glutathione S-transferase M1	GSTM1	1p13.3	-1.4	-1.4
Glutathione S-transferase M2 (muscle)	GSTM2	1p13.3	-1.4	-1.5
myeloid/lymphoid or mixed-lineage leukemia 4	MLL4	19q13.1	-1.6	-1.6

NDRG family member 2	NDRG2	14q11.2	-1.6	-1.5
retinol binding protein 1, cellular	RBP1	3q23	-1.8	-1.7
Spermidine/spermine N1-acetyltransferase	SSAT	Xp22.1	-1.8	-1.4
stearoyl-CoA desaturase (delta-9-desaturase)	SCD	10q23-q24	-1.9	-1.4
selenoprotein O	SELO	22q13.33	-1.3	-1.4
transforming growth factor, beta receptor II (70/80kDa)	TGFBR2	3p22	-1.6	-1.4
Homo sapiens hypothetical protein LOC284591	---	1p36.33	1.4	1.6
amyotrophic lateral sclerosis 2 (juvenile) chromosome region	ALS2CR9	2q33	1.9	1.6
KIAA0537 gene product	ARK5	12q24.11	1.4	1.5
ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G isoform 2	ATP6V1G2	6p21.3	1.4	1.3
BAI1-associated protein 2	BAIAP2	17q25	1.4	1.4
beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)	BECN1	17q21	1.3	1.3
hypothetical protein FLJ38993	FLJ38993	1q42.13	1.5	1.7
KIAA0379 protein	KIAA0379	3p25.1	1.5	1.4
hypothetical protein LOC285812	LOC285812	---	1.5	1.5
Ras-associated protein Rap1	LOC51277	2p24.1	1.5	1.7
protein associated with Myc	PAM	13q22	1.5	1.5
protein tyrosine phosphatase, receptor type, T	PTPRT	20q12-q13	1.5	1.3
raft-linking protein	RAFTLIN	3p25.1	1.3	1.4
semaphorin 4F	SEMA4F	2p13.1	1.4	1.4
synapsin II	SYN2	3p25	1.3	1.4
synaptotagmin XIII	SYT13	11p12-p11	1.3	1.4
T-cell activation leucine repeat-rich protein	TA-LRRP	1p22.2	1.5	1.3

Figure 4. A, Clustered image map (CIM) of the hierarchical cluster analysis of the 375 genes identified as being differentially expressed in BA11. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression. B, Cluster set plots displaying the average expression profile (light green line) of all genes in each of the clusters, along with the minimum and maximum deviation around the mean (black vertical lines). C, Principal component analysis (PCA) based on the differentially expressed genes. The first three components accounted for 63.15% of the total variance. The depressed suicides, suicides, and controls show separation on the first component. D, Graphical representation showing the percentage of the most common gene ontology terms within each of the clusters in BA11.

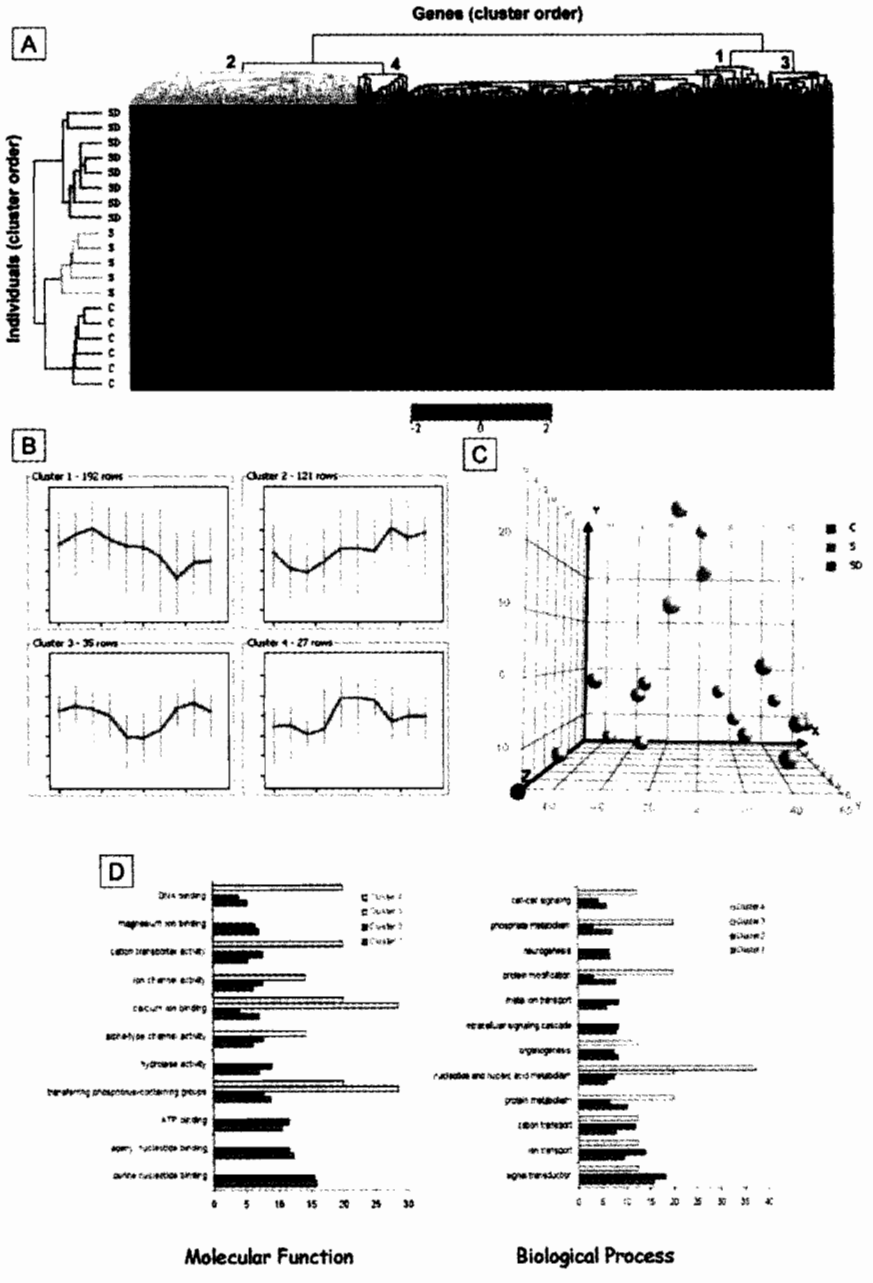


Figure 5. Motor cortex (BA4) analysis. A, Clustered image map (CIM) of the hierarchical cluster analysis of the 163 genes identified as being differentially expressed genes in the BA4. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression. Majority of the genes (99 or ~60%) were misregulated in depressed-suicides in relation to controls. Of these, 63 were up-regulated and 36 were down-regulated, in relation to controls. B, Cluster set plots displaying the average expression profile (light green line) of all genes in each of the clusters, along with the minimum and maximum deviation around the mean (black vertical lines). C, Principal component analysis (PCA) based on the differentially expressed genes. The first three components accounted for 61.16% of the total variance. Components 1, 2, and 3 accounted for 36.35, 19.96, and 4.85 % of the variance, respectively. The depressed-suicides, suicides, and controls show separation on the first component. D, Graphical representation showing the percentage of the most common gene ontology terms within each of the clusters.

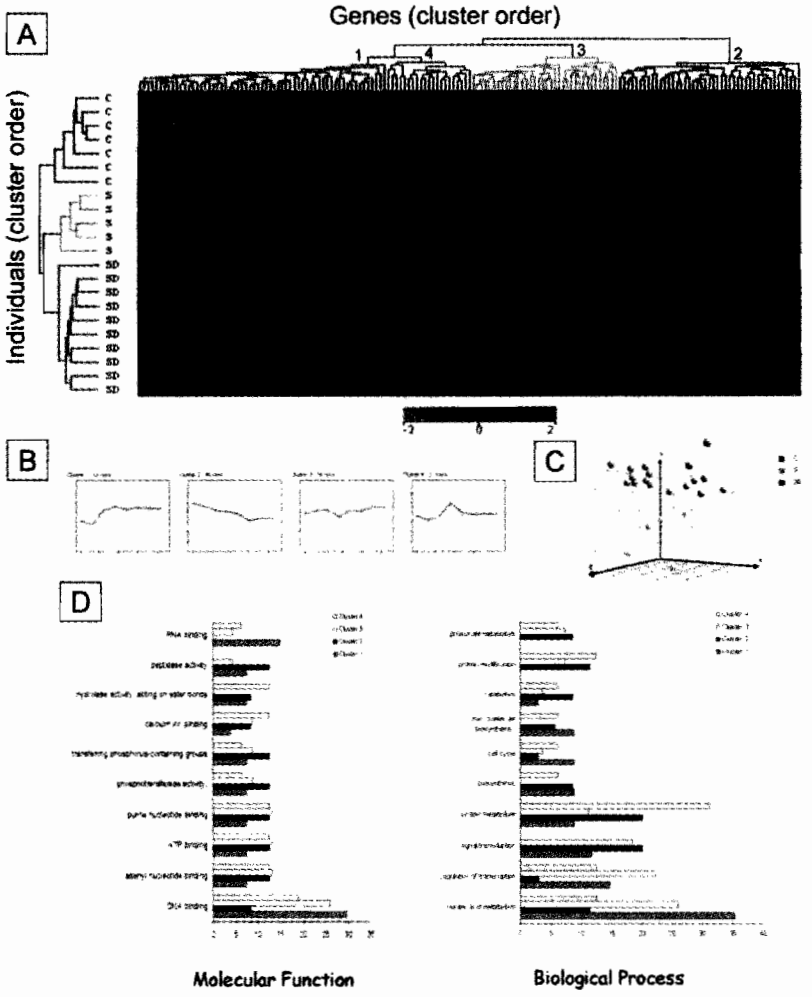


Figure 6. Dorsolateral prefrontal cortex (BA8,9) analysis. A, Clustered image map (CIM) of the hierarchical cluster analysis of the 282 genes identified as being differentially expressed genes in the BA8,9. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression. The majority of genes were misregulated in suicides in relation to controls (140 or ~50%) and depressed-suicides (75 or ~25%). B, Cluster set plots displaying the average expression profile (light green line) of all genes in each of the clusters, along with the minimum and maximum deviation around the mean (black vertical lines). C, Principal component analysis (PCA) based on the differentially expressed genes. The first three components accounted for 65.57% of the total variance. Components 1, 2, and 3 accounted for 43.62, 17.24, and 4.71 % of the variance, respectively. The depressed-suicides, suicides, and controls show separation on the first component. D, Graphical representation showing the percentage of the most common gene ontology terms within each of the clusters.

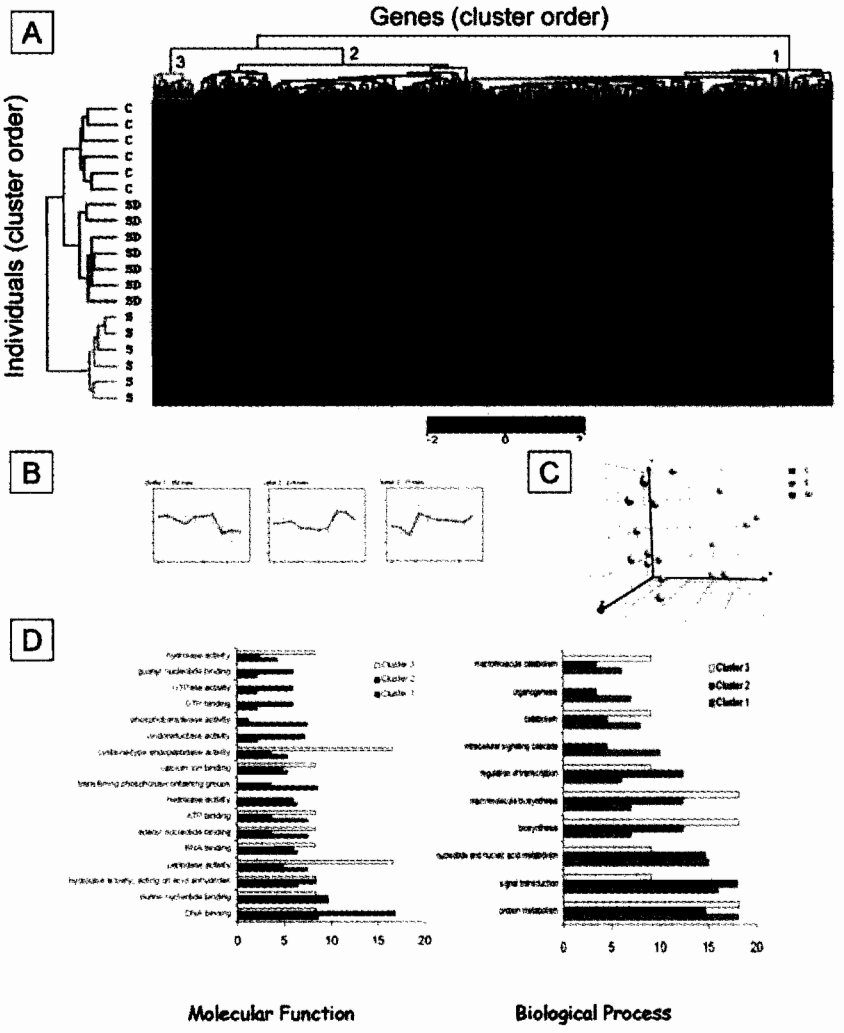


Figure 7. Graphic representations of the observed changes in SSAT expression. Raw Affymetrix data (MAS 5.0) illustrating the differential expression of SSAT in the BA4, BA8,9 and BA11.

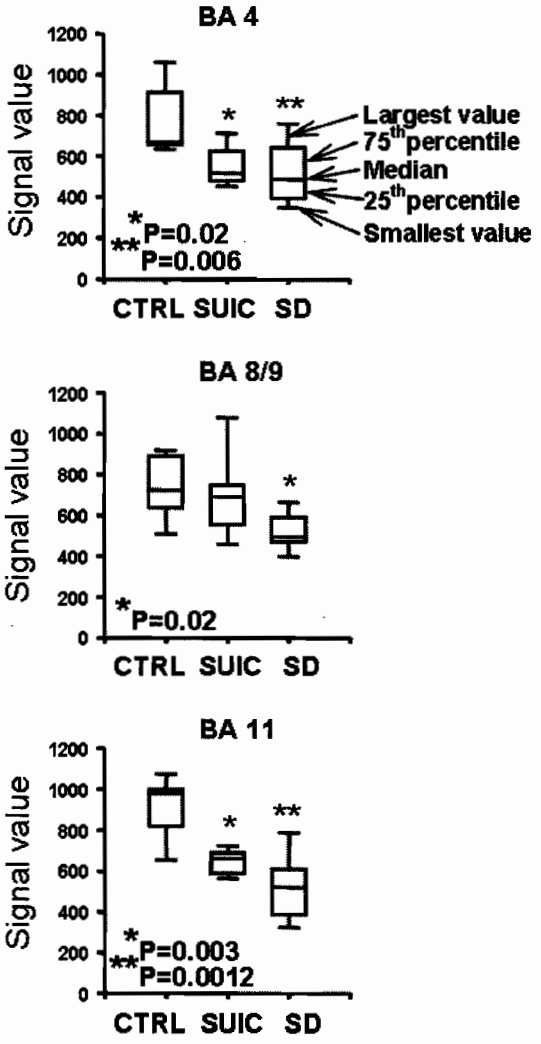
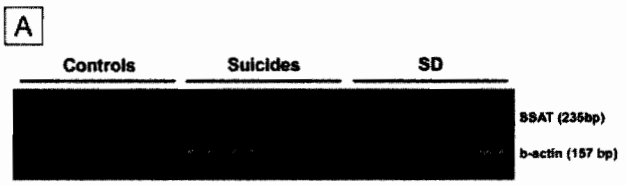


Table 5. Summary of the genes identified as differentially expressed in at least two of the three regions and their chromosomal location.

Gene Title	Gene Symbol	Chromosomal Location
calpain, small subunit 1	CAPNS1	19q13.13
Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	10q23-q24
hypothetical protein FLJ20700	FLJ20700	19p13.3
actinin, alpha 1	ACTN1	14q24.1-q24.2
coatomer protein complex, subunit alpha	COPA	1q23-q25
hypothetical protein MGC10940	MGC10940	9q33.1
SET translocation (myeloid leukemia-associated)	SET	9q34
spermidine/spermine N1-acetyltransferase	SSAT	Xp22.1
likely ortholog of mouse la related protein	LARP	5q33.2
trans-golgi network protein 2	TGOLN2	2p11.2
transportin-SR	TRN-SR	7q32.3
Homo sapiens LOC285843 (LOC285843), mRNA	---	---
citron (rho-interacting, serine/threonine kinase 21)	CIT	12q24
Homo sapiens Alu repeat (LNX1) mRNA sequence	---	---
spermatogenesis associated, serine-rich 2	SPATS2	12q13.12
p53 target zinc finger protein	WIG1	3q26.3-q27
period homolog 3 (Drosophila)	PER3	1p36.23
ELK3, ETS-domain protein (SRF accessory protein 2)	ELK3	12q23
hypothetical protein MGC15396	MGC15396	17q21.33
protocadherin 19	PCDH19	Xq13.3
Homo sapiens, Similar to nuclear localization signals binding protein 1, clone MGC:21810 IMAGE:4183576, mRNA, complete cds	---	---
cathepsin B	CTSB	8p22
oligodendrocyte transcription factor 1	OLIG1	21q22.11
hypothetical protein FLJ32029	FLJ32029	11q13.3
hypothetical protein DKFZp761L1417	DKFZp761L1417	7q22.1
Homo sapiens transcribed sequences	---	---

Figure 8. A, Agarose gel stained by ethidium bromide showing SSAT and β-actin PCR products after reverse transcription and PCR amplification. Bands illustrated as an example in this figure are from BA4. B, Distribution of the SSAT342A/C locus in a sample of suicides victims (n=181) and matched controls (n=80). C, Graphical representation of the relative SSAT mRNA levels in a group of controls (ctrl), a group of suicides (suic) and a group of suicides with major depression (sd)(% of β-actin). Semi-quantitative analysis by reverse transcription polymerase chain reaction (RT-PCR) of SAT mRNA levels was performed in the motor cortex (BA4), dorsolateral prefrontal cortex (BA8,9) and orbital cortex (BA11). Asterisks indicate significant differences in comparisons with the control group.



