ORIGINAL ARTICLE

Increased *BDNF* Promoter Methylation in the Wernicke Area of Suicide Subjects

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Context: Brain-derived neurotrophic factor (BDNF) plays a pivotal role in the pathophysiology of suicidal behavior and *BDNF* levels are decreased in the brain and plasma of suicide subjects. So far, the mechanisms leading to downregulation of *BDNF* expression are poorly understood.

Objectives: To test the hypothesis that alterations of DNA methylation could be involved in the dysregulation of *BDNF* gene expression in the brain of suicide subjects.

Design: Three independent quantitative methylation techniques were performed on postmortem samples of brain tissue. *BDNF* messenger RNA levels were determined by quantitative real-time polymerase chain reaction.

Setting: Academic medical center.

Patients or Other Participants: Forty-four suicide completers and 33 nonsuicide control subjects of white ethnicity.

Main Outcome Measures: The DNA methylation degree at *BDNF* promoter IV and the genome-wide DNA methylation levels in the brain's Wernicke area.

Results: Postmortem brain samples from suicide subjects showed a statistically significant increase of DNA methylation at specific CpG sites in *BDNF* promoter/exon IV compared with nonsuicide control subjects ($P < .001$). Most of the CpG sites lying in the −300/+500 region, on both strands, had low or no methylation, with the exception of a few sites located near the transcriptional start site that had differential methylation, while genome-wide methylation levels were comparable among the subjects. The mean methylation degree at the 4 CpG sites analyzed by pyrosequencing was always less than 12.9% in the 33 nonsuicide control subjects, while in 13 of 44 suicide victims (30%), the mean methylation degree ranged between 13.1% and 34.2%. Higher methylation degree corresponded to lower *BDNF* messenger RNA levels.

Conclusions: *BDNF* promoter/exon IV is frequently hypermethylated in the Wernicke area of the postmortem brain of suicide subjects irrespective of genome-wide methylation levels, indicating that a gene-specific increase in DNA methylation could cause or contribute to the downregulation of *BDNF* expression in suicide subjects. The reported data reveal a novel link between epigenetic alteration in the brain and suicidal behavior.

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BRAIN-DERIVED NEUROTROPHIC factor (BDNF), a member of the neurotrophin family, plays a key role in the development and survival of neurons in the central nervous system.[1] BDNF binds to a specific tyrosine kinase receptor (tropomyosin-related kinase B receptor [trkB]) and regulates many functions related to neuron development such as neurite outgrowth, synthesis of differentiating factors, and morphological plasticity.[1] In adulthood, *BDNF* is involved in neural homeostasis and in processes related to neuronal plasticity and connectivity, including learning and memory,[2,3] drug addiction,[4] response to social stress, aggressiveness, and anxietylike behaviors.[5,6] 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 including depression and bipolar disorder.[7] Single-nucleotide polymorphisms of the *BDNF* gene have also been associated, although with conflicting results, with different psychiatric conditions, including schizophrenia and suicidal behavior.[8-10]

Recent studies demonstrate that BDNF levels are decreased in the brain (prefrontal cortex and hippocampus) of suicide victims,[11,12] suggesting that *BDNF* plays a role in the pathophysiological aspects of suicidal behavior. The expression of the hu-

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man *BDNF* gene is controlled by a complex regulatory region, well conserved in mouse and rat,[13] that includes at least 9 promoters each driving transcription of *BDNF* messenger RNA (mRNA) transcripts containing 1 of the nine 5′ noncoding exons.[14] *BDNF* promoter IV has been shown to be highly regulated during development, and epigenetic mechanisms play a key role in such regulation.[15-17] The DNA methylation state of CpG sites within mouse promoter/exon IV is correlated with the expression of *BDNF* in the developing mouse forebrain.[15] Very interestingly, the neuronal activity-dependent activation of the *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.[18,19] Moreover, promoter IV is the major target of chromatin changes associated with alteration of *BDNF* expression in mouse models of neuropsychiatric disorders.[6,20] However, to our knowledge, no data on the methylation state of the *BDNF* gene, either in human mental illness or in suicide victims, have been reported so far.

In the present study, we quantitatively analyzed the DNA methylation degree of 4 CpG sites within the human *BDNF* promoter/exon IV in the postmortem brain (Wernicke 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 a different *BDNF* methylation degree. Our results showed that DNA methylation levels at *BDNF* promoter IV were increased in suicide victims compared with 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 postmortem samples of brain tissue extracted from the Wernicke area obtained from 44 suicide completers and 33 nonsuicide control subjects. Most of the autoptic samples were collected within 12 to 26 hours of the subject's death (postmortem interval information is reported in the eTable, http://www.archgenpsychiatry.com), in the course of autopsy at the Institute of Forensic Medicine, University of Ljubljana, between 1999 and 2005, and stored at –80°C in the care of the Biological Bank of the Institut za Varovanje Zdravja, Ljubljana, Slovenia. This study was performed according to the ethical requirements of the institution. All studied subjects were of white ethnicity and all were right handed. Data on subjects' sex, age, drug history, and cause of death were gathered from the subjects' records. Data on psychiatric diagnoses were obtained from the physician's note for autopsy, and in the cases in which a coroner's inquest was done in the presence of relatives, their testimony was taken into consideration. A detailed description of the subjects analyzed in this study is reported in the eTable.

