Abstract

Background: Suicide represents a major health concern, especially in developing countries. While many demographic risk factors have been proposed, the underlying molecular pathology of suicide remains poorly understood. A body of evidence suggests that aberrant DNA methylation and expression is involved. In this study, we examined DNA methylation profiles and concordant gene expression changes in the prefrontal cortex of Mexicans who died by suicide.

Methods: In collaboration with the coroner’s office in Mexico City, brain samples of males who died by suicide (n = 35) and age-matched sudden death controls (n = 13) were collected. DNA and RNA were extracted from prefrontal cortex tissue and analyzed with the Infinium Methylation480k and the HumanHT-12 v4 Expression Beadchips, respectively.
Results: We report evidence of altered DNA methylation profiles at 4430 genomic regions together with 622 genes characterized by differential expression in cases vs controls. Seventy genes were found to have concordant methylation and expression changes. MetaCore-enriched analysis identified 10 genes with biological relevance to psychiatric phenotypes and suicide (ADCY9, CRH, NFATC4, ABCC8, HMGA1, KAT2A, EPZA2, TRRAP, CD22, and CBLN1) and highlighted the association that ADCY9 has with various pathways, including signal transduction regulated by the cAMP-responsive element modulator, neurophysiological processes regulated by the corticotrophin-releasing hormone, and synaptic plasticity. We therefore went on to validate the observed hypomethylation of ADCY9 in cases vs control through targeted bisulfite sequencing.

Conclusion: Our study represents the first, to our knowledge, analysis of DNA methylation and gene expression associated with suicide in a Mexican population using postmortem brain, providing novel insights for convergent molecular alterations associated with suicide.

Keywords: Epigenomics/transcriptomics, Mexico, suicide, postmortem human brain
from the Forensic Sciences Institute. All subjects were Mexicans who lived in Mexico City at the time of death, and to reduce ethnic variation and stratification effects, we selected subjects that descended from 2 Mexican generations. This study included male subjects: (1) who died by suicide (cases, n=35); or (2) who died suddenly without prolonged agonal state (controls, n=13). The cause and manner of death were determined by the coroner’s office, in accordance with the National Code of Criminal Procedures (2016), after evaluating autopsy results, circumstances of death, data from extensive toxicological testing, police reports, family interviews, and medical records. Groups were matched for age and postmortem interval. The exclusion criteria for both groups included age >65 years old, comorbid medical illness, undetermined cause of death, incomplete forensic records, and poor DNA and RNA quality. Sample characteristics are presented in supplementary Table 1. Information on socio-demographic data (age, years of education, marital status, occupational status), circumstances of the death (method, toxicology reports, suicide note), and clinical information (medical and psychiatric reports from the hospital as well as death certificates) for each donor was obtained through the complete coroner’s records. To ensure comparability of the 2 groups, all individuals were subject to a consensus diagnosis between a pathologist, a psychologist, a criminologist, and a psychiatrist based on the DSM-5 criteria and using proxy-based testimonies as described elsewhere (Romero-Pimentel et al., 2018). Demographic and clinical data were compared between groups and considered for final analyses (supplementary Table 1).

For identification and dissection of Brodmann area 9, which corresponds to the dorsolateral prefrontal cortex, we used well-characterized neuroanatomical maps (Haines, 2000; Nolte, 2002). Briefly, the second prefrontal gyrus and precentral gyrus were identified to landmark the area of interest. In all cases, 3 cm³ of left hemisphere gray matter tissue was carefully dissected and immediately snap-frozen, whereas 1.5 cm³ of the tissue was stored in RNAlater (Qiagen, Singapore) at −80°C until further processing. This study was conducted in accordance with the ethical principles of the last Declaration of Helsinki and was processed. This study was conducted in accordance with the National Code of Criminal Procedures (2016), after evaluating autopsy results, circumstances of death, data from extensive toxicological testing, police reports, family interviews, and medical records. Groups were matched for age and postmortem interval. The exclusion criteria for both groups included age >65 years old, comorbid medical illness, undetermined cause of death, incomplete forensic records, and poor DNA and RNA quality. Sample characteristics are presented in supplementary Table 1. Information on socio-demographic data (age, years of education, marital status, occupational status), circumstances of the death (method, toxicology reports, suicide note), and clinical information (medical and psychiatric reports from the hospital as well as death certificates) for each donor was obtained through the complete coroner’s records. To ensure comparability of the 2 groups, all individuals were subject to a consensus diagnosis between a pathologist, a psychologist, a criminologist, and a psychiatrist based on the DSM-5 criteria and using proxy-based testimonies as described elsewhere (Romero-Pimentel et al., 2018). Demographic and clinical data were compared between groups and considered for final analyses (supplementary Table 1).