B

		Controls	Suicides
SSAT342	A	20 (0.25)	20 (0.11)
	C	60 (0.75)	161 (0.89)

(OR =2.68; CI=1.35-5.33; p=0.005)

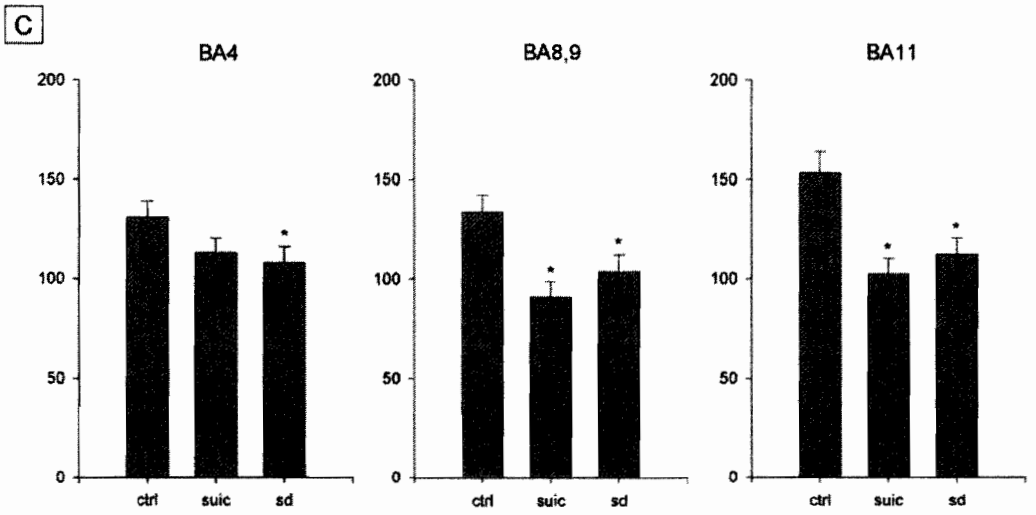
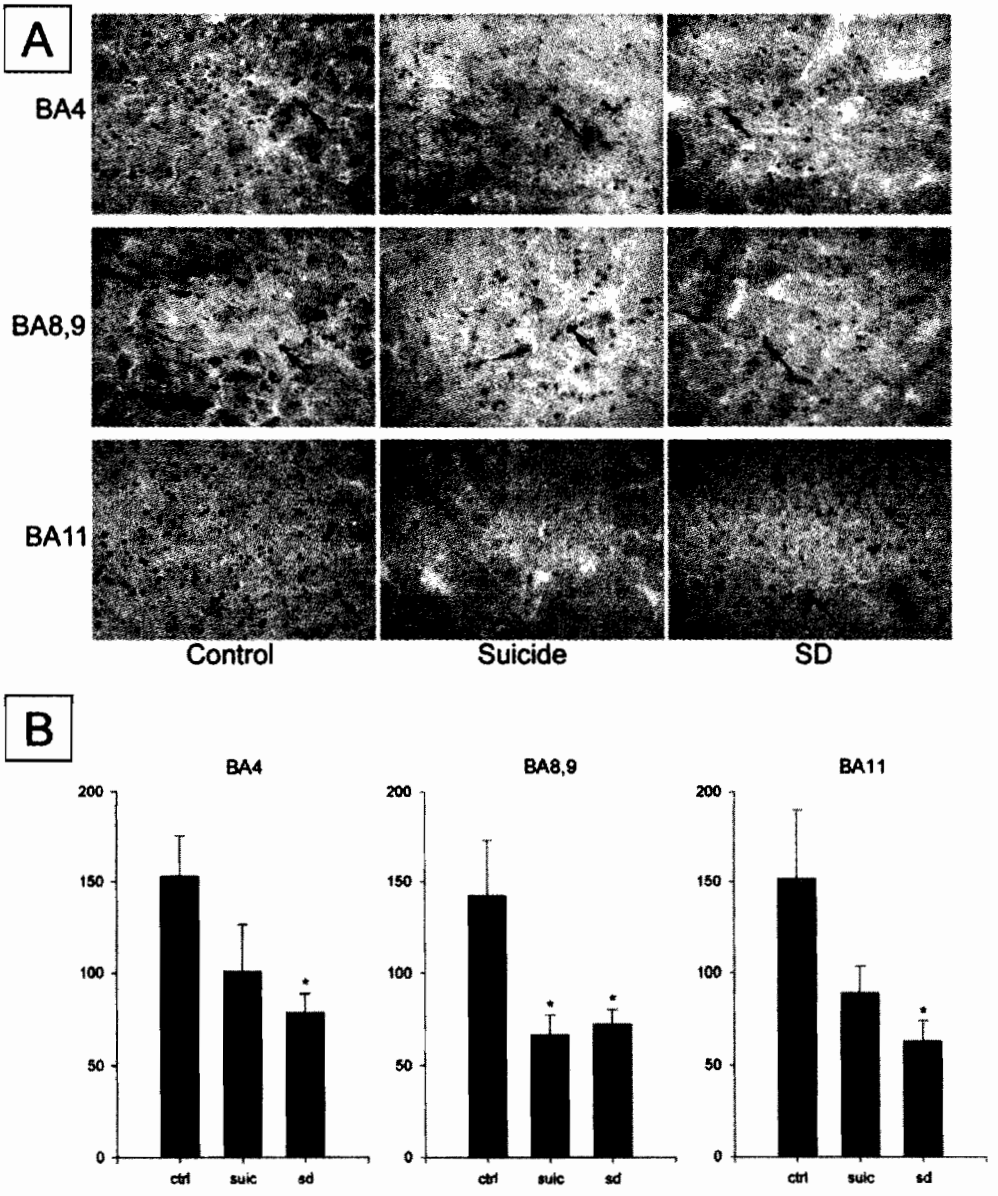


Figure 9. Confirmation of the SSAT gene expression differences at the protein level. A, immunohistochemical staining photomicrograph of adjacent brain sections using an anti-SSAT polyclonal antibody (1:75) in the motor cortex (BA4), the dorsolateral prefrontal cortex (BA8,9) and the orbital cortex (BA11) of a control, a suicide and a suicide with major depression. Arrows indicate examples of positive immunohistochemical staining. B, Western blot analysis using an anti-SSAT polyclonal antibody (1:1 000) in the motor cortex (BA4), dorsolateral prefrontal cortex (BA8,9) and orbital cortex (BA11). Asterisks indicate significant differences in comparisons with the control group.



CHAPTER 6

PATTERNS OF GENE EXPRESSION IN THE LIMBIC SYSTEM OF SUICIDES WITH AND WITHOUT MAJOR DEPRESSION

This work was accepted as:
A Sequeira, T Klempan, L Canetti, J ffrench-Mullen, C Benkelfat, GA Rouleau, G Turecki. Patterns of gene expression in the limbic system of suicides with and without major depression. Mol Psychiatry. 2007.

CONTRIBUTION BY AUTHOR:

Sequeira: performed the experiments, did the analysis, interpreted the results and wrote the paper.

Kemplan: did part the analysis, interpretation of the results and helped writing the paper.

Canetti: performed part of the experiments.

Ffrench-Mullen and Benkelfat: helped in the design of the study and collecting the data.

Casero: provided the anti-SSAT antibody and helped for the discussion of results.

Rouleau: design and interpretation of the results.

Turecki: Design, writing of the paper and supervision.

PREFACE

After focusing on gene expression changes implicated in suicide and major depression in cortical areas in the previous chapter, the focus of this study was limbic regions. Besides the frontal cortical areas, the limbic system has also been previously associated with mood disorders and with the control of emotions and aggressivity.

Following the encouraging results obtained in chapter 5 and the identification of new targets involved in suicide and depression, we conducted another similar study using this time limbic system areas such as the amygdala, hippocampus and the anterior (BA24) and posterior cingulate (BA29). We used in this study the same microarray platform and the same subjects as for chapter 5

These regions are neuroanatomically very different and the results in terms of differentially expressed genes were less homogenous than when comparing only cortical areas. We were however able to confirm some of the results observed in chapter 5. We also found molecular themes specifically altered in the limbic system in suicides and in major depressed suicides.

ABSTRACT

The limbic system has consistently been associated with the control of emotions and with mood disorders. The goal of this study was to identify new molecular targets associated with suicide and with major depression using oligonucleotide microarrays in the limbic system (amygdala, hippocampus, anterior cingulate gryus (BA24) and posterior cingulate gyrus (BA29)). A total of 39 subjects were included in this study. They were all male subjects and comprised 26 suicides (depressed suicides=18, non depressed suicides=8) and 13 matched controls. Brain gene expression analysis was carried out on human brain samples using the Affymetrix HG U133 chip set. Differential expression in each of the limbic regions showed group-specific patterns of expression, supporting particular neurobiological mechanisms implicated in suicide and depression. Confirmation of genes selected based on their significance and the interest of their function with reverse transcriptase-polymerase chain reaction showed consistently correlated signals with the results obtained in the microarray analysis. Gene ontology analysis with differentially expressed genes revealed an overrepresentation of transcription and metabolism-related genes in the hippocampus and amygdala, whereas differentially expressed genes in BA24 and BA29 were more generally related to RNA-binding, regulation of enzymatic activity and protein metabolism. Limbic expression patterns were most extensively altered in the hippocampus, where processes related to major depression were associated with altered expression of factors involved with transcription and cellular metabolism.

Additionally, our results confirm previous evidence pointing to global alteration of gabaergic neurotransmission in suicide and major depression, offering new avenues in the study and possibly treatment of such complex disorders. Overall, these data suggest that specific patterns of expression in the limbic system contribute to the etiology of depression and suicidal behaviors and highlight the role of the hippocampus in major depression.

INTRODUCTION

Suicide is an important public health problem that has a strong association with psychopathology and particularly with mood disorders [1,2]. There is a large body of evidence suggesting that neurobiological factors play a role increasing predisposition to suicide [3,4]. In spite of some overlap, this neurobiological predisposition seems to be, to a considerable extent, different from that mediating risk to major depression or other psychopathological processes commonly present in suicide completers [5].

Neuroimaging studies have produced a substantial body of knowledge about alterations of the limbic system in mood disorders. In the amygdala, alterations in cerebral blood flow and metabolism [6], asymmetry of amygdalar volumes [7], as well as smaller [8-10] and larger volumes [11-14] have been observed in depressed subjects when compared to normal controls. In the hippocampus, volumetric analysis studies have also revealed reduced volumes in subjects suffering from major depression in some [7,14-21] but not all studies [10,22-24] comparing depressed patients versus controls. Alterations in the cingulate cortex have also been observed by many authors. Among these, a smaller anterior cingulate (or Brodmann Area 24: BA24) was observed in depressed patients when compared to controls [9,25,26] and altered activity has also been reported [27-29]. Brodmann area 29 (BA29) or posterior cingulate, has been associated mainly to response to antidepressant treatment with changes in metabolic response associated with the

different treatments [29-32]. The observed changes in these limbic regions may modulate the risk of suicidal behavior through their influence on depression or stress response. In particular, the involvement of the hippocampus in depression and suicidal behavior has been inferred from studies revealing morphological changes of this structure in response to stress hormones, although these changes may often be reversible markers of an ongoing stress or depression-related process [33].

Also, post-mortem examinations have produced a large body of evidence of the implication of limbic areas in suicide. Molecular studies in port-mortem hippocampi of suicides have pointed out to altered levels of 5-HT2A receptor [34], CREB [35], ERK1/2 MAP kinases [36,37], PKC isozymes [37]. Additionally, in a recent study, Rosel et al. observed altered levels of 5-HT2A and 5-HT4 receptors and their respective intracellular signaling systems IP_3 and cAMP in hippocampi of suicides when compared to controls [38]. A number of recent investigations have highlighted the potential for identification of genetic predisposing factors for depression [39] and suicidal behavior [40], including WFS1 [41 42] and p75NTR [43], among others. However, these studies have focused on expression levels of one or several genes at a time and compared suicides with or without psychopathology to controls, not providing an idea of the overall changes taking place in relation to suicide versus the ones related to major depression. While many of these findings may be biologically relevant, they are difficult to confirm in the absence of larger studies seeking to replicate these findings in similar clinical samples with

comprehensive coverage of the variants at a particular candidate locus. It is therefore important that genome-wide analyses of gene expression accompany these focused efforts to better understand the relative significance of previously reported findings and direct attention toward particular biochemical pathways and processes.

A growing number of microarray-based investigations have been conducted in recent years, however relatively few have examined complex behavioral phenotypes, especially in humans. There is good evidence for differential gene expression underlying complex phenotypes, as in the case of avoidance learning in rodents [44]. In this study, using oligonucleotide microarrays for high throughput analysis of mRNA levels in the limbic system (hippocampus, amygdala, BA24 and BA29), we compared the expression patterns of male suicides with and without major depression and psychiatrically normal controls.

MATERIALS AND METHODS

Subjects

Subjects were all males of French-Canadian origin, a homogeneous population with a well-known founder effect[45]. Both cases and controls were age and post-mortem interval matched. All subjects died suddenly without a prolonged agonal state or protracted medical illness. Brain samples were obtained from the Quebec

Suicide Brain Bank and were collected with post-mortem intervals of less than 36 hours at autopsy. Amygdala, hippocampus, anterior cingulate gryus (BA24) and posterior cingulate gyrus (BA29) were sampled at 4 °C and snap-frozen in liquid nitrogen before storage at -80 °C. This study was approved by our local IRB and informed consent was obtained from next of kin.

All subjects were psychiatrically characterized by psychological autopsies, which are validated methods to reconstruct psychiatric history by means of extensive proxy-based interviews, as outlined elsewhere[46]. The sample consisted of subjects who committed suicide during an episode of major depression (SMD; n=18); suicide victims (S; n=10) with no lifetime history of major depression; and matched controls (C; n=13) with no history of suicidal behavior or a major psychiatric diagnosis.

Microarray analysis

Sample processing, RNA extraction, RNA quality control and gene expression by microarray were performed at Gene Logic Inc (Gaithersburg, MD, www.genelogic.com). All microarray data and clinical information were embedded into Gene Logic's Genesis 2.0 software as a component of its Bioexpress® system. RNA samples used in the current study had a minimum A260/A280 ratio of 1.9. The samples were further checked for evidence of degradation and integrity. Samples had a minimum 28S/18S ratio of > 1.6 (2100-

Bioanalyzer, Agilent Technologies, Palo Alto, Calif). We used the Human Genome U133 Set, which consists of two GeneChip arrays with ~45,000 probesets representing >39,000 transcripts derived from ~33,000 well substantiated human genes (available at http://www.affymetrix.com).

GeneChip analysis was performed in Genesis 2.0 (Gene Logic Inc.) and with Microarray Analysis Suite (MAS) 5.0, Data Mining Tool (DMT) 2.0, and Microarray Database software (available at http://www.affymetrix.com). All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 100.

Several microarray RNA integrity indicators were used in this study to filter samples for quality for final analysis. Principal Component Analysis (PCA) was used to quickly identify outlier arrays. Microarray quality control parameters included the following: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, and consistent β-actin and GAPDH 5'/3' signal ratios. Outlier arrays-subjects were excluded on a region basis without any subject being excluded from all the regions. Similar number of subjects were included in the final analysis across the three regions (amygdala: C=8, S=6 SMD=14; hippocampus: C=6, S=6, SMD=10; BA24: C=7, S=5, SMD=9; BA29:C=8, S=7, SMD=10).

Data analysis

For a gene to be included in the final analysis, it had to be "present" (according to MAS 5.0) in at least 75 % of the subjects in at least one of the three groups in order to reduce the chances of false positive results and to exclude bad probe sets. Expression data was analyzed using Genesis 2.0 (GeneLogic Inc., Gaithersburg, MD) and AVADIS software (Strand Genomics, Redwood City, Calif). Gene expression values were floored to 1 and then $\log_2$-transformed.

ANOVAs were performed for each gene to identify statistically significant gene expression changes. To identify differences between the suicides with major depression (SMD), suicides (S), and controls (C), statistically significant genes were subjected to a post-hoc t-test for the contrasts SMD versus C, S versus C, and SMD versus S. Two criteria were used in all to determine whether a gene was differentially expressed. First, a gene had to have an ANOVA *P*-value of less than or equal to 0.01. Second, for a given contrast a gene had to have a fold change/*P*-value combination of at least 1.3 fold change in either direction and $P \leq 0.01$.

Cluster analysis was performed using average-linkage hierarchical cluster analysis with a correlation metric. Both expression patterns in individuals and genes were clustered. Principal component analysis (PCA) was performed based on the initial gene sets and on the selected genes (according to our significance criteria). PCA based on the initial gene set did not discriminate the groups. PCA based on the selected genes showed discrimination of the three groups.

Gene ontology analysis was conducted for differentially expressed genes using the enrichment algorithm integrated in the Database for Annotation, Visualization and Integrated Discovery (DAVID 2.0). DAVID is a web-based application that allows users to access relational databases for functional annotations [47].

Real-time PCR

Total RNA was re-extracted from 10-20 mg of frozen tissue in the four areas examined using Trizol (Invitrogen Corp, Carlsbad, Calif). These samples were independent from those used in the microarray assays, and were obtained from adjacent tissue dissections. Quality of the RNA was established using OD measurements and evaluation on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), with samples below an RNA integrity number (RIN) of 5 excluded from further analysis. Synthesis of cDNA from 1 μg of total RNA was carried out by oligo(dT)-priming using SuperScript II reverse transcriptase (Invitrogen Corp). Real-time analysis of expression results for genes of interest (SSAT1, SSAT2, OATL1, SYT4, ADCY8, APLP2, and BACE1) was carried out using TaqMan gene expression assays (Applied Biosystems, Foster City, Calif) with 5 ng of cDNA template in a 15 μl reaction volume. The PCR reactions were run on an ABI 7900HT Real-Time PCR system (Applied Biosystems) according to manufacturer specified conditions (ABI TaqMan Gene Expression Assays Protocol, Rev E). Fold changes between groups were

evaluated using relative quantitation (delta Ct method) with β-actin and GAPDH endogenous controls (demonstrating low variation from microarray analysis), and all real-time reactions carried out in triplicate. Real-time results were analyzed using the SDS software (Applied Biosystems, v.2.2.1), with automatic computation of baseline and threshold fluorescence levels. Gene expression Ct values below that of endogenous controls were discarded from analyses and outlier removal was performed in cases where the standard deviation of Ct values exceeded 0.3 cycles. Student's *t*-tests and Pearson's correlations (between microarray and real-time comparative fold changes) were employed in statistical analysis (SPSS, v.12.0, Chicago, Il). The genes chosen for validation were selected on the basis of their involvement in stress response, signal transduction and neurotransmission, and on the strength of findings from microarray analysis.

RESULTS

No significant difference were observed in terms of age, pH and post-mortem interval between the groups (Mean age: 35.6 (C), 35.2 (S), 34.1 (SMD); Mean pH: 6.4 (C), 6.3 (S), 6.5 (SMD); Mean PMI: 23.5 (C), 24.3 (S), 24.2 (SMD). The effect of age and post-mortem interval on quality control parameters like β-actin and GAPDH 5'/3' ratios and the number of present calls was evaluated. No significant correlation was observed between post-mortem interval and any of the quality control variables in our sample in all the regions studied. Figure 1 shows

the correlations between pH and PMI (post-mortem interval) with 5'/3' ratios for β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in hippocampus. Similar relationships were also observed in the other limbic areas..

Initial filtering in the amygdala, which consisted of selecting genes called "present" (according to MAS 5.0) in at least 75% of the subjects in at least one group, resulted in 15007 genes that were included in the analysis. The ANOVA and post-hoc analysis resulted in 182 differentially expressed genes. Among these, 127 genes were significant for the SMD-C comparison, 51 for the S-C comparison and 33 for the SMD-S comparison (Figure 2). The intersections show the number of significant genes common for two comparisons. The PCA plot and the clustered image map show the degree of discrimination between the groups using the differentially expressed genes (Figure 3a and b). Gene ontology analysis based on the differentially expressed genes revealed an overrepresentation of genes implicated in the regulation of transcription and in nucleic acid metabolism in the amygdala (Table 2). For instance, X-box binding protein 1 (XBP1), a transcription factor gene whose expression was previously shown to be implicated in bipolar disorder [48], was upregulated in the two suicide groups and even more among the suicides without major depression (Table 3). Also, several genes playing a role in the regulation of second messenger systems were differentially expressed in the amygdala, among these, there were several protein kinase and protein phosphatases genes, as well as cAMP dependent kinases (Table 3).

In the hippocampus, 14537 genes passed the initial filtering and were included in the analysis. A total of 429 genes were differentially expressed between the three groups, 35 for the SMD-C comparison, 120 for the S-C comparison and 359 for the SMD-S comparison (Figure 2). It is noteworthy that in contrast to the other regions, in the hippocampus the vast majority of genes were significantly different between the two groups of suicides, with and without major depression. Accordingly, the hierarchical clustering analysis showed the biggest separation between the two groups of suicides and the clustered image map clearly shows two groups of genes differentially expressed between the two suicide groups (Figure 4a). Also, in the PCA, the 20 genes contributing most to the fist component of the PCA are differentially expressed between these two groups, as reflected by their separation in the tri-dimensional space (Figure 4b). Gene ontology analysis revealed an overrepresentation of genes implicated mainly in the regulation of transcription and in nucleic acid binding and metabolism (Table 2). Overrepresented ontology categories corresponded to several kinases, phosphatases, one phosphodiesterase and one adenylate cyclase (ADCY8), all implicated in intracellular signaling cascades and second messenger systems (Table 2). Several genes implicated in neurotransmission were also altered in the hippocampus such as the genes coding for the multiple coiled-coil GABABR1-binding protein (MARLIN1), the inositol 1,4,5-triphosphate receptor type 1 (ITPR1), the cannabinoid receptor 2 (CNR2) and the leptin receptor (LEPR). Finally, three genes coding for proteins involved in the biosynthesis and catabolism of polyamines were also differentially expressed in the hippocampus:

spermine/spermidine N-acetyltransferase 2 (SSAT2), spermine synthase (SMS), and ornithine aminotransferase-like 1 (OATL1).