pH MEASUREMENT AND DRUG ANALYSIS

Blood was taken from the subjects to perform general toxicological screening using gas chromatography–mass spectrometry. The analysis of pH of the homogenate was performed using the automated analyzer 865 (Bayer Leverkusen, Germany, now Siemens, Malvern, Pennsylvania). A 50- to 100-mg sample of liquid nitrogen–pulverized tissue was mixed with distilled deionized water in a 10% (weight to volume ratio) solution. To confirm blood data, we also performed drug analysis on homogenates, using the REMEDI column-switching LC instrument (Bio-Rad Laboratories, Hercules, California), which detects about 700 drugs or metabolites, including most illicit substances and drugs of abuse or their direct metabolites.[21]

DNA AND RNA EXTRACTION FROM TISSUES

DNA and RNA were extracted, from each sample, from a portion of liquid nitrogen–pulverized tissue. DNA was prepared using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the instruction manual. Total RNA was extracted from tissues using TRI Reagent solution (Invitrogen, Carlsbad, California), according to the manufacturer's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis (eFigure). RNA quality and quantity assessment was performed by an ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, Delaware). All samples showed a quality ratio (260: 280-260:230) between 1.8 and 2.2, which is considered an optimal range.[22] Negative controls were obtained by performing polymerase chain reaction (PCR) on samples that were not reverse transcribed but otherwise identically processed.

BISULFITE TREATMENT

Sodium bisulfite conversion of genomic DNA (2 μg) was obtained using Epitect Bisulphite kit (Qiagen), following the manufacturer's instructions. Amplicons used for the different methylation analyses were obtained from appropriate amounts of bisulfite-treated genomic DNA.

DNA METHYLATION ANALYSIS

Pyrosequencing

Pyrosequencing technology[23] was used for DNA methylation quantitative analysis of the *BDNF* gene and was performed using the PSQ 96MA instrument (Biotage AB, Uppsala, Sweden), following the manufacturer's protocol. The reactions were assayed on the PSQ 96MA using the single-nucleotide polymorphism analysis software. Global DNA methylation analysis was performed using the PyroMark LINE-1 assay kit (Biotage AB), according to the manufacturer's instructions. Long interspersed nucleotide elements (LINE-1) represent about 15% of the human genome; thus, quantitative DNA methylation analysis of LINE-1 may be considered a surrogate analysis of global DNA methylation.[24] Primer sequences and detailed protocol for *BDNF* and global methylation analysis can be found in the supplementary Methods section.

Bisulfite Genomic Sequencing (Cloning Technology)

Two microliters of each sample were used as a 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). Amplifications and the cloning procedure were performed as previously described.[25] At least 20 independent clones were sequenced to determine the methylation pattern of individual molecules.

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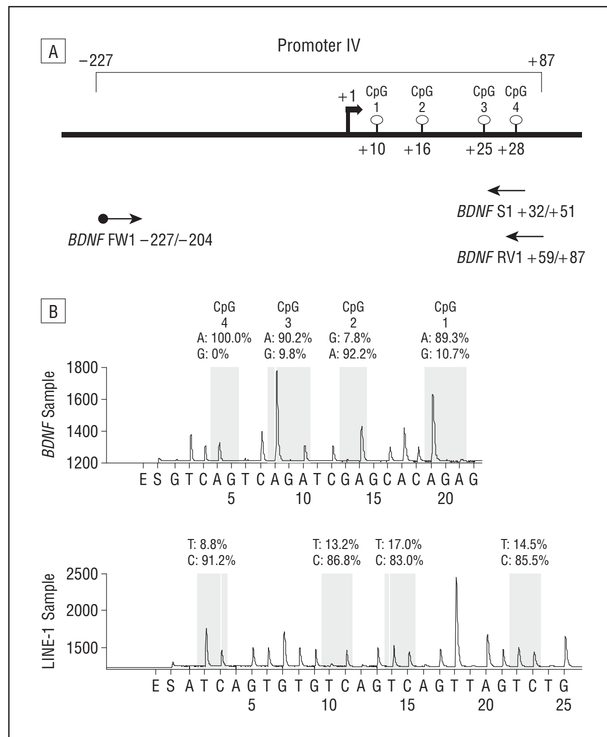

Figure 1. Pyrosequencing analysis of the *BDNF* gene. A, Structure of the human *BDNF* gene promoter IV. The transcriptional start site (+1) is indicated by an arrow. The positions of the CpG sites analyzed (open circles) and of the primers used (arrows) for pyrosequencing analysis are indicated. *BDNF*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

MassARRAY Platform

The MassARRAY platform (Sequenom, San Diego, California) uses matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base–specific cleavage (MassCLEAVE; Sequenom). A detectable pattern was then analyzed for methylation status. The MassCLEAVE biochemistry was performed as previously described.[26] Mass spectra were acquired by using MassARRAY Compact MALDI-TOF mass spectrometry (Sequenom) and the spectra methylation ratios were generated by EpiTYPER software (version 1.0; Sequenom). Further technical information and primer sequences can be found in the supplementary Methods section. Presence of a CpG island in the genomic region analyzed was assessed by using CGplot software (http://www.ebi.ac.uk/emboss/cpgplot/).