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**Genome-Wide DNA Methylation Analysis on the Infinium HumanMethylation450 BeadChip**

Genomic DNA was isolated from 25 mg of tissue using the manufacturer’s instructions of the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and tested for purity and degradation using the NanoDrop 2000 spectrometer (Thermo Fisher, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. Genomic DNA (200 ng) from each sample was treated with sodium bisulfite using the EZ DNA methylation Kit (Zymo Research, CA, USA) following the manufacturer’s standard protocol. DNA methylation was quantified using the Infinium HumanMethylation450 BeadChip Array (Illumina, Inc., San Diego, CA, USA), as previously described (Pidsley et al., 2013). Bisulfite conversion and initial methylation signal detection quality control were performed at the Microarray Core Facility in the National Institute of Genomic Medicine (UMI) located in Mexico City.

Cases and controls were randomized among the BeadChip to avoid batch effects. Pre-processing and analysis of raw microarray data were conducted within R (ver 3.4) using the Chip Analysis Methylation Pipeline Bioconductor package (Morris et al., 2014). Sample methylation quality control (QC) was assessed by plotting log median methylated and unmethylated signals. Only samples that passed QC measures (>1% of sites P >.05) were included. Probes were excluded if they showed intensities indistinguishable from the background (detection P >.05) in at least 1% of samples and bead count <3 in 5% of the samples as well as if they were non-specific, showed cross-reactivity, and if they hybridized to single nucleotide polymorphisms.

Sex chromosomes with CpG probes were used to confirm sample sex. Single value decomposition analysis was used to detect technical batches and covariates. For the annotation of probes, the University of California, Santa Cruz (UCSC) RefGene name from illumina’s annotation file and enhanced annotation to the UCSC Known Gene were used. All annotations used the human February 2009 (GRCh37/hg19) assembly. Beta (β) values were calculated as the ratio of methylated signal to the sum of unmethylated and methylated signals at each CpG site, and log, transformed β values were used for the remainder of pre-processing steps (Du et al., 2010). Technical batches and covariates were detected and corrected for using single value decomposition analysis via the ComBat method before differential methylation analysis (Johnson et al., 2007).

After these procedures, 474 958 CpG sites were extracted. Differentially methylated regions (DMRs) were identified between cases and controls using the DMRCate method (Peters et al., 2015). DMRCate fits a limma linear model with empirical Bayes adjustment for each individual CpG site. We implemented the default smoothing parameters with bandwidth λ = 1000 bp and scaling factor C = 2. Nominal P < .05 was used to denote significant DMRs between controls and cases and corrected for genome-wide multiple testing using the Benjamini-Hochberg procedure with false discovery rates (FDR) of 0.1. Also, a mean fold change of ± 0.01 was set as a cutoff value to decrease the number of significant DMRs and to identify sites with more biologically relevant methylation differences.

**Genome-Wide mRNA Gene Expression Analysis on the HumanHT-12 v4 Expression BeadChip**

Total RNA was extracted from 25 mg of tissue using the RNeasy Kit (Qiagen), according to the manufacturer’s instructions. NanoDrop 2000 spectrometer (Thermo Fisher) and Agilent Bioanalyzer 2100 were used to assess RNA quality across samples, with an RNA integrity number cutoff of 6. RNA samples were then sent to UMI, where an additional RNA QC was conducted. Complementary RNA was prepared using standard Illumina Whole-Genome protocols and was hybridized to Human HT-12 v4 Expression BeadChips (Illumina, USA). Differential gene expression analysis was conducted on the Human HT-12 v4 Expression BeadChip (Illumina), which provides accurate genome-wide expression coverage on up to 47 000 well-known genes, gene candidates, and splice variants. Initial QC of raw probe signals was conducted in GenomeStudio by UMI.

