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BDNF GENE METHYLATION IN BRAIN OF SUICIDE SUBJECTS

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Introduction

Overview of Epigenetics

The word "epigenetic" has been used historically to refer to a heritable phenotype not coded by a change in DNA sequence. Cellular differentiation is a classic example in which epigenetic mechanisms play a critical role. Because all cells in an organism contain the same DNA, the ability to form distinct cell types with unique functions is achieved by the selective activation and silencing of individual genes across the genome. This epigenetic process is coordinated in large part through control of chromatin structure. Increasing evidence now indicates that changes in chromatin structure not only mediate these heritable epigenetic phenomena but also that the same types of changes in chromatin occur also in mature, postmitotic cells where they contribute to health and diseases (Nestrel 2009).

Related concepts are epialleles which refer to epigenetic variants of a genetic allele and epigenetic polymorphisms which have been described as variations of epigenetic patterns across individuals. For example, DNA methylation polymorphisms involve variations of DNA methylation across the genome. The concepts of the molecular aspects of epigenetics are evolving rapidly. Three molecular mechanisms, which interact with each other, have been shown to be involved. One is methylation of DNA which usually, but not always, depending on the position of the methylation change relative to the position of the transcription start site, inhibits gene transcription (Jone *et al*., 2001) DNA gets methylated at sites where there is cytosine by the enzymatic addition of a methyl group, using S-adenosylmethionine as the methyl group source, to the carbon-5 position of cytosine. The majority of 5'-methylcytosine in mammalian DNA is present in 5'-CpG-3' dinucleotides. Non-CpG sequences may also exhibit methylation, but generally at a much lower frequency (Castello *et al*., 2001). The methylation of CpG sites within the human genome is catalyzed by four well-

documented DNA methyltransferases (DNMTs) named DNMT1, DNMT2, DNMT3A and DNMT3B (Brueckner *et al.,* 2004). DNA methylation has many functions like silencing of transposable elements, defense against viral sequences and transcriptional repression of certain genes. Eukaryotic DNA is intimately associated with proteins to form a highly ordered and condensed DNA: protein complex called chromatin. The most abundant proteins in chromatin are a family of small basic proteins called histones. Histones can be modified by different ways like acetylation, methylation, and phosphorylation of the histone tail domains. This alters chromatin structure, leading to regulation of gene transcription. Histone modifications lead to dynamic transitions between gene activation and silencing. These "on-off" transcription states are due to differences in histone modification leading to the formation of accessable, euchromatic (on) or condensed, heterochromatic (off) states (Jenuwein *et al.,* 2001). A third epigenetic mechanism in gene expression has been described more recently: small RNAs derived from cleavage of double-stranded RNA can silente genes at transcriptional and post-transcriptional levels.Small RNAs are 21 to 28 nucleotides long and include micro RNAs (miRNAs) and small interfering RNA(siRNAs). RNA-mediated gene silencing is important in maintaining chromosomal structure,genome defense and gene regulation (Almeida *et al.,* 2005).

Environment, epigenetics and human behaviour.

Common diseases involve interactions between genes and environment and epigenetic mechanisms are thought to be an interface between genes and the environment in psychiatric disorders (Bjornsson *et al.,* 2004). The environmental factors may impinge on epigenetic mechanisms in gene expression by many ways. Thus, dietary levels of methionine, the metabolic precursor of S-adenosylmethionine during prenatal and early postnatal life, influences susceptibility to chronic adult diseases because dietary methionine influences

DNA methylation. Environmental factors also modulate proteins like enzymes associated with DNA methylation and histone modification. Other proteins like polycomb proteins (proteins that maintain many genes important for development in a repressed state) also mediate the epigenetic effects of the environment on gene expression (Cao *et al.,*2002).

Psychosocial factors have been described as important environmental factors involved in the pathogenesis of the idiopathic mental disorders and many experimental evidence from studies on animals that psychological factors can modify behaviour by epigenetic mechanisms. For example, it has been shown in cross-fostering studies in the rat, that non-genomic transmission of maternal behaviour and stress responses occur from one generation to the next (Francis *et al.,* 1999). In two strains of inbred mice it was shown that strain-related behavioral differences may result from environmental factors acting during prenatal and postnatal development, rather than from genetic differences in the offspring. In a study conducted on pup licking and grooming by rat mothers (Weaver *et al.,* 2004), increased frequency of these maternal behaviours increased the number of hippocampal glucocorticoid receptors in the pups, causing better regulation of glucocorticoid secretion.

The increased glucocorticoid receptors in the pups were due to changes in DNA methylation and histone acetylation associated with the glucocorticoid receptor gene promoter. These differences emerged over the first week of life, were reversed with cross-fostering, and persisted into adulthood.

Very interestingly, hypermethylation of glucocorticoid receptor was also observed in human brain in subjects that had received childhood abuse (McGowan *et al.,* 2009.) , thus translating in humans what previously observed in rat.

Other clear evidences demonstrating that environment exposure is able to drive methylation changes in humans come from the milestones works addressing the phenotipic differences in monozygotic (MZ) twins.

Recent studies showed that although twins are epigenetically indistinguishable during the early years of life, older monozygous twins exhibited remarkable

differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation, affecting their gene-expression portrait. These findings indicate how an appreciation of epigenetics in missing from our understanding of how different phenotypes can be originated from the same genotype.

It is also worth to note that monozygotic twins sharing the same experiences show much more similarities in epigenetic patterns during the life with respect of MZ twins that have lived apart (Fraga *et al., 2005*).

Epigenetics of neuropsychiatric disorders

Pathophysiological changes in the brain that are associated with psychiatric disorders or animal models of these conditions include gross differences in the sizes of specific brain regions, alterations in the morphology of subpopulations of neurons, neurochemical changes at the synaptic cleft, alterations in intracellular signalling and changes in the regulation of gene expression. Most psychiatric disorders share important features, including a substantial genetic predisposition and a contribution from environmental factors (Kendler *et al.,* 2001). Another common attribute of psychiatric conditions is long-lasting behavioural abnormalities. An important challenge in psychiatric research has been to identify the molecular basis of stable changes in behaviour that account for both the symptoms of mental illness and their reversal during treatment. The regulation of gene expression has been proposed as one molecular mechanism that could mediate stable adaptations and maladaptations in the brain (Hyman *et al.,* 1993). Changes in mRNA levels have been documented in specific brain regions both in animal models of psychiatric illness and in human brains, and have been related to altered behaviour. However, it has been difficult to identify the molecular mechanisms that underlie such stable changes in gene expression; virtually all reported changes in transcription factors and other nuclear regulatory proteins in animal models revert to normal within hours or days of chronic perturbation.

Recent research has raised the notion that epigenetic mechanisms, which exert lasting control over gene expression without altering the genetic code, could mediate stable changes in brain function. Historically, the field of epigenetics has focused on how cellular traits can be inherited without a change in DNA sequence. Studies of epigenetic mechanisms that underlie heritable transmission have flourished in the fields of developmental and cancer biology, where the continuity of unique patterns of gene expression between parent and daughter cells is crucial. These studies have converged on a set of common enzymatic modifications to chromatin structure that can up or down regulate gene expression in a manner that is transmissible to daughter cells. These mechanisms also regulate gene expression in neurons, but, as most neurons do not divide, chromatin modifications are instead sustained within individual cells.

Recent discoveries show that the epigenetic regulation of neurobiological adaptations are associated with long-lasting behaviours in animal models of psychiatric conditions and in the brains of humans with these disorders. There are plenty of recent evidences indicating that altered methylation is present in brains of schizophrenic or bipolar patient. Some genes have been found aberrantly hypermethylated and underexpressed in schizophrenic or bipolar subjects compared to normal control. These genes are very important for the main pathways regulating neuronal plasticity and emotional responses such dopaminergic, serotoninergic and gabaergic systems.

Many work have focused on epigenetic alterations at the reelin promoter gene. Post-mortem studies of patients with schizophrenia reveal significant down-regulation of reelin expression in several brain regions that is not associated with neuronal loss. The reelin promoter contains a large CpG island, indicating that DNA methylation might be important for regulating its exspression. Furthermore, in recent postemortem studies on brain from person diagnosed with schizopherenia revealed that expression of glutamic acid decarboxylase 67 (GAD67), an enzyme critical for synthesis of the inhibitory neurotransmitter GABA, is significantly reduced in interneurons. Animal models of reelin haploinsufficiency exhibit reduced GABAergic inhibitory tone and a deficit in

sensorimotor gating, hallmarks of schizophrenia (Tueting *et al.,* 1999; Qui *et al .,* 2006). As a whole, these results suggest that an aberrant decrease in reelin and GAD67 gene expression could contribute to the pathogenesis of schizophrenia (Jonathan 2006).

Moreover DNA methylation defects have been linked to members of a large group of human neuropsychiatric diseases that are associated with triplets instability. Recently, an aberrant CpG methylation has been identified in the first intron of FXN gene, upstream from the triplet expansion, in patients with Friedrereich Ataxia. In particular, our group found hypermethylation of the region in four FRDA patients compared with four healthy subjects. In this study we found a strong correlation between CpG methylation degree of specific CpG sites with both the size of the triplet expansion and the age of onset (Castaldo *et al.,* 2008).

May depression have an epigenetic origin?

The potential role of epigenetic alteration as cause of depression has been studied both in animal model and in humans.

Chronic social defeat stress, an animal model of depression that mimics many kind of human depression, also alters chromatin regulation of BDNF gene. Prolonged exposure to an aggressor induces lasting changes in mouse behaviour such as social avoidance, which are reversed by cronic treatment with antidepressants. At a molecular level, chronic dafeat stress in mice induce substained downregulation of the expression of two variants of BDNF, BDNF III and BDNF IV, in the hippocampus. These changes are reversed only after treatment with the antipressant imipramine. Chronic defeat stress induces robust and long-lasting increases in H3-K27 dimethylation, a repressive modification, specifically at the promoters of the downregulated BDNF trascripts.

In humans, mood disorders are among the most prevalent forms of mental illness. Severe forms of depression affect 2%–5% of the U.S. population, and up to 20% of the population suffer from milder forms of the illness. Depressionis almost twice as common in females than males. Another roughly 1%–2% are afflicted by

bipolar disdisorder (also known as manic-depressive illness),which affects females and males equally. Mood disorders are recurrent, life threatening (due to the risk for suicide), and a major cause of morbidity worldwide (Blazer *et al.,*2000). Depression has been described by mankind for several millenia. Most of the major symptoms of depression observed today were recognized in ancient times, as were the contributions of innate predispositions and external factors in causing the illness.The accients also recognized a large overlap of depression with anxiety and excessive alcohol consumption, both of which are well established today. Indeed, similarities between ancient descriptions of depression and those of the modern era are striking, yet it wasn't until the middle part of the 19 th century that the brain became the focus of efforts understand the pathophysiology of this disorder.