Analysis in BA24 was conducted on 14556 genes after the initial filtering and revealed 84 genes as differentially expressed between the three groups. Post-hoc analysis identified 52 genes as differentially expressed for the SMD-C comparison, 20 genes for the S-C comparison and 27 genes for the SMD-S comparison (Figure 2). A good separation and clustering of the groups was observed with the PCA and the hierarchical clustering analysis using the differentially expressed genes (Figure 5a and b). Gene ontology analysis of the differentially expressed genes revealed an overrepresentation of binding and binding related functions such as RNA and nucleic acid binding, as well as enzymatic regulation (Table 2). Interestingly, the beta-site APP-cleaving enzyme 1 (BACE1) known to play an important role in neurodegeneration and in Alzheimer disease [49] was downregulated in the depressed suicide group when compared to controls, suggesting a possible role for this enzyme in the neurobiology of depression. Two gabaergic receptors were also differentially expressed in opposite ways in BA24. While the gamma-aminobutyric acid (GABA) A receptor, alpha 1 (GABRA1) was highly upregulated among the depressed suicides versus the controls, the gamma-aminobutyric acid (GABA) A receptor, beta 1 (GABRB1) showed an important down regulation for that same comparison. Suicides without major depression showed intermediate levels, suggesting possible quantitative effects on the phenotype.

After filtering, 15032 genes were included in the analysis for BA29, 83 of which were differentially expressed according the defined criteria. More specifically, 19 genes were significant for the SMD-C comparison, 40 for the S-C comparison and 40 for the SMD-S comparison (Figure 2). Differentially expressed genes used for the PCA and the hierarchical clustering were able to discriminate the groups which showed a good separation and an accurate clustering (Figure 6a and 6b). Gene ontology analysis was performed with the differentially expressed genes but only 69 genes were fully annotated and were included in the enrichment analysis. Probably due to the small number of genes, only two ontology terms were significant in BA29 corresponding to Organismal movement and Protein metabolism. One of these metabolic genes, the spermidine/spermine N1-acetyltransferase gene (SSAT1), was differentially expressed between the two suicide groups.

Because some of the subjects in the three groups had a history of substance dependence or abuse, possibly implicating more chronic substance-related gene expression changes, whereas other subjects had negative history of substance-related disorders, but had a positive toxicological result for alcohol or cocaine (Table 1), which may be associated to more acute substance-related gene expression alterations, we performed an analysis of covariance to control for these 2 different effects on our gene expression findings. The majority of genes differentially expressed remained significant following this analysis, suggesting

that the most of our findings are more directly related to depression and suicide. Table 3 shows the corrected p-values (*P) for genes of interest. Some genes (Table 4) were significantly affected ($P \leq 0.01$) by the history of dependence/abuse of substance (amygdala=2; hippocampus=8; BA24=0; BA29=2) and presence according to the toxicology screening (amygdala=4; hippocampus=4; BA24=1; BA29=1). Due however to the limited number of subjects per category, further analysis will need to be done in a larger sample to investigate the role played by those genes in substance abuse and intoxication.

Similarities in terms of common differentially expressed genes were explored between the three regions. Few similarities in terms of differentially expressed genes between the regions were observed (Amygdala-Hippocampus (1 gene), Amygdala-BA24 (0 genes), Amygdala-BA29 (2 genes), Hippocampus-BA24 (0 genes), Hippocampus-BA29 (2 genes), BA24-BA29 (2 genes)). This is however not surprising in the case of such distinct functional and neuroanatomical areas, More overlap was seen for instance in a previous study by our group exploring expression in cortical regions [50].

Evaluation of the microarray fold changes was performed in independent samples collected from adjacent tissue from that used for the microarray assay in a total of 11 controls, 7 non-depressed suicides, and 13 depressed suicides using real-time PCR. The genes selected for RT-PCR validation were chosen based on known function and the significance of microarray findings. Real-time analyses

confirmed primary group-level changes in expression for all seven genes selected for further study from the initial set of significant genes: SSAT1 (BA29), SSAT2 (hippocampus), OATL1 (hippocampus), SYT4 (hippocampus), ADCY8 (hippocampus), APLP2 (amygdala), and BACE1 (BA24). The precision as measured by agreement between results of replicate real-time runs was high (meanΔCtSD=0.13), with 3% of samples showing a ΔCtSD in excess of 0.3 cycles. The fold changes at the validation stage ranged from 1.11 - 1.62 fold, and several of these changes exceeded those from the microarray analysis (ADCY8 and SSAT2). For four of the seven genes, the strongest fold change resulted from the contrast between the suicide groups with (D) and without (S) depression: OATL1 (microarray FC (S/D = 6/10) = 1.50, RT FC (S/D = 5/7) = 1.19), SSAT1 (microarray FC (S/D = 7/10) = 1.45, RT FC (S/D = 5/8) = 1.19), SSAT2 (microarray FC (S/D = 6/10) = -1.36, RT FC (S/D = 4/7) = -1.57), and SYT4 (microarray FC (S/D = 6/10) = -1.69, RT FC (S/D = 5/6) = -1.11). The BACE1 gene showed reduced expression in the depressed suicide group compared with controls (C): BACE1 (microarray FC (D/C = 9/7) = -1.32, RT FC (D/C = 7/9) = -1.14). Finally, two genes were confirmed as differentially regulated between the non-depressed suicide and control groups: ADCY8 (microarray FC (S/C = 6/6) = 1.46, RT FC (S/C = 5/6) = 1.62) and APLP2 (microarray FC (S/C = 6/8) = 1.35, RT FC (S/C = 6/6) = 1.33). Overall, as seen in other studies using microarrays and brain tissue [51], correlations between relative fold changes seen using microarray and real-time analyses for the seven genes were good (mean R=0.377, SD=0.179). All genes apart from OATL1 and BACE1 demonstrated significant

approach-wise correlations in calculated fold changes at the $P<0.05$ level, across all groups. The gene to gene variation in the magnitude of fold changes and correlations is likely due to replication at the tissue rather than RNA level, and the use of an overlapping, but non-identical sample set for confirmation.

DISCUSSION

Microarray analysis was performed in four limbic areas of suicides with and without major depression and psychiatrically normal controls. The vast majority of expression changes were observed in the amygdala (182 probe sets), and particularly, in the hippocampus (429 probe sets, of 761 across all four regions). In contrast, fewer differentially expressed genes were observed in the two cingulate cortex regions (BA24=84; BA29=83). Selected genes for validation with an alternative method (RT-PCR) showed consistently correlated signals with the results obtained in the microarray analysis, supporting the ability of the study design to identify candidate molecular target that may be involved in the neurobiology of major depression and suicide.

We used a design that allowed us to control for the presence of suicide (suicides with major depression vs. suicides without major depression) and therefore, to identify processes that may be more likely attributed to major depression. As such, in our design, the identification of processes exclusively attributed to

suicide is confounded by the presence of psychopathology. Nevertheless, by having a normal control group, we had the ability to identify molecular processes that may be related to suicidality independently of major depression. Having a comparison group with MDD that did not die by suicide would have allowed us to fully separate effects attributed to suicide. However, for such a group to be comparable, it would have been necessary to include subjects who were affected with MDD prior to death, or at least in the last 6-month. Collecting a group that would be demographically comparable with the suicide group and that died while affected with MDD is operationally very challenging. While some studies investigating the neurochemistry of suicide and major depression have included a group of MDD controls, these typically had a lifetime history of depression and not necessarily depression at the time of death. It is unclear the level of impact that an episode of depression that may have occurred several years earlier could have on current gene expression patterns.

Few studies have explored global expression changes between groups of suicides and psychiatrically normal controls, some have however focused on a particular psychiatric diagnosis, such as bipolar disorder or schizophrenia, and included within these groups subjects that died by suicide[52-59]. Sibille et al recently compared expression patterns in BA9 and BA47 of depressed suicides versus psychiatrically normal controls matched on the basis of sex, age, post-mortem interval, and race [51]. Despite the number of transcripts investigated they observed no significant evidence of differences in gene expression that correlated with

major depression and suicide. In limbic regions structural abnormalities have been observed consistently in the past decades in psychiatric disorders [60-62] and recent expression studies in suicides have confirmed altered expression of genes implicated in neurotransmission and intracellular signalling in some limbic areas of depressed suicides [38] in agreement with the observed results in the present paper[34-37,63-66].

One potential limitation of human brain microarray studies is the possible confounding effect on gene expression by psychoactive drugs. In spite of the presence of psychopathology in suicides, most of the cases used in this study were not actively treated prior to death. Accordingly, only two cases had a history of antidepressant treatment. This is consistent with data from the literature that indicates that most suicide completers were not properly treated prior to death [67,68]. In our sample, as expected for suicides, several subjects had a history of substance dependence/abuse, primarily involving alcohol. This was the case in the two suicide groups, and to a lesser degree, also in the control group. However, after controlling for the history of alcohol and/or cocaine dependence/abuse or the presence of substance intoxication as per toxicological results, the group effect on the expression differences observed for most genes remained significant, suggesting that the gene expression patterns observed are not due to the effect of alcohol or cocaine. Nevertheless, for certain genes, a specific effect of acute or chronic substance exposure was observed. The effect alcohol and cocaine on the expression of those genes in the context of suicide and depression should be

further explored. Another possible confounding factor to take into consideration is the heterogeneity of the tissue as using regional gene expression data actually compares transcripts contained in the majority of neuronal and glial populations. Another approach would be to use a combination of laser capture microdissection and array technologies. This was done in an elegant paper using neurons from the entorhinal cortex of schizophrenics with interesting results [69]. However on the other hand, it is hard to extrapolate single cell results to the whole population of cells in a given area of the brain and to complex conditions such as major depressive disorder and suicide.

One of the challenges with microarray studies is to process the large amounts of information generated. Differentially expressed genes were fully annotated using electronic databases (NetAffix) and further explored using gene ontology and enrichment algorithms (DAVID), which constitutes an interesting way to summarize and functionally explore microarray data despite some problems concerning vague higher functional classifications. In our study we implemented stringent significance criteria in the identification of differentially expressed genes (minimum 1.3-fold change, ANOVA and t-test *P*-values<0.01) in order to reduce the complexity of the data and enrich for genes which are biologically relevant to suicide and depression. The combination of the ANOVA and subsequent t-tests is commonly referred to as Fisher protected LSD test. In the case of gene expression studies of complex traits such as psychiatric disorders, it has been suggested to use less stringent criteria in the initial analysis stages [70], and

the use of only t-tests or Fisher protected LSD test has been commonly accepted in psychiatric brain expression studies [50,56,57] as well as in other research areas [71].

Multiplicity is always a concern when performing microarray analysis and many correction procedures have been developed to address it such as the Bonferroni method or FDR based methods [72]. However these methods as commonly used, assume independence of the hypotheses being tested [73]. This is far from reality in the context of gene expression in brain tissue, where there is extensive multicollinearity between probe-sets as a result of genes in pathways, or those implicated in a particular molecular function, which can be regulated in a coordinated manner. For instance, many researchers have found not genes but families of genes or pathways as being implicated in psychiatric disorders in the past [54,56,57,70,74-79]. One additional consideration for this research was the distinction between depressed and non-depressed suicides, permitting the evaluation of depression specific effects on gene expression separately from suicide specific effects.

The large number of differentially expressed genes in the hippocampus was mainly due to differences between the two groups of suicides. As processes related to the neurobiology of suicide should be controlled for when comparing both suicide groups, the observed expression differences in this region are probably more specifically associated to biological mechanisms related to major depression. This supports recent findings indicating an important role of the

hippocampus in depression and in the anti-depressant response to pharmacotherapy [80,81]. Also, altered size and impaired function of several regions, but more particularly the hippocampus, have been found in depression in a number of recent clinical imaging studies (for a meta-analysis see Campbell [82]). Furthermore, the expression changes observed in the hippocampus were observed in genes implicated in second messenger systems, such as kinases, phosphatases and the adenylate cyclase 8 gene (ADCY8), supporting previous evidences of synaptic plasticity alterations in mood disorders [83,84]and in antidepressant response beyond the neurotransmitter and receptor level [85]. Recent molecular studies in post-mortem brains have also revealed alterations in second messenger systems in hippocampi of suicides and depressed suicides [37,38,64,86].

The observation of a disproportionate number of genes identified as differentially expressed between the suicide groups in the hippocampus is clearly reflected in the PCA, with the top ten eigen values for the first component arising from genes differentially expressed between the depressed and non-depressed suicide groups. This contrast between suicide groups is consistent with the prominent role of the highly plastic hippocampus in depression and stress response [20,82], discriminating between depressive and non-depressive states for a sub-set of differentially expressed genes. A gene ontology analysis of probes that were significantly differentially expressed between the two suicide groups in the hippocampus reveals a strong over-representation of factors involved in transcriptional regulation and metabolism, similar to the result including all comparisons in the

hippocampus but with a much higher degree of significance. The enrichment of factors involved in metabolic processes may outline a differential stress response between the suicide groups, while the preponderance of transcriptional regulators may act on diverse networks of additional genes, enhancing the apparent difference between these two groups in the hippocampus. In addition, the gene expression pattern in the hippocampus may be broadly related to long-term changes caused by a recurrent depressive state, this being contrary to changes brought about by a more transient and excitable state for the non-depressed suicides. A more focused and critical investigation of the hippocampus in further suicide victims will help to discriminate between these possibilities.

Amongst the genes validated in the four limbic regions, several play fundamental roles in generalized neurotransmission, as in the case of amyloid precursor protein (APP)-like protein 2 (APLP2), the absence of which in knockout mice is associated with a reduction in both density and number of docked vesicles at the active zone [87]. Both APLP2 and APP are processed by the product of the also identified BACE1 gene [88], exposing a pathway considered of primary importance in the pathogenesis of Alzheimer's disease. The apparent upregulation of synaptotagmin 4 (SYT4, a presynaptic calcium-sensor and regulator of synaptic release) gene expression in the hippocampus of depressed suicide victims parallels findings in the SYT4 knockout mouse, which displays reduced levels of anxiety and depression-like behavior, as well as altered short-term plasticity (CA1) and hippocampal-dependent memory [89,90], and the upregulated

hippocampal expression of ADCY8 in non-depressed suicides compared with controls integrates with a convincing body of research demonstrating perturbed cyclic amp (cAMP) signaling in bipolar disorder [91], depression [92], and suicide [93] [94,95]. These genes constitute interesting avenue for further investigation.

Several gabaergic system genes were identified as differentially expressed in this investigation, in both BA24 and hippocampus, drawing attention to a possible gabaergic dysfunction in the limbic system of suicidal and depressive individuals. Two genes are of particular interest, Gamma-aminobutyric acid (GABA) A receptor, alpha 1 (GABRA1) and Gamma-aminobutyric acid (GABA) A receptor, beta 1 (GABRB1) (Table 3), as GABA receptors mRNA levels have been previously associated with depression and suicidality [96]. Inhibitory neurotransmission in the mammalian brain is mainly accomplished by gabaergic neurons, responsible for the release of gamma-amminobutyric acid (GABA) [97]. GABA neurotransmission modulates the activity of noradrenergic, dopaminergic and serotonergic systems [97]. Clinical data have pointed out to an alteration in gabaergic neurotransmission in mood disorders and more specifically in major depression [98]. Thus, CSF and plasma studies have observed altered levels of GABA in depressed patients. Also, SSRIs are known to increase GABA levels in the brain of depressed patients [99]. Finally, our results also confirm the recent findings of another group concerning gene expression abnormalities in GABA signal transmission in the cerebral cortex of subjects who had suffered from depression and more specifically GABA(A) and GABA(B) alterations in

individuals who died by suicide suggesting their potential role in suicidality [54]. Our findings together with previous studies clearly point to an alteration of gabaergic neurotransmission in suicide and major depression in the context of suicide and offers new potential pharmacological targets for the treatment of such complex disorders.

Real-time PCR has been widely used in the confirmation of findings from microarray studies due to its high sensitivity and precision. However, while these advantages may be significant, a number of technique and analysis-specific parameters capable of greatly influencing the final result have recently been discussed [100], including factors such as quality of the template, the reverse transcription step, selection of endogenous controls, and the data analysis approach employed. Current research has emphasized the need for multiple control genes for relative quantification [101] owing to the inconstancy in expression of what were previously considered "housekeeping" genes. Rigorous sample quality control and two reference genes were used in this study, with the real-time fold changes and correlations between microarray and real-time data supporting the validation of the most significant results from the microarray component. The correlation between microarray and real-time data could at first sight seem modest, but one has to take into account the fact that this RT-PCR assays were not carried out in the same RNA sample, but rather on RNA extracted from additional samples from the same subjects obtained from adjacent tissue to that used for in the original microarray experiments. This is different from what is usually done in

most microarray studies, which use the same RNA sample. By doing our procedure, we are not only validating the microarray result, but also the intra-subject reproducibility. Finally, because the purpose of the RT-PCR was solely a technical replication of the results obtained in the microarray, the biological replication was obtained by using multiple samples from different subjects, a slightly less conservative criteria for RNA quality was used for the RT-PCR than for the microarray. As our RT-PCR results correlate positively with the microarray data, even when using adjacent samples from the same subjects for both experiments, we can consider with an acceptable degree of confidence that issues related to RNA integrity were unlikely responsible for our results.

Global microarray experiments should be regarded as screening assays capable of shedding light onto biological processes involved in complex conditions such as those investigated in this study. As such, the sets of differentially expressed genes obtained in each region constitute interesting targets for future studies focused on suicide and major depression. This investigation represents to our knowledge the first gene expression study on suicides and depressed suicides examining genome wide alterations in limbic brain regions. The expression profile in the hippocampus underlies the importance of this structure in major depression in the context of suicide, and implicates a fundamental role for second messenger systems in this important public health problem. Additionally, our results confirms previous evidence pointing to global alteration of gabaergic neurotransmission in suicide and major depression, offering new avenues in the

study, and possibly treatment, of such complex conditions. Further studies are warranted to confirm these results at the mRNA but also at the protein and functional level, as well as the link between molecular alterations with symptoms and risk factors associated with suicide and with major depression.

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Table 1 Demographic and clinical characteristics of the subjects included in the analysis. (SMD: suicide with major depression; MDD: major depressive disorder; OCD: obsessive compulsive disorder; BPD: borderline personality disorder; APD: antisocial personality disorder).

Group	Age	PMI	Cause of death	DSM-IV (six months diagnosis)	Toxicological findings
C	32	26.5	Cardiac arrest	Alcohol intoxication, cannabis abuse	
C	31	24	Cardiac arrest	Alcohol dependence	
C	19	32	Car accident		
C	47	12	Cardiac arrest	Alcohol abuse	
C	30	30	Cardiac arrest		
C	28	27	Car accident		
C	41	24	Myocardial Infarction		
C	31	29.5	Car accident		
C	46	19.5	Myocardial Infarction		
C	21	24	Cardiac arrest	Social phobia	
C	27	20.5	Cardiac arrest		
C	51	15	Car accident	Alcohol dependence	Alcohol
C	55	24	Car accident		
S	26	69	Hanging		
S	38	23	Hanging	APD, Alcohol dependence, cocaine dependence	Alcohol
S	21	21	Asphyxiation	OCD, alcohol dependence	Alcohol
S	31	32.5	Hanging		
S	29	26.5	Hanging		
S	36	25	Hanging		
S	51	21	Self inflicted gun shot	Alcohol dependence, alcohol intoxication	
S	33	18	Hanging	BPD	
S	42	27	Carbon monoxide		
S	30	27	Stabbing	Paranoid Schizoprenia	
SMD	22	11.5	Hanging	MDD, Alcohol dependence and intoxication	Alcohol, cocaine
SMD	19	29.5	Hanging	MDD	
SMD	53	29	Hanging	MDD, Alcohol intoxication, alcohol dependence	
SMD	42	21	Drowning	MDD, Alcohol intoxication	
SMD	45	20.5	Self inflicted gun shot	MDD, Pathologial gambling	
SMD	39	25.5	Hanging	MDD	
SMD	49	32	Hanging	MDD, Alcohol abuse and intoxication	
SMD	26	34	Hanging	MDD	Cocaine
SMD	40	22	Hanging	MDD	
SMD	39	19	Overdose	MDD	
SMD	26	21.5	Carbon monoxide	MDD, Alcohol abuse, cocaine dependence	Cocaine
SMD	35	31	Hanging	MDD, Alcohol dependence, alcohol intoxication	
SMD	53	14	Carbon monoxide	MDD, BPD	
SMD	53	33.5	Hanging	MDD, Adjustment disorder	
SMD	18	27	Carbon monoxide	MDD	
SMD	22	20	Hanging	MDD	
SMD	40	23	Hanging	MDD,Alcohol dependence	
SMD	28	20	Hanging	MDD, Alcohol dependence	Alcohol

Figure 1 Graphical representation of the correlation between pH and PMI (post-mortem interval) with5'/3' ratios for β-actin and GAPDH (glycerraldehyde-3-phosphate dehydrogenase) in hippocampus. Similar relationships are observed in the other limbic areas.