Pre-processing steps and differential gene expression analysis were performed in R using the limma Bioconductor package (Ritchie et al., 2015). Only the subset of samples that appeared in our mRNA methylation analysis were included for whole-genome expression analysis (25 cases and 6 controls). Probe signals were detected with the propexp function and normalized with the normalize Between Arrays function. Probe filtering was conducted using a detection P < .05 in at least 20% of samples cut off to denote retained probes, where 27 453 gene probes were preserved for downstream analysis. Differentially expressed genes were identified through linear regression analyses. All analyses were conducted with log₂-transformed values. Only probes with a log fold change (LFC) of at least > ±0.3, between
cases and controls, were included for further investigation. LFC is the log-ratio of a gene expression value in 2 different conditions, and it is calculated as the ratio of the difference between the final value and the initial value over the initial value. Finally, genes that contained DMRs with ±0.01 mean β fold change and that appeared in our differential expression analysis were identified using GeneOverlap package within R.

**Sensitivity Analyses**

We performed sensitivity analyses to determine the effects of toxicology and psychiatric condition on our methylation and expression findings. For each sensitivity analysis, we adjusted the original models to include each additional covariate and then ran linear regressions to compare the original and adjusted LFCs. Altogether, 4 sensitivity analyses were run.

**Investigating the Effects of Brain Cell and Genetic Heterogeneity**

To address the possibility of confounding effects of brain cell composition, Cell EpigencType Specific marker was used to estimate brain cellular heterogeneity in all samples. The mapper was designed for quantification and normalization of differing neuronal proportions in genome-wide DNA methylation data sets (Quinivano et al., 2013). To check for potential genetic heterogeneity, we extracted CpG sites that were population-specific SNPs using the MethylSNP R package (LaBarre et al., 2019). The R package extracts CpG sites that are present in 3 discrete levels of methylation: fully methylated, fully unmethylated, and 50% methylation, which correspond to genotypes CC, TT, and CT. Then a principal component analysis was performed in the population-specific SNPs data to identify sample outliers.

**Pathway Analysis**

The overlapping differentially expressed and methylated gene set was input into pathway analysis package MetaCore version 6.27 (GenoGo, Thomson Reuters, New York, NY, USA) to build top biological networks and list the associated biological processes. P = .05 was used as a cutoff to determine significant pathway enrichment. MetaCore analysis is based on MetaBase (http://metadatabase.org), a 100% manually curated integrated database of mammalian biology that contains over 6 million experimental findings on protein-protein, protein-DNA, protein-RNA, and protein-compound interactions, metabolic, signaling pathways, and others (Bolser et al., 2012).

**Targeted Bisulfite Sequencing for Technical Validation**

In our selection of the region that we chose to validate, through targeted bisulfite sequencing, we looked at a DMR that was the most overrepresented in our Metacore enrichment analysis. The DMR in ADYC9 not only fit this criterion but was also the most hypomethylated region. Therefore, DNA methylation across the ADYC9-associated DMR (spanning chromosome 16:4102293-4103533) was selected. The region encompasses 3 CpG sites (cg00701890, cg16774375, cg02910037), which overlap with those measured by the 450k array. A single amplicon (222 bp) was amplified with primers designed using the Methyl Primer Express software (ThermoFisher Scientific) and using genome assembly GRCh37/hg19. Specific details for primer design and amplicon library preparation are included in the supplementary Methods.
relative to controls (mean β fold change = 0.20, \(P = 1.22 \times 10^{-122}\)). The 100 most significant DMRs (\(P < 3.16 \times 10^{-301}\)) are provided in supplementary Table 2.