Epidemiologic studies show that roughly 40%–50% of the risk for depression is genetic (Sanders *et al.,*1999; Fava and Kendler, 2000). This makes depression a highly heritable disorder, at least as heritable as several mon complex medical conditions (type II diabetes, hypertension,asthma, certain cancers), which are often thought of as genetic. Yet, the search for specific genes that confer this risk has been frustrating, with no genetic abnormality being identified to date with certainty. The difficulty in finding depression vulnerability genes parallels the difficulty in finding genes for other psychiatric disorders and, in fact, for most common complex disdisordereases. There are many reasons for this difficulty, whichare reviewed elsewhere (Burmeister *et al.,*1999), including the fact that depression is a complex phenomenon with many genes possibly involved. Thus, any single gene might produce a relatively small effect and would therefore be difficult to detect experimentally. It is also possible that variants in different genes may contribute to depression in each family, which further complicates the search for depression genes.In addition, vulnerability to depression is only partly genetic, with no genetic factors also being important. Nongenetic factors as diverse as stress and emotional trauma, viral infections, and even stocastic (or random) processes during brain development have been implicated in the etiology of depression (Akibetweenskal *et al.,*2000; Fava and Kendler, 2000). Depressive

syndromes, indistinguishable from major depression defined by DSMIV and by their response to standard antidepressant treatments, occur in the context of innumerable medical conditions such as endocrine disturbances (hyper- or hypocortisolemia, hyper- or hypothyroidism), collagen vascular diseases, Parkinson's disease, traumatic head injury, certain cancers, asthma, diabetes,and stroke.The role of stress warrants particular comment. Depression is often described as a stress-related disorder, and there is good evidence that episodes of depression often occur in the context of some form of stress. However, stress per se is not sufficient to cause depression. Most people do not become depressed after serious stressful experiences, whereas many who do become depressed so after stresses that for most people are quite mild. Conversely, severe, horrendous stress, such as that experienced during combat, rape, or physical antidepresabuse, does not typically induce depression, but instead causes post-traumatic stress disorder (PTSD) that is distinct from depression based on symptomatology, treatment, and longitudinal course of illness. This underscores the view that depression in most people is caused by interactions between a genetic predisposition and some environmental factors, which makes the mechanisms of such interactions an important focus of investigation (Nestler *et al.,* 2002).

Suicide

Depression is associated with an increased number of suicide attempts and increased lethality (Bostwick *et al.,* 2000; Soloff *et al.,* 2000). Suicide accounts for almost 2% of the world's deaths. In most of the developed world, suicide is among the top 10 leading causes of death for individuals of all ages, and is the third leading cause of death among adolescents, after motor vehicle accidents and homicide (Mao *et al.,*1990; Mann *et al.,* 2002). Several arguments suggest that suicidal behavior is a disorder of its own, although psychiatric disturbances, including depression, are major contributing factors. Autopsy studies of suicide victims have identified a high rate of major depressive disorder (MDD) as one of

the main causes of increased mortality among suicide victims. The presence of psychopathology is a strong predictor; however, only a minority of people with such diagnoses commits suicide, which indicates that there is a certain predisposition to suicide that is independent of the main psychiatric disorders (Mann *et al.,*;1998; Turecki *et al.,* 2005). Despite the devastating impact of depression and suicide on numerous lives, there is still a dearth of knowledge concerning the mechanisms underlying their pathogenesis. Overwhelming evidence points to altered synaptic and structural plasticity in patients with depression and in suicidal patients. In fact, it has been proposed that depression/suicide results from an inability of the brain to make appropriate adaptive responses to environmental stimuli as a result of impaired synaptic plasticity and structural plasticity. Support for this comes from a variety of studies in major depressed/suicidal subjects demonstrating altered brain structure, such as reduction in cell number, density, cell body size, neuronal and glial density in frontal cortical or hippocampal brain areas and decrease in parahippocampal cortex cortical/laminar thickness (Cotter *et al.,*2001; Cotter *et al.,* 2002; Rajkowska *et al.,* 2007). Furthermore, depression is associated with negative impact on learning and memory; and stress, a major factor in depression and suicide, hinders performances on hippocampal-dependent memory tasks and impairs induction of hippocampal long-term potentiation. These studies clearly demonstrate impaired structural and functional plasticity in depression and suicide; however, the precise molecular and cellular nature of events that lead to such altered plasticity in these disorders remains unclear. Survival and development of neurons in the central nervous system (CNS) depends on the influence of a variety of extracellular signals. One set of signals is provided by neurotrophins.

The role of neurotrophins in the suicide

Function of neurotrophins and their receptors (TrK) in brain development

The role of neurotrophins in directing brain growth and neuronal functioning is being increasingly recognized. Neurotrophins not only play an important role in cellular proliferation, migration, and phenotypic differentiation and/or maintenance in the developing central nervous system, CNS (Lewin *et al.,* 1996; McAllister *et al.,* 2001), but their presence is required in the adult CNS for maintenance of neuronal functions, structural integrity of neurons, and neurogenesis (Sofroniew *et al.,* 1990; Cooper *et al.,* 1996) which suggests that neurotrophins are biologically significant over the entire life span. In addition, a number of studies have demonstrated that neurotrophic factors regulate structural, synaptic, and morphological plasticity to modulate the strength or number of synaptic connections and neurotransmission (McAllister *et al.,* 1999; Thoenen *et al.,* ;2000). Thus, a pathological alteration of the neurotrophic factor system may not only lead to defects in neural maintenance and regeneration and, therefore, structural abnormalities in the brain, but may also reduce neural plasticity and, therefore, impair the individual's ability to adapt to crisis situations.

Mammalian neurotrophins are homodimeric proteins that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT) 3, and NT 4/5. Most functions of neurotrophins are mediated by the tropomycin receptor kinase (Trk) family of tyrosine kinase receptors. The interaction of neurotrophins with the Trk receptors is specific: NGF binds with TrkA, BDNF and NT 4 both bind with TrkB, and NT 3 binds with the highest affinity to TrkC but is also capable of signaling through TrkA and TrkB. In addition to the full length TrkB receptor, several noncatalytic truncated TrkB isoforms have also been identified; these isoforms lack the signaling domain, preventing the

induction of a signal transduction mechanism. Binding of a neurotrophin to the appropriate Trk receptor leads to the dimerization and transphosphorylation of tyrosine residues in the intracellular domain of the Trk receptors and subsequent activation of cytoplasmic signaling pathways (Chao *et al.,* 2006; Reichardt *et al.,* 2006).

Of various neurotrophins, BDNF has attracted a great deal of interest as a functional candidate gene in various mental disorders. The *Bdnf* gene lies on the reverse strand of chromosome 11p13 and encodes a precursor peptide pro-BDNF(Seidah *et al.,*1996). In fact, all neurotrophins, including BDNF, are synthesized as a pre-pro-neurotrophin precursor that undergo post translational modifications before giving rise to mature homodimeric protein. The pro-BDNF is produced in endoplasmic reticulum, which is accumulated in trans-Golgi network via Golgi apparatus. It has been suggested that pro-BDNF binds to sortilin in the Golgi, which facilitates the correct folding of the mature domain. The mature domain of BDNF binds to carboxypeptidase E, thereby sorting BDNF to the regulated secretary pathway (Lu *et al.,* 2005). A substitution of valine (Val) to methionine (Met) at codon 66 in the prodomain impairs this sorting of BDNF (Egan *et al.,* 2003).

The expression of the *BDNF* gene is tightly regulated by neuronal activity, through mechanisms dependent on calcium (Mellstrom *et al.,* 2004).

In addition to BDNF, the function of a receptor for BDNF is also regulated in an activity-dependent manner. TrkB is primarily localized in the synaptic sites. Further localization of TrkB occurs at the synaptic sites after neuroanal activity. Neuronal activity, therefore, is critical for synthesis and intracellular targeting of TrkB receptors. Thus, BDNF release and expression of TrkB receptors in a coordinated fashion are important for optimal synaptic response (Lu *et al.,* 2005).

Structure of BDNF gene

Previous studies have revealed that the human *BDNF* gene consists of seven putative 5' exons and one protein-coding exon (Liu *et al.,* 2005; Aoyama *et al.,* 2001). However, the expression patterns of different exons have not been thoroughly studied and possible linkage of these exons to separate promoters has not been investigated. On the other side recent studies show that the human *BDNF* gene, extending over 70 kb, contains 11 exons. The 3' exon encodes all or most of the protein depending on the 5' exon used. Independent of the 5' exon usage, two separate polyadenylation signals in exon IX can beutilized in *BDNF* transcripts. In addition, a new data showed that the human *BDNF* gene comprises nine functional promoters (Figure1). The structures of the human *BDNF* gene and transcripts determined in these study are in good agreement with the results obtained for the rat and mouse *BDNF* genes (Aid *et al.,* 2007). Some differences are present, though. First, human *BDNF* contains two more exons than rodent *BDNF*. Compared to the rat and mouse genes (Aid *et al.,* 2007) there is an additional exon, exon Vh, linked to a promoter between exons V and VI. Human *BDNF* exon VIIIh, which is not linked to a separate promoter, is also not present in rodent *BDNF*. Furthermore, cryptic splicing donor and acceptorsites are used in human exon IX leading to transcripts containing exons IXbd and IXabd. These transcripts have not been detected in rodents. All this adds more complexity to the regulation of human *BDNF*. Second, in most cases, the usage of alternative promoters in the human *BDNF* gene leads to the expression of transcripts with different 5' UTRs and with the protein-coding region in the common 3' exon IXd. However, usage of an alternative upstream in-frame translation start site containing exon I, VII, or VIII could potentially lead to human BDNF prepro-proteins with longer N-termini. Only the translation initiation codon within exonI is characteristic of rodent *BDNF* genes, suggesting that there are more BDNF protein isoformsin human than in rodents. Third, although the transcription initiation sites are generally in good agreement with the respective regions in rodents, we identified more transcription start sitesfor human exons II and IV than

had been reported previously for the rodent *BDNF* respective exons. Fourth, in contrast to the rodent *BDNF* genes we found that exon VIII of the human*BDNF* is not used as a 5' exon as determined by the 5' RACE analysis. We show that in human the rarely used exon VIII of *BDNF* is exclusively spliced to exon V. Exon V can also be spliced to exon IXd without including exon VIII. Exon VIII was not detected in any transcript other than the ones starting with exon V, pointing to a possible functional regulation between the usage of a certain promoter and subsequent splicing.This kind of splicing regulation is especially interesting provided the notion that exon VIII of *BDNF* contains one of the alternative ATGs that may lead to the synthesis of a prepro-BDNF protein with an alternative N-terminus.

