Increased DNA methylation in the suicide brain

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Clinical studies find that childhood adversity and stressful life events in adulthood increase the risk for major depression and for suicide. The predispositions to either major depression or suicide are thought to depend on genetic risk factors or epigenetic effects. We investigated DNA methylation signatures postmortem in brains of suicides with diagnosis of major depressive disorder. DNA methylation levels were determined at single C-phosphate-G (CpG) resolution sites within ventral prefrontal cortex of 53 suicides and nonpsychiatric controls, aged 16 to 89 years. We found that DNA methylation increases throughout the lifespan. Suicides showed an 8-fold greater number of methylated CpG sites relative to controls ($P<2.2\times10^{-16}$), with greater DNA methylation changes over and above the increased methylation observed in normal aging. This increased DNA methylation may be a significant contributor to the neuropathology and psychopathology underlying the risk of suicide in depression.

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Introduction

The World Health Organization estimates that nearly 1 million people die from suicide yearly (World Health Organization figures, 2012). Psychological autopsies find that about 90% of suicides in Western nations have a psychiatric illness at the time of suicide. Of those, about 60% are suffering from a mood disorder. Suicide risk is influenced by a wide range of factors. Although some genetic risk factors have been identified, environmental events such as childhood adversity or repeated exposure to life-threatening situations typical in war and active combat have also been reported to contribute to the increased risk of suicidal behavior.

Exposure to stress can confer lasting biological and behavioral effects, such as altered responses to stress, throughout the lifespan, and as such these effects may be mediated by epigenetic factors like DNA methyla-
tion (through addition of a methyl group to cytosine residues). Studies indicate that epigenetic alterations may contribute to the risk of suicide. For example, DNA methylation levels at the NGFI-A binding site of the human glucocorticoid receptor (hGR) promoter and at the rRNA promoter are increased in suicides with a history of childhood adversity compared with either controls and suicides without history of adversity. In both cases, hypermethylation was associated with decreased gene expression in hippocampus. History of childhood abuse in suicides has also been associated with site-specific hypermethylation in hippocampal hGR1B and 1C promoters. Moreover, serotonergic and γ-aminobutyric acid (GABA)ergic systems have been extensively studied in the context of suicide. The C allele of the 5-HT _2A_ receptor gene polymorphism is hypermethylated in leukocytes of suicide attempts though not in prefrontal cortex (PFC) of suicides. Increased DNA methylation was detected in the GABA _α_ receptor _α_1 gene in prefrontal cortex of suicides, together with increased DNA methyltransferase 3B (DNMT3B) in various brain regions such as the amygdala and brain stem. In the prefrontal cortex of suicides, promoter, hypermethylation, and lower gene expression were found for tyrosine kinase B (TrKB1). Hypermethylation of the brain-derived neurotrophic factor (BDNF) promoter IV has been reported in Wernicke’s area in suicides versus controls. Less methylation of specific CpGs in the promoter region of the S-adenosylmethionine decarboxylase (AMID1) and arginase (ARG2) genes was correlated with increased gene expression in Brodmann Area 44 in suicides compared with nonsuicide controls.

While the majority of these studies involved candidate genes previously implicated in suicide-related neuropathologies, Labonté et al utilized a genome-wide technique to examine DNA methylation changes in the hippocampi of suicide completers versus nonpsychiatric, sudden-death comparison subjects. A total of 366 differentially methylated promoters (273 hypermethylated and 93 hypomethylated) were found, including genes involved in learning, memory, and behavior. In the present study, we performed DNA methylation profiling on the orbital prefrontal cortex of 53 suicides and controls with a diagnosis of major depressive disorder (N=25) and in nonpsychiatric controls (N=28) focusing on DNA methylation perturbations associated with aging. Although a number of studies have reported directional epigenetic perturbations associated with aging in a multitude of tissue and organ systems including the human brain, to date no study has examined the dynamics of age-related epigenetic perturbations in suicide. These data represent a genome-scale study of DNA methylation dynamics associated with aging and suicide in postmortem human brain.