### Effects of Altered DNA Methylation on Gene Expression

To understand the possible biological effects of altered methylation, we analyzed gene expression using HumanHT-12 v4 Expression BeadChips. Comparison between cases and controls revealed 621 differentially expressed genes (LFC ≥ ±0.3; Figure 2B). To determine whether DNA methylation differences, between cases and controls, led to a functional impact on transcription, we investigated the overlap of DNA methylation and gene expression data using GeneOverlap package within R. Statistical overlap significance was calculated with the exact hypergeometric test (\(P < .05\)) (Shen, 2020). The Venn diagram in Figure 2C shows 70 regions that show altered DNA methylation and altered gene expression, and all overlapped with unique genes (exact hypergeometric test \(P < 2.58 \times 10^{-24}\); supplementary Table 3). From the list of differentially methylated regions, 38 (54%) probes indicated hypermethylation and 32 (46%) probes indicated hypomethylation in suicide cases. Noteworthy, B-Cell Receptor CD22 (CD22) located in chromosome 19:35818807-35820181 (GRCh37/hg19) and NLR Family Pyrin Domain Containing 3 (NLRP3) located in chromosome 1: 247578552-247580106 (GRCh37/hg19) were the most significantly hypermethylated regions between suicide cases and controls (\(P = 5.89 \times 10^{-36}\), mean β fold change = .056, FDR = 0.1, and \(P = 8.21 \times 10^{-15}\), mean β fold change = .045, FDR = 0.1, respectively). Solute Carrier Family 25 Member 34 (SLC25A34) located in chromosome 1:16062361-16063471 (GRCh37/hg19) and Adenylate Cyclase 9 (ADCY9) located in chromosome 16: 4102293-4103533 were, on the other hand, genes with altered expression and the highest hypomethylation after correcting for multiple testing (\(P = 5.47 \times 10^{-20}\), mean β fold change = −.034 and \(P = 8.85 \times 10^{-14}\), mean β fold change = −.032, q = 0.10, respectively).

Next, to determine the effect of toxicology and psychiatric condition and our methylation and expression findings, we performed a series of sensitivity analyses. Overall, adjusting our models for these variables had minimal effects on our results (supplementary Figure 1).

### Brain Cell and Genetic Heterogeneity

The individual proportion of neuronal and non-neuronal cells did not have any specific, significant effects on our primary Table 1.

| Demographics and Clinical Data of Samples Considered in the Epigenomic and Transcriptomic Overlap Analysis |
|----------------------------------------------------------|
| Control (n = 6) | Cases (n = 25) | Statistical analysis |
|---|---|---|
| **Age** | 29.50 (18,40) | 30 (16,61) | U = 72.00, \(P = .89\) |
| **PMI (h)** | 16 (11,19) | 12 (3,16) | U = 36.50, \(P = .53\) |
| **RIN** | 6.9 (5.2, 7.2) | 5.8 (4.4, 8.1) | U = 62.50, \(P = .54\) |
| **Cause of death** | Accidental death 100% (n = 6) | Hanging: 92% (n = 32) | \(X^2 = 1.4, df = 2, P = .48\) |
| | | Jumping: 4% (n = 1) | \(X^2 = 5.4, df = 1, P = .02\) |
| | | Cutting: 4% (n = 1) | |
| **Years of education** | 10.5 (9, 16) | 9 (6, 16) | U = 43, \(P = .10\) |
| **Occupational status** | | | |
| Student | 0% (n = 0) | 8% (n = 2) | \(X^2 = 7.4, df = 2, P = .028\) |
| Employed | 100% (n = 6) | 80% (n = 20) | |
| Unemployed | 0% (n = 0) | 12% (n = 3) | |
| **Marital status** | | | |
| Single | 0% (n = 0) | 52% (n = 13) | \(X^2 = 5.4, df = 1, P = .02\) |
| Married | 100% (n = 6) | 48% (n = 12) | |
| **Psych. family history** | | | |
| Yes | 0% (n = 0) | 0% (n = 0) | |
| No | 100% (n = 6) | 96% (n = 24) | \(X^2 = 0.8, df = 1, P = .39\) |
| Unknown | 0% (n = 0) | 4% (n = 1) | |
| **Previous episodes of suicide attempt** | | | |
| Yes | 0% (n = 0) | 12% (n = 3) | \(X^2 = 18, df = 4, P = .001\) |
| No | 100% (n = 6) | 88% (n = 24) | |
| **Toxicology** | | | |
| Alcohol | 0% (n = 0) | 52% (n = 13) | \(X^2 = 6.10, df = 2, P = .04\) |
| Cocaine | 0% (n = 0) | 4% (n = 1) | |
| Negative | 100% (n = 6) | 44% (n = 11) | |
| **Psych. disorder (DSM-5)** | | | |
| Depressive disorder | 35.29% (n = 12) | 0% (n = 0) | \(X^2 = 18, df = 4, P = .001\) |
| Substance-related disorder | 52.94% (n = 18) | 28.57% (n = 2) | |
| Psychotic disorder | 2.94% (n = 1) | 0% (n = 0) | |
| Personality disorder | 5.88% (n = 2) | 14.29% (n = 1) | |
| None | 2.94% (n = 1) | 57.14% (n = 3) | |
| **Postmortem note** | | | |
| Yes | n/a | 8% (n = 2) | |
| No | n/a | 92% (n = 23) | |