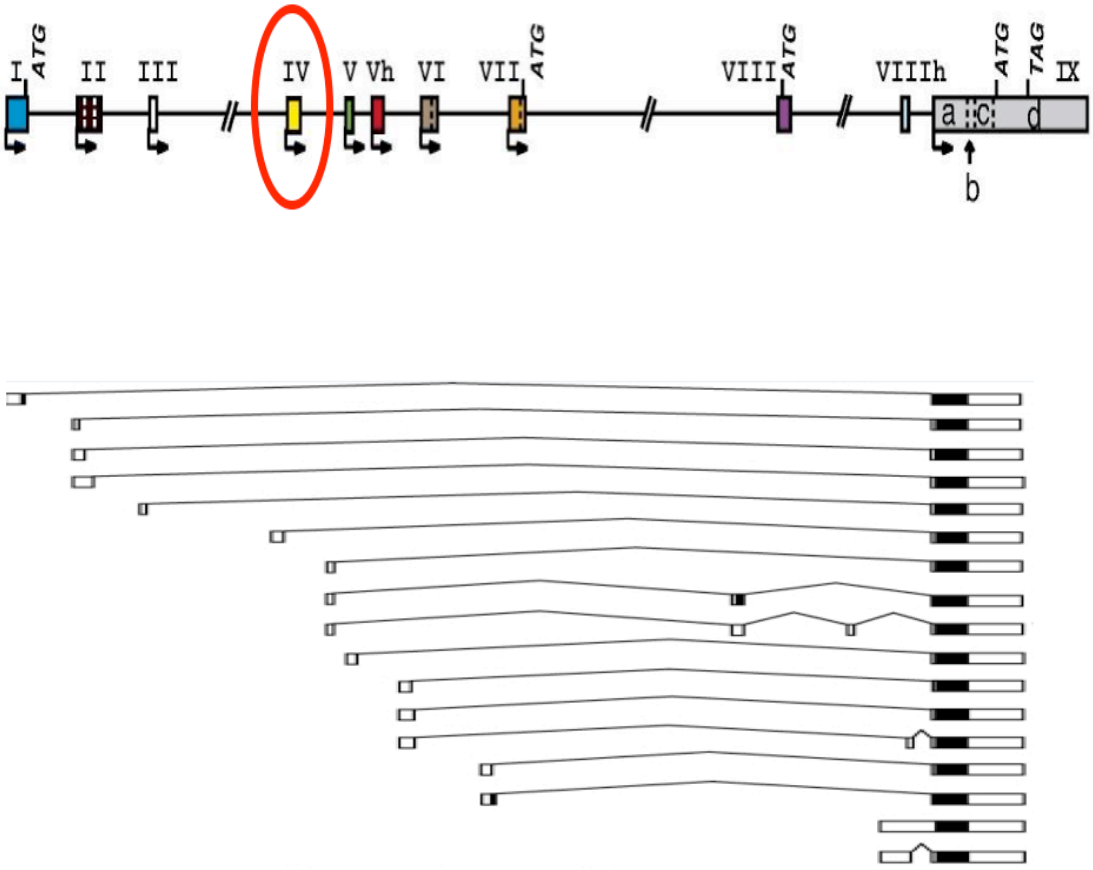

Figure 1. Structure and alternative transcripts of the human *BDNF*.

Structure of human BDNF gene (top). Exons are shown as boxes and introns as lines. Filled boxes and open boxes indicate the translated regions of the exons and the untranslated regions of the exons, respectively. Arrows indicate the transcription start sites. ATG and TAG mark the positions of the translational start and stop codons, respectively. Vertical dashed lines indicate alternative splicing sites for the respective exons. *BDNF* exon IX is divided into regions "a", "b", "c", and "d" as indicated in the box marking the position of exon IX. Novel exons are in green (V), red (Vh), purple (VIII), and light blue (VIIIh). The red circle indicate the trascript studied in this work. Alternative transcripts of the human *BDNF* gene (bottom).

BDNF gene and Suicide

The role of BDNF in depression has gained broad attention because many pre-clinical and clinical studies provide direct evidence suggesting that modulation in expression of BDNF could be involved in behavioral phenomenon associated with depression. Based on these studies, the neurotrophin hypothesis of depression was proposed, which suggests that stress and depression is associated with decreased expression of BDNF and that antidepressants alleviate depressive behavior by increasing its level (Duman *et al.,* 2000; Duman *et al.,* 2002). This is quite relevant given that BDNF plays a key role in development and survival of neurone in the central nervous system, that it is involved in synaptic plasticity and, as earlier mentioned, compromised synaptic and structural plasticity have been shown to be associated with depression. Because stress and depression are the major contributory factors in suicide, recently, a number of studies have attempted to investigate the role of BDNF in suicidal behavior.

In adulthood, BDNF is involved in neural homeostasis and in processes related to neuronal plasticity and connectivity including learning and memory (Tyler *et al.,* 2002; Yamada *et al.,* 2002), drug addiction (Bolanos *et al.,* 2004), response to social stress, aggressiveness and anxiety-like behaviours (Berton *et al.,* 2006; Tsankova *et al.,* 2006). Alteration of BDNF expression in specific neurons may reduce neural plasticity, therefore impairing the ability to respond to stressors, and contributes to different neurodegenerative and neuropsychiatric disorders.

Recent studies demonstrate that BDNF levels are decreased in brain (prefrontal cortex, and hippocampus) of suicide victims (Dwivedi *et al.,* 2003- 2005) suggesting that BDNF plays a role in the pathophysiological aspects of suicidal behaviour. The expression of human BDNF gene is controlled by a complex regulatory region, well conserved in mouse and rat (Aid *et al.,* 2007) that includes at least nine promoters each driving transcription of BDNF mRNA transcripts containing one of the nine 5' noncoding exons. BDNF promoter IV has been shown to be highly regulated during development, and epigenetic mechanisms play a key role in such regulation (Dennis *et al.,* 2005; Mellios *et al.,* 2009). DNA

methylation state of CpG sites within mouse promoter/exon IV is correlated with the expression of BDNF in developing mouse forebrain. Very interestingly, the neuronal activity-dependent activation of BDNF gene is mediated by decreased CpG methylation of BDNF promoter IV and the release of a chromatin repressor complex containing MeCP2 methyl binding protein (Chen *et al.,* 2003; Zhou *et al.,* 2006). Moreover, promoter IV is the major target of chromatin changes associated with alteration of BDNF expression in mouse models of neuropsychiatric disorders (Tsankova *et al.,* 2006 and 2007) However, no data on the methylation state of BDNF gene, either in human mental illness or in suicide victims, have been reported so far.

Aim of the thesis

In this work we tested the hypothesis that alteration of DNA methylation cuold be involved in the disregulation of BDNF gene expression in the brain of suicide subjects. To this aim, we analyzed, by three independent quantitive techniques, the DNA methylation degree of large region of human BDNF gene in post-mortem brain (Wernicke's area) of 44 suicide completers and 33 control subjects. We also determined the global DNA methylation state in the same samples and the BDNF mRNA levels in some samples that displayed different BDNF methylation degree. Our results showed that DNA methylation levels at BDNF promoter IV were increased in suicide victims compared to normal control subjects, irrespective of global DNA methylation degree, and that the amount of BDNF transcript IV was lower in samples displaying a higher BDNF promoter IV methylation.

Methods

Study Subjects

The study was performed on post-mortem samples of brain tissue extracted from the Wernicke area obtained from 44 suicide and 33 non suicide control subjects. Most of autoptic samples were collected within 12-26 hours of subject's death (PMI information are reported in Table 1), in the course of autopsy at the Institute of Forensic Medicine, University of Ljubljana, in a period of time comprised between 1999 and 2005, and stored at -80°C c/o the Biological Bank of the Inštitut za Varovanje Zdravja, Ljubljana (Slovenia). This study was performed according to Ethical issues of such Institution. All studied subjects were of Caucasian ethnicity and all were right handed. A detailed description of the subjects analyzed in this study as data on subjects', sex, age and cause of death were gathered from the subject's record are reported in Table 1. Data on psychiatric diagnoses were obtained from the doctor's note for autopsy and in the cases in which coroner's inquest was done in the presence of relatives also their testimony was taken into consideration (Table 2 and Table 3).

pH Measurement and Drug Analysis

Blood was taken from the subjects in order to perform general toxicological screening using gas chromatography-mass spectrometry (GC-MS).

The analysis of pH of the homogenate was performed using the automated analyzer 865 (Bayer, now Siemens). A 50-100 mg sample of liquid nitrogen pulverized tissue was mixed with distilled deinoinzed water in a 10% (W/V) solution. To confirm blood data we performed drug analysis also on homogenates, using the REMEDI column switching L C (Biorad Lab) which deteets about 700

drugs or metabolites including most of illicit substances and drungs of abuse or their diredt metabolites (Zhou *et al.,* 2006).

DNA and RNA extraction from tissues

DNA and RNA were extracted, from each sample, from a portion of liquid nitrogen pulverized tissue. DNA was preparated using QIAamp DNA Mini Kit (Qiagen) following the instruction manual. Total RNAs were extracted from tissues using TRI REAGENT® (Invitrogen) solution, according to the manufacture's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis. RNA quality and quantity assessment was performed by ND-1000 NanoDROP spectrophotometer. All samples showed a quality ratio (260:280 – 260:230) between 1,8 and 2,2 which is considered optimal range (Tsankova *et al.,* 2007*)*.
Negative controls were obtained by performing the PCR on samples that were not reversed-transcribed but otherwise identically processed.

Bisulfite treatment

Sodium bisulphite conversion of genomic DNA (2 mg) was obtained using Epitect® Bisulphite kit (Qiagen) following the purchaser's instructions. Amplicons used for methylation analysis were obtained from 50 ng of bisulfite treated genomic DNA.

DNA Methylation analysis

Pyrosequencing. Pyrosequencing technology (Tost *et al.,* 2007) was used for DNA methylation quantitative analysis that was performed using the PSQ 96MA instrument from Pyrosequencing (Biotage AB, Uppsala, Sweden) following the manufacturers protocol. The reactions were assayed on the PSQ™ 96MA using the SNP analysis software. Primers used for PCR were: BDNF FW1 (position from nucleotides -227 to -204) 5'-tttgTtggggTtggaagtgaaaaT-3' (5'-Biotinylated); BDNF RV1 (from +59 to +87) 5'-cccatcaacRaaaaactccatttaatctc-3'. Capital T represent thymidines that derive from the the conversion of cytidines after bisulfite treatment while t represent original thymidines. R stands for G or A. Amplifications were carried out on 10 ng of bisulphite treated DNA using HotStarTaq DNA polymerase (Qiagen) under the following conditions: 15 min at 95°C, followed by 50 cycles of 30 sec at 95°C, 52 sec at 59°C, and 1 min at 72°C, then a final elongation of 10 min at 72°C, in a final reaction volume of 30 ml. PCR final products (314 bp) were then used for Pyrosequencing reactions; sequencing primer (BDNF 2) was 5'-acaaaaaaatttcatactaa-3' (position from +32 to +51). Target CpGs were evaluated by analysis of the resulting pyrograms. Guanine (since a reverse sequencing primer was used) was incorporated during pyrosequencing if the template CpG was methylated, while adenine was incorporated if the template CpG was unmethylated. Thus the proportion of G : A incorporated is stoichiometrically proportional to the degree of methylation at that CpG site in the template DNA.