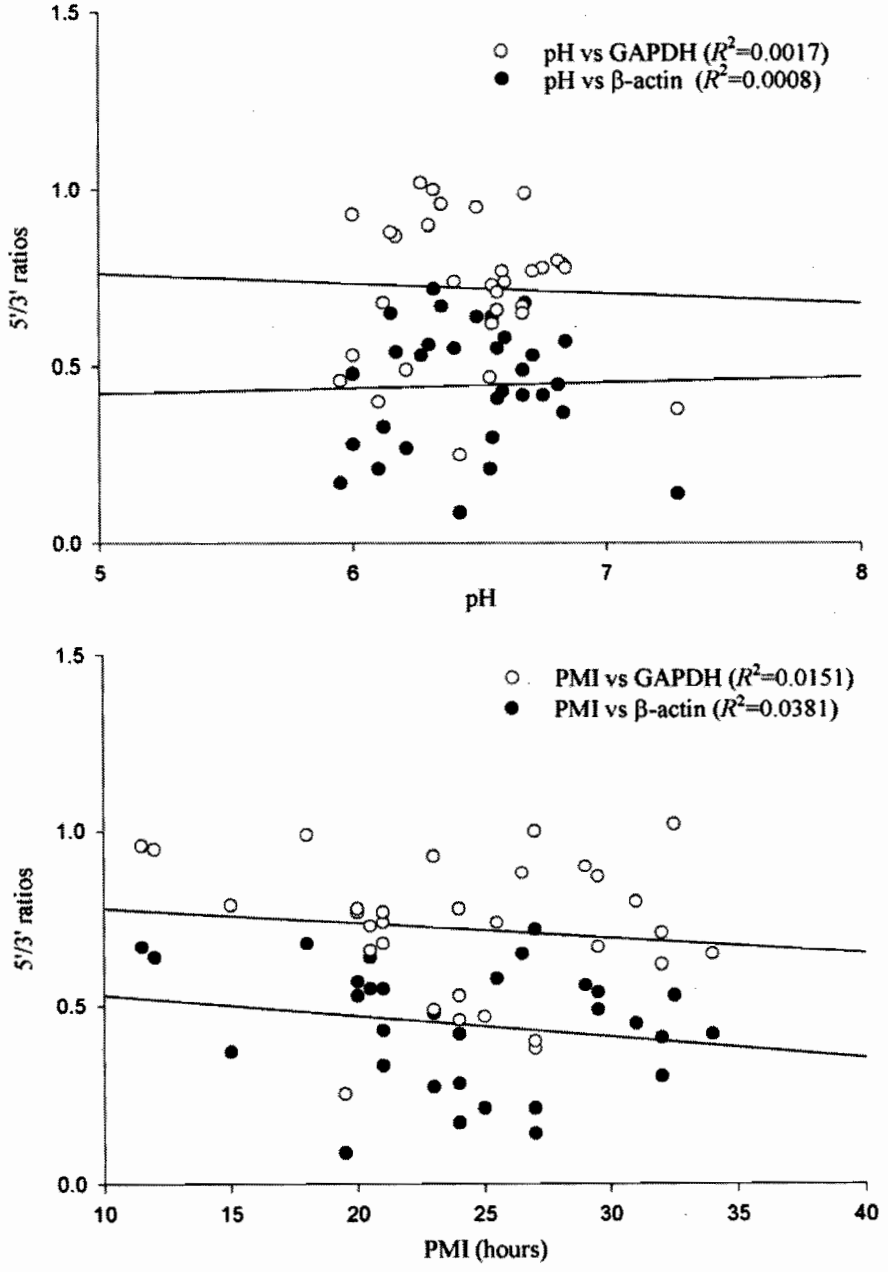

Figure 2 Venn diagrams showing the number of genes identified as differentially expressed and the overlap of genes between the different comparisons in the amygdala, hippocampus, BA24 and BA29. The intersections of the circles indicate the number of genes in common between contrasts.

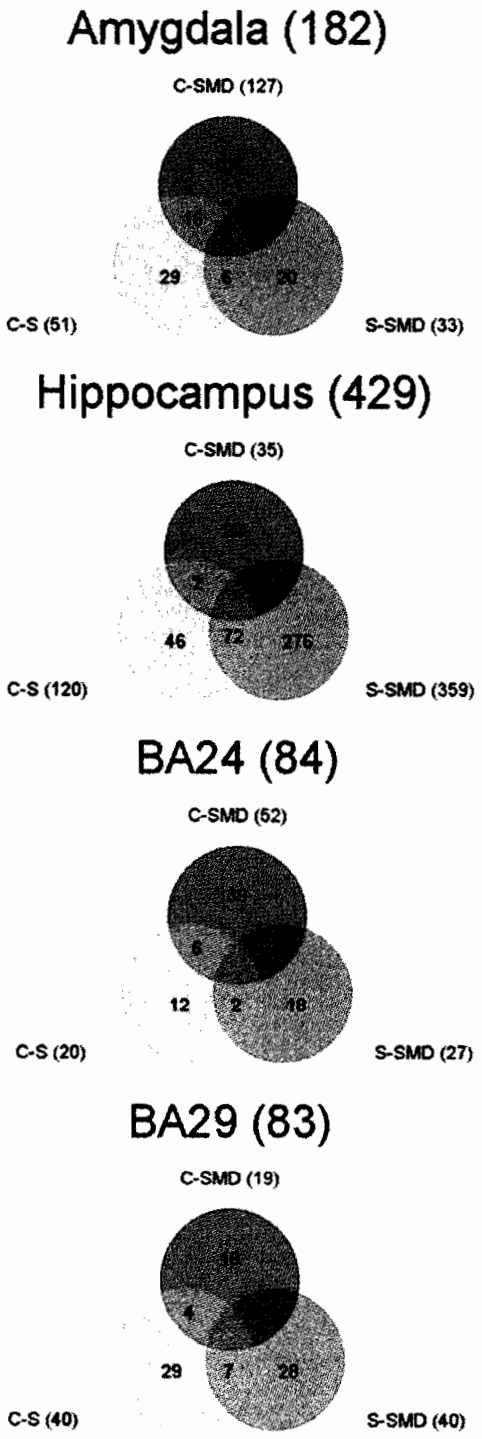

Figure 3 Amygdala gene expression patterns. (a) Clustered image map (CIM) of the differentially expressed genes in the amygdala. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression. (b) Principal component analysis (PCA) based on the differentially expressed genes.

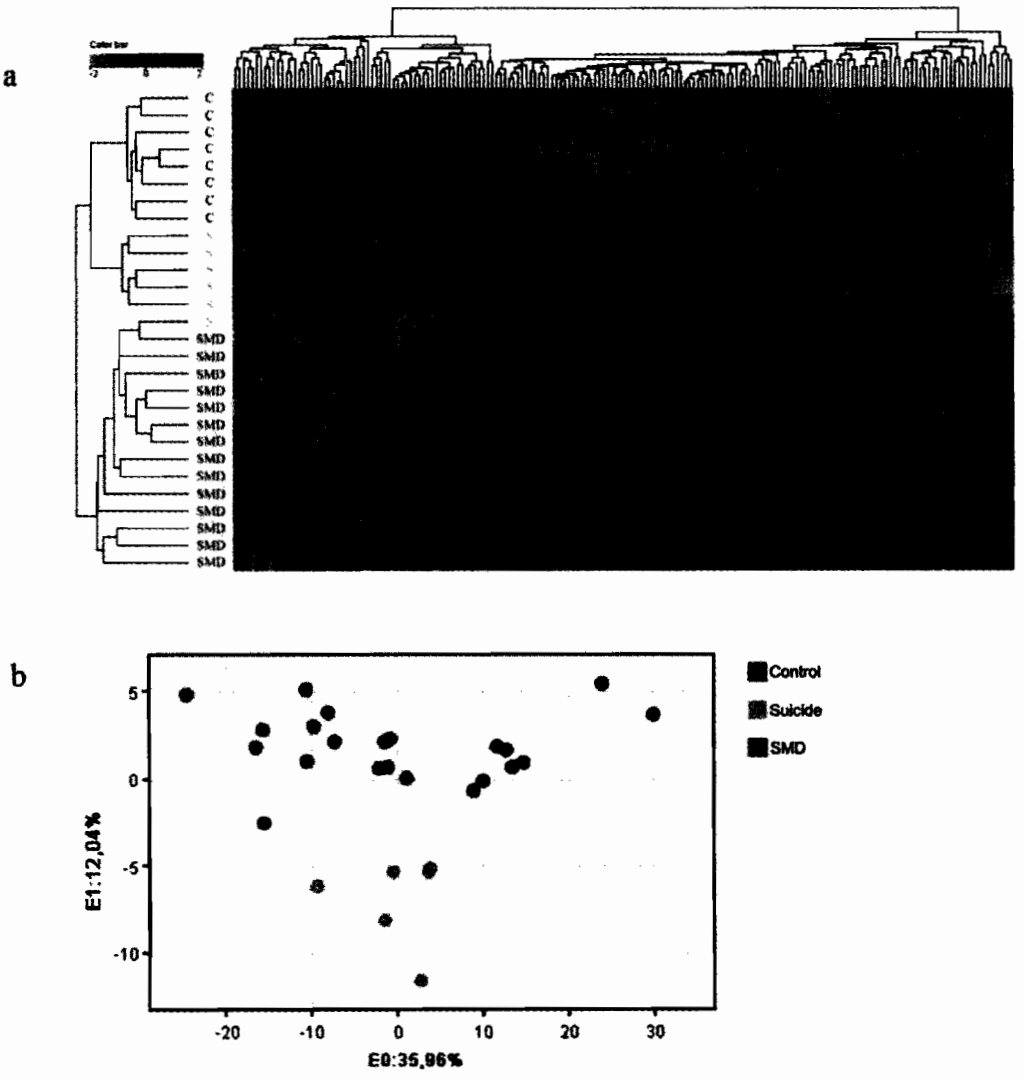

Figure 4 Hippocampus gene expression patterns. (a) Clustered image map (CIM) of the differentially expressed genes in the hippocampus. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression. (b) Principal component analysis (PCA) based on the differentially expressed genes.

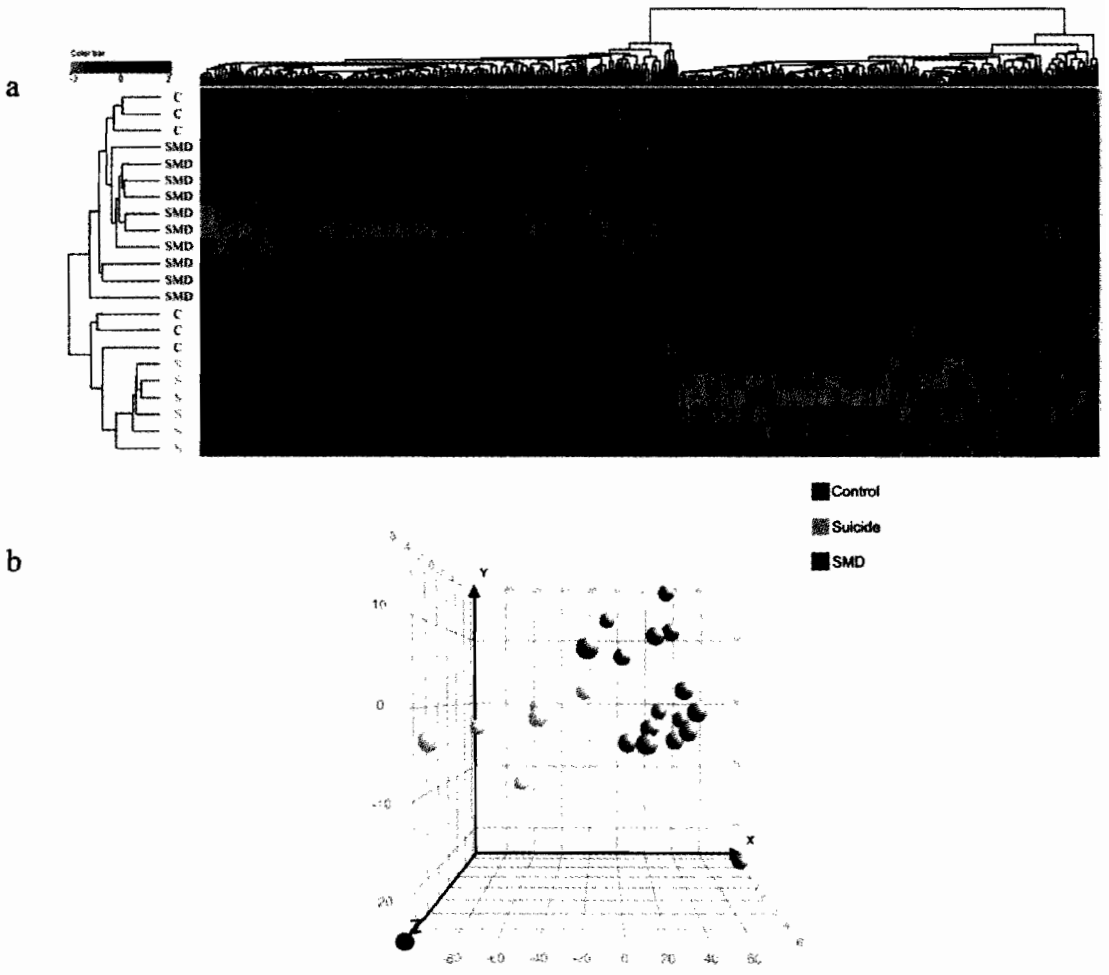

Figure 5 BA24 gene expression patterns. (a) Clustered image map (CIM) of the differentially expressed genes in BA24. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression. (b) Principal component analysis (PCA) based on the differentially expressed genes.

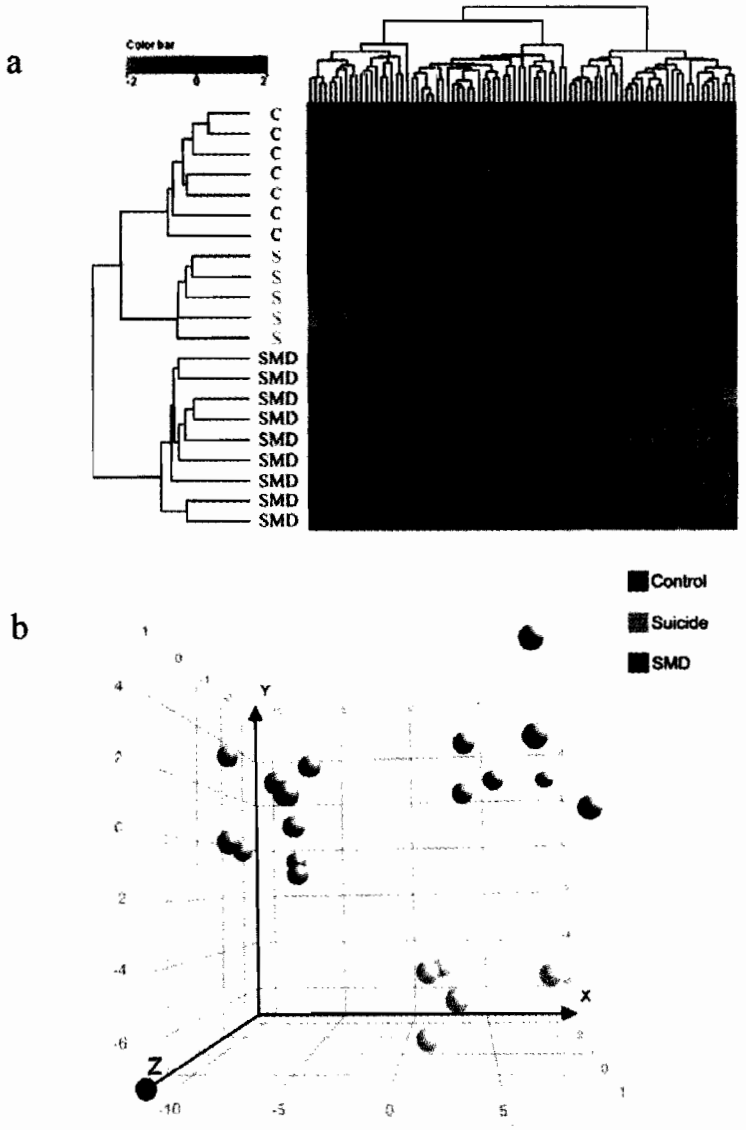

Figure 6 BA29 gene expression patterns. (a) Clustered image map (CIM) of the differentially expressed genes in BA29. Both expression patterns in individuals and genes were clustered. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression. (b) Principal component analysis (PCA) based on the differentially expressed genes.

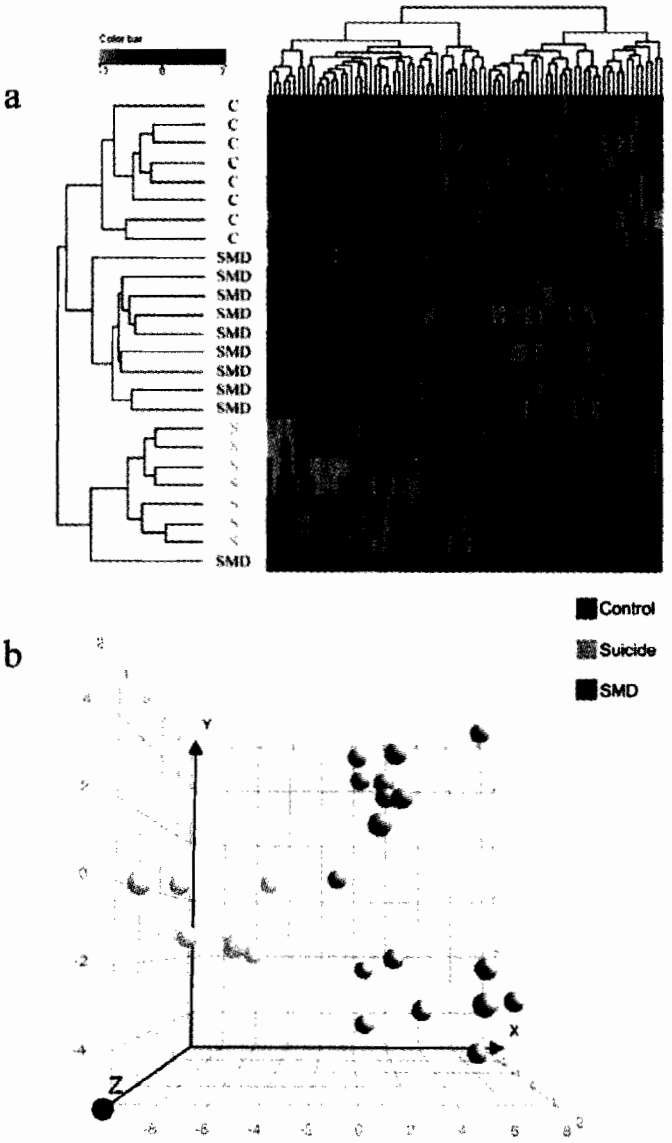

Table 2 Enriched gene ontology terms calculated using the differentially expressed genes per region. DAVID genes correspond to the total number of unique DAVID annotated genes. The percentage represents the number of differentially expressed genes belonging to a given category over the total number of DAVID annotated genes.