### Materials and methods

#### Samples and subjects

Human tissue specimens were obtained according to an institutional approved protocol. Tissue was coded and samples had no person identifiers. The brains were collected at forensic autopsies. With informed consent, family members were interviewed as part of a psychological autopsy to obtain demographic information and any evidence of psychiatric or somatic disease. At least one family member per sample, for both cases and controls, agreed to be interviewed for the purpose of a psychological autopsy, which generated an Axis I and Axis II diagnosis. Depressed-suicide cases (N=25) were selected according to the following criteria: death by suicide, diagnosis of major depressive disorder (DSM-IV), absence of psychotropic or illegal drugs on toxicological screens, death without prolonged agonal state or protracted medical illness. Controls (N=28) did not have an Axis I psychiatric disorder, were drug-free and died suddenly without a prolonged agonal period from causes other than suicide. Due to the potentially confounding effect of pharmacological treatments in epigenetic studies, only brain samples with toxicological screens that ruled out recent consumption of medications (including illicit and psychotropic drugs) were investigated.

Distribution of age at death in suicides and nonpsychiatric, sudden death controls were comparable (Figure 1), averaging 47±17 and 52±17 years among suicides and controls, respectively. All suicides also had a diagnosis of major depressive disorder. Brain pH ranged from 5.4 to 6.9 and the average postmortem interval (PMI) was 15±7 hours. Methylation patterns did not differ significantly by sex, pH, and PMI within specimens examined. Toxicological screens of body fluids and, in the majority of cases, brain tissue were used to rule out exposure to medications within the last 3 months ante-mortem that might alter DNA methylation.
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**Sample dissection**

A total of 53 cortical specimens from ventral prefrontal cortex were examined (Table I). The right cerebral hemisphere was sliced coronally at intervals of 2 to 5 cm, and the slices were rapidly frozen in Freon (dichlorodifluoromethane or 1,1,1,2 tetrafluoroethane). Frozen specimens were then stored at -80°C until use. Prefrontal cortex Brodmann area 47 (BA47) was dissected from frozen brain sections that had been transferred from -80 to -20°C for 2 h prior to gray matter sampling.

**Illumina Infinium HumanMethylation27 BeadChip**

CpG methylation of 27 578 CPG sites was determined using Illumina Infinium HumanMethylation27 BeadChip, described previously. In accordance with the manufacturer's protocol, 1 ug of genomic DNA was bisulfite converted with the DNA Methylation Bisulfite Kit (Zymo). Samples were then subject to whole-genome amplification (WGA) and enzymatic fragmentation. Each sample was then hybridized to the locus-specific oligos on the BeadChip: T for the unmethylated state and C for the methylated state. Single base extension was then performed with labeled dideoxynucleotides (ddNTPs) that can be measured and quantified. Methylation β values were quantified, by the intensity of the C allele over the combined intensities of the C and T alleles. To avoid confusion between the β value and parameters in the linear models used in our statistical analyses, hereafter we refer to the β value as methylation value. The Infinium assay is an extension of the GoldenGate assay. Methylation values for all samples were exported from GenomeStudio and samples with call rates below 95% were eliminated from subsequent analyses.

**Illumina Infinium HumanMethylation QC and analysis**

In addition to call-rate filtering, further data filtering was applied based on the following criteria: (i) probes that overlap with potential SNPs (annotated by dbSNP130) to eliminate methylation signal variability due to interindividual allelic variability; (ii) probes on sex chromosomes to avoid methylation bias in gender; and (iii) probes with detection P-values ≥0.05 in over 10% of the depressed suicide cases and control samples, respectively. The total number of target CpG sites after filtering was 20 493. CpG sites with coefficient of variation (CV) >0.1 (15 249 CpGs) were considered as informative and used for all subsequent analyses.

A linear regression model using centered age ($x_{i1}$) and phenotype ($x_{i2}$) as the predictor variables and methylation value ($y_i$) as the response variable was fit by the R function `aov`:

$$y_i = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \epsilon_i,$$

where $i$ denotes target CpGs. In order to eliminate potential age bias, we transformed standard age data to centered age (as $x_i = x_i - \bar{a}$, where $j$ corresponds to a specific sample). The sum of squares for the three variables in the model ($x_{i1}$, $x_{i2}$, and $x_{i1} x_{i2}$) was computed by `aov`, and the ratio of each variable was calculated. The contribution of each variable to DNA is demonstrated by the distribution of the ratio of all target CpGs. Significance of correlation between age and increase in DNA methylation among suicide cases compared with controls was computed using a t-statistic of $\beta_j$, and the corresponding degrees of freedom. We also defined a null linear model

$$y_i = \beta_1 x_{i2} + \epsilon_i.$$

We conducted an Analysis of Variance (ANOVA) F-test of the two models and computed $P$-values for a combined age and phenotype effect on DNA methylation for each CpG site. Since the filtered CpG sites from the HumanMethylation27 BeadChip were 15 249, the $P$-values generated by the ANOVA F-test were corrected for multiple testing using the Benjamini-Hochberg linear step-up procedure.