Global DNA methylation analysis was performed using the PyroMarkTM LINE-1 assay kit (BiotageAB, Uppsala, Sweden). Long Interspersed Nucleotide Elements (LINE-1) represent about 15% of human genome; thus, quantitative DNA methylation analysis of LINE-1 may be considered a surrogate analysis of global DNA methylation (Yang *et al.,* 2004). The conditions and primers applied were according to manufacturer's instructions. PCR Amplifications were carried out using the LINE-1 Forward and Reverse primers on 120 ng of bisulphite treated DNA, using HotStarTaq DNA polymerase (Qiagen), in a final reaction volume of

25 ml. Methylation was quantified by Pyrosequencing analysis using the sequencing primer provided by the LINE-1 assay reagent kit.

***Bisulphite genomic sequencing*:** 2 mL of each sample were used as template in PCR reactions using the following primers: BDNF FW2 5'-gggggaggattaaTtgagTTagttTtg -3' (position from nucleotides -358 to -332) and BDNF RV2 5'- cccatcaacRaaaaactccatttaatctc -3'(from nucleotides +59 to +87), PCR final products (445 bp) were then used for the analysis of the BDNF promoter IV. Amplifications were carried out on 10 ng of bisulphite treated DNA using HotStarTaq DNA polymerase (Qiagen) under the following conditions: 15 min at 95°C, followed by 50 cycles of 30 sec at 95°C, 52 sec at 58,3°C, and 1 min at 72°C, then a final elongation of 10 min at 72°C before holding at 4°C, in a final reaction volume of 30 ml. Confirmation of PCR product quality and freedom from contamination was established on 2% agarose gels with ethidium bromide staining. PCR final products were then cloned into the pGEM-T-easy vector provided by Promega pGEM®-T-Easy Vector System II (Promega Italia, Milan, Italy) following the supplier's procedures. Plasmid DNA was purified using the Qiagen plasmid Mini Kit. The purified plasmids were sequenced in both directions using T7 and Sp6 primers. At least 20 independent clones were sequenced to determine the methylation pattern of individual molecules (Lembo *et al.,* 2003). Sequencing was performed at the CEINGE Sequencing Core Facility.

SEQUENOM MassARRAY platform: This system utilizes MALDI-TOF mass spectrometry in combination with RNA base specific cleavage (MassCLEAVE). A detectable pattern is then analyzed for methylation status. PCR primers to analyze BDNF promoter IV (Upper and Lower strands), designed by using Methprimer (www.urogene.org/methprimer), were: L2F, 5'-aggaagagagTTGATTTTTTTAGAGTTTGTTTAATAGGA-3'; L2R, 5'-cagtaatacgactcactatagggagaaggctAACATAAAATCTCCCTACCTCTACC-3'; L1F, 5'-aggaagagagTGTATTTTTTTTGTTTTGTAGTAAAGAAGT-3', L1R, cagtaatacgactcactatagggagaaggctAAAACTTTTTATTTATCTTAACTACCCTAA; U2F, 5'-aggaagagagTTTTTTTGTTGTAGAATAGAAGGAGT-3'; U2R, 5'-cagtaatacgactcactatagggagaaggctAAAAAACCCAAACAATAACAAACCT-3;

U1F, 5'-aggaagagagGGTTGGAAGTGAAAATATTTGTAAAAG-3'; U1R, 5'-cagtaatacgactcactatagggagaaggctACTCCTTCTATTCTACAACAAAAAAAT-3'. For reverse primer, an additional T7 promoter tag for in vivo transcription was added, as well as a 10-mer tag on the forward primer to adjust for melting-temperature differences. Sequences of these tags are indicated in lower case. The MassCLEAVE biochemistry was performed as previously described (Ehrich et al., 2005), Mass spectra were acquired by using a MassARRAY Compact MALDI-TOF (Sequenom) and spectra's methylation ratios were generated by the Epityper software v1.0 (Sequenom).

Presence of CpG island in the genomic region analyzed was assessed by using CGplot software (http://www.ebi.ac.uk/emboss/cpgplot/).

Real-time RT-PCR

1 µg of total RNA of each sample was reverse-transcribed with the QuantiTect® Reverse Transcription (Qiagen) using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions. To design the qPCR assay we used a Human ProbeLibrayTM system (Exiqon, Denmark). Using the free ProbeFinder assay design software, which is an integrated part of the package, we chose the best probes and primers pair. To amplify a fragment for real time PCR of BDNF mRNA, we entered its accession number (ENST00000395980) on the assay design page of the ProbeFinder software. ProbeFinder generated an intron-spanning assay identifying the exon-exon boundaries within submitted transcript. We chose an amplicon of 112 nucleotides generated by a couple of primers located in the first exon of transcript originating by promoter IV and in the third common coding exon, respectively. The number of probe was "human 31" (according to the numbering of Exiquon's Human ProbeLibrary kit) and the primer sequences were: BDNF forward 5'-gccttgatggttactttgacaag -3'; BDNF reverse 5'-agccttcatgcaaccaaagt -3'. The same procedure was used to choose both probes and primers for housekeeping genes: GAPDH, accession number

NM_002046,3, probe “60”; UBC, accession number ENSG00000150991, custom probe; HPRT1, accession number ENSG00000165704, probe “73”; and RNA Polymerase II (RP II), accession number X63564.1, probe “65”. The primers sequences were: GAPDH fw 5’-agccacatcgctcagacac -3’; GAPDH rv 5’-gcccaatacgaccaaatcc-3’; UBC fw 5’- atttgggtcgcggttcttg -3’; UBC rv 5’-tgccttgacattctcgatggt-3’; HPRT1 fw 5’-tgaccttgatttattttgcatacc-3’; HPRT1 rv 5’-cgagcaagacgttcagtcct-3’; RP II fw 5’-tgtcattgcagaggatgtgaa-3’; RP II rv 5’-gcccagacttctgcatgg-3’.

Relative Quantitative TaqMan PCR was performed in Chromo4 Detector, MJ Research in 96-well plates using a final volume of 20 µl. For PCR we used 8 µl of 2,5x RealMasterMixTM Probe ROX (Eppendorf AG, Germany) 200 nM of each primer, 200 nM probe and cDNA generated from 200 ng of total RNA. The conditions used for PCR were 2 min at 95°C, and then 45 cycles of 20 sec at 95°C and 1 min a 60°C. Each reaction was performed in duplicate.

The strategy used for the normalization of quantitative real-time RT-PCR data was the use of geometric averaging of multiple internal control genes in according with the method proposed by Vandesompele *et al*. (20….) We have evaluated 4 housekeeping genes (GAPDH, HPRT I, UBC and RP II) that represent accurate control for mRNA expression analysis of post-mortem brain samples. For each housekeeping gene we measured the gene-stability (M) and ranking them using the geNORM algorithm (Vandesompele *et al* 2002; Barrachina *et al.,* 2006; Hanninen *et al.,* 2009). Stepwise exclusion of the gene with the highest M value allowed ranking of the tested genes according to their expression stability. We applied at each sample the delta-Ct formula for transforming Ct values to relative quantities ($Q = E^{(minCt - sampleCt)}$) as described (Vandesompele *et al.,* 2002). We calculated the Normalization Factor (NF) based on the geometric mean of quantity obtained by the transformation of Ct data. A sample, arbitrarily chosen among control subjects RNAs (920/04), was used as a reference sample. Amplification over 32 cycles were considered out of linear range. (Soong *et al.,* 2003).

Statistical analysis

We performed statistical analyses with the SPSS software package (version 13.0). We used the One-way variance analysis (ANOVA) to compare methylation levels between sample groups. p values <0.05 were considered statistically significant. Mann-Whitney U Test was used to compare non Gaussian distribution. ANCOVA was used to assess the possible influence of gender and age.

Results

Extensive methylation analysis of BDNF promoter IV in Wernicke's area of suicide victims and normal control subject.

To investigate a possible correlation between DNA methylation state of BDNF gene and suicidal behaviour, we analyzed genomic DNA samples derived from post-mortem brain samples extracted from the Wernicke's area of 44 suicide subjects (21 men and 23 women; age range 15 to 79 yrs) and 33 non suicide control (16 men and 17 women; age range 13 to 76 yrs). A detailed description of the subjects analyzed in this study is reported in Table 1, psychiatric diagnoses, medication and toxicological findings are reported in Table 2 and Table 3.

First the methylation analysis was peformed in a wide genomic region for both upper and lower strands, by two independent quantitative methylation analyses, bisulfite genomic sequencing (molecular cloning technique) and a mass spectrometry based methylation analysis (MassARRAY). These analyses were performed in a subgroup of subjects (24) including 13 suicide completers and 11 control subjects of different ages and sex. We analyzed by bisulfite genomic sequencing the genomic region from –203 to +58 (upper strand) encompassing the TSS of the BDNF gene and including 16 CpG sites (Figure 2). The results showed that the analyzed BDNF gene region was mainly unmethylated or low methylated and that the differentially methylated sites were essentially those lying in the close proximity of TSS. The results indicated that sites +10, +16, +25 and +28 were differentially methylated between suicide and control subjects. In addition this analysis showed that CpG site -93 was hypermethylated in almost all samples irrespective of groups (suicide and control subjects), age or gender. Finally, by MassARRAY we analyzed the genomic region from -219 to + 405 on upper strand, including 30 CpG sites, and the region from -289 to +512 on lower strand, including 34 CpG sites (Figure 3).

