Increased BDNF Promoter Methylation in the Wernicke Area of Suicide Subjects

Simona Keller, PhD; Marco Sarchiapone, MD; Federica Zarrilli, PhD; Alja Videtić, PhD; Angelo Ferraro, PhD; Vladimir Carli, MD, PhD; Silvana Sacchetti, PhD; Francesca Lembo, PhD; Antonella Angiolillo, PhD; Nikolina Jovanovic, MD; Francesco Pisanti, MD; Rossella Tomaiuolo, MD, PhD; Antonella Monticelli, MD; Jože Balazić, MD; Alec Roy, MD; Andrej Marusic, MD†; Sergio Cocozza, MD; Alfredo Fusco, MD, PhD; Carmelo B. Bruni, MD, PhD; Giuseppe Castaldo, MD, PhD; Lorenzo Chiariotti, MD; Joze Balazic, MD; Alec Roy, MD; Andrej Marusic, MD†; Sergio Cocozza, MD; Alfredo Fusco, MD, PhD; Carmelo B. Bruni, MD, PhD; Giuseppe Castaldo, MD, PhD; Lorenzo Chiariotti, MD

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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man BDNF gene is controlled by a complex regulatory region, well conserved in mouse and rat, that includes at least 9 promoters each driving transcription of BDNF messenger RNA (mRNA) transcripts containing 1 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. The DNA methylation state of CpG sites within mouse promoter/exon IV is correlated with the expression of BDNF in the developing mouse forebrain. 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. Moreover, promoter IV is the major target of chromatin changes associated with alteration of BDNF expression in mouse models of neuropsychiatric disorders. However, to our knowledge, no data on the target of chromatin changes associated with alteration of BDNF expression 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 non-suicide control subjects. Most of the autopsic 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.

### 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. 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 Bisulfite 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 was used for DNA methylation quantitative analysis of the BDNF gene and was performed using the PSQ 96/96MA instrument (Biotage AB, Uppsala, Sweden), following the manufacturer’s protocol. The reactions were assayed on the PSQ 96/96MA 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. 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′-ggggaggatttaTtgagTTgtg-3′ (position from nucleotides −358 to −332) and BDNF RV2 5′-ccctacaaraaaaactcatt-tactc-3′ (from nucleotides +59 to +87). Amplifications and the cloning procedure were performed as previously described. At least 20 independent clones were sequenced to determine the methylation pattern of individual molecules.

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**Toxicology**

Toxicology measures were performed to determine the type and amount of drugs or metabolites in the subjects' blood samples. These analyses were performed using gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). The GC-MS method was used for the detection of volatile organic compounds (VOCs), including alcohols, ketones, and hydrocarbons. The LC-MS/MS method was used for the detection of non-volatile organic compounds (NVOCs), including drugs and metabolites. The results of these analyses were used to determine the possible effects of drugs on the subjects’ mental状态 and behavior.

**Methylation Analysis**

DNA methylation analysis was performed using the PyroMark Bisulfite kit (Qiagen). The bisulfite conversion of DNA was followed by quantitative PCR analysis using the PyroMark Q24 instrument (Qiagen). The results were used to determine the methylation status of the BDNF gene in the postmortem brain samples.

**Global Methylation Analysis**

Global methylation analysis was performed using the PyroMark Q24 instrument (Qiagen). The results were used to determine the overall DNA methylation pattern in the postmortem brain samples.
STATISTICAL ANALYSES

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, 13-76 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 1A). 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-
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 3A, 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 $/H_11001$ and $/H_11001$ (Figure 3B). A similar, but not statistically significant, trend to hypermethylation was found for the other 2 CpG sites tested ($/H_11001$ and $/H_11001$). 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 $/H_11001$ and $/H_11001$ ($P = .002$ 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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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 \textit{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 \textit{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 \textit{BDNF} promoter (Mann-Whitney \textit{U} test, \( P = .30 \) and \( .14 \), respectively).

Overall, our results show that \textit{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 \textit{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 \(H11001\) \(58\) (upper strand) encompassing the TSS of the \textit{BDNF} gene and including 16 CpG sites (Figure 4). The results showed that the analyzed \textit{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 \(H11001\) \(10\), \(H11001\) \(16\), \(H11001\) \(25\), and \(H11001\) \(28\) 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,
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.

**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-
BDNF samples in the midrange (Figure 6).

dium-methylated samples, being the mildly methylated

subjects. The main conclusions of our work are (1) suicide

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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.