REAL-TIME REVERSE TRANSCRIPTION–PCR

One microgram of total RNA of each sample was reverse transcribed with the QuantiTect Reverse Transcription kit (Qiagen) using an optimized blend of oligo-dT and random primers, according to the manufacturer's instructions. Primer sequences and detailed protocol for *BDNF* and the internal control genes' quantitative mRNA analysis can be found in the supplementary Methods section. The strategy used for the normalization of quantitative real-time reverse transcription–PCR (RT-PCR) data was geometric averaging of multiple internal control genes according to the Vandesompele et al[27] method. We evaluated 4 housekeeping genes (*GAPDH*, *HPRT I*, *UBC*, and *RP II*) that represent accurate controls for mRNA expression analysis of postmortem brain samples.[27-29] For each housekeeping gene, we measured the gene stability and ranked it using the geNorm algorithm.[27] Stepwise exclusion of the gene with the highest gene stability value allowed ranking of the tested genes according to their expression stability. We applied to each sample the delta Ct formula for transforming Ct values to relative quantities ($Q = E^{(\text{minCt}-\text{sampleCt})}$), as described elsewhere.[27] We calculated the normalization factor based on the geometric mean of quantity obtained by the transformation of Ct data. A sample (sample ID 920/04), arbitrarily chosen among control subjects' RNA, was used as a reference sample. Amplification over 32 cycles was considered out of linear range.[30]

STATISTICAL ANALYSES

We performed statistical analyses with the SPSS software package (version 13.0; SPSS, Chicago, Illinois). We used 1-way analysis of variance to compare methylation levels between sample groups. *P* values $<.05$ were considered statistically significant. The Mann-Whitney *U* test was used to compare nongaussian distribution. Analysis of covariance was used to assess the possible influence of sex and age.

RESULTS

METHYLATION ANALYSIS OF *BDNF* PROMOTER IV IN THE WERNICKE AREA OF SUICIDE VICTIMS AND NORMAL CONTROL SUBJECTS

To investigate a possible correlation between the DNA methylation state of the *BDNF* gene and suicidal behavior, we analyzed genomic DNA samples derived from postmortem brain samples extracted from the Wernicke area of 44 suicide completers (21 men and 23 women; age range, 15-79 years) and 33 nonsuicide controls (16 men and 17 women; age range, 13-76 years). A detailed description of the subjects analyzed in this study, including psychiatric diagnoses, medication, and toxicological findings, is reported in the eTable.

We chose to analyze the methylation status of 4 CpG sites (+10, +16, +25, and +28) located downstream the transcription initiation site of promoter IV of the *BDNF* gene (**Figure 1**A). 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 to assess the precise degree of methylation of each CpG site. 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 the **Table**. The analysis was repeated 3 times and each value did not differ significantly (±0.7%). The mean methylation degree of the 4 CpG sites in each subject is also reported in the Table and graphically shown in **Figure 2**. Overall, the results showed that the mean methylation degree of the 4 CpG sites was always less than 12.9% in the 33 nonsuicide control subjects, while in 13 of 44 sui-

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Table. Methylation Analysis Data

Sample ID[a]	Subject	Degree of Methylation					
		CpG 1	CpG 2	CpG 3	CpG 4	Mean	Global
156/05	MC	8.4	4.3	7.9	3.5	6.025	83.48
202/02	MC	0	19	0	0	4.75	87.24
437/02	MC	11.2	14	0	0	6.3	85.36
142/00	MC	5.1	0	9.6	0	3.675	83.96
146/00	MC	8.6	0	15	0	5.9	88.75
604/01	MC	0	0	10.7	0	2.675	83.45
545/02	MC	0	10.9	17.5	0	7.1	87.87
201/02	MC	0	0	26.7	0	6.675	87.41
454/02	MC	0	0	11	0	2.75	85.6
447/02	MC	34.9	0	16.7	0	12.9	84.41
471/02	MC	6.2	4.5	9.2	0	4.975	88.8
487/02	MC	0	0	0	0	0	84.4
576/02	MC	3.3	1.6	5.7	0	2.65	85.45
626/02	MC	4	2.6	6.3	9.7	5.65	88.42
708/02	MC	4.4	2	4.8	1.5	3.175	89.92
457/02	MC	8.4	2.5	5.3	2.6	4.7	84
72/00	FC	0	7.6	8.4	0	4	90.55
138/00	FC	6.5	3.3	7	0	4.2	86.25
931/04	FC	0	4.4	7.1	3	3.625	86.52
94/05	FC	4.4	3.7	5.1	6.5	4.925	88.75
28/05	FC	0	6.5	12.8	6.9	6.55	88.02
773/03	FC	0	16.5	0	14.4	7.725	87.24
404/05	FC	0	0	0	15	3.75	85.82
372/05	FC	16.2	8.8	0	0	6.25	84.85
136/00	FC	19	11.2	0	10.2	10.1	81.95
149/05	FC	0	10.3	15.8	11.2	9.325	86.2
498/05	FC	0	0	0	12.8	3.2	88.5
485/02	FC	0	0	0	7.7	1.925	90.2
547/01	FC	0	0	13.1	7.6	5.175	86.45
907/04	FC	6.8	5	10.6	0	5.6	81.82
79/05	FC	0	0	12.8	0	3.2	87.3
882/04	FC	3.8	2	4.8	0	2.65	82.41
593/02	FC	12.4	10.7	12.1	7.2	10.6	86.52
674/04	MS	19.7	8.4	24.7	9.7	15.625	87.23
179/04	MS	11.2	6.8	11.8	0	7.45	89.52
727/03	MS	37.1	0	47.2	0	21.075	88.91
447/03	MS	45	25.6	42.2	24	34.2	86.75
315/02	MS	0	0	0	0	0	84.35
920/04	MS	5.8	4.3	6.9	0	4.25	90.2
1015/04	MS	13	7.2	14.9	6.5	10.4	86.32
578/02	MS	0	0	7.2	0	1.8	82.85
110/04	MS	0	0	18.2	0	4.55	83.4
22/02	MS	6.9	4.8	10.7	0	5.6	80.3