Table 1 . Description of the analyzed subjects

SAMPLE ID	TYPE	AGE	CAUSE OF DEATH	PMI	PH
156/05	MC	13	external cause of death: assault	≤24	5.5
202/02	MC	20	transport accident	≤24	-
437/02	MC	20	sudden natural death	≤24	-
142/00	MC	45	sudden natural death	≤24	6.8
146/00	MC	47	external cause of accidental injury-fall	≤24	-
604/01	MC	20	transport accident	≤24	-
545/02	MC	20	accidental poisining by noxious substances	≤24	6.9
201/02	MC	60	natural heart death	24	6.5
454/02	MC	62	sudden natural death	≤24	7.1
447/02	MC	55	external cause of accidental injury-accidental drowning	24	-
471/02	MC	71	transport accident	≤24	6.9
487/02	MC	55	sudden natural death	≤24	-
576/02	MC	62	sudden natural death	≤24	-
626/02	MC	64	sudden natural death	≤24	6.8
708/02	MC	65	sudden natural death	≤24	-
457/02	MC	60	sudden natural death	≤24	-
72/00	FC	46	sudden natural death	≤24	-
138/00	FC	38	trombemboly	≤24	-
931/04	FC	42	transport accident	≤24	6.9
94/05	FC	21	sudden natural death	≤24	7.1
28/05	FC	44	complication of medical and surgical care	≤24	7.0
773/03	FC	39	transport accident	≤24	6.8
404/05	FC	34	transport accident	24	6.7
372/05	FC	49	transport accident (byciclist)	≤24	6.5
136/00	FC	76	sudden natural death	≤24	-
149/05	FC	55	external cause of accidental injury-fall	24	7.1
498/05	FC	65	transport accident	≤24	7.2
485/02	FC	64	external cause of accidental injury-accidental drowning	≤24	-
547/01	FC	76	trombemboly	≤24	-
907/04	FC	70	brain bleeding	24	7.0
79/05	FC	53	trombemboly	≤24	6.9
882/04	FC	59	sudden natural death	≤24	7.1
593/02	FC	71	sudden natural death	≤24	-
674/04	MS	22	intention self harm-hanging	≤24	7.0
179/04	MS	15	intentional self harm-jump from high place	≤24	6.7
727/03	MS	19	intentional self harm-poisoning	24	7.0
447/03	MS	23	intentional self harm-shooting	≤24	6.8

315/02	MS	22	intentional self harm-shooting	≤24	-
920/04	MS	19	intentional self harm-shooting	26	6.8
1015/04	MS	19	intentional self harm-shooting	≤24	7.0
578/02	MS	25	intentional self harm-shooting	46	-
110/04	MS	19	intentional self harm-jump under train	≤24	6.9
22/02	MS	60	intentional self harm-hanging	≤24	-
888/99	MS	24	intentional self harm-shooting	≤24	-
598/01	MS	19	intentional self harm-hanging	≤24	-
190/02	MS	52	intentional self harm-other poisoning	≤24	-
288/02	MS	52	intentional self harm-poisoning	≤24	6.9
371/02	MS	52	intentional self harm-hanging	≤24	-
199/02	MS	56	intentional self harm-jump from high place	≤24	-
665/01	MS	69	intentional self harm-hanging	24	6.7
540/02	MS	77	intentional self harm-poisoning	≤24	6.8
579/02	MS	56	intentional self harm-hanging	≤24	6.4
555/02	MS	59	intentional self harm-poisoning	≤24	6.8
584/02	MS	56	intentional self harm-hanging	≤24	-
84/04	FS	44	intentional self harm-hanging	≤24	7.1
78/04	FS	27	intentional self harm-jump from high place	≤24	5.9
103/05	FS	21	intentional self harm-drowning	24	-
515/01	FS	44	intentional self harm-poisoning	≤24	-
456/03	FS	49	intentional self harm-drowning	26	7.0
703/02	FS	38	intentional self harm-jump from high place	≤24	-
617/03	FS	41	intentional self harm-cutting with sharp object	≤24	7.1
427/04	FS	25	intentional self harm-jump from high place	≤24	6.9
524/02	FS	21	intentional self harm-hanging	≤24	-
690/01	FS	15	intentional self harm-jump from high place	24	-
799/04	FS	14	intention self harm-hanging	≤24	6.8
22/00	FS	28	intention self harm-shooting	≤24	-
101/00	FS	40	intentional self harm-jump from high place	≤24	-
63/02	FS	71	intentional self harm-hanging	≤24	-
108/02	FS	64	intentional self harm-poisoning	≤24	-
668/03	FS	57	intentional self harm-drowning	≤24	6.9
99/04	FS	58	intentional self harm-drowning	≤24	6.8
43/02	FS	65	intentional self harm-drowning	≤24	-
228/02	FS	71	intentional self harm-poisoning	≤24	-
259/02	FS	55	intentional self harm-hanging	≤24	-
498/02	FS	60	intentional self harm-poisoning	≤24	-
489/02	FS	79	intentional self harm-drowning	≤24	-
679/02	FS	51	intentional self harm-hanging	≤24	-

Table 2. Relationship between psychiatric conditions and Methylation/Expression levels of BDNF gene in Suicide subjects.

Psychiatric diagnosis	Total N° of subjects	N° of subjects with Hypermethylation at BDNF gene
Schizophrenia	4	1
Bipolar disorder	2	0
Major depressive disorders	12	3
Schizoaffective disorder	2	0
Other	2	0
No diagnosed psychiatric illness	22	9

Table 3. Relationship between Drug use and Methylation/Expression levels of BDNF gene in Suicide subjects

Toxicological findings	Total N° of subjects	N° of subjects with Hypermethylation at BDNF gene
CARBROMAL	1	0
ZOLPIDEM	1	0
OLANZEPINE	1	0
DIAZEPAM	3	1
IMIPRAMINE	3	0
CITALOPRAM	6	2
NONE	29	10

sites were essentially those lying in the close proximity of TSS. The results indicated that sites +10, +16, +25 and +28 were differentially methylated between suicide and control subjects. In addition this analysis showed that CpG site -93 was hypermethylated in almost all samples irrespective of groups (suicide and control subjects), age or gender. Finally, by MassARRAY we analyzed the genomic region from -219 to + 405 on upper strand, including 30 CpG sites, and the region from -289 to +512 on lower strand, including 34 CpG sites (Figure 3). This analysis showed that also in this wider genomic region most of CpG sites were unmethylated or low methylated in both suicide and control subjects and that, on the upper strand, sites +10, +16, +25 and +28 remain the differentially methylated CpG sites. Differences in methylation degree of these same sites detected on lower strand were slighter but a higher methylation level was detected at some of these CpG sites where the correspondent sites on upper strand were hypermethylated. Frequent hypermethylation of -93 on upper strand site was confirmed in these experiments. Slight differences in methylation degree at some CpG sites observed in the analysis performed by the different technical approaches were possibly due to the different sensitivity of the methods. Finally, we analyzed, by a further independent and very sensitive technique, the methylation status of four CpG sites (+10, +16, +25 and +28) located downstream the transcription initiation site of promoter IV of the BDNF gene (Figure 4A). These sites are embedded in a small CpG island (located from -99 to + 101) spanning the transcriptional start site (TSS). A quantitative methylation analysis was performed using the pyrosequencing technology in order to assess the precise degree of methylation of each CpG site.

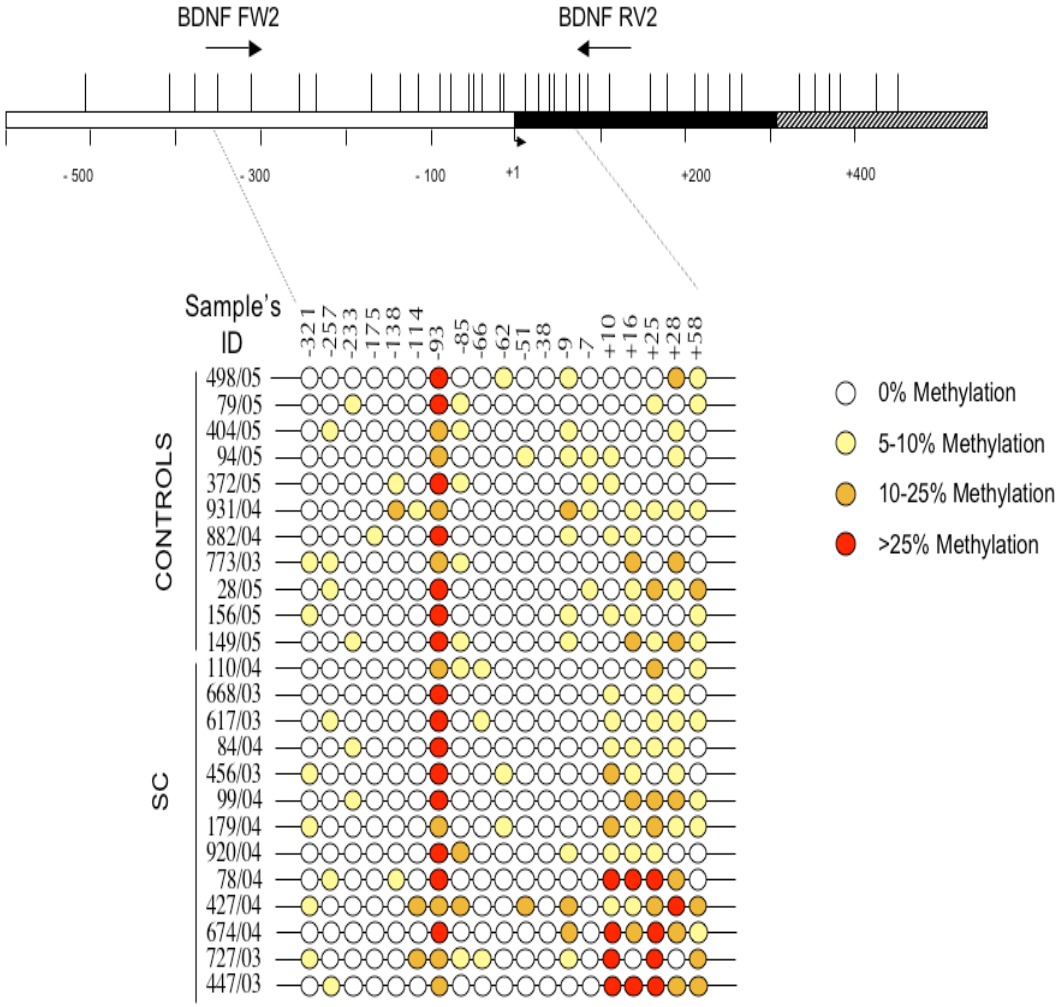

Figure 2. Methylation analysis by genomic bisulfite sequencing. Top: Diagramatic representation of BDNF gene (promoter/exon IV). Regulatory upstream region (open box), exon IV (black box) and intron IV (striped box) are indicated. Vertical bars represent the relative positions of each CpG site. TSS is indicated by an arrow. The relative positions of the primer utilized for amplification (BDNF FW2 and BDNF RV2) are indicated. Bottom: The methylation degree of each CpG site (circles) is indicated by different colours as specified in the Figure. SC = suicide completers; Controls = non suicide subjects.