CATEGORY	TERM	Genes	Total DAVID genes	%	*P*-value
Amygdala					
Biological Process	Intracellular signaling cascade	11	135	8.1	0.028
Biological Process	Cell proliferation	11	135	8.1	0.049
Biological Process	Transcription	17	135	12.6	0.038
Biological Process	Regulation of biological process	22	135	16.3	0.035
Biological Process	Nucleotide and nucleic acid metabolism	23	135	17	0.038
Biological Process	Cellular process	43	135	31.9	0.043
Biological Process	Metabolism	46	135	34.1	0.046
Molecular Function	Adenyl nucleotide binding	12	135	8.9	0.045
Molecular Function	Protein binding	23	135	17	0.000
Hippocampus					
Biological Process	Regulation of transcription, DNA-dependent	35	348	10.1	0.034
Biological Process	Regulation of transcription	35	348	10.1	0.048
Biological Process	Regulation of metabolism	38	348	10.9	0.035
Biological Process	Regulation of biological process	53	348	15.2	0.009
Biological Process	Nucleotide and nucleic acid metabolism	58	348	16.7	0.003
Molecular Function	Zinc ion binding	29	348	8.3	0.011
Molecular Function	Transition metal ion binding	33	348	9.5	0.005
Molecular Function	DNA binding	40	348	11.5	0.012
Molecular Function	Ion binding	43	348	12.4	0.020
Molecular Function	Metal ion binding	43	348	12.4	0.020
Molecular Function	Protein binding	44	348	12.6	0.001
Molecular Function	Nucleic acid binding	63	348	18.1	0.001
Molecular Function	Catalytic activity	95	348	27.3	0.002
BA24					
Molecular Function	Enzyme regulator activity	7	70	10	0.003
Molecular Function	RNA binding	8	70	11.4	0.000
Molecular Function	Protein binding	11	70	15.7	0.030
Molecular Function	Nucleic acid binding	15	70	21.4	0.036
Molecular Function	Binding	34	70	48.6	0.008
BA29					
Biological Process	Organismal movement	5	66	7.6	0.032
Biological Process	Protein metabolism	14	66	21.2	0.036

Table 3 Selected differentially expressed genes in amygdala, hippocampus, BA24 and BA29. For a gene to be considered as differentially expresses it had to have an ANOVA $P \leq 0.01$ and for a given contrast a fold change/*P*-value combination of at least 1.3 fold change in either direction and $P \leq 0.01$. Corrected p-values (**P*) are shown after controlling for the possible confounder effect of substance dependence/abuse.

Probe set	Gene Title	Symbol	Cytoband	C	S	SMD	*P	SMD-C		S-C		S-SMD	
Amygdala								*P*	FC	*P*	FC	*P*	FC
208704_x_at	Amyloid beta (A4) precursor-like protein 2	APLP2	11q23-q25	1058	1415	1225	0.013	0.06	1.16	0.00	1.35	0.06	1.16
203006_at	Inositol polyphosphate-5-phosphatase, 40kDa	INPP5A	10q26.3	204	288	265	0.008	0.01	1.29	0.00	1.41	0.33	1.10
225278_at	Protein kinase, AMP-activated, beta 2 non-catalytic subunit	PRKAB2	1q21.1	219	275	305	0.001	0.00	1.40	0.01	1.27	0.21	-1.10
225011_at	Protein kinase, cAMP-dependent, regulatory, type II, alpha	PRKAR2A	3p21.3-p21.2	368	419	486	0.001	0.00	1.32	0.06	1.14	0.02	-1.16
206687_s_at	Protein tyrosine phosphatase, non-receptor type 6	PTPN6	12p13	51	31	36	0.012	0.01	-1.43	0.01	-1.61	0.34	-1.12
200670_at	X-box binding protein 1	XBP1	22q12.1	74	99	92	0.022	0.01	1.23	0.01	1.33	0.42	1.08
Hippocampus													
206811_at	Adenylate cyclase 8 (brain)	ADCY8	8q24	52	76	64	0.013	0.10	1.20	0.00	1.46	0.06	1.22
226463_at	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C, isoform 1	ATP6V1C1	8q22.3	580	425	609	0.002	0.66	1.05	0.01	-1.36	0.00	-1.43
204311_at	ATPase, Na+/K+ transporting, beta 2 polypeptide	ATP1B2	17p13.1	294	486	339	0.009	0.37	1.14	0.00	1.66	0.02	1.46
206586_at	Cannabinoid receptor 2 (macrophage)	CNR2	1p36.11	99	143	99	0.006	0.90	-1.01	0.00	1.44	0.01	1.46
213275_x_at	Cathepsin B	CTSB	8p22	296	236	364	0.002	0.07	1.26	0.22	-1.24	0.00	-1.56
227961_at	Cathepsin B	CTSB	8p22	564	358	650	0.006	0.26	1.25	0.16	-1.49	0.00	-1.86
203710_at	Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	3p26-p25	229	201	347	0.004	0.01	1.56	0.55	-1.11	0.00	-1.73
211323_s_at	Inositol 1,4,5-triphosphate receptor, type 1	ITPR1	3p26-p25	110	101	155	0.014	0.02	1.42	0.64	-1.08	0.01	-1.54
227095_at	Leptin receptor	LEPR	1p31	94	91	67	0.015	0.01	-1.44	0.67	-1.04	0.02	1.38
238600_at	Multiple coiled-coil GABABR1-binding protein	MARLIN1	4p16.1	420	303	476	0.000	0.08	1.14	0.04	-1.43	0.00	-1.63
205669_at	Neural cell adhesion molecule 2	NCAM2	21q21.1	54	65	45	0.013	0.09	-1.23	0.14	1.19	0.01	1.46
229463_at	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	9q22.1	368	277	487	0.002	0.07	1.29	0.10	-1.42	0.00	-1.83
236095_at	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	9q22.1	209	188	264	0.002	0.01	1.27	0.33	-1.11	0.00	-1.41
227820_at	Ornithine aminotransferase-like 1	OATL1	Xp11.3-p11.23	150	196	135	0.020	0.41	-1.13	0.02	1.33	0.01	1.50
236300_at	Phosphodiesterase 3A, cGMP-inhibited	PDE3A	12p12	104	131	91	0.011	0.26	-1.15	0.04	1.27	0.00	1.46
203680_at	Protein kinase, cAMP-dependent, regulatory, type II, beta	PRKAR2B	7q22	207	240	318	0.001	0.00	1.53	0.15	1.17	0.02	-1.30
227728_at	Protein phosphatase 1A	PPM1A	14q23.1	519	379	579	0.001	0.13	1.11	0.02	-1.42	0.00	-1.57
235061_at	Protein phosphatase 1K (PP2C domain containing)	PPM1K	4q22.1	215	194	319	0.016	0.03	1.58	0.90	-1.03	0.00	-1.62
208617_s_at	Protein tyrosine phosphatase type IVA, member 2	PTP4A2	1p35	476	672	527	0.016	0.33	1.12	0.01	1.44	0.01	1.29
225272_at	Spermidine/spermine N1-acetyltransferase 2	SSAT2	17p13.1	636	509	694	0.005	0.35	1.08	0.03	-1.26	0.00	-1.36
202043_s_at	Spermine synthase	SMS	Xp22.1	109	136	154	0.011	0.00	1.40	0.05	1.24	0.23	-1.13
205551_at	Synaptic vesicle glycoprotein 2B	SV2B	15q26.1	936	775	1055	0.008	0.12	1.13	0.13	-1.22	0.00	-1.39
225721_at	Synaptopodin 2	SYNPO2	4q26	179	258	175	0.012	0.90	-1.02	0.02	1.47	0.00	1.50
223529_at	Synaptotagmin IV	SYT4	18q12.3	1579	1353	2359	0.001	0.03	1.45	0.24	-1.16	0.00	-1.69

225204_at	T-cell activation protein phosphatase 2C	TA-PP2C	12q24.11	333	231	331	0.006	0.88	-1.02	0.01	-1.46	0.01	-1.43
225213_at	T-cell activation protein phosphatase 2C	TA-PP2C	12q24.11	377	228	347	0.002	0.36	-1.09	0.01	-1.71	0.00	-1.57
BA24													
224335_s_at	Beta-site APP-cleaving enzyme 1	BACE1	11q23.2-q23.3	339	303	253	0.015	0.00	-1.32	0.37	-1.10	0.02	1.20
206678_at	Gamma-aminobutyric acid (GABA) A receptor, alpha 1	GABRA1	5q34-q35	301	244	175	0.020	0.00	-1.72	0.29	-1.21	0.06	1.42
207010_at	Gamma-aminobutyric acid (GABA) A receptor, beta 1	GABRB1	4p12	510	478	714	0.007	0.00	1.42	0.72	-1.05	0.00	-1.49
229773_at	Synaptosomal-associated protein, 23kDa	SNAP23	15q15.1	83	63	78	0.013	0.40	-1.07	0.00	-1.33	0.03	-1.25
BA29													
221482_s_at	Cyclic AMP phosphoprotein, 19 kD	ARPP-19	15q21.2	1000	1197	873	0.014	0.13	-1.22	0.04	1.21	0.01	1.48
242482_at	Protein kinase, cAMP-dependent, regulatory, type I, alpha	PRKAR1A	17q23-q24	107	80	86	0.014	0.02	-1.24	0.01	-1.33	0.38	-1.07
203455_s_at	Spermidine/spermine N1-acetyltransferase	SSAT	Xp22.1	230	279	199	0.008	0.16	-1.18	0.08	1.23	0.00	1.45

Table 4 Genes significantly affected by the history of substance dependence or abuse (chronic substance effects) and positive substance intoxication as per toxicological result for alcohol or cocaine (acute substance effects) in the amygdala, hippocampus, BA24 and BA29. Corrected *P*-values (**P*), after controlling for the possible confounder effect of substance dependence/abuse and *P*-values associated with the history of substance dependence or abuse (Chronic) and positive toxicological result (Acute) for alcohol or cocaine are shown.

							Substance effects					
				Groups			Chronic			Acute		
Probeset	**Gene Title**	**Symbol**	**Cytoband**	**df**	**F**	***P****	**df**	**F**	***P***	**df**	**F**	***P***
Amygdala												
219855_at	Nudix (nucleoside diphosphate linked moiety X)-type motif 11	NUDT11	Xp11.22	2	11,525	0,000	1	8,874	0,007	1	6,104	0,021
223497_at	KIAA1411	KIAA1411	6q12-q13	2	10,511	0,001	1	2,388	0,136	1	12,133	0,002
225919_s_at	Chromosome 9 open reading frame 72	C9orf72	9p21.2	2	3,574	0,044	1	9,640	0,005	1	0,771	0,389
226406_at	Chromosome 18 open reading frame 25	C18orf25	18q21.1	2	9,360	0,001	1	0,103	0,752	1	16,885	0,000
239425_at	Full length insert cDNA clone ZC34E11		1	2	11,306	0,000	1	1,846	0,187	1	10,178	0,004
239437_at	Transcribed locus		6	2	7,152	0,004	1	5,937	0,023	1	14,912	0,001
Hippo												
202780_at	3-oxoacid CoA transferase 1	OXCT1	5p13.1	2	14,357	0,000	1	5,016	0,039	1	10,543	0,005
204258_at	Chromodomain helicase DNA binding protein 1	CHD1	5q15-q21	2	11,834	0,001	1	7,676	0,013	1	10,944	0,004
210176_at	Toll-like receptor 1	TLR1	4p14	2	12,077	0,001	1	1,749	0,204	1	9,115	0,008
212262_at	Quaking homolog, KH domain RNA binding (mouse)	QKI	6q26-27	2	20,340	0,000	1	10,686	0,005	1	6,355	0,022
219269_at	Hypothetical protein FLJ21616	FLJ21616	8p21.1	2	10,775	0,001	1	12,002	0,003	1	1,707	0,209
223880_x_at	Chromosome 20 open reading frame 24	C20orf24	20q11.23	2	12,852	0,000	1	9,985	0,006	1	8,341	0,010
227450_at	Hypothetical protein FLJ32115	FLJ32115	12p12.3	2	17,002	0,000	1	10,596	0,005	1	10,475	0,005
228549_at	KIAA0792 gene product	KIAA0792		2	9,882	0,001	1	9,793	0,006	1	0,415	0,528
229966_at	Ewing sarcoma breakpoint region 1	EWSR1	22q12.2	2	11,193	0,001	1	10,866	0,004	1	3,943	0,063
235366_at	Zinc finger protein 10	ZNF10	12q24.33	2	11,702	0,001	1	13,546	0,002	1	3,837	0,067
91816_f_at	Ring finger and KH domain containing 1	RKHD1	19p13.3	2	11,926	0,001	1	8,789	0,009	1	1,095	0,310
BA24												
52837_at	KIAA1644 protein	KIAA1644		2	8,426	0,003	1	0,309	0,586	1	9,014	0,008
BA29												
204544_at	Hermansky-Pudlak syndrome 5	HPS5	11p14	2	13,681	0,000	1	14,823	0,001	1	9,748	0,005
241876_at	Mdm4, transformed 3T3 cell double minute 4, p53 binding protein (mouse)	MDM4	1q32	2	12,587	0,000	1	11,142	0,003	1	1,647	0,214

CHAPTER 7

GLOBAL BRAIN GENE EXPRESSION ANALYSIS LINKS GLUTAMATERGIC AND GABAERGIC ALTERATIONS TO SUICIDE AND MAJOR DEPRESSION

This work was submitted as:
A Sequeira, T Kemplan, F Mamdani, L Canetti, J ffrench-Mullen, C Benkelfat, GA Rouleau, G Turecki. Global brain gene expression analysis links glutamatergic and GABAergic alterations to suicide and major depression.

CONTRIBUTION BY AUTHOR:

Sequeira: performed the experiments, did the analysis, interpreted the results and wrote the paper.

Kemplan, Mamdani: did part of the analysis and experiments, interpretation of the results and helped writing the paper.

Canetti: performed part of the experiments.

Ffrench-Mullen and Benkelfat: helped in the design of the study and collecting the data.

Rouleau: design and interpretation of the results.

Turecki: Design, writing of the paper and supervision.

PREFACE

After the interesting results found in certain cortical and subcortical areas, as presented in chapters 5 and 6, we wanted to explore expression changes occurring all across the brain. We investigated 17 regions with a slightly bigger sample in order to find not only individual genes differentially expressed but common themes dysregulated in a global manner. For this we exploited the functionalities of ErmineJ, a new gene ontology tool and combined the data of the 17 regions to identify the most common themes and then screen our differentially expressed genes to extract the most interesting targets.

ABSTRACT

Context: Most studies investigating the neurobiology of major depression and suicide have focused on the serotonergic system. While it seems clear that serotonergic alterations play a role in the pathogenesis of these conditions, dysfunction in additional neurotransmitter systems and other molecular alterations may also be implicated. Microarray expression studies are excellent screening tools to generate hypotheses about these additional molecular processes that may be at play. However, they can produce a substantial number of false positive results. We hypothesized that biological processes that are globally altered across different brain regions that are known to be implicated in the neurobiology of suicide and major depression are likely to represent valid global molecular alterations.

Objective: To carry out a global brain gene expression study identifying molecular processes that are differentially expressed across brain regions of relevance to suicide and major depression.

Design: Gene expression analysis using the *Affymetrix* HG-U133 A and B chip set in 17 cortical (Brodman areas (BA) 4, 6, 8-9, 10, 11, 20, 21, 38, 44, 45, 46, 47) and subcortical (BA24, BA29, amygdala, hippocampus, nucleus accumbens) brain regions from suicides with and without major depression and controls. Global ontological profiling analysis to identify across-region altered biological

processes and validation of genes representative of these processes using independent samples from the same subjects.

Subjects: Total mRNA for microarray analysis was obtained from 663 brain samples isolated from 39 subjects, including 26 suicide cases and 13 controls diagnosed by means of psychological autopsies.

Main Outcome Measures: Gene expression measures from microarrays, Gene Score Resampling results, and semi-quantitative RT-PCR.

Results: We observed the highest number of suicide specific alterations in prefrontal cortical areas and hippocampus. Global ontological profiling revealed alterations of synaptic neurotransmission and intracellular signaling and resulted in the observation that Glutamatergic (GLU) and GABAergic related genes were globally altered. Semi-quantitative RT-PCR results investigating expression of GLU and GABA genes from independent samples obtained from the same subjects were consistent with microarray data.

Conclusions: The observed results represent the first overview of global gene expression changes in the brain of suicide victims with and without major depression and suggest a global brain alteration of GLU and GABA genes in these conditions.

INTRODUCTION

Suicide accounts for almost 2% of the world's deaths, as in most developed countries it is the leading cause of death for males younger than 40 years of age [1]. Suicide is a complex condition which is frequently, but not exclusively, associated with depressive disorders. Although it is clear that these conditions are mediated by specific neurobiological processes [2;3], the precise molecular alterations and the brain circuits involved in suicide and major depression remain largely unknown.

The suicide brain is believed to have a complex pattern of neurochemical alterations involving several neurotransmitter systems and different brain regions [4]. While most of the attention to date has focused on the possible dysregulation of the serotonergic system, and to a lesser extent, the noradrenergic neurotransmitter system [56;7;7-11], there is also evidence implicating other neurotransmitters, such as the dopaminergic [12-15], polyaminergic [16], glutamatergic [17-20] and GABAergic systems [4;5;21-23]. In addition, several studies have also investigated the role of signal transduction and other molecular systems [24;2526;27]. Imaging studies of subjects with major depression and/or suicidal behavior using functional magnetic resonance and positron emission tomography have pointed to possible dysfunction of prefrontal neuronal circuits and subcortical areas of the brain. Among the latter, particularly those involved in the limbic system [28-34]. The complexity of neurotransmitter systems interacting in many distinct neuroanatomical regions

underlines the need of a more comprehensive and inclusive approach monitoring alterations in different regions of the brain.

Microarray technology offers the possibility of parallel monitoring expression levels of several thousands to virtually all genes based on the hybridization of nucleotide probes mounted on high density arrays to a target nucleotide sequence [35;36]. Recently this technology was implemented in psychiatry to study gene expression changes in postmortem brain tissue from psychiatric patients (for a review see [37]) and from suicide completers [38;3916].

One of the major shortcomings of microarray experiments is the level of false-positive results. While different statistical approaches exist to correct for type I errors [40-42], independent replication, both internal and external, is the method of choice to determine the accuracy of results [43]. We hypothesized that biological processes that are globally altered across different brain regions believed to be implicated in the neurobiology of suicide and major depression are likely to represent valid global molecular alterations. Therefore, in this study, we conducted a global gene expression survey in 17 cortical and subcortical brain areas of male suicides versus matched psychiatrically normal controls aiming at the identification of molecular pathways that are differentially expressed, consistently, across these brain regions.

METHODS

SUBJECTS AND DIAGNOSTIC PROCEDURES

Brain tissue was obtained from the Quebec Suicide Brain Bank. All samples used in the present study were from male subjects of French-Canadian origin, a homogeneous population with a well-known founder effect [44]. Cases and controls were group-matched for age and post-mortem interval. To be included in this study, suicides and controls had to die suddenly, with no medical or paramedic intervention, and with no prolonged agonal period. Brains were dissected at 4 °C and snap-frozen in liquid nitrogen before storage at -80 °C. This study was approved by our IRB and signed informed consent was obtained from next of kin.

The subjects were psychiatrically diagnosed by means of psychological autopsies, which are validated methods to reconstruct psychiatric history by means of extensive proxy-based interviews, as outlined elsewhere 45. In total, we analyzed 663 brain samples isolated from 39 subjects throughout the 17 regions, including suicides who died in the process of a major depressive episode (SMD; N=16); suicide victims with no history of depressive disorders (S; N=10); and group-matched population controls (C; N=13) who died suddenly from causes other than suicide. While this represents the total sample used in this study, there was some variability between regions following outlier exclusion (see below).

SAMPLE PROCESSING AND RNA QUALITY CONTROL

RNA samples used in this study had a minimum A260/A280 ratio of > 1.9. The samples were further checked for evidence of degradation and integrity. Samples had a minimum 28S/18S ratio > 1.6 (2100-Bioanalyzer, Agilent Technologies). We used the Human Genome U133 AB set, containing around 45,000 probesets representing >39,000 transcripts derived from around 33,000 human genes (http://www.affymetrix.com).