(continued)

cide victims (30%), the mean methylation degree ranged between 13.1% and 34.2% (Figure 2 and the Table). To establish whether the observed differences in the mean methylation degree between suicide and nonsuicide subjects were statistically significant, we performed 1-way analysis of variance. As shown in **Figure 3**A, significant differences were clearly discernible in the mean methylation status of the region analyzed between cases and controls (1-way analysis of variance, $F = 13.7$; $P = .001$). By the same method, we analyzed the relationship between the methylation degree of each of the 4 CpG sites and suicidal behavior. Statistically significant differences between cases and controls were found for the CpG sites +10 and +25 (Figure 3B). A similar, but not statistically significant, trend to hypermethylation was found for the other 2 CpG sites tested (+16 and +28). We also analyzed the data by a nonparametric test (Mann-Whitney *U* test), obtaining similar results. Statistically significant differences between cases and controls were found for the mean methylation of the region (Mann-Whitney *U* test, $P = .001$) and for CpG sites +10 and +25 ($P = .002$ and .001, respectively). No effect of sex or age was found by analysis of covariance (data not shown).

Next, because the global DNA methylation state of specific brain areas could vary among individuals, we addressed whether an increased methylation degree of *BDNF* promoter/exon IV in suicide victims was associated with higher methylation levels throughout the genome or whether it was a specific feature of the *BDNF* gene. We studied global methylation in all suicide and control samples by quantitatively analyzing the methylation of LINE-1, using pyrosequencing. A representative pyro-

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Table. Methylation Analysis Data (continued)

Sample ID[a]	Subject	Degree of Methylation					
		CpG 1	CpG 2	CpG 3	CpG 4	Mean	Global
888/99	MS	0	7.6	13.8	0	5.35	81.62
598/01	MS	37.9	17.6	31.6	24.5	27.9	90.45
190/02	MS	0	0	16.7	0	4.175	84.75
288/02	MS	35.8	0	40.8	0	19.15	85.12
371/02	MS	0	0	22.1	0	5.525	81.23
199/02	MS	0	0	26.8	0	6.7	80.82
665/01	MS	49.4	0	43.5	25	29.475	86.92
540/02	MS	9.3	0	10.6	0	4.975	87.21
579/02	MS	7.9	0	8.6	0	4.125	85.7
555/02	MS	0	0	18.1	0	4.525	82.72
584/02	MS	4.2	0	5	2	2.8	89.95
84/04	FS	9.9	5.5	13.1	3.5	8	86.05
78/04	FS	34.8	19.1	33.5	17.4	26.2	80.25
103/05	FS	0	14.5	0	15.3	7.45	83.25
515/01	FS	6.5	6.1	8.4	27.6	12.15	82.09
456/03	FS	19.3	6.4	7.3	2.9	8.975	86.05
703/02	FS	24.6	22.5	42.2	18.1	26.85	87.35
617/03	FS	9.3	3,6	5.3	0	4.55	88.54
427/04	FS	4.9	2	7.4	59	18.325	85.5
524/02	FS	5.6	2.7	10.6	0	4.725	84.65
690/01	FS	9.6	0	8.3	0	4.475	90.52
799/04	FS	8	5.6	8.9	3.8	6.575	85.25
22/00	FS	17.6	0	20	0	9.4	89.54
101/00	FS	16.6	14.6	17.1	10.4	14.675	88.45
63/02	FS	13.9	0	18.4	0	8.075	82.25
108/02	FS	10.5	0	11.2	0	5.425	86.98
668/03	FS	3.6	0	6	4.1	3.425	89.32
99/04	FS	0	12.7	18.4	11.5	10.65	82.52
43/02	FS	27.6	25.1	0	21	18.425	86.45
228/02	FS	0	17.6	0	16.3	8.475	86.76
259/02	FS	36	4.6	6.2	5.7	13.125	87.87
498/02	FS	22.6	8.8	16.9	8.9	14.3	87.24
489/02	FS	9.5	4.8	13.4	4	7.925	86.58
679/02	FS	10.7	7.8	9.8	0	7.075	83.85

Abbreviations: FC, female control; FS, female suicide victim; MC, male control; MS, male suicide victim.
[a] Sample ID: progressive number/year of death.

gram is shown in Figure 1B, bottom panel. Results, summarized in the Table, 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 were not associated with suicidality, sex, or age (data not shown). Finally, we verified whether psychopathological conditions or past or current use of specific medications could influence the *BDNF* gene methylation state. Drug history indicated that no subject used psychoactive substances during life other than those detected by toxicological screens and reported in the eTable. We found no significant correlation between any of these variables and the methylation degree at the *BDNF* promoter (Mann-Whitney *U* test, $P = .30$ and .14, respectively).