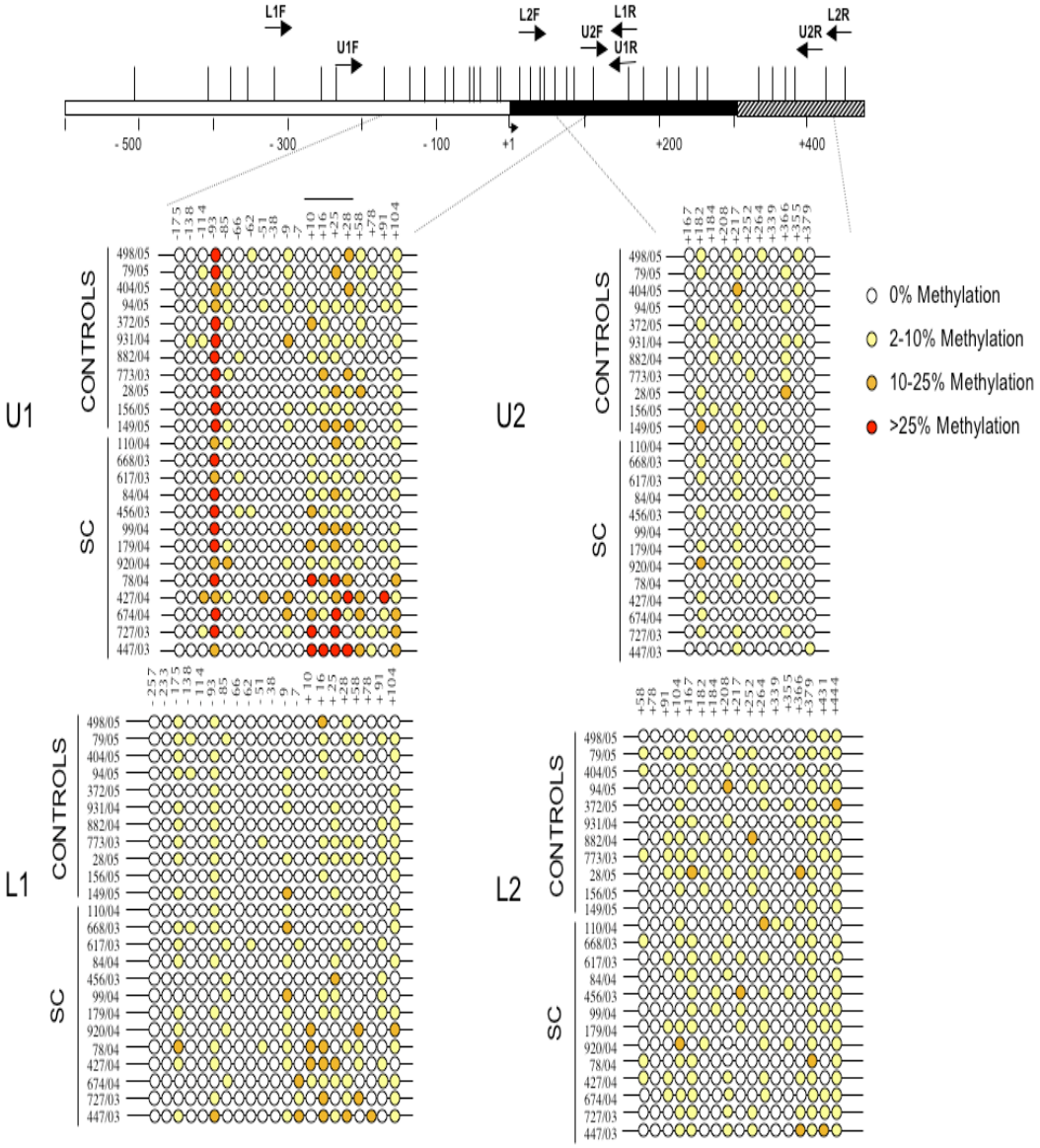

Figure 3. Methylation analysis by MassARRAY. Top: Diagramatic representation of BDNF gene (promoter/exon IV) as in Figure 4. The relative positions of the primer utilized for amplification of amplicons U1 (upper strand from -218 to + 160), U2 (upper strand from +134 to +405), L1 (lower strand from -289 to +163) and L2 (lower strand from +33 to +512) are indicated by arrows on top. Bottom: Comprehensive view of methylation state of BDNF gene. The methylation degree of each CpG site (circles) belonging to the amplicons U1, U2, L1 and L2, is indicated by different colours as specified in the Figure. CpG sites analyzed by MALDI-TOF were 14/19 (U1), 7/11 (U2), 16/21 (L1) and 15/17 (L2). For CpG sites not analyzable by the assay, data from other procedures or average of two adjacent CpG sites are indicated. Each subject is identified by sample ID. SC = suicide completers; Controls = non suicide subjects.

A representative pyrogram is shown in Figure 1B. Full raw data, indicating the methylation degree of each CpG site in each sample, are reported in Table 4. The analysis was repeated three times and each value did not differ significantly (+/- 0.7%). The mean methylation degree of the four CpG sites in each subject is also reported in Table 4 and graphically shown in Figure 5. Overall, the results showed that the mean methylation degree of the 4 CpG sites was always under 12.9% in the 33 non-sucide control subjects while, in 13/44 cases (30%) among the suicide victims, the mean methylation degree ranged between 13.1 and 34.2% (Figure 5 and Table 4). In order to establish whether the observed differences in the mean methylation degree between suicide and non suicide subjects were statistically significant, we performed one-way variance analysis (ANOVA). As shown in Figure 6A, significant differences are clearly discernible in the mean methylation status of the region analyzed between cases and controls (One-way ANOVA, $F=13.7$, $p<0.001$). By the same method, we analyzed the relationship between the methylation degree of each of the four CpG sites and suicidal behaviour. Statistically significant differences between cases and control groups were found for the CpG sites +10 and +25 (Figure 6B). A similar, but not statistically significant, trend to hypermethylation was found for the other two CpG sites tested (+16 and + 28). We analyzed the data also by a non parametric test (Mann-Whitney U Test) obtaining similar results. Statistically significant differences between cases and control groups were found for the average methylation of the region (Mann-Whitney U Test $p = 0.001$) and for CpG sites +10 and +25 ($p = 0.002$ and 0.001 respectively). No effect of gender and age was found by ANCOVA analysis (data not shown).

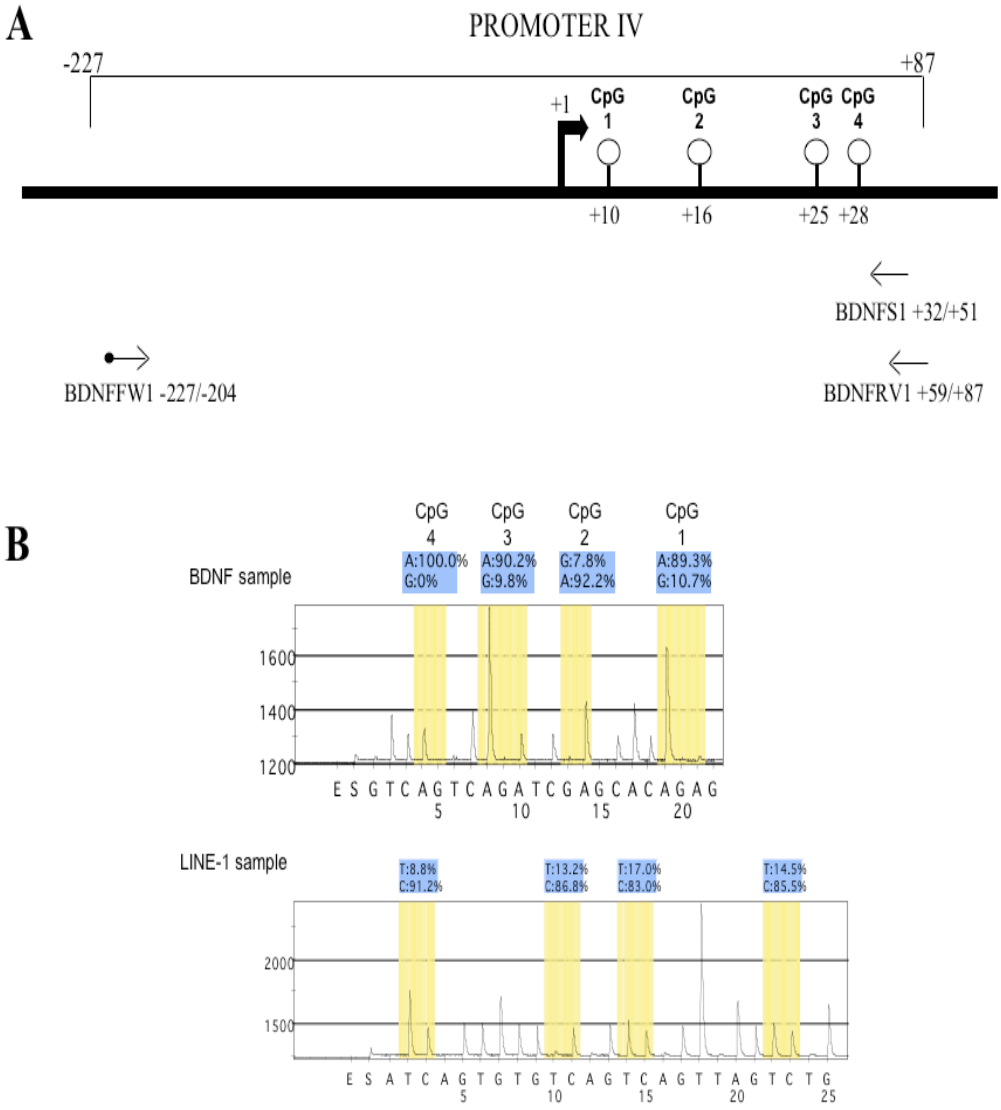

Figure 4. Pyrosequencing analysis of BDNF gene. A: Structure of human BDNF gene, promoter IV. The transcriptional start site (+1) is indicated by an arrow. The positions of the CpGs analyzed (open circle) and of the primers used (arrows) for pyrosequencing analysis are indicated. BDNF FW1 (biotinylated) and BDNF RV1: amplification primers; BDNF S1: sequencing primer. B. Top panel: representative pyrogram for pyrosequencing analysis of BDNF. The four targeted cytosines are enclosed in shaded squares (as reverse strand was read, G peaks indicate methylated cytosine while A indicates unmethylated cytosine, and the order of the CpGs analyzed is inverted). Bottom panel: representative pyrogram of global methylation (LINE-1). Four CpG sites were analyzed (shaded squares); in this case as forward strand was read, C peaks indicate methylation while T peaks indicate no methylation.

Table 4. Methylation analysis data. Degree of methylation of each of the four CpG sites analyzed, their average value, and degree of DNA global methylation for each sample are reported.