Expression data was analyzed using Genesis 2.0 (GeneLogic Inc, Gaithersburg, MD) and AVADIS (Strand Genomics, Redwood City, Calif). Several RNA integrity measures were used in this study to detect samples with poor RNA quality before final analysis. Microarray quality control parameters used included the following: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, and consistent β-actin and GAPDH 5'/3' signal ratios. Problematic arrays were also detected using principal component analysis (PCA). Outlier subjects/arrays were excluded on a region specific basis, without any subject being excluded from all the regions.

SEMI-QUANTITATIVE RT-PCR

For technical validation of differentially expressed genes, we used semi-quantitative RT-PCR using RNA extracted from additional samples that were

collected in each brain region from tissue adjacent to that used in the microarray expression study. Reverse transcription was performed in a total volume of 40µl with 2µg of total mRNA using M-MLV reverse transcriptase (Gibco, Burlington, Ontario) and oligo(dT)16 primers. PCR amplification was carried out using the AmpliTaq Gold from Applied Biosystems (Foster City, California). mRNA-specific primers, were designed using Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) in order to avoid amplification of contaminating genomic DNA. Primer sequences are available upon request. PCR products were visualized using ethidium bromide staining after electrophoresis in a 3% agarose gel. Images were digitalized and analyzed using Gene Tools (Syngene, Cambridge). Experiments were carried in parallel in triplicate and β-actin was used as an internal control gene.

ANALYSIS

Analyzed genes were "Present" (according to MAS 5.0) in at least 75 % of the subjects in at least one of the groups. Gene expression values were floored to 1 and then $\log_2$-transformed. ANOVAs were performed for each gene to identify statistically significant gene expression changes. Statistically significant genes were subjected to a post-hoc t-test and fold-change analysis (FC) in order to identify differences between the suicides with major depression (SMD), the suicides (S), and the controls (C). For a gene to be considered as differentially expressed it had to have an ANOVA *P*-value of less than or equal to 0.01 and a

fold change/*P*-value combination of at least a 1.3 fold change in either direction. Post-hoc analyses were carried out using the Fisher protected LSD test with a *P*-value set at 0.01.

Cluster analysis was performed using average-linkage hierarchical cluster analysis with a correlation metric. Both expression patterns in individuals and genes were clustered. Principal component analysis (PCA) was performed based on the initial gene sets and on the selected genes (according to our significance criteria).

Functional ontological profiling of the expression changes was performed across all 17 regions using the Gene Score Resampling (GSR) method implemented in the ErmineJ software (version 2.1.8, Columbia University, NY) and that examines the distributions of scores (FC or P-values) across the whole array 46. The parameters used were the following: Maximum gene set size: 300; Minimum gene set size: 5; with the mean of replicates, 5000 iterations and full resampling. The rank and P-value computed by ErmineJ were used to calculate the most overrepresented ontologies across all regions. The distribution pattern of the ErmineJ calculated P-values in the different regions of the brain was examined by hierarchical clustering using AVADIS, with the normalized negative log of the P-values as the input. Further annotations were conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2006 47.

RESULTS

GLOBAL ANALYSIS

Demographic and clinical characteristics of the subjects included in this study are shown in Table 1. No significant differences were observed between the groups for the different demographic measures such as age (Mean age: 35.6 (C), 35.2 (S), 34.1 (SMD)), the post-mortem interval (PMI), or the brain tissue pH. Furthermore, no significant correlation was observed in our sample as a whole between quality control parameters such as noise (RawQ), number of genes detected as present across arrays, scale factor, β-actin and GAPDH 5'/3' ratios and pH or PMI in several regions tested (data not shown). This suggests that RNA quality from our tissue was acceptable, probably reflecting our brain recruitment procedures, which are limited to sudden death without medical intervention, prolonged agonal periods or extended PMI.

Overall, 251,206 probe sets passed the initial filtering criteria and were included in the analysis across the 17 regions with an average of around 15,000 probe sets per region. A summary of the analyzed and the differentially expressed probe sets per region and per comparison are shown in Table 2 and in Figures 1. The region with the least probe sets analyzed was BA10 with 11,935 and the one with the most was BA45 with 15,886 probe sets. A total of 5,868 probe sets were significantly altered after the ANOVA at the $P \leq 0.01$ level. Finally, 4,472 probe

sets were differentially expressed across the regions after Fisher protected LSD tests and fold-change (FC) filtering. There was substantial variation between regions in terms of total number of differentially expressed genes, ranging from 83 in BA29 to 636 in BA10. In addition to BA10, the other region with the largest number of differentially expressed probe sets was BA46 with 626. BA46 and BA10 are two prefrontal cortex regions that are anatomically close and previously associated with suicidal behaviors and with major depression. On the other hand, the two limbic regions that are part of the cingulated cortex, BA24 and BA29, had the least number of differentially expressed probe sets, 84 and 83 respectively.

FUNCTIONAL PROFILING

In order to identify biological and molecular pathways that were differentially expressed across the regions investigated in this study, we initially used ErmineJ [46] to generate a list of overrepresented ontologies per brain region. Subsequently, hierarchical clustering analyses were carried out to identify those biological processes that were commonly represented across all the brain regions (Figure 2). The purpose of this approach was to identify biological functions that were globally altered in depression and/or suicide. We then selected the 10 most commonly overrepresented ontologies based on the rankings and P-values in the 17 brain areas from the GSR analysis in ErmineJ . In order, from more to less commonly represented, these were signal transduction, intracellular signalling cascade, cell organization and biogenesis, protein localization, protein transport,

establishment of protein localization, transmission of nerve impulse, small GTPase mediated signal transduction, synaptic transmission and vesicle-mediated transport. The corresponding probe sets to these ontologies were functionally annotated using DAVID, and 568 unique genes were identified and classified. As showed in Table 3, most of these genes corresponded to genes implicated in cell communication processes and related subcategories of functions such as intracellular signalling cascade, signalling transduction, transmission of nerve impulse, and more specifically, synaptic transmission.

As intracellular signalling cascade, signalling transduction and transmission of nerve impulse are parent nodes related to synaptic transmission, a more specialized molecular function that is of particular interest to the neurobiological investigation of major depression and suicide, we explored more specifically the genes related to synaptic transmission. A total of 57 genes corresponded to this category (Table 4) and consisted in several pre-synaptic proteins (SYN2, SYPL1, SNAP25, SYT1, SYT5, SNPH) and signal transduction genes such as the mitogen-activated protein kinase 1 (MAPK1) and the 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP). However, it was remarkable that a large proportion of these 57 genes (22 out of 57 or 38.6%) corresponded to genes implicated in GABAergic or glutamatergic neurotransmission or in the transport of these neurotransmitters (Table 4).

PATHWAYS GLOBALLY DIFFERENTIALLY EXPRESSED

In order to more specifically explore GABAergic and glutamatergic genes that were differentially expressed across the different brain regions, we interrogated our database of differentially expressed genes using the probe sets extracted from DAVID.

GABAergic genes

A total of 27 GABAergic-related probe sets were differentially expressed across the regions, many corresponding in fact to probe sets for the same genes as represented in Figure 3. For instance, one gene, the Gamma-aminobutyric acid (GABA) A receptor, delta (GABRD) gene was differentially expressed in BA6, BA44, BA45, BA46 and the GABA(A) receptor-associated protein like 1 gene (GABARAPL1) was differentially expressed in BA10, BA20 and BA46 (Figure 3). Part of the ventrolateral prefrontal cortex, BA46 was of particular interest with a total of six GABAergic genes differentially expressed (GABARAPL1, GABRA5, GABRB1, GABRD, GABRG1, GABRG2). Also noteworthy, the majority of the differentially expressed GABAergic probe sets (19 out of 27) corresponded to different subunits of GABA A receptors, particularly the alpha, beta, delta, gamma and rho subunits. As seen in Figure 3, a clear pattern of dysregulation was observed in terms of genes and regions implicated with a majority of GABAergic genes being up-regulated (red) among the suicides with major depression. For instance, in the hippocampus, all differentially expressed GABAergic genes were clearly up-regulated in suicides with major depression

(GABARAPL1, GABARA4 and GABARB1) and with low expression among suicides without history of depressive disorders, suggesting a depression specific effect. Also seen in Figure 3, a total of 10 GABA(A) receptor beta probe sets were differentially expressed and were generally up-regulated among the depressed suicides. The same was observed for the GABA(A) receptor–associated protein like 1 (GABARAPL1) which was up-regulated in the depressed suicide group in BA20, hippocampus and BA46 and BA10. In summary, a striking number of probe sets corresponding to GABA(A) receptors or GABA(A) receptor-associated binding protein were altered between the three groups and the majority were up-regulated among the suicides with major depression and with lower expression levels among the suicides without major depression or the controls, suggesting their role in molecular processes that may be more specific to the pathophysiology of major depressive disorder.

Glutamatergic genes

A total of 28 probe sets corresponding to genes implicated in glutamatergic neurotransmission were differentially expressed across the regions. A graphical representation of the gene expression changes between the three groups can be seen in Figure 4. A good proportion of these probe sets (7) corresponded to the Glutamate-ammonia ligase (glutamine synthase) gene (GLUL) that codes for an enzyme implicated in glutamate recycling. GLUL probe sets were found consistently down-regulated among the depressed suicides in the prefrontal cortex (BA44 BA45, BA46) and the amygdala (Figure 4). Also of particular interest, 7

out of 28 probe sets (Figure 4) correspond to one of four subtypes (AMPA1, AMPA2, AMPA3, AMPA4) of the glutamate AMPA receptor that was differentially expressed in several brain cortical (BA10, BA21, BA46) and subcortical areas (hippocampus, nucleus accumbens, amygdala). The majority of the glutamatergic related probe sets correspond to ionotropic NMDA (GRINA, GRIN2A, GRINL1A) and AMPA (GRIA3,GRIA4, GRIA1, GRIA2) receptors with the latest being consistently up-regulated among the suicides with major depression versus the controls or the suicides without history of major depression (Figure 4). Also noteworthy in Figure 4, the glutamate receptor metabotropic 3 (GRM3) was consistently down-regulated among the suicides with major depression versus the controls and the suicides without major depression in two areas of the prefrontal cortex BA46 and BA47 and two areas of the parietal cortex BA38 and BA20. In brief, we observed a global up-regulation of AMPA receptors in the suicide with major depression group and a global down-regulation of the GRM3 receptor and the glutamine synthase (GLUL) genes expression in the suicide with major depression group.

VALIDATION OF MICROARRAY GENE EXPRESSION RESULTS

Differential expression of GABAergic and glutamatergic ANOVA results was also confirmed by semi-quantitative RT-PCR on independent samples, adjacent to those used for the microarrays, from the same subjects in the brain areas where

differential expression was observed in the microarray experiments (Table 5). Out of the 16 genes/regions tested, 12 were also significantly differentially expressed according to the semi-quantitative RT-PCR data and in concordance in terms of the direction of the changes between the groups with the microarrays (Table 5).

COMMENT

In this study, gene expression was investigated using genome-wide microarrays in 17 suicide brain areas thought to be involved in the neurobiology of suicide and major depression, comparing suicides with and without major depression to psychiatrically normal controls. This is, to our knowledge, the first large-scale brain expression study aiming at identifying global brain alterations associated to suicide and major depression. The extent of the expression changes observed was considerably different between the diverse brain areas investigated, with certain areas such as those that comprise the prefrontal cortex and hippocampus accounting for the majority of expression changes. This is consistent with what one would expect according to neuroanatomical studies of depression and suicide and is also consistent with previous studies looking at discrete brain regions [4;48-54]. The analysis also revealed that genes involved in cell communication processes were globally altered. Among genes involved in synaptic transmission, a striking number of GABAergic receptor subunit genes were generally up-regulated among

the suicides with major depression, but with lower expression levels among the 2 other groups. We also observed for the suicide with major depression group a general up-regulation of AMPA receptor subunit genes and a global down-regulation of the GRM3 receptors and the glutamine synthase gene expression. Our study suggests the presence of consistent alterations of several genes coding for components of the same pathway across different brain regions.

The HG-133AB chipset contains around 44 000 probe sets many of which may not be expressed at biologically significant or detectable levels and they represent mostly noise. Accordingly, Jongeneel et al. estimated that between 10 to 15 thousands transcript are actually expressed in several types of human cell lines [55]. For that reason and in order to reduce the multiplicity problem, we used a combination of filtering methods in order to include in our analysis only transcripts that were actually expressed and reliably detectable. This approach efficiently allows to significantly reduce the total number of analyzed probe sets without notably decreasing the number of truly positive genes [56]. This resulted in an average of around 15 000 probe sets analyzed per region in accordance with the number of expected genes expressed in any type of cell [55]. Second, in order to control for type I errors, we also used a combination of stringent *P*-value thresholds ($\leq$0.01 both at the ANOVA and post-hoc test), as well as a fold change of at least 1.3 in either direction. Most importantly, by focusing on results that replicate across several different brain regions, which constitute partially

independent experiments, we are likely to have significantly reduced the occurrence of type I errors in our study.

The current approach led to the identification of 4 472 differentially expressed probe sets over the 17 brain regions (Table 2). As expected, and in accordance with the neuroanatomical and post-mortem biomarkers literature, three prefrontal cortex areas, BA8,9, BA10, BA46, and the hippocampus, had the higher number of differentially expressed probe sets confirming the implications of those regions in the pathophysiology of suicide and major depression (Table 2). These four areas have been well characterized and have been previously shown as implicated in suicidal behaviors and depression in numerous post-mortem studies [4;57-69]. In vivo, neuroimaging studies have also pointed to alterations in the prefrontal cortex and the hippocampus in patients suffering from major depression [50;70;29;52;70-72]. Our study provides in a genomic scale, potential molecular targets that may account for those alterations in the brains of suicide victims with and without major depression.

Functional analysis using gene ontologies [73] was performed across the 17 regions using a new tool (ErmineJ) that efficiently addresses many of the limitations and problems of the initial gene ontology tools [74], by implementing more comprehensive algorithms and the possibility of performing analyses in parallel. This global functional ontological profiling revealed specific functional ontologies commonly overrepresented in all the regions investigated in this study,

and further investigation showed that an important proportion of genes belonged to cell-communication processes. Among these, a remarkable number of probe sets corresponded to genes that are part of the to GABAergic and glutamatergic neurotransmitter systems.

L-glutamic acid (glutamate) and GABA are respectively the main excitatory and inhibitory neurotransmitters in the central nervous system [75]. Growing evidence has supported alterations in both of these neurotransmitter systems in major depression [22;23;76-78] and suicide [17;19;20;78;79]. Sanacora et al. [21] using a magnetic resonance spectroscopy protocol observed elevated levels of glutamate and lower levels of GABA in the occipital cortex of subjects diagnosed with major depression. Furthermore, Hasler et al. [80] demonstrated that abnormal reductions in glutamate/glutamine and GABA concentrations are present in the prefrontal cortex of unmedicated depressed patients. Our results are also in concordance with those of Choudary et al. [18], who performed a gene expression study in the cingulate and prefrontal cortex brain areas of suicides and depressed suicides using one of the chips (HG-U133A) of the microarray we used in our study. Interestingly, their results point to similar alterations in glutamate recycling (glutamine synthase), glutamate receptors subunits (GRIA1, GRIA3, GRIK1, GRM3) and GABA receptors subunits (GABARB3, GABRD, GABARG2). Also, recently, Merali et al. [78] observed altered levels of GABA(A) receptor subunits (α1, α3, α4 and δ) in the BA10 of depressed suicide victims versus non-depressed controls.

Since glutamine synthase (GLUL), which is responsible of the recycling of glutamate by its conversion into glutamine, was down-regulated in the prefrontal and the parietal cortices of suicides with major depression, but not in suicides without major depression, while the majority if ionotropic glutamatergic receptors subunits were up-regulated in these brain regions, one possible interpretation of our results is that increased ionotropic glutamatergic neurotransmission may be, either directly or indirectly, related to depressive psychopathology. This hypothesis, if true, is in agreement with the observation that a single dose of Ketamine, an NMDA antagonist, is sufficient to produce a rapid and long lasting antidepressant effect [81]. Glutamate seems to mediate stress-induced neuronal atrophy in the hippocampus [82]. In addition, although not always consistent, there are different lines of evidence, comprising peripheral studies [83], postmortem brain studies [20], and in-vivo imaging studies [84] reporting glutamatergic dysfunction in major depression. Interestingly, glutamatergic neurotransmission is closely controlled by intracellular levels of polyamines, spermine and spermidine being specific modulators of NMDA and AMPA receptors activity [85;85-90]. Polyamines, and more specifically SSAT, the rate limiting enzyme in the catabolism of polyamines, were associated with suicide and depression in a previous study by our group [16]. Polyamines modulate GABAergic and glutamatergic neurotransmission, and were also found to be altered in the present study. In light of these observations, it is important to consider the polyamine-glutamatergic

systems as a possible target for future strategies for the treatment of major depression.

In this study, even though possible limitation regarding pre- and post-mortem factors such as agonal factors and post mortem interval were experimentally controlled, the conclusions presented here are to be taken with caution and need to be confirmed in an independent and larger sample. Although microarray technology have proven its efficacy in studying mood disorders [16;18;91], in this study, microrrays were only used as an initial screening technique validated by means of more robust and classical techniques and further investigated by genetics of gene expression. The study design does not allow to clearly differentiating the alterations solely related to suicide from those specific to major depression. This would be possible to resolve by including a group of matching patients with major depression not death by suicide, but such a group would be too difficult to obtain due to the demographic characteristics of suicide victims and depressed patients. Nevertheless, our results are interesting as they shed light into the molecular alterations taking place at the moment of their death in the brains of suicides and depressed suicides.

In conclusion, this is, to our knowledge, the first study attempting to determine global brain expression changes taking place in the brain of suicide victims with and without major depression. We observed global changes in genes implicated in synaptic transmission, and more specifically, in genes involved in GABAergic

and glutamatergic neurotransmission. Further studies are warranted in order to examine in detail region specific alterations, to validate the observed changes using complementary approaches and to investigate possible genetic factors related to the observed alterations.

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Table 1: Demographic and clinical characteristics of the subjects included in the study.

Group	Age	PMI	Cause of death	DSM-IV (six months diagnosis)	Toxicology
C	51	15	Motor vehicle crash	Alcohol dependence	Alcohol
C	31	24	Cardiac arrest	Alcohol dependence	
C	19	32	Motor vehicle crash		
C	47	12	Cardiac arrest	Alcohol abuse	
C	30	30	Cardiac arrest		
C	28	27	Motor vehicle crash		
C	41	24	Myocardial Infarction		
C	31	29.5	Motor vehicle crash		
C	46	19.5	Myocardial Infarction		
C	21	24	Cardiac arrest		
C	27	20.5	Cardiac arrest		
C	32	26.5	Cardiac arrest	Cannabis abuse	
C	55	24	Motor vehicle crash		
S	38	23	Hanging	Alcohol dependence, cocaine dependence	Alcohol
S	21	21	Asphyxiation	OCD, Alcohol dependence	Alcohol
S	31	32.5	Hanging		
S	29	26.5	Hanging		
S	33	18	Hanging		
S	26	69	Hanging		
S	30	27	Stabbing	Paranoid schizophrenia	
S	36	25	Hanging		
S	51	21	Self inflicted gun shot	Alcohol dependence	
S	42	27	Carbon monoxide		
SMD	28	20	Hanging	MDD, alcohol dependence	Alcohol
SMD	22	11.5	Hanging	MDD, alcohol dependence	Alcohol, cocaine
SMD	53	14	Carbon monoxide	MDD	
SMD	26	34	Hanging	MDD	Cocaine
SMD	40	23	Hanging	MDD, alcohol dependence	
SMD	19	29.5	Hanging	MDD	
SMD	53	29	Hanging	MDD, alcohol dependence	
SMD	42	21	Drowning	MDD	
SMD	45	20.5	Self inflicted gun shot	MDD, pathological gambling	
SMD	35	31	Hanging	MDD, alcohol dependence	
SMD	39	25.5	Hanging	MDD	
SMD	49	32	Hanging	MDD, alcohol abuse	
SMD	40	22	Hanging	MDD	
SMD	53	33.5	Hanging	MDD	
SMD	18	27	Carbon monoxide	MDD	
SMD	22	20	Hanging	MDD	

C = control, S = suicide, SMD = suicide with major depression, MDD = major depressive disorder.