Overall, our results show that *BDNF* promoter/exon IV is hypermethylated in the postmortem brain Wernicke area of suicide subjects compared with normal control subjects irrespective of global methylation levels, suggesting that a gene-specific increase in DNA methylation could cause or contribute to downregulation of *BDNF* expression in suicide subjects.

EXTENDED METHYLATION ANALYSIS BY MOLECULAR CLONING AND MassARRAY

To extend the methylation analysis to a wider genomic region and to both upper and lower strands, we performed 2 additional 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 (n = 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 4**). 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 proximity of the TSS. The results confirmed that sites +10, +16, +25, and +28 were differentially methylated between suicide and control subjects, showing methylation values comparable with those obtained by pyrosequencing analysis. In addition, to confirm the pyrosequencing data,

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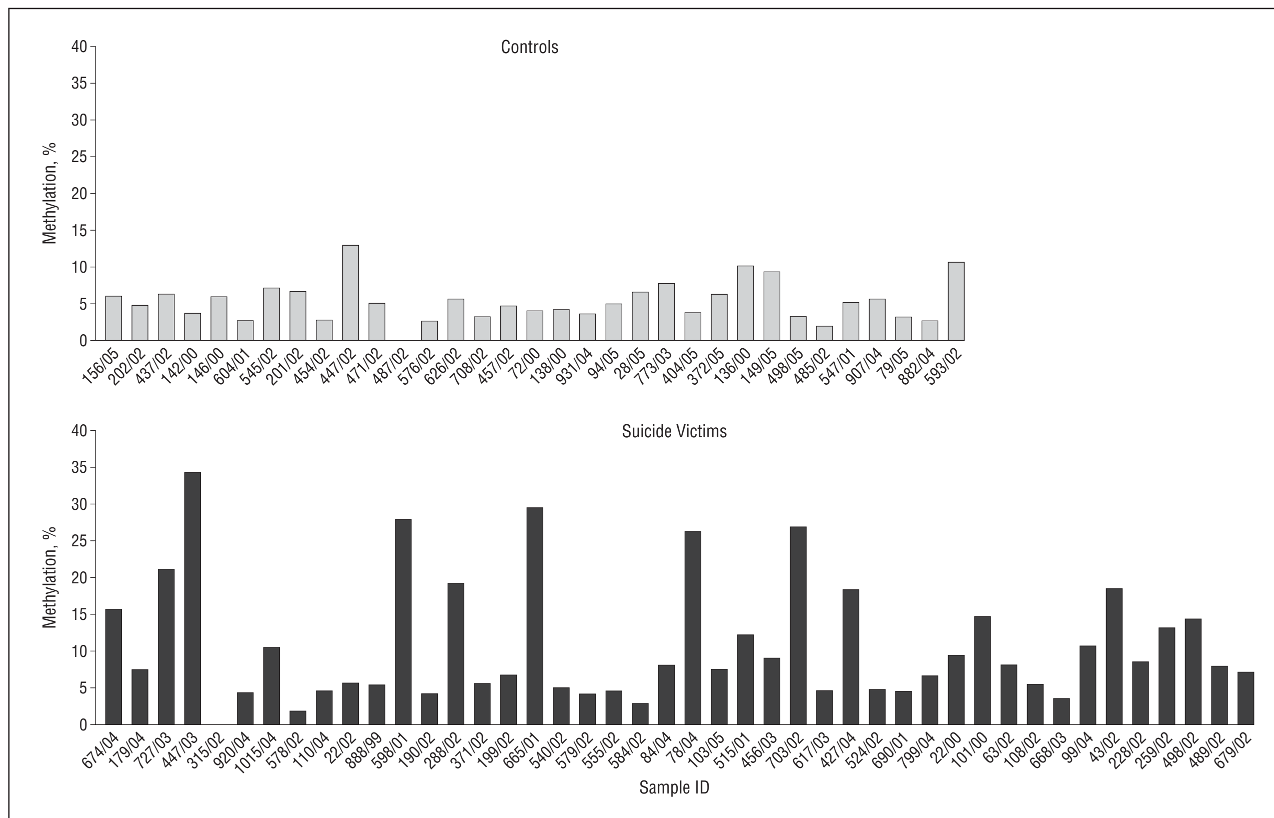

Figure 2. Percentage of *BDNF* promoter IV methylation in suicide subjects and nonsuicide controls. Each subject is identified by a sample ID. For each sample, the percentage of methylation represents the mean methylation degree of the 4 CpG sites analyzed.

this analysis showed that CpG site −93 was hypermethylated in almost all samples irrespective of group (suicide and control subjects), age, or sex. Finally, by MassARRAY, we analyzed the genomic region from −219 to +405 on the upper strand, including 30 CpG sites, and the region from −289 to +512 on the lower strand, including 34 CpG sites (**Figure 5**). This analysis showed that also in this wider genomic region most of the 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 remained differentially methylated. Differences in the methylation degree of these same sites detected on the lower strand were slighter but a higher methylation level was detected at some of these CpG sites where the correspondent sites on the upper strand were hypermethylated. Finally, frequent hypermethylation of −93 on the upper strand site was confirmed in these experiments. Slight differences in the methylation degree at some CpG sites observed in the analysis by the different technical approaches were possibly due to the different sensitivity of the methods.