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 and problem solving. 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. 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, 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) and in the plasma 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. 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. 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, chronic social defeat stress, and antidepressant administration. 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. Maya Vetencourt et al 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
Our study, relating the permethylated in suicide completers in different brain areas. 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 BDNF methylation state of the SC indicates suicide completers; controls, nonsuicide subjects.

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.

Study, we did not find any relationship between BDNF methylation or expression and diazepam or fluoxetine consumption in the study subjects. McGowan et al 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 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. In particular,
controls, 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. 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 +23 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.

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. 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. 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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Author Affiliations: Dipartimento di Biologia e Patologia Cellulare e Molecolare and Istituto di Endocrinologia ed Oncologia Sperimentale del CNR (Drs Keller, Sacchetti, and Monticelli and Profs Lembo, Chiariotti, Coccozza, Fusco, and Bruni), Dipartimento di Biochimica e Biotecnologie Mediche (Dr Tomaiuolo and Prof Castaldo), Dipartimento di Chimica Farmaceutica e Tosicologica, Facoltà di Farmacia (Profs Lembo and Chiariotti), Università degli Studi di Napoli Federico II, CEINGE–Biotecnologie Avanzate (Drs Keller, Sacchetti, Ferraro, and Tomaiuolo and Profs Fusco, Castaldo, and Chiariotti), NOGEC (Naples Oncogenomic Centre), European School of Molecular Medicine (SEMM) (Drs Keller, Ferraro, and Sacchetti and Profs Fusco and Chiariotti), Patologia Clinica ASL Napoli 3 (Dr Pisanti), Naples, Dipartimento di Scienze per la Salute, Università del Molise, Campobasso (Prof Sarchiapone and Drs Carli and Jovanovic), Fondazione Leonardo per le Scienze Mediche, Montegrotto, Padova (Prof Sarchiapone and Drs Carli and Jovanovic), Facoltà di Scienze MFN, Università del Molise, Isernia (Drs Zarrilli and Angiolillo), Italy; University of Ljubljana, Ljubljana (Drs Videtič and Balazic), and Health Research Department, PINT, University of Primorska, Koper (Dr Marusic), Slovenia; National Prevention of Suicide and Mental Ill-Health (NASP) at Karolinska Institutet, Stockholm, Sweden (Dr Carli); Department of Psychiatry, School of Medicine, University of Zagreb, Zagreb, Croatia (Dr Jovanovic), and Department of Veteran Affairs East Orange, East Orange, New Jersey (Dr Roy).

Correspondence: Lorenzo Chiariotti, MD, PhD, Dipartimento di Biologia e Patologia Cellulare e Molecolare,
12. Dwivedi Y, Mondal AC, Rizavi HS, Conley RR. Suicide brain is associated with
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Author Contributions:

Dr s Keller and Sarchiapone contributed equally to this work.

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1. Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in

2. Tyler WJ, Alonso M, Bramham CR, Pozzo-Miller LD. From acquisition to con-
solidation: on the role of brain-derived neurotoxic factor signaling in hippocamp-

3. Yamada K, Misuno M, Nabeshima T. Role for brain-derived neurotoxic factor in

4. Bolaños CA, Nestler EJ. Neurotrophic mechanisms in drug addiction. Neuro-

D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ.

phorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic

7. Zilman N, Renthal W, Kurum AE. Epigenetic regulation in psychiatric

8. Fitzpatrick RM, Rivera JD, Herold DA. Broad spectrum drug identification directly from
1999;45(8, pt 1):1224-1234.

9. Feige S, Pfaff M. DNA integrity and the effect on the real-time qRT-PCR


for estimating global DNA methylation using bisulfite PCR of repetitive DNA

phorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic

13. Zilman N, Renthal W, Kurum AE. Epigenetic regulation in psychiatric

14. We thank Vittorio Enrico Avvedimento, MD, PhD, Pasquale De Luca, PhD, and Andrea De Bartolomeis, MD, PhD, for helpful scientific discussions and useful suggestions and G. Oriani, MD, PhD, for promoting collaboration among the participant groups. We dedicate this study to our beloved friend and colleague Jared Marusci, who largely contributed to the design and implementation of this research project. The absence of his brilliant mind constitutes a great loss for suicidology.

REFERENCES


11. Dvirvedi Y, Rizavi HS, Conley RR, Roberts RC, Tammenga CA, Pandey GN. Altered gene expression of brain-derived neurotoxic factor and receptor tyro-


17. Mellios N, Huang HS, Baker SP, Galdzicka M, Gins M, Abramian S. Molecular determinants of disrupted GABAergic gene expression in the prefrontal cor-
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