SAMPLE ID	SUBJECT	CpG 1	CpG 2	CpG 3	CpG 4	AVERAGE
156/05	MC	8,4	4,3	7,9	3,5	6,025
202/02	MC	0	19	0	0	4,75
437/02	MC	11,2	14	0	0	6,3
142/00	MC	5,1	0	9,6	0	3,675
146/00	MC	8,6	0	15	0	5,9
604/01	MC	0	0	10,7	0	2,675
545/02	MC	0	10,9	17,5	0	7,1
201/02	MC	0	0	26,7	0	6,675
454/02	MC	0	0	11	0	2,75
447/02	MC	34,9	0	16,7	0	12,9
471/02	MC	6,2	4,5	9,2	0	4,975
487/02	MC	0	0	0	0	0
576/02	MC	3,3	1,6	5,7	0	2,65
626/02	MC	4	2,6	6,3	9,7	5,65
708/02	MC	4,4	2	4,8	1,5	3,175
457/02	MC	8,4	2,5	5,3	2,6	4,7
72/00	FC	0	7,6	8,4	0	4
138/00	FC	6,5	3,3	7	0	4,2
931/04	FC	0	4,4	7,1	3	3,625
94/05	FC	4,4	3,7	5,1	6,5	4,925
28//05	FC	0	6,5	12,8	6,9	6,55
773/03	FC	0	16,5	0	14,4	7,725
404/05	FC	0	0	0	15	3,75
372/05	FC	16,2	8,8	0	0	6,25
136/00	FC	19	11,2	0	10,2	10,1
149/05	FC	0	10,3	15,8	11,2	9,325
498/05	FC	0	0	0	12,8	3,2

485/02	FC	0	0	0	7,7	1,925
547/01	FC	0	0	13,1	7,6	5,175
907/04	FC	6,8	5	10,6	0	5,6
79/05	FC	0	0	12,8	0	3,2
882/04	FC	3,8	2	4,8	0	2,65
593/02	FC	12,4	10,7	12,1	7,2	10,6
674/04	MS	19,7	8,4	24,7	9,7	15,625
179/04	MS	11,2	6,8	11,8	0	7,45
727/03	MS	37,1	0	47,2	0	21,075
447/03	MS	45	25,6	42,2	24	34,2
315/02	MS	0	0	0	0	0
920/04	MS	5,8	4,3	6,9	0	4,25
1015/04	MS	13	7,2	14,9	6,5	10,4
578/02	MS	0	0	7,2	0	1,8
110/04	MS	0	0	18,2	0	4,55
22//02	MS	6,9	4,8	10,7	0	5,6
888/99	MS	0	7,6	13,8	0	5,35
598/01	MS	37,9	17,6	31,6	24,5	27,9
190/02	MS	0	0	16,7	0	4,175
288/02	MS	35,8	0	40,8	0	19,15
371/02	MS	0	0	22,1	0	5,525
199/02	MS	0	0	26,8	0	6,7
665/01	MS	49,4	0	43,5	25	29,475
540/02	MS	9,3	0	10,6	0	4,975
579/02	MS	7,9	0	8,6	0	4,125
555/02	MS	0	0	18,1	0	4,525
584/02	MS	4,2	0	5	2	2,8
84/04	FS	9,9	5,5	13,1	3,5	8
78/04	FS	34,8	19,1	33,5	17,4	26,2
103/05	FS	0	14,5	0	15,3	7,45
515/01	FS	6,5	6,1	8,4	27,6	12,15
456/03	FS	19,3	6,4	7,3	2,9	8,975
703/02	FS	24,6	22,5	42,2	18,1	26,85
617/03	FS	9,3	3,6	5,3	0	4,55

427/04	FS	4,9	2	7,4	59	18,325
524/02	FS	5,6	2,7	10,6	0	4,725
690/01	FS	9,6	0	8,3	0	4,475
799/04	FS	8	5,6	8,9	3,8	6,575
22/00	FS	17,6	0	20	0	9,4
101/00	FS	16,6	14,6	17,1	10,4	14,675
63/02	FS	13,9	0	18,4	0	8,075
108/02	FS	10,5	0	11,2	0	5,425
668/03	FS	3,6	0	6	4,1	3,425
99/04	FS	0	12,7	18,4	11,5	10,65
43/02	FS	27,6	25,1	0	21	18,425
228/02	FS	0	17,6	0	16,3	8,475
259/02	FS	36	4,6	6,2	5,7	13,125
498/02	FS	22,6	8,8	16,9	8,9	14,3
489/02	FS	9,5	4,8	13,4	4	7,925
679/02	FS	10,7	7,8	9,8	0	7,075

Sample ID: progressive number/year of death. Abbreviation used: MC, male-control; MS, male-suicide victim; FC, female-control; FS, female-suicide victim.

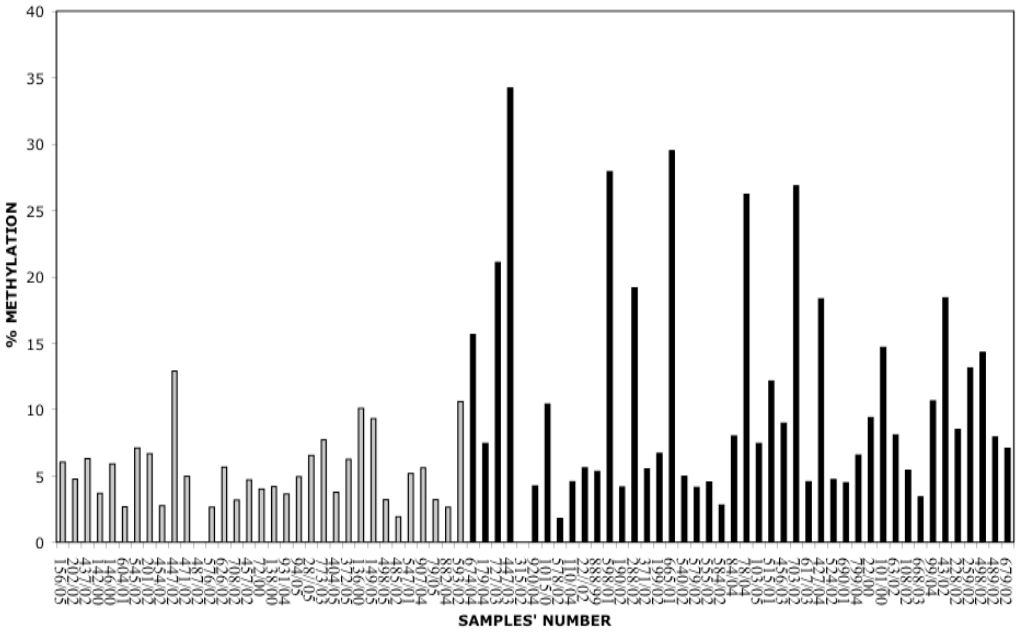

Figure 6. Percentage of BDNF promoter IV methylation in sucide subjects and non suicide controls. For each sample the percentage of methylation represents the average of the methylation degree of the 4 CpG sites analyzed. Grey columns: control samples; black columns: suicide victims.

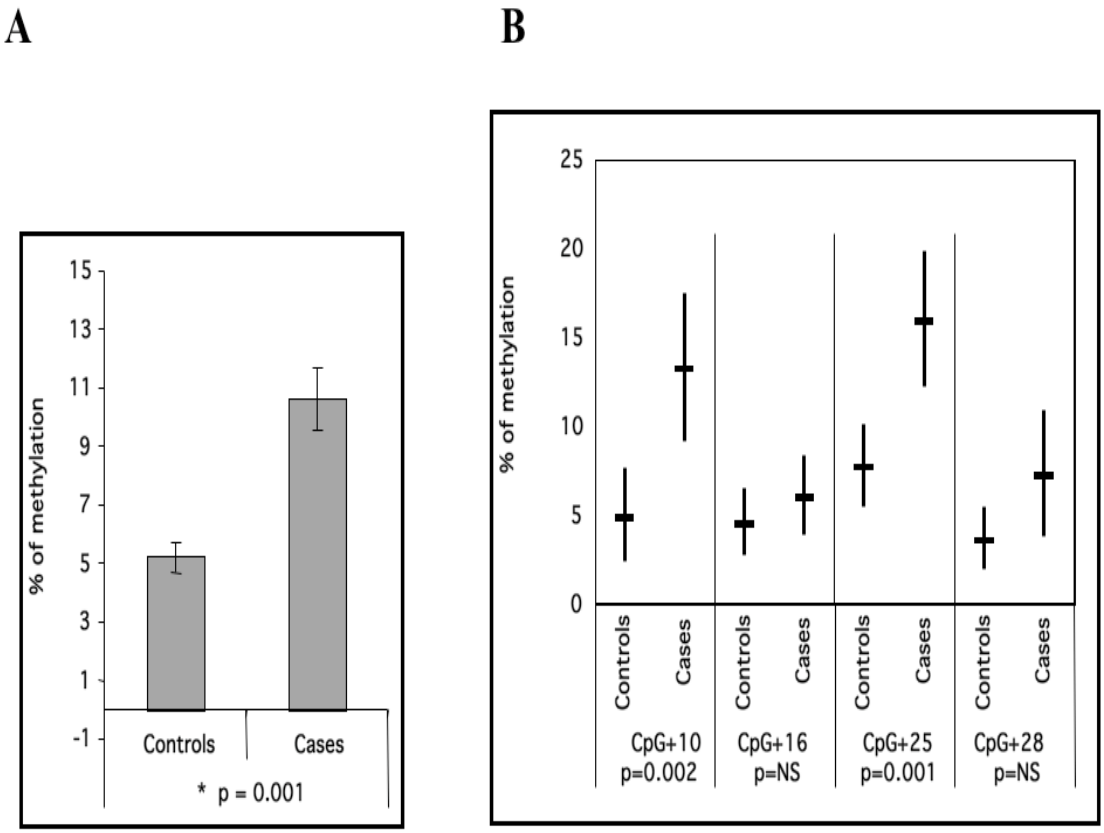

Figure 6. Statistical analysis of methylation status of 4 CpG residues in the region of the promoter IV of the BDNF gene in suicide and control subjects. a) Average methylation of the four CpGs. Values represent mean +/- SE. b) Methylation status of individual CpG. Values represent mean and 95% CI

Global genomic methylation analysis

Next, because it is known that global DNA methylation state of specific brain's areas could vary among individuals, we addressed the question whether an increased methylation degree of BDNF promoter/exon IV in sucide victims associates with higher methylation levels throughout the genome or whether it is a specific feature of the BDNF gene. For this purpose we studied global methylation in all suicide and control samples by quantitatively analyzing the methylation of the long interspersed nucleotide elements (LINE-1), using pyrosequencing. A representative pyrogram is shown in Figure 7. Results, indicate that global methylation ranged between 81% and 90% among individuals. However, no correlation was found between global methylation and BDNF promoter IV methylation degree (data not shown). Moreover, global methylation levels did not associate with suicidality, gender and age (data not shown). Finally, we verified whether psycopathological conditions or the past and actual use of specific medications, could influence the BDNF gene methylation state. Drug history examination indicated that no subject made use of psycoactive substances during life other than those detected by toxicological screens and reported in Table 2 and Table 3. We found no significant correlation between any of these variables and the methylation degree at BDNF promoter (Mann-Whitney U Test p = ns). Overall, our results show that the BDNF promoter/exon IV is hypermethylated in post-mortem brain Wernicke'area of suicide subjects compared to normal control subjects irrespective of global methylation levels, suggesting that a gene-specific increase in DNA methylation could cause or contribute to down-regulation of BDNF expression in suicide subjects.

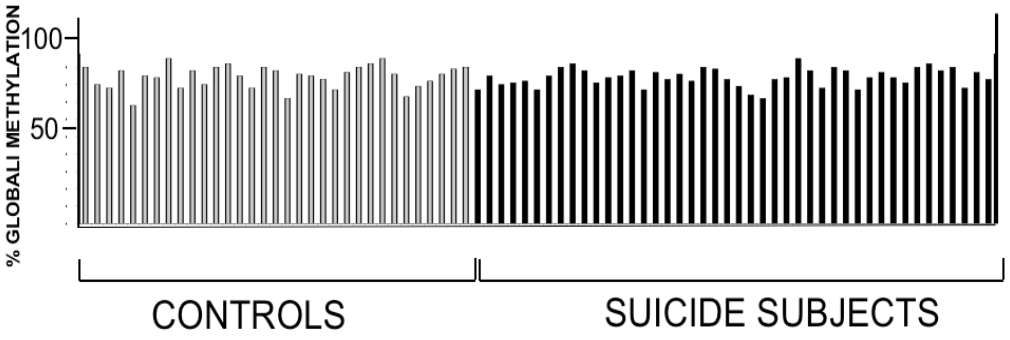

Figure 7. Global DNA Methylation Analysis. Percent of global genomic dna methylation in brain wernicke's area of suicide and control subjects as assessed by line-1 pyrosequencing analysis.