Table 2: Summary of results of the differential expression analysis in 17 brain areas of controls and suicides with and without major depression.

	Analyzed	ANOVA	Significant	C-SMD	C-S	SMD-S
BA4 (C=6; S=5; SMD=8)	14632	276	202	128 (66 up; 62 down)	55 (28 up; 27 down)	62 (35 up; 27 down)
BA6 (C=7; S=6; SMD=13)	15266	434	286	216 (105 up; 111 down)	76 (31 up; 45 down)	46 (20 up; 26 down)
BA8,9 (C=6; S=5; SMD=9)	14854	690	589	73 (33 up; 40 down)	302 (71 up; 231 down)	411 (147 up; 264 down)
BA10 (C=6; S=6; SMD=7)	11935	692	636	604 (436 up; 168 down)	41 (30 up; 11 down)	69 (31 up; 38 down)
BA11 (C=6; S=5; SMD=9)	14410	178	152	85 (19 up; 66 down)	33 (11 up; 22 down)	77 (50 up; 27 down)
BA20 (C=5; S=5; SMD=6)	13944	304	255	77 (36 up; 41 down)	68 (21 up; 47 down)	173 (50 up; 123 down)
BA21 (C=9; S=6; SMD=5)	14129	223	204	161 (42 up; 119 down)	20 (8 up; 12 down)	64 (37 up; 27 down)
BA38 (C=7; S=5; SMD=6)	14395	249	203	66 (37 up; 29 down)	119 (68 up; 51 down)	68 (16 up; 52 down)
BA24 (C=7; S=5; SMD=9)	15243	167	84	52 (27 up; 25 down)	20 (5 up; 15 down)	27 (15 up; 12 down)
BA29 (C=8; S=7; SMD=10)	15032	126	83	19 (7 up; 12 down)	40 (13 up; 27 down)	40 (24 up; 16 down)
Amy (C=8; S=6; SMD=14)	15007	256	182	127 (92 up; 35 down)	51 (24 up; 27 down)	33 (17 up; 16 down)
Hippo (C=6; S=6; SMD=10)	14495	518	429	35 (16 up; 19 down)	120 (60 up; 60 down)	359 (196 up; 163 down)
NAcc (C=6; S=6; SMD=10)	15232	178	140	22 (8 up; 14 down)	60 (10 up; 50 down)	91 (11 up; 80 down)
BA44 (C=12; S=6; SMD=13)	15788	275	140	88 (32 up; 56 down)	24 (15 up; 9 down)	48 (30 up; 18 down)
BA45 (C=11; S=6; SMD=12)	15886	196	101	60 (13 up; 47 down)	21 (8 up; 13 down)	27 (17 up; 10 down)
BA46 (C=7; S=7; SMD=12)	15655	839	623	140 (37 up; 103 down)	193 (148 up; 45 down)	470 (373 up; 97 down)
BA47 (C=9; S=7; SMD=12)	15303	267	163	102 (48 up; 54 down)	29 (13 up; 16 down)	51 (29 up; 22 down)
Total	251206	5868	4472			

C = control, S = suicide, SMD = suicide with major depression, Amy = amygdala, Hippo = hippocampus, NAcc = nucleus accumbens.

Figure 1: Pie charts representing the distribution of the differentially expressed genes in **(A)** prefrontal cortical areas and in **(B)** other cortical and subcortical brain areas. The number of chips per group that passed quality control assessment is also given.

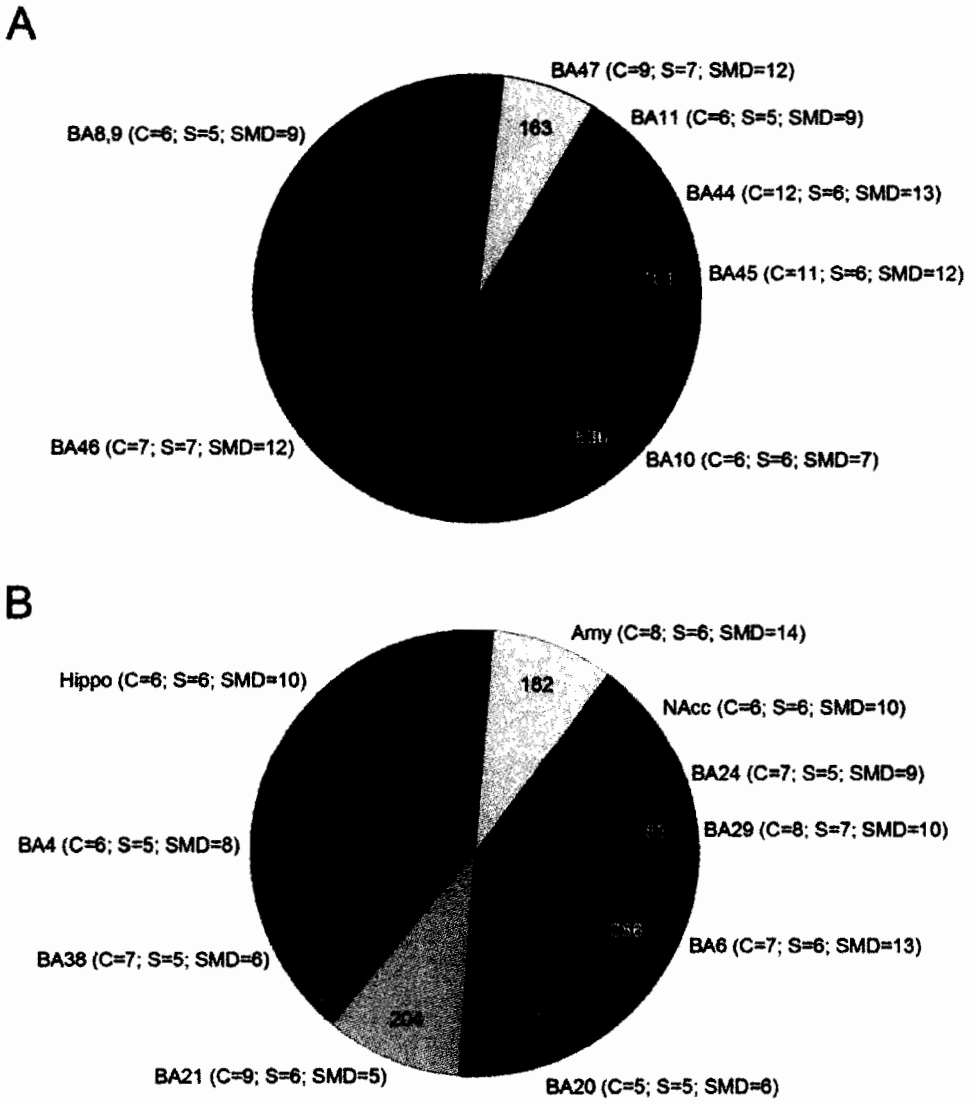

Figure 2: Clustered image map (CIM) of the hierarchical cluster analysis of the distribution pattern of ErmineJ calculated *P*-values across all the regions studied. Both ontological categories and the regions were clustered. The color and intensity indicate level of significance: red spectrum colors indicate very highly significant gene ontologies (0.001), yellow colors indicate highly significant gene ontologies (0.01) and white colors represent no significance.

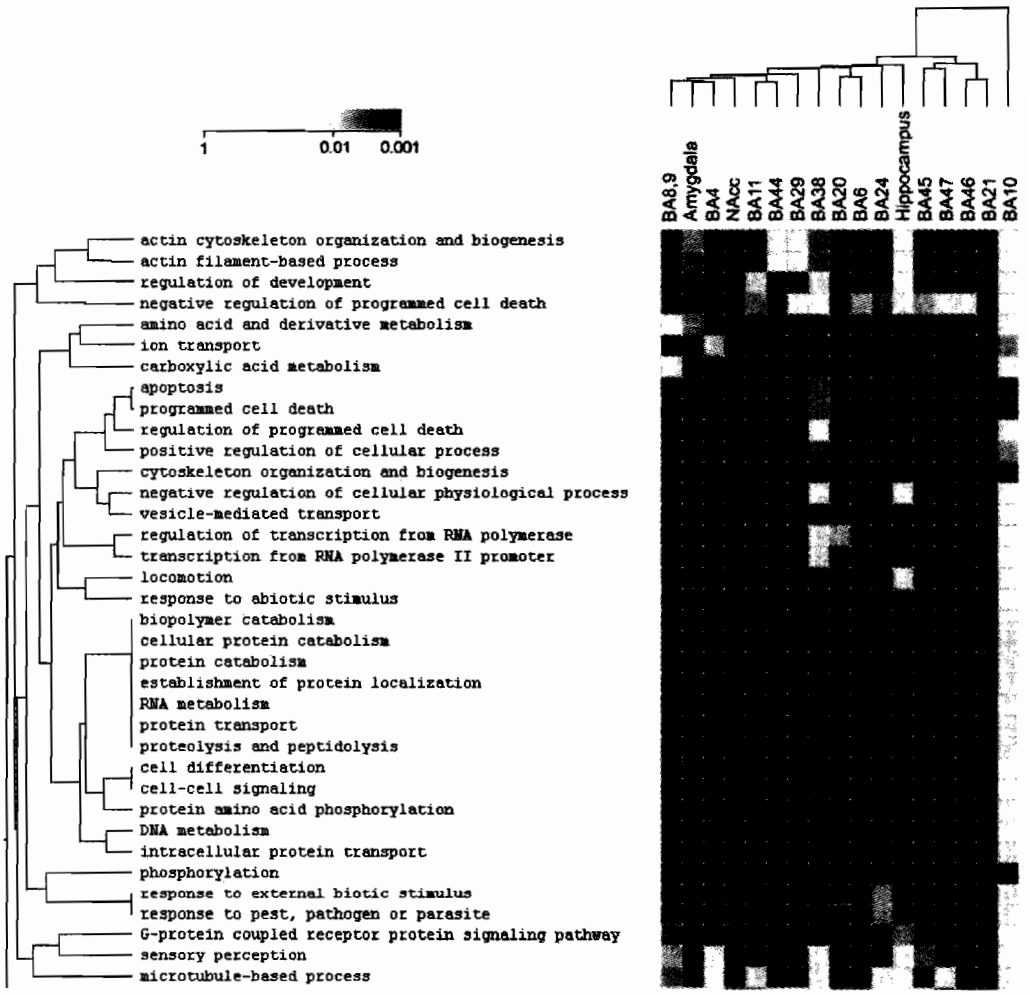

Table 3: The 568 unique genes identified in our database of differentially expressed genes were regrouped using DAVID (2006) according to their function.

Category	Term	Count	%
Biological Process	signal transduction	237	41.73%
Biological Process	intracellular signaling cascade	138	24.30%
Biological Process	cell organization and biogenesis	132	23.24%
Biological Process	protein localization	79	13.91%
Biological Process	protein transport	77	13.56%
Biological Process	establishment of protein localization	77	13.56%
Biological Process	transmission of nerve impulse	61	10.98%
Biological Process	small GTPase mediated signal transduction	59	10.39%
Biological Process	synaptic transmission	57	10.03%
Biological Process	vesicle-mediated transport	55	9.68%

Table 4: Differentially expressed genes directly implicated in synaptic transmission as determined using DAVID (2006).

Name	Symbol	Cytoband	Entrez Gene
2',3'-cyclic nucleotide 3' phosphodiesterase	CNP	17q21	1267
4-aminobutyrate aminotransferase	ABAT	16p13.2	18
5-hydroxytryptamine (serotonin) receptor 2a	HTR2A	13q14-q21	3356
adenylate cyclase activating polypeptide 1 (pituitary) receptor type i	ADCYAP1R1	7p14	117
amphiphysin (stiff-man syndrome with breast cancer 128kda autoantigen)	AMPH	7p14-p13	273
apolipoprotein e	TOMM40	19q13	10452
cocaine- and amphetamine-regulated transcript	CART	5q13.2	9607
cortistatin	APITD1	1p36.22	378708
discs, large (drosophila) homolog-associated protein 1	DLGAP1	18p11.3	9229
double c2-like domains, alpha	DOC2A	16p11.2	8448
drebrin 1	DBN1	5q35.3	1627
dystrobrevin, alpha	DTNA	18q12	1837
gaba(a) receptor-associated protein like 1	GABARAPL1	12p13.2	23710
gamma-aminobutyric acid (gaba) a receptor, alpha 1	GABRA1	5q34-q35	2554
gamma-aminobutyric acid (gaba) a receptor, alpha 4	GABRA4	4p12	2557
gamma-aminobutyric acid (gaba) a receptor, alpha 5	GABRA5	15q11.2-q12	2558
gamma-aminobutyric acid (gaba) a receptor, beta 1	GABRB1	4p12	2560
gamma-aminobutyric acid (gaba) a receptor, delta	GABRD	1p|1p36.3	2563
gamma-aminobutyric acid (GABA) A receptor, gamma 1	GABRG1	4p12	2565
gamma-aminobutyric acid (gaba) a receptor, gamma 1	GRIA2	4q32-q33	2891
gamma-aminobutyric acid (gaba) a receptor, gamma 2	GABRG2	5q31.1-q33.1	2566
gamma-aminobutyric acid (gaba) b receptor, 2	GABBR2	9q22.1-q22.3	9568
gamma-aminobutyric acid (gaba) receptor, rho 1	GABRR1	6q14-q21|6q13-q16.3	2569
glutamate dehydrogenase 1	GLUD1	10q23.3	2746
glutamate receptor, ionotrophic, ampa 3	GRIA3	Xq25-q26	2892
glutamate receptor, ionotropic, ampa 1	GRIA1	5q33|5q31.1	2890
glutamate receptor, ionotropic, ampa 2	GRIA2	4q32-q33	2891
glutamate receptor, ionotropic, kainate 1	GRIK1	21q22.11	2897
glutamate receptor, ionotropic, n-methyl d-aspartate 2a	GRIN2A	16p13.2	2903
glutamate receptor, metabotropic 3	GRM3	7q21.1-q21.2	2913
glutamate-ammonia ligase (glutamine synthetase)	GLUL	1q31	2752
gtp cyclohydrolase 1 (dopa-responsive dystonia)	GCH1	14q22.1-q22.2	2643
mitogen-activated protein kinase 1	MAPK1	22q11.2|22q11.21	5594
myelin basic protein	MBP	18q23	4155
myelin oligodendrocyte glycoprotein	MOG	6p22.1	4340
nad(p)h dehydrogenase, quinone 1	NQO1	16q22.1	1728
neuronal pentraxin ii	NPTX2	7q21.3-q22.1	4885
neuropeptide y	NPY	7p15.1	4852
pallidin homolog (mouse)	PLDN	15q21.1	26258
peripheral myelin protein 22	PMP22	17p12-p11.2	5376
phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	PIK4CA	22q11.21	5297
piccolo (presynaptic cytomatrix protein)	PCLO	7q11.23-q21.3	27445
potassium large conductance calcium-activated channel, subfamily m, beta member 4	KCNMB4	12q	27345
potassium voltage-gated channel, kqt-like subfamily, member 2	KCNQ2	20q13.3	3785
rab14, member ras oncogene family	RAB14	9q32-q34.11	51552
s100 calcium binding protein, beta (neural)	S100B	21q22.3	6285
sodium channel, voltage-gated, type x, alpha	SCN10A	3p22-p21	6336
solute carrier family 1 (glial high affinity glutamate transporter), member 2	SLC1A2	11p13-p12	6506
solute carrier family 1 (glial high affinity glutamate transporter), member 3	SLC1A3	5p13	6507
solute carrier family 6 (neurotransmitter transporter, creatine), member 8	SLC6A8	Xq28	6535
solute carrier family 6 (neurotransmitter transporter, gaba), member 1	SLC6A1	3p25-p24	6529
synapsin ii	SYN2	3p25	6854
synaptophysin-like 1	SYPL1	7q22.2	6856
synaptosomal-associated protein, 25kda	SNAP25	20p12-p11.2	6616
synaptotagmin i	SYT1	12cen-q21	6857
synaptotagmin v	SYT5	19q|11p	6861
syntaphilin	SNPH	20p13	9751

Figure 3: Clustered image map (CIM) of the hierarchical cluster analysis of the GABAergic differentially expressed genes across the 17 regions investigated. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression.

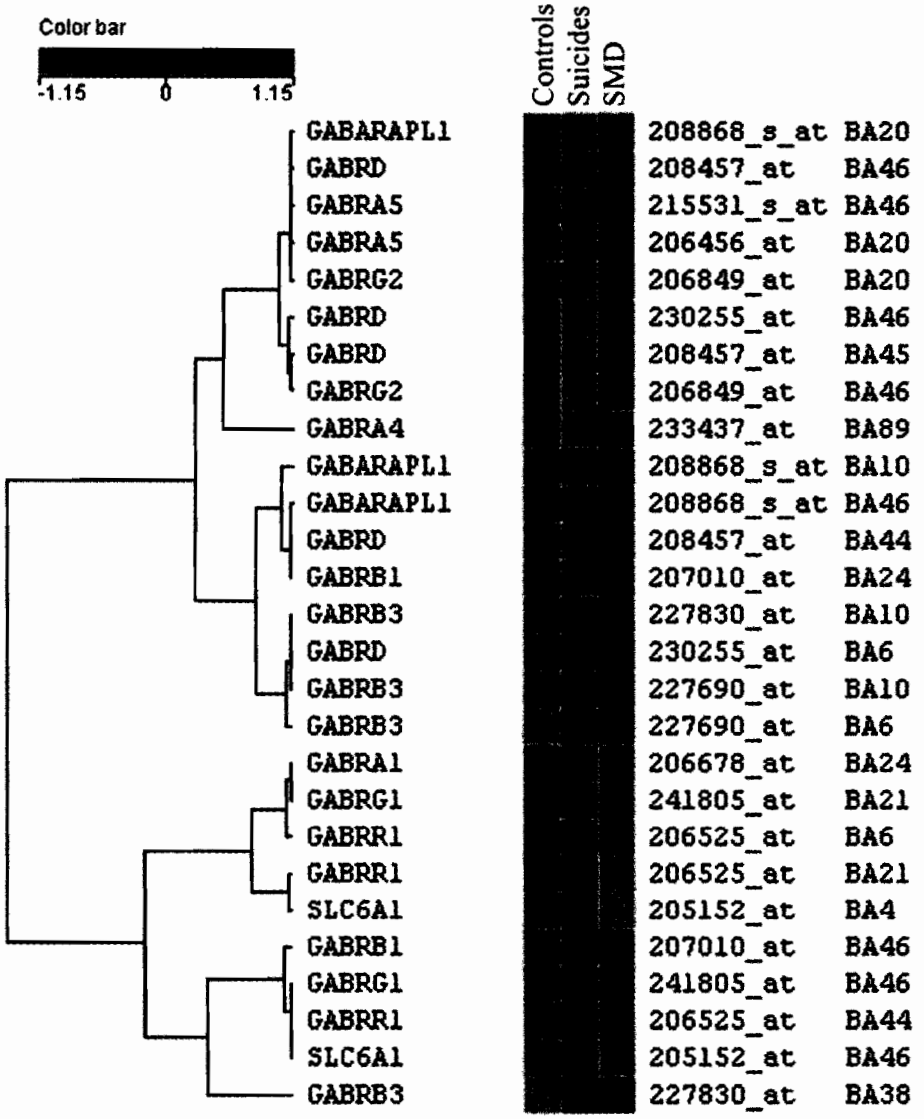

Figure 4: Clustered image map (CIM) of the hierarchical cluster analysis of the glutamatergic system differentially expressed genes across the 17 regions investigated. The color and intensity indicate direction and level of change: blue spectrum colors indicate down-regulated expression, while red spectrum colors indicate up-regulated expression.