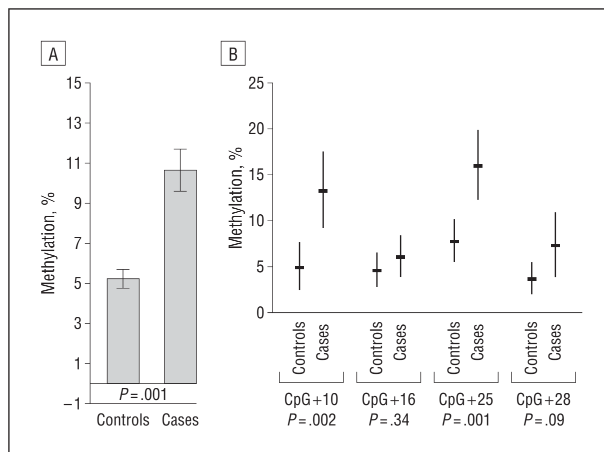

Figure 3. Statistical analysis of the methylation status of 4 CpG residues in the region of promoter IV of the *BDNF* gene in suicide and control subjects. A, Average methylation of the 4 CpG sites. Values represent mean (SE). B, Methylation of individual CpG sites. Values represent mean and 95% confidence interval.

RELATIONSHIP BETWEEN *BDNF* EXPRESSION AND *BDNF* PROMOTER IV METHYLATION DEGREE

To assess whether hypermethylation of *BDNF* promoter IV (CpG sites +10, +16, +25, and +28) was associated with 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 the suicide group and 6 from the control group) with a low level of methylation at *BDNF* promoter IV, 9 samples (4 from the suicide group and 5 from the control group) showing intermediate methylation levels, and 5 samples from suicide subjects with the highest methylation degree (**Figure 6**). For this quantitative RT-PCR assay, we used specific prim-

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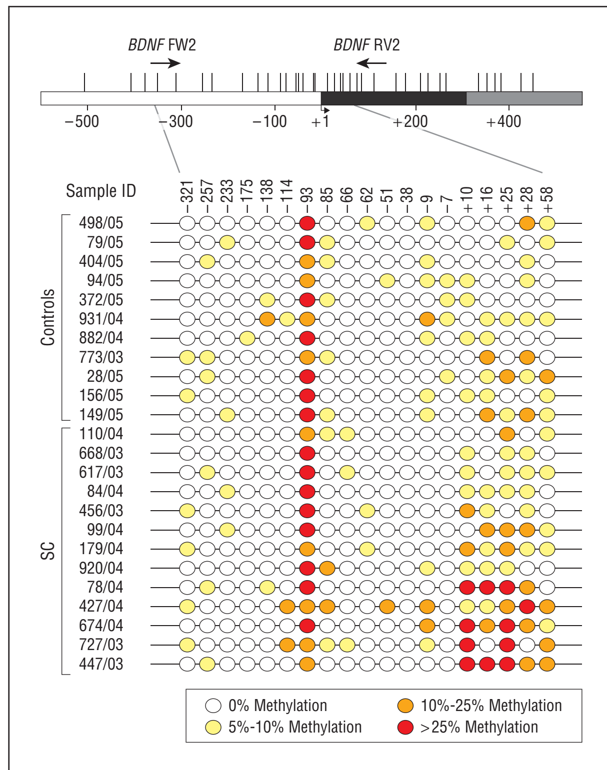

Figure 4. Methylation analysis by genomic bisulfite sequencing. Top panel: Diagrammatic representation of *BDNF* gene (promoter/exon IV). Regulatory upstream region (open box), exon IV (black box), and intron IV (gray box) are indicated. Vertical bars represent the relative positions of each CpG site. The transcriptional start site is indicated by an arrow. The relative positions of the primers used for amplification (*BDNF* FW2 and *BDNF* RV2) are indicated. Bottom panel: The methylation degree of each CpG site (circles). Each subject is identified by a sample ID. SC indicates suicide completers; controls, nonsuicide subjects.

ers for the analysis of *BDNF* mRNA transcript originating from promoter IV. The analyzed samples showing a high-methylated *BDNF* promoter IV expressed lower levels of *BDNF* mRNA as compared with the low- and medium-methylated samples, being the mildly methylated samples in the midrange (Figure 6).

Taken together, our data indicate that a higher-methylated *BDNF* promoter IV in the Wernicke area of the brain correlates with suicidal behavior and suggests that increased DNA methylation levels of *BDNF* promoter IV can negatively regulate *BDNF* expression.

COMMENT

We analyzed, by 3 independent sensitive quantitative methods, the DNA methylation degree at *BDNF* promoter IV and the global DNA methylation levels in the Wernicke area of the brain of 44 suicide subjects and 33 nonsuicide control subjects. The main conclusions of our work are (1) suicide subjects showed a statistically significant increase of DNA methylation at *BDNF* promoter IV and this higher methylation degree corresponded to a lower level of *BDNF* transcript IV; (2) such an increase in CpG methylation was gene specific since it was not accompanied by an increase of global DNA methylation; and (3) global DNA methylation levels in the Wernicke area varied among individuals but did not correlate with suicidal behavior and were not dependent on sex or age.