Relationship between BDNF expression and BDNF promoter IV methylation degree.

In order to assess whether hypermethylation of BDNF promoter IV (CpG sites +10, +16, +25 and +28) was associated to a decreased gene expression, we analyzed by real time RT-PCR the BDNF mRNA (transcript IV) levels in the same subgroup of subjects. We analyzed 13 samples from the suicide victims and 11 from control subjects; in particular 10 samples (4 from suicide and 6 from control group) with low level of methylation at BDNF promoter IV, 9 samples (4 from suicide and 5 from control group) showing intermediate methylation levels and 5 samples from suicide subjects with the highest methylation degree (Figure 8). For this quantitative RT-PCR assay we used specific primers for the analysis of BDNF mRNA transcript originating from promoter IV. The analyzed samples showing a high methylation degree of BDNF promoter IV expressed lower levels of BDNF mRNA as compared to the low and medium methylated samples beeing the mildly methylated samples in the mid-range (Figure 8).

Taken together, our data indicate that a higher methylation degree of BDNF promoter IV in brain (Wernicke's area) correlates with suicidal behaviour and suggest that increased DNA methylation levels of BDNF promoter IV can negatively regulate BDNF expression.

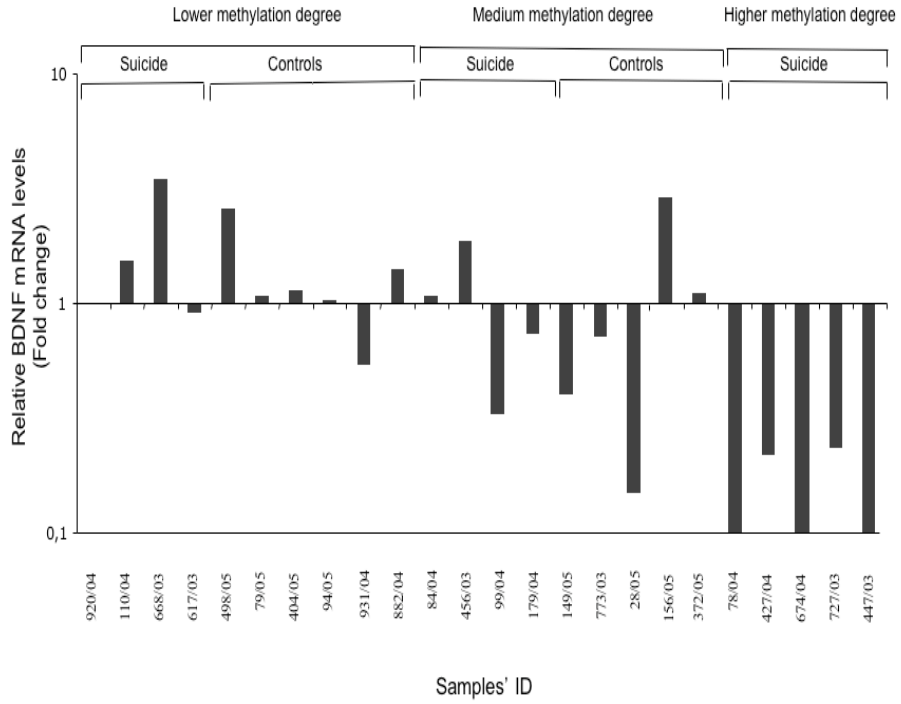

Figure 8. Relative mRNA levels of BDNF transcript IV. The BDNF expression was normalized using 4 internal control genes (as described in Methods) and relative expression levels are shown. In parentheses, the pH value of each sample is indicated. No significant correlation was found between pH values and mRNA expression or methylation degree (Mann-Whitney U Test p = ns). The cut off line (bottom) indicates that detection was over 32 cycles.

Comment

We analyzed, by three independent sensitive quantitative methods, the DNA methylation degree at BDNF promoter IV and the global DNA methylation levels in the brain (Wernicke's area) of 44 suicide subjects and 33 non-suicide control subjects. The main conclusions of our work are: i) suicide subjects showed a statistically significant increase of DNA methylation at BDNF promoter IV and higher methylation degree corresponded to lower BDNF transcript IV; ii) such increase in CpG methylation was gene-specific since it was not accompanied by an increase of global DNA methylation; iii) global DNA methylation levels in Wernicke's area varied among individuals but did not correlate with suicidal behaviour, and were not dependent on gender or age.

The present work and the relative conclusions represent an absolute novelty for several aspects. In fact, the present study is novel in examining the possibility that BDNF hypermethylation could be associated with suicidality. This study was performed on samples of brain tissue obtained from the Wernicke's area of suicide completers and control subjects who died for other causes. Wernicke's area has been chosen for its function in understanding word meaning and semantic thinking and for its critical involvment with human language and with associative and integrative functions. This fact is consistent with several findings of neurocognitive alterations in suicide attempters, such as an impairment in decision making (Jollant *et al.,* 2005, 2007) and problem solving (Speckens *et al.,* 2005) Wernicke's area and its connections with other brain structures represent a unique feature of human brain, as suicidal behaviour is peculiar to humans, and may influence many factors including human social behavior (Rilling *et al.,*2008). Completed suicide cannot be considered a sudden and casual death but is the outcome of a process which involves a wide spectrum of thoughts, communications and acts. It is consistent with this assumption that in suicidal behaviour gene expression may be altered in a cortical area which has a highly specialized integrative and associative functions. Moreover post-mortem studies reported an age-related expression of BDNF in the temporal cortex (Webster *et*

al., 2006), suggesting that this neurotrophin is important in the early development of the temporal cortex. Suicidal behaviour has been found to be associated with early traumatic experiences and this link could be based on early modifications in the expression of the BDNF gene.

The choice to analyze the methylation state of BDNF gene derived from previous evidences that BDNF mRNA and protein levels are decreased in different post-mortem brain's areas (hyppocampus and frontal cortex) (Dwivedi *et al.,* 2003,2005) and in plasma (Kim *et al.,* 2007) of suicide victims compared to non suicide controls. Human BDNF expression is controlled by a very complex regulatory region including nine different transcription initiation sites driven by corresponding promoters (Pruunsild *et al.,* 2007). We chose to analyze DNA methylation of promoter IV because it has been previously established that the equivalent rat and murine promoters are strongly regulated during development and in adult neurons and that epigenetic mechanisms play a critical role in such transcriptional regulation (Tsankova *et al.,* 2009; Dennis *et al.,* 2005; Mellios *et al.,* 2009). Of particular relevance are the observations that the epigenetic state of promoter IV may be also modulated in mouse and rat brain by several exogenous factors such as membrane depolarization-induced calcium influx (Chen *et al.,* 2003; Zhou *et al.,* 2006) chronic social defeat stress and antidepressant administration (Tsankova *et al.,* 2006). These observations reinforce the growing hypothesis that complex epigenetic mechanisms, which may be modified by environment and may regulate gene activity without altering the DNA code, have long-lasting effects within mature neurons and are implicated in the regulation of human complex behaviour, including psychiatric disorders (Tsankova *et al.,* 2007; Onishchenko *et al.,* 2008; Szyf *et al.,* 2008) Maya Vetencourt *et al* (Maya Vetencourt *et al.,* 2008) showed that cortical administration of diazepam prevents the fluoxetin-mediated BDNF activation in the visual cortex raising the interesting question whether changes of BDNF expression or methylation state in suicide subjects may be influenced by drug consumption. However, in this study we did not find any relationship between BDNF methylation or expression with diazepam or fluoexetin consumption of the study subjects. McGowan *et al*.

(McGowan *et al.,* 2008) demonstrated that in the brain of 11 suicide subjects with history of early childhood abuse, the rRNA gene was downregulated and hypermethylated compared to control subjects. Very interestingly, Ernst *et al.* (Ernst *et al.,* 2009) found that trkB is hypermethylated in sucide completers in different brain's areas. Our study, that relates BDNF gene methylation state to suicidal behaviour, strongly supports the conclusions of these studies and provides a possible mechanism responsible for the reduction of BDNF levels observed in the brain of suicide subjects. In the near future it will be very interesting to extend the methylation analysis of BDNF gene to other brain's areas involved in suicidal behaviour.

Previous studies revealed that the methylation state of specific CpG sites in the BDNF rat and mouse promoter IV may play a critical role in BDNF gene regulation. In particular, it has been shown that the CpG sites -128 and +19 of the rat BDNF promoter IV may mediate, when methylated, the binding to MeCP2 which, in turn, is responsible for trascriptional repression (Chen *et al.,* 2003; Zhou *et al.,* 2006). However, the role of DNA methylation in the control of BDNF expression has not been previously investigated in human tissues. In the present work we show that CpG sites adjacent to the TSS of human BDNF transcript IV may play a role in the regulation of BDNF expression. In particular, statistical analysis showed that the methylation state of CpG sites +10 and +25 associates with suicidal behaviour. Moreover, we showed that an higher methylation degree of these sites is associated with lower BDNF mRNA levels suggesting that, at least in part, DNA methylation is involved in BDNF transcriptional regulation in human brain. Because we found much lower BDNF trancript IV mRNA levels in samples showing 20-30% methylation of four CpG sites in BDNF promoter IV compared to samples showing 3-5% methylation, we hypothesize that other mechanisms, including additional epigenetic mechanisms and/or lack of transcription factors, may contribute to such a strong repression. In the near future, it will be very interesting to determine the relative role, in the regulation of BDNF expression, of CpG sites lying in the different BDNF gene promoters in order to study the possible association of epigenetic state of other promoters with

suicidal behaviour. DNA methylation and other epigenetic factors could also provide some explanation to reported conflicting data on the association of the BDNF gene polymorphims with suicidal behaviour.

In this study, we found a relatively low rate of suicide completers with a psychiatric disease. It cannot be excluded that in a few cases psychiatric conditions were present but were undiagnosed. However, the rate of diagnosed psychiatric diseases among suicide completers in our sample is comparable to the rate reported by other studies (Rockett *et al.,* 2009). Moreover, the geographical region where the sample was collected has high suicide rates and it has been suggested that genetic component(s) might have an effect on increased suicide rates (Marusic *et al.,* 2005). This genetic component possibly acts through personality features such as impulsive aggression. Overall, our study reinforces the mounting hypothesis that DNA methylation is involved in psychiatric conditions and deviant human behaviours and represents one of the first demonstration that alteration of gene-specific DNA methylation in human brain associates with suicidal behaviour.

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