**Table I.** Sample description with gender and diagnosis distributions.
DNA methylation changes with respect to factors like age and gender were evaluated with a simple linear model $y_i = \beta_0 + \beta_1 x_i + \epsilon_i$ (where $x_i$ refers to age or gender). $P$-values for a specific variable were estimated with a one-sided t-test. For each factor, we tested two null hypotheses: (i) DNA methylation does not increase with the confounding variable; and (ii) DNA methylation does not decrease with the confounding variable.

Gene ontology and pathway analysis

Genes with significant target CpGs were imported into Ingenuity Pathway Analysis Software (Ingenuity Systems, http://www.ingenuity.com/). Gene ontology analysis was performed and enriched function categories were produced using Ingenuity Knowledge Base Genes as the reference set. Enrichment $P$-values for biological functions were corrected for multiple testing using the Benjamini-Hochberg linear step-up procedure, with the threshold for significant functions set a priori at $P<0.05$.

Validation of Illumina methylation assay at selected CpG sites by bisulfite pyrosequencing

Methylation data from the Illumina assay was validated by quantifying methylation levels at randomly selected CpG sites by bisulfite pyrosequencing. The %CpG methylation at selected sites proximal to the genes Eya2 (eyes absent homolog 2), Megf11 (multiple EGF-like-domains 11), Lmna (lamin A/C), Glud1 (glutamate dehydrogenase 1), Erbb3 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3) and Slc18a2 (solute carrier family 18 member 2) were quantified by bisulfite conversion of genomic DNA followed by PCR amplification and pyrosequencing (Figure 2). 500 ng of genomic DNA from the same brain region and the batch of DNA used in the Illumina methylation assay was bisulfite-treated using the EpiTect Bisulfite kit (Qiagen) and stored at -20°C until further analysis. The bisulfite converted DNA was amplified by PCR using the PyroMark PCR kit (Qiagen) according to the manufacturer's instructions and PCR primers provided in Table II. PCR and sequencing primers were selected using the PyroMark Assay Design software (Version 2.0; Qiagen) and tested for linearity using standards with known methylation levels. PCR products were sequenced using a PyroMark Q96 MD system (Qiagen) following the manufacturer’s suggested protocol. Percent methylation at each CpG site was quantified using the PyroMark CpG software (Version 1.0.10, Qiagen).

Results

DNA methylation data analysis which included age and diagnosis, as well as interaction of these factors, revealed that CpG methylation variability in major depression suicides and controls is predominantly attributable to aging (Figure 3). These analyses are based on examination of 15 249 CpG sites in common across all 53 samples (referred to as “target” CpG sites through application of probe filtering, described in Methods). While diagnostic state (or phenotype) also accounted for some of the methylation variability in the data, age was the dominant factor (Figure 3).

Given these observations, we next investigated age-related DNA methylation patterns in major depression suicide and control groups separately. In each diagnostic group (ie, suicide or control) a simple linear model with age as the covariate was applied to all target CpG sites. All significant CpG sites showed an increase in DNA methylation with increasing age (Figure 4A). In contrast, no CpG site showed a decrease in DNA methy-
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DNA methylation patterns are significantly impacted by ageing. Among the samples, the variance component attributed to age is greater than that of diagnosis (phenotype) and age×phenotype interaction. Box plot shows the contribution of age, phenotype, and interaction of age×phenotype variance components, that account for the total methylation variability.