To our knowledge, 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 area of suicide completers and control subjects who died of other causes. The Wernicke area was chosen for its function in understanding word meaning and semantic thinking and for its critical involvement with human language and associative and integrative functions. This fact is consistent with several findings of neurocognitive alterations in suicide attempters, such as an impairment in decision making[31,32] and problem solving.[33] The Wernicke area and its connections with other brain structures represent a unique feature of the human brain, as suicidal behavior is peculiar to humans, and may influence many factors, including human social behavior.[34] Completed suicide cannot be considered a sudden and casual death but is the outcome of a process that involves a wide spectrum of thoughts, communications, and acts. It is consistent with this assumption that in suicidal behavior gene expression may be altered in a cortical area that has highly specialized integrative and associative functions. Moreover, postmortem studies reported an age-related expression of *BDNF* in the temporal cortex,[35] suggesting that this neurotrophin is important in the early development of the temporal cortex. Suicidal behavior 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 the *BDNF* gene derived from previous evidence that *BDNF* mRNA and protein levels are decreased in different postmortem brain areas (hippocampus and frontal cortex)[11,12] and in the plasma[36] of suicide victims compared with nonsuicide controls. Human *BDNF* expression is controlled by a very complex regulatory region including 9 different transcription initiation sites driven by corresponding promoters.[14] We chose to analyze the 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.[6,15-17] 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,[18,19] chronic social defeat stress, and antidepressant administration.[6] 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 behavior, including psychiatric disorders.[20,37,38] Maya Vetencourt et al[39] showed that cortical administration of diazepam prevents the fluoxetine hydrochloride–mediated *BDNF* activation in the visual cortex, raising the interesting question of whether changes of *BDNF* expression or methylation state in suicide subjects may be influenced by drug consumption. However, in this

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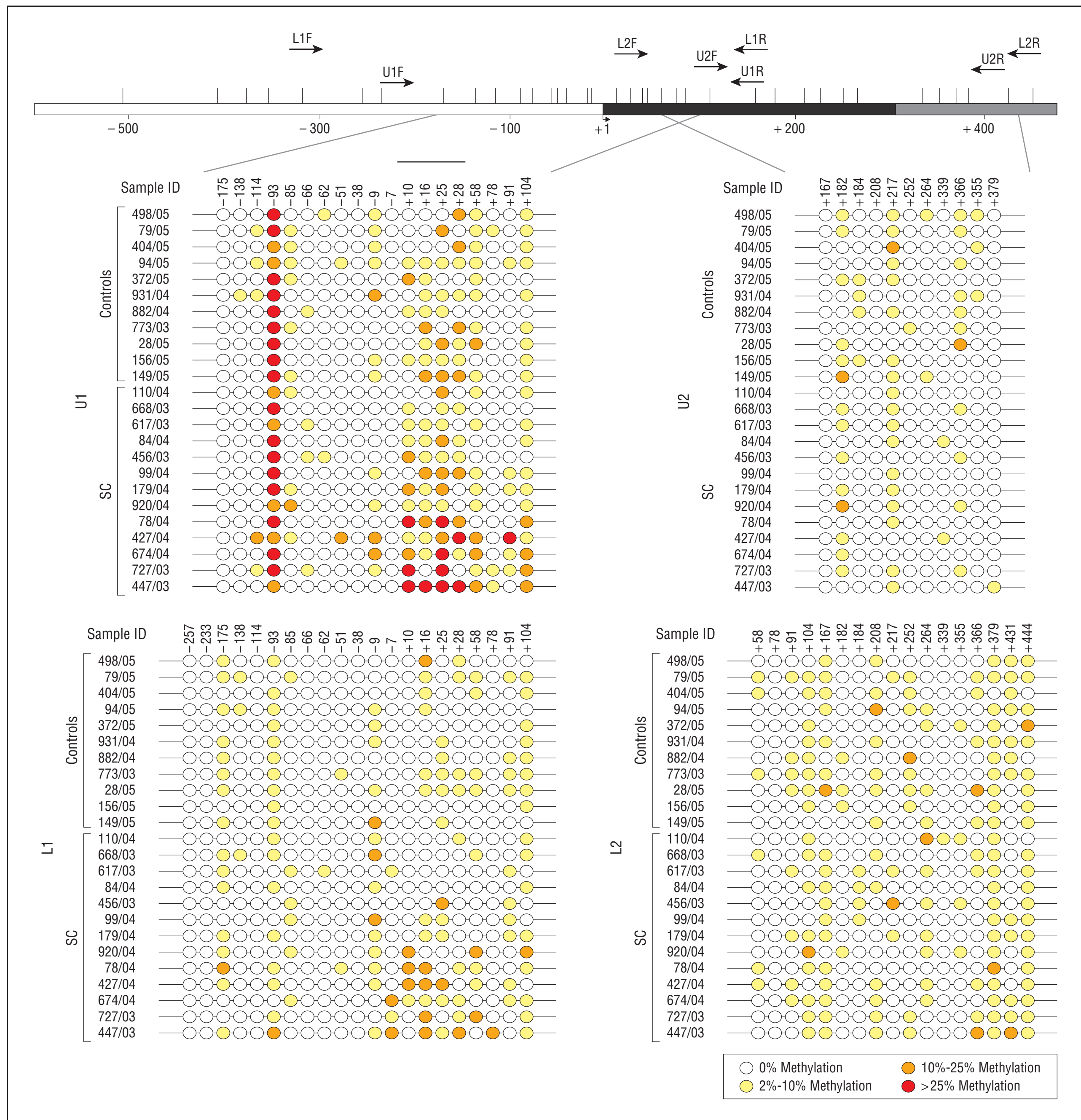