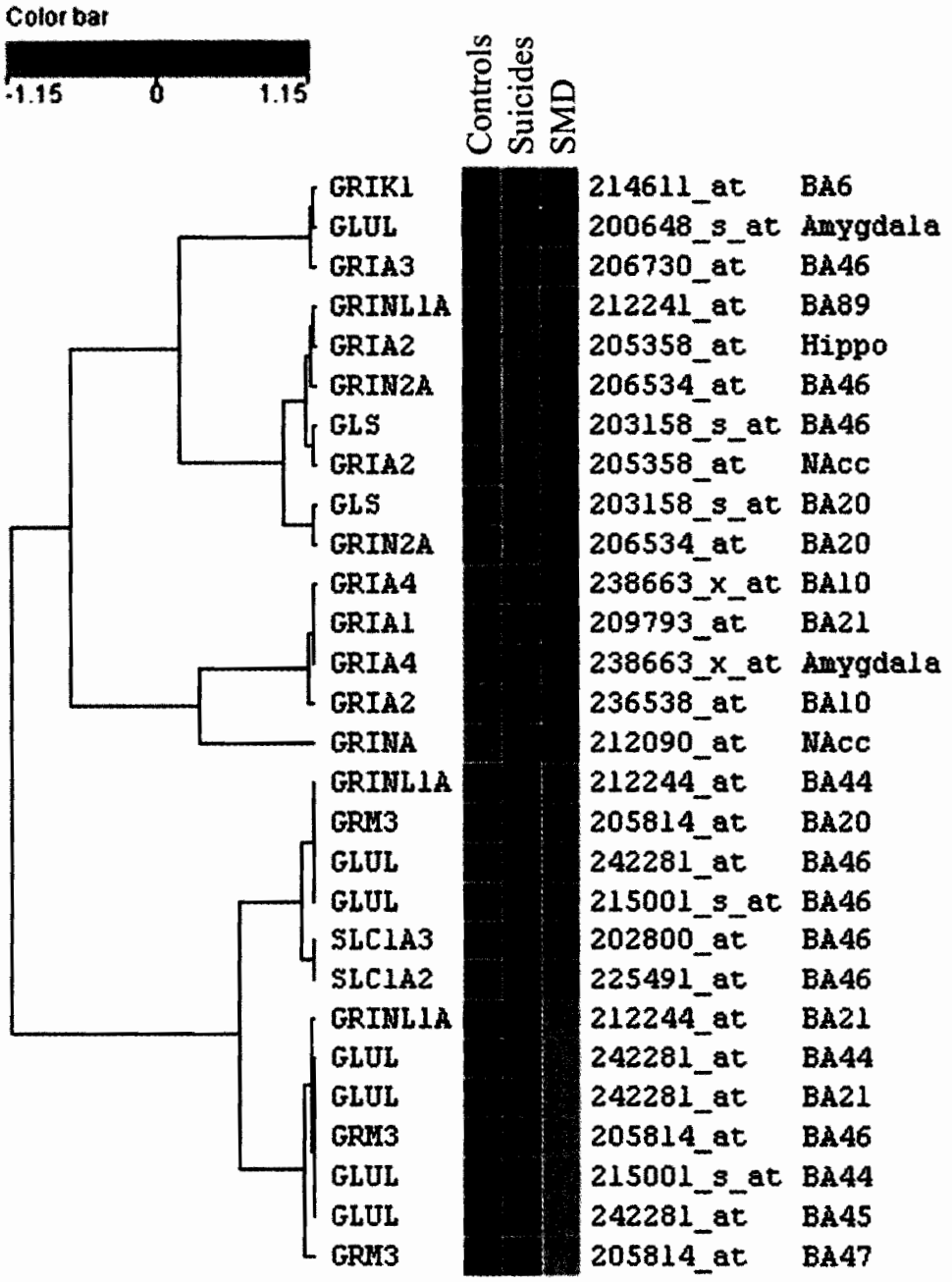

Table 5: Confirmation of the microarray results in independent samples from the same subjects/areas by semi-quantitative RT-PCR. All genes showed similar changes in terms of direction to that observed in the microarray. In bold are those genes with statistical significant differences also in the semi-quantitative RT-PCR experiments.

		SemiQ RT-PCR				Affymetrix			
Gene	Region	Control	Suicide	SMD	*P*	Control	Suicide	SMD	*P*
GABARAPL1	BA46	135.35	102.88	171.73	0.13	197.566	181.244	261.768	0.002
GABARD	**BA45**	**70.96**	**49.53**	**69.86**	**0.04**	**265.784**	**190.645**	**273.365**	**0.002**
GABARD	**BA46**	**196.79**	**92.43**	**109.35**	**0.04**	**405.741**	**300.856**	**443.493**	**0.003**
GABARG1	BA21	64.17	85.35	61.89	0.13	262.739	215.787	170.116	0.002
GABARG1	**BA46**	**35.92**	**46.04**	**31.30**	**0.02**	**120.571**	**174.447**	**107.812**	**0.000**
GABRG2	**BA46**	**52.95**	**36.15**	**48.91**	**0.04**	**416.956**	**267.694**	**418.698**	**0.007**
GABRR1	**BA44**	**195.05**	**376.23**	**138.60**	**0.01**	**23.6967**	**35.345**	**19.1808**	**0.007**
GLS	BA46	56.56	48.36	56.43	0.12	231.561	163.459	243.658	0.002
GLUL	**Amy**	**93.46**	**76.52**	**68.49**	**0.02**	**789.678**	**438.535**	**422.579**	**0.002**
GLUL	BA21	131.67	118.04	107.04	0.08	463.524	442.932	265.696	0.002
GLUL	**BA45**	**142.64**	**128.99**	**108.66**	**0.02**	**338.71**	**307.803**	**197.303**	**0.002**
GLUL	**BA46**	**128.30**	**184.69**	**87.91**	**0.01**	**251.503**	**361.113**	**190.146**	**0.001**
GRIA1	**BA21**	**71.30**	**67.63**	**142.03**	**0.04**	**118.831**	**150.885**	**180.902**	**0.004**
GRIA3	**BA46**	**82.77**	**48.63**	**51.32**	**0.05**	**132.681**	**93.5443**	**100.471**	**0.006**
GRM3	**BA46**	**70.75**	**52.57**	**38.63**	**0.02**	**286.41**	**281.21**	**241.308**	**0.004**
SLC6A1	BA4	150.61	156.09	140.69	0.58	648.13	648.05	478.61	0.002

CHAPTER 8

DISCUSSION

6 DISCUSSION

In this project the molecular basis of suicide was explored using two approaches. First, the hypothesis-driven association study design using a candidate gene approach which takes advantage of the knowledge accumulated about the physiopathology of this complex trait and the second a more exploratory approach aiming to find new molecular targets implicated in suicide: genome-wide gene expression analysis. The French-Canadian population from Quebec studied is a genetically homogenous population with a known and well documented founder effect (Scriver, 2001). The low genetic background of this population makes it an ideal candidate for case-control association studies in which population stratification can sometimes lead to spurious positive associations (Pritchard et al., 2000). The initial population of about 60 000 people has now grown to a population of around 6 million people with low migratory influence, making this population suitable for the study of complex multigenic traits. The fact that this population not only shares a common socio-economic and cultural background, but also a homogenous genetic background increases the power of detecting genetic determinants for suicide especially when using case-control candidate gene association studies, but also gene expression analysis. The reason being that by investigating subjects from this young (approximately 12 generations) and isolated population it is possible that we may have reduced, in our study, total variability in gene expression patterns.

In light of our review of the literature in chapter 2, other possible confounding factors for gene expression can be identified. First gender differences in gene expression are important and can dampen expression changes associated with any studied phenotype. Second, other possible confounding factors such as the quality of RNA can now effectively be measured to detect any damage to RNA due to long post-mortem interval or prolonged agonal state. Also with the numerous microarray platforms available nowadays and the increased accessibility to the technology, bigger samples can now be analyzed in order to overcome the effect of confounding variables. Thus, when factors affecting the quality of the data are experimentally or statistically addressed, microarrays will likely be of great help in the identification of new molecular targets or new functional pathways implicated in mood disorders.

ASSOCIATION STUDIES

In chapters 3 and 4, we assessed the influence of genetic variation of possible, new target genes on susceptibility to suicide. Wolfram and alpha 2A genes are two very interesting candidates given their possible modulation of psychiatric disorders accompanied with suicidal behaviours in the case of Wolfram and the negative feedback exerted on serotonergic and adrenergic firing rates in the case of the alpha 2A adrenergic receptor. Both studies were conducted on a relatively large sample of fully characterized suicides and normal controls and with the

advantage of being from a genetically homogenous population decreasing the chances of erroneous results due to population stratification. In the case of the Wolfram, a significant association was found between the 611R non-synonymous polymorphism and suicide and we also found evidence for a possible mechanism for this association, as carriers of the R allele had higher levels of impulsivity, suggesting a role for this gene in the modulation of impulsivity in suicide. Other studies failed to find a significant association between genetic variants of this gene and suicide in an Australian sample (Crawford et al., 2002) and with psychiatric disorders in a Spanish sample (Martorell et al., 2003). However, their samples were relatively small and from different populations and they didn't have personality measures.

For the alpha2A adrenergic receptor gene, even though no significant association was found, the rare functional variant N251K was only found among the suicide victims suggesting a possible role in at least a proportion of suicide cases. This possibility has to be further explored in a significantly larger sample. Another group failed to replicate our finding in a slightly larger sample from Spain as they did not observe the rare 251K variant in their sample.

GENE EXPRESSION STUDIES

These association studies were very useful in making us realize the limitations of candidate gene approaches where one is investigating the implication of one gene

at the time in a complex multigenic trait such as suicide. Even though the Wolfram and alpha2A genes were very interesting candidates, each implicated gene is expected to have a marginal influence on the final outcome, suicide. Previous work on the neurobiology of suicide has demonstrated a plethora of neurotransmitter systems implicated in suicide and also underlined the importance of a more comprehensive approach involving a multiplicity of genes (Gross-Isseroff et al., 1998). With the completion of the human genome sequence (Venter et al., 2001;Lander et al., 2001) and the development of high throughput expression screening technologies (Schena et al., 1995;Lockhart et al., 1996), whole genome strategies have become accessible for the study of complex multigenic traits. In this light, research for new target genes implicated psychiatric disorders has shifted recently from candidate gene approaches to whole genome approaches such as genome scans, genome-wide association studies and global gene expression screening by means of microarrays.

We carried the gene expression projects using a design that allowed us to control for the presence of suicide (suicides with major depression vs. suicides without major depression) and therefore, to identify processes that may be more likely attributed to major depression. Meanwhile, the identification of processes exclusively attributed to suicide can be estimated by comparing suicides without major depression against controls. Having a comparison group with major depression that did not die by suicide would have allowed us to fully separate effects attributed to suicide from those of major depression. However, for such a

group to be comparable, we would have needed to include subjects who were affected with major depression prior to death, or at least in the six months prior to death. Collecting a control group that would be demographically comparable with the suicide group, which is a relatively young sample, and that died while affected with major depression is operationally very challenging and could take a very long time. Studies investigating the molecular changes in suicide and major depression have included a group of major depression "controls" who died of causes other than suicide. However, these subjects typically have a lifetime history of depression and not necessarily depression at the time of death, not too mention that they tend to have prolonged agonal periods and agonal complications. It is unclear the level of impact that an episode of depression that may have occurred several year earlier could have on current gene expression patterns. Since the impact a depressive episode occurring several years prior to death could have on current gene expression patterns is unclear it would be wise to not include such a group, so as not to introduce any confounding factors.

Making sense of the massive amount of data generated by genome wide approaches such as microarrays is particularly challenging. This issue was intensified in this project by looking at multiple brain regions of the same subjects. We used gene ontology information (Ashburner et al., 2000) to explore the overall functional profile of the genes associated with our phenotype. Many ontological tools proposed in the recent years were explored and used (www.geneontology.org/go.tools.shtml), the majority of these tools presenting

some computational biases and intrinsic drawbacks (Khatri and Draghici, 2005). However, new tools for functional ontological profiling such as ErmineJ (Lee et al., 2005) or GSEA (Kim and Volsky, 2005) introduced in chapter 2, utilize an approach more suitable to find processes and pathways distributed across the transcriptome, while allowing for subtle changes at the level of individual genes. For the global analysis in chapter 7 for instance, ErmineJ was crucial in the identification of molecular systems implicated in suicide and depression.

In Chapter 5 we conducted a gene expression study using the HG-U133AB chipset containing around 44 000 probe sets and covering virtually all the genome in cortical areas of male sample of non depressed and depressed suicides, as well as controls. Using this approach we identified SSAT (spermine/spermidine N1-acetyltransferase gene), a gene involved in polyamine metabolism, as being down regulated in three cortical brain areas (BA4, BA8,9 and BA11) of suicides with and without major depression (Sequeira et al., 2006). These results were validated and further explored using RT-PCR, immunohistochemistry and Western-Blots in adjacent samples. SSAT being involved in the catabolism of polyamines, these findings suggest that polyamines play a role in the neurobiology of suicide and depression. Additionally, a variant located in the SSAT polyamine responsive element regulatory region (SSAT342A/C) revealed a significant effect of genotype on SSAT brain expression levels and further investigation of this variant in an independent sample of male suicides and controls showed a higher frequency of the SSAT342C allele among suicide cases suggesting that this allele

may increase predisposition to major depression and suicide. In that sense, using an animal model of depression, Zomkowski et al. showed that the polyamine putrescine has an antidepressant effect supporting the idea that alterations of the polyamine system are involved in depression (Zomkowski et al., 2006).

Confirmation and follow up of the expression changes included RT-PCR, Western blots and immunohistochemistry. RT-PCR assays were carried out on RNA extracted from new samples from the same subjects obtained from adjacent tissue to that used for the original microarray experiments. This is different from what is usually done in most microarray studies, which use the same RNA sample. By doing our procedure, we are not only validating the microarray result, but also the intra-subject reproducibility. Validation at the protein level is also important as changes at the mRNA level not always lead to altered protein levels. However, in the case of SSAT the validation of successful but at the mRNA and at the protein level using two methods.

In order to continue the investigation of expression alterations in the suicide brain, in chapter 6 we conducted a gene expression investigation in another region often associated with the control of emotions, the limbic system. Changes observed in the cingulated areas studied (BA24 and BA29) were relatively small whereas changes in the amygdala and particularly in hippocampus, shown to be affected in depressed patients, were more important. In the hippocampus, gene ontology analysis revealed an alteration among depressed suicides of processes related to

transcription and cellular metabolism. Overall, these data suggest that specific patterns of expression in the limbic system could contribute to the etiology of depression and suicidal behaviors and highlight the role of the hippocampus in major depression.

In chapter 7, we explored expression patterns associated with suicide and depression in suicides in a global comprehensive way by integrating the data from 17 cortical (Brodman areas (BA) 4, 6, 8-9, 10, 11, 20, 21, 38, 44, 45, 46, 47) and subcortical (BA24, BA29, amygdala, hippocampus, Nucleus Accumbens) brain regions in order to find functional themes altered. As expected from previous functional imaging studies, in this global study the highest number of suicide-specific alterations was observed in prefrontal cortical areas and hippocampus in agreement with previous observations implicating those regions in suicide and depression. We used an approach in this study slightly different as we proceed to find not genes but themes or pathways that were altered not in one region globally across the regions investigated. This global ontological profiling revealed common functions such as synaptic neurotransmission and intracellular signalling and lead us to the observation that Glutamatergic and GABAergic related genes were globally altered and more particularly in prefrontal cortical areas. Some of these results were validated using RT-PCR, confirming the implication of these systems. This result constitute the first overview of global molecular changes in the brain of suicide victims and suggests an alteration of genes implicated

synaptic neurotransmission and more specifically of genes involved in GABAergic and Glutamatergic neurotransmission in suicide and depression.

Other evidences point also to alterations of GABAergic and glutamatergic neurotransmission such as the observation by Sanacora et al. (Sanacora et al., 2004) of elevated levels of glutamate and lower levels of GABA in the occipital cortex of subjects diagnosed with major depression. Additionally, findings from Choudary et al. (Choudary et al., 2005), who performed a gene expression studies in cingulate and prefrontal cortex brain areas of suicides and depressed suicides, confirm our observations as they point to similar alterations in glutamate recycling (glutamine synthase), glutamate receptors (GRIA1, GRIA3, GRIK1, GRM3) and GABA receptors (GABARB3, GABRD, GABARG2). Also, another group observed recently altered mRNA levels of GABA(A) receptor subunits (α1, α3, α4 and δ) in the BA10 of depressed suicide victims versus non-depressed controls (Merali et al., 2004) adding more evidences for a role of GABA in the depressed suicide phenotype. Finally, evidence supporting this finding came also from a recent clinical trial using a NMDA antagonist for the treatment of major depression patients that were treatment resistant to other types of pharmacotherapy (Zarate, Jr. et al., 2006). The authors observed an impressive and rapid (within hours) clinical response underlying the importance of the GABAergic system in the physiopathology of major depression.

Overall, while candidate gene association studies are of limited power for the study of complex multigenic phenotypes such as suicide, gene expression studies can be really helpful in finding molecular correlates to phenotypic manifestations of psychiatric disorders and suicide. This is with the condition of properly controlling for confounding factors experimentally or statistically. Validation at the mRNA and protein level is also essential and further insight can then be obtained about the causes of the expression changes by performing genetic of gene expression analysis in order to find genetic variants responsible for the observed changes.

In conclusion, the genetic association studies performed helped us exploring the involvement of candidate genes in suicide and lead us to the identification of a polymorphism in the Wolfram gene possibly conferring susceptibility to suicide. Also, using microarrays to investigate genome wide changes in gene expression lead us to the discovery of a new molecular target for depression and suicide (SSAT), and provided evidence of the implication of the GABAergic and glutamatergic systems in physiopathology of suicide and depression.

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APPENDICES

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April 11, 2006

Dr. Gustavo Turecki
Douglas Hospital
6875 LaSalle Boulevard
Verdun, Quebec
H4H 1R3

Dear Dr. Turecki:

We are writing in response to your request for continuing review for the study A04-M09-00 entitled "Behavioral and Biological Risk Factors for Suicide Completion in Major Depression".

The progress report was reviewed and we are pleased to inform you that full Board re-approval for the study was provided on **April 10, 2006**, valid until **April 9, 2007**. The certification of annual review has been enclosed.

We ask you to take note of the investigator's responsibility to assure that the current protocol and consent document are deposited on an annual basis with the Research Ethics Board of each hospital where patient enrollment or data collection is conducted.

Should any modification or unanticipated development occur prior to the next review, please advise the IRB promptly.

Yours sincerely,

P. Celeste Johnston

Celeste Johnston, DEd, RN
Co-chair
Institutional Review Board

cc: A04-M09-00
Mr. S. Levy – MUHC/MGH
Ms. L. Bourgon, DHC

Hôpital Douglas Hospital

Ouverts d'esprit. Depuis ... ans.
Caring with Open Minds. For ... Years.

August 4th, 2006

Dr. Gustavo Turecki
Douglas Hospital Research Centre
FBC Pavilion

Subject: **Protocol 04/15** *Predisposition to Suicide: A Brain Expression Study*
Annual Renewal

Dear Dr. Turecki;

Thank you for the annual report you submitted for approval for the above protocol. As Chairperson, I have examined your report and found it satisfactory. I therefore give expedited approval to this annual renewal request since it is complete and it meets REB requirements.

This study is re-approved for a one-year period.

Sincerely yours,

for:
Serge Gauthier, M.D., F.R.C.P(c)
Chairperson
Douglas Hospital Research Ethics Board
/lb

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Hôpital Douglas Hospital

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May 3rd, 2006

Dr. Gustavo Turecki
Douglas Hospital Research Centre
FBC pavilion

Subject: **Protocol entitled:** *A04-B09-00 Entitled: Behavioural and Biological Risk Factors for Suicide Completion in Major Depression*
Annual Renewal by McGill IRB

Dear Dr. Turecki;

Thank you for informing the REB that there have been no changes to the protocol and consent forms for the above study.

As Chairperson, I therefore acknowledge the decision from the IRB to re-approve this protocol for a one-year period.

Sincerely yours,

for:
Serge Gauthier, M.D., F.R.C.P(c)
Chairperson
Douglas Hospital Research Ethics Board
/lb