Figure 3. DNA methylation patterns are significantly impacted by ageing. Among the samples, the variance component attributed to age is greater than that of diagnosis (phenotype) and age×phenotype interaction. Box plot shows the contribution of age, phenotype, and interaction of age×phenotype variance components, that account for the total methylation variability.

| Gene  | Region | Primer sequence | Biotinylated strand | Amplicon size | #CpG analyzed |
|-------|--------|-----------------|---------------------|---------------|---------------|
| EYA2  | F      | GAGTTTTTAGGAGTGTATTTGTTATAGT | Forward              | 154           | 3             |
|       | R      | TTCCTCCTACAACCAAACCAT       |                      |               |               |
|       | S      | CCAACATAAACCTAT            |                      |               |               |
| MEGF11| F      | GGGTAGGTTAGGATAGGGATTTAGAT | Reverse              | 156           | 3             |
|       | R      | ACCCTCTCTCTTAAACTCTAAATTTTC |                      |               |               |
|       | S      | GGGAATTTAGATAGGG            |                      |               |               |
| LMNA  | F      | ATTTAAAGGTTTTTGTGAGGTTTTGA | Reverse              | 142           | 1             |
|       | R      | CCTCTCTATCTTCTTCTTCTTCTTCT |                      |               |               |
|       | S      | GGGAATTGATGATG              |                      |               |               |
| GLUD1 | F      | ATGGGGAAGGAGGAGGATTTAGT     | Reverse              | 182           | 5             |
|       | R      | ACCACCCCAAACCTTCTTCAAATATAT |                      |               |               |
|       | S      | AGTATAATGTTGTAGGG           |                      |               |               |
| ERBB3 | F      | AGGTGTAAGGGTTGCTTTTAGGAAAGT | Reverse              | 166           | 3             |
|       | R      | AATTACTACCCCAACCTACT       |                      |               |               |
|       | S      | TTTGGAGAAATAATTAGG          |                      |               |               |
| SLC18A2| F     | GGGTGAAGGTTGTTTTTAGGAA     | Forward              | 200           | 4             |
|       | R     | AACACCAACCAAACCTTATAACCTCC |                      |               |               |
|       | S     | CTTTAAACCTCAATATCCTAT      |                      |               |               |

Table II. Primers used in validation of Illumina methylation data via bisulfite pyrosequencing. The F, R, and S in the region column correspond to the forward, reverse, and sequencing primers.
group had substantially more CpG sites that undergo increased CpG methylation compared with controls (Figure 4C). These results indicate that there is much greater DNA methylation change across the lifespan in the prefrontal cortex in major depression suicides.

CpG sites that exhibited age-dependent DNA methylation were mapped to the human reference genome to find the associated genes. This identified a total of 17 and 136 genes uniquely to the control and major depression suicide groups, respectively, with 34 genes in common in the two groups. To distinguish the biological functions in major depression suicide and controls, genes that were uniquely mapped to either the suicide or control groups, a comparison of biological functions

Figure 4. Aging and methylation in suicide brains. (A) Scatter plot showing DNA methylation is correlated with chronological age. (B) Venn diagram of significant target CpG sites with positive correlation between methylation and aging ($P<0.05$ corrected for multiple testing using Benjamini-Hochberg linear step-up procedure). (C) Box plot showing that the increase in the number of CpG sites in suicide brains is not attributed to slight differences in age distribution of suicide cases vs controls. The plot represents results of permutation test via resampling of suicide and control samples in a series of 10-year age intervals (exhausting all 63 possible suicide sample combinations and 1000 random sampling of age-matched controls), confirming significantly greater number of methylation changes in suicide brains ($P<0.001$).

Figure 5. Bar chart showing comparison of suicide and control diseases and functions via Ingenuity Pathway analysis.
via Ingenuity Pathway Analysis software was employed. Genes showing increased methylation in major depression suicide were significantly associated with behavior, cell cycle, cell death and survival, cellular and embryonic development (Figure 5), and correspondingly genes from the controls were significantly associated with cell cycle, cell-to-cell signaling and interaction, and cellular growth and proliferation.