Figure 5. Methylation analysis by MassARRAY (Sequenom, San Diego, California). Top panel: Diagrammatic representation of the *BDNF* gene (promoter/exon IV) as in Figure 4. The relative positions of the primers used for amplification of amplicons U1 (upper strand from −218 to +160), U2 (upper strand from +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 panel: Comprehensive view of methylation state of the *BDNF* gene. The methylation degree of each CpG site (circles) belonging to the amplicons U1, U2, L1, and L2 is indicated. CpG sites analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry 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 the average of 2 adjacent CpG sites are indicated. Each subject is identified by a sample ID. SC indicates suicide completers; controls, nonsuicide subjects.

study, we did not find any relationship between *BDNF* methylation or expression and diazepam or fluoxetine consumption in the study subjects. McGowan et al[40] demonstrated that in the brain of 11 suicide subjects with history of early childhood abuse, the ribosomal RNA gene was downregulated and hypermethylated compared with control subjects. Very interestingly, Ernst et al[41] found that trkB is hypermethylated in suicide completers in different brain areas. Our study, relating the *BDNF* gene methylation state to suicidal behavior, 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 the *BDNF* gene to other brain areas involved in suicidal behavior.

Previous studies revealed that the methylation of specific CpG sites in the rat and mouse *bdnf* promoter IV may play a critical role in *BDNF* gene regulation.[15,18] In particu-

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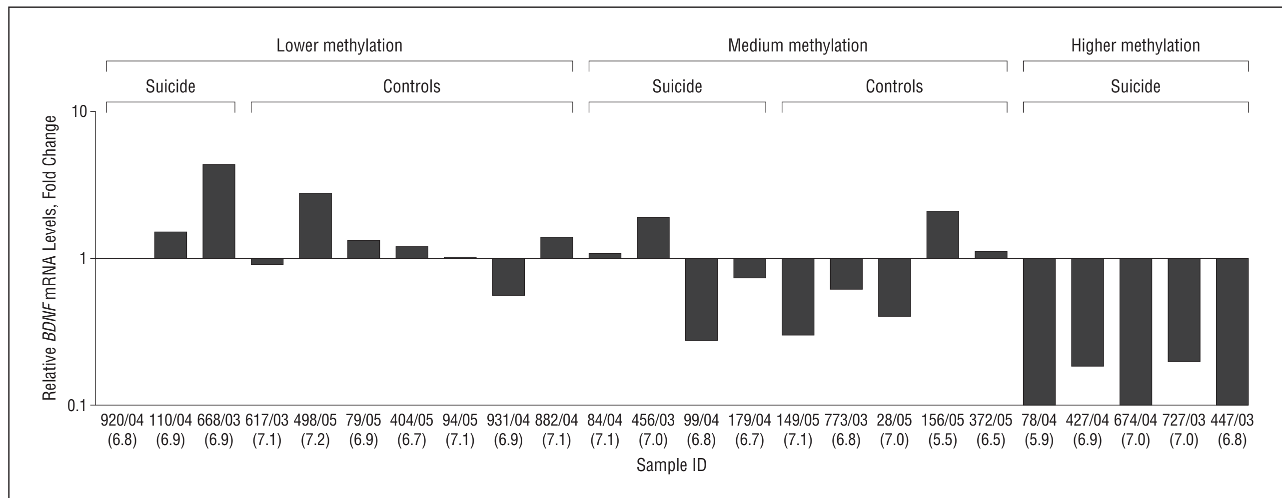

Figure 6. Relative messenger RNA (mRNA) levels of *BDNF* transcript IV. The *BDNF* expression was normalized using 4 internal control genes (as described in the "Methods" section) and relative expression levels are shown. The pH value of each sample is indicated in parentheses. No significant correlation was found between pH values and mRNA expression or methylation degree (Mann-Whitney *U* test, $P = .25$ and .21, respectively). The cutoff line (bottom) indicates that detection was over 32 cycles.

lar, 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 transcriptional repression.[18,19] 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 of CpG sites +10 and +25 is associated with suicidal behavior. Moreover, we showed that a 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 the human brain. Because we found much lower *BDNF* transcript IV mRNA levels in samples showing 20% to 30% methylation of 4 CpG sites in *BDNF* promoter IV compared with samples showing 3% to 5% methylation, we hypothesize that other mechanisms, including additional epigenetic mechanisms and/or lack of transcription factors, may contribute to such a strong repression. 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 to study the possible association of the epigenetic state of other promoters with suicidal behavior. DNA methylation and other epigenetic factors could also provide some explanation for reported conflicting data on the association of *BDNF* gene polymorphisms with suicidal behavior.[9,10]

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 with the rate reported by other studies.[42] 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.[43] 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 behaviors and represents one of the first demonstrations that alteration of gene-specific DNA methylation in the human brain is associated with suicidal behavior.

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