Discussion

The present study shows increased age-related DNA methylation perturbations in prefrontal cortex in major depression suicide compared with nonpsychiatric controls. These perturbations involve a gain of methylation during aging, indicated by an 8-fold greater degree of methylation across the lifespan in major depression suicides, pointing to a mechanistic link between aberrant hypermethylation in suicide and related psycho- and neuropathologies, aging, and cellular senescence. Although this study cannot establish a causal relationship between increased DNA methylation and risk for suicide, it does show a remarkable increase in the numbers of cytosine sites in suicide brains as compared with age- and sex-matched controls across the lifespan. It should be noted that the suicides in the present study also had a diagnosis of mood disorder, which in general is found in about 60% of suicides. Thus, these findings may underlie the neuropathology related to mood disorder and/or suicide. Accumulating evidence from postmortem studies reveals differences in morphology and neurotransmitter and trophic indices in the brains of suicides and controls, such as a reduction in neuronal density.27-29 Similarly, there is much evidence from postmortem studies indicating loss of neurons or glia in mood disorders.23,30-32 There are both structural and functional imaging data showing abnormalities in the brain associated with suicidal behavior and with mood disorders. The prefrontal cortex is both thinner and hypoactive in major depression and in association with suicidal behavior, independently of mood disorder.32 Future studies need to examine the effects of major depression and suicide separately.

Our findings suggest that some of the same molecular and cellular factors associated with aging may also contribute to the neuropathology of suicide, mood disorders, and related neuropathologies. The etiological basis of mood disorders appears to be more environmental and less genetic and familial as patients get older. The influence of epigenetic factors on suicide or depression may be greater as patients age relative to the impact of genetic factors. Aging is associated with cellular senescence involving telomere shortening, oxidative stress, and accumulation of genetic and epigenetic modifications.34-36 In particular, epigenetic modifications, such as DNA methylation and post-translational modifications of histone proteins, are central to cellular functioning, and those associated with aging can, in a subtle and progressive fashion, negatively influence proper cellular functioning. Such perturbations will likely have a cumulative effect on neuronal functioning and have the potential to lead to neuropathology, severe mental illness or even suicide.

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Aumento de la metilación del ADN en el cerebro de pacientes suicidas

Los estudios clínicos han encontrado que la niñez adversa y los acontecimientos vitales estresantes en la adultez aumentan el riesgo para la depresión mayor y para el suicidio. Se piensa que la predisposición tanto para la depresión como para el suicidio depende de factores de riesgo genético o de efectos epigenéticos. Se investigaron los signos de metilación del ADN en cerebros postmortem de pacientes suicidas con diagnóstico de depresión mayor. Los niveles de metilación del ADN se determinaron en resolución única de Citosina-Fosfato-Guanina (CfG) en la corteza prefrontal ventral de 53 pacientes suicidas y de controles no psiquiátricos con edades entre 16 y 89 años. Se encontró que la metilación del ADN aumenta a lo largo de la vida. Los pacientes suicidas mostraron un número 8 veces mayor de sitios CfG metilados en relación con los controles (p < 2,2x10^-6), con cambios mayores en la metilación del ADN, por sobre lo observado en el envejecimiento normal. Este aumento en la metilación del ADN puede contribuir de manera significativa a la neuropatología y a la psicopatología que están a la base del riesgo de suicidio en la depresión.

Augmentation de la méthylation de l'ADN dans le cerveau des suicidés

D’après des études cliniques, le malheur dans l’enfance et les événements de vie stressant à l’âge adulte augmentent le risque de dépression caractérisée (majeure) et de suicide. Les prédispositions à la dépression caractérisée ou au suicide dépendent probablement de facteurs de risque génétiques ou d’effets épigénétiques. Nous avons analysé des signatures postmortem de la méthylation de l’ADN dans des cerveaux de suicidés diagnostiqués comme ayant eu un épisode dépressif caractérisé. Nous avons déterminé les taux de méthylation de l’ADN avec une résolution CpG (C-phosphate-G) à site unique dans le cortex préfrontal ventral de 53 suicidés et témoins non psychiatriques âgés de 16 à 89 ans. Nous avons trouvé que la méthylation de l’ADN augmente tout au long de la vie. Le nombre de sites CpG méthyliés étaient multipliés par 8 chez les suicidés par rapport aux témoins (p < 2,2 x 10^-6), avec des changements de méthylation de l’ADN plus importants, allant au-delà de l’accroissement de la méthylation observé dans le vieillissement normal. Cette augmentation de la méthylation de l’ADN peut participer significativement à la neuropathologie et à la psychopathologie qui sous-tend le risque de suicide dans la dépression.
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