**Abbreviations**: \(df\), degrees of freedom; PMI, postmortem interval; RIN, RNA integrity number; \(U\), Mann–Whitney \(U\) test; \(X^2\), chi-squared test. Median (maximum, minimum) are shown.
findings (supplementary Table 4). Our genetic heterogeneity analysis also showed that population-specific SNPs for each sample formed 1 cluster with no outliers (supplementary Figure 2).

Pathway Analysis

To shed light on the potential biological effects of the 70 genes where expression and methylation was significantly altered in suicide cases vs controls (supplementary Table 3), a pathway analysis was performed based on the Metacore database. The most enriched gene ontology processes, after FDR adjustment (<0.05), included regulation of synaptic plasticity ($P=1.02 \times 10^{-05}$), notochord cell differentiation ($P=1.06 \times 10^{-05}$), and the negative regulation of permeability maintenance of brain blood-barrier ($P=1.06 \times 10^{-05}$) (Figure 3A). In addition, Metacore biological processes analysis indicated that these differentially methylated and expressed genes were also highly correlated with (1) inflammation regulated by the macrophage migration inhibitory factor ($P=9.20 \times 10^{-05}$), (2) signal transduction regulated by the cAMP-responsive element modulator also known as cAMP responsive element modulator (CREM) pathway ($P=1.97 \times 10^{-05}$), and (3) neurophysiological process regulated by the corticotrophin-releasing hormone ($P=3.35 \times 10^{-02}$) (Figure 3B). However, these pathways did not survive the FDR adjustment. The main pathways are shown in Table 2.

Technical Validation

We next sought to technically validate our Illumina 450K data using targeted bisulfite sequencing. For this, we overlapped the list of genes identified as network objects from active Metacore data to select a DMR for validation. Ten genes were identified as actively enriched Metacore terms. What was most notable, ADCY9 was enriched in 4 terms and showed the highest negative LFC after correcting for multiple testing ($P=8.85E-14$, mean $\beta$ fold change $=0.032$, $q=0.10$) (Table 3). Therefore, differential methylation of the ADYC9 region (chromosome 16:4102293-4103533 [GRCh37/hg19]), in our genome-wide analysis was selected for validation. Consistent with the microarray analysis, targeted bisulfite sequencing revealed ADYC9 to be significantly hypomethylated in suicide cases vs controls ($P<0.001$, Student’s $t$ test). Also, we obtained a significant Pearson’s correlation coefficient of $r=0.40$ with a $P<.009$ among the level of CpGs methylation assessed by targeted bisulfite sequencing and microarray methods (Figure 4).
Discussion

To our knowledge, this represents the first genome-wide study that integrates differential methylation and expression analyses in postmortem human brain tissue from Latin American individuals who died by suicide. In this study, we identified several differentially methylated regions, with fold changes >±0.01 between cases and controls. While a cutoff of >±0.01 may appear relatively low, subtle methylation differences in multiple functional gene networks are often reported in studies of psychiatric disorders. Together, these subtle differences in many genes are thought to reflect the complex etiology of suicidal behavior (Labonte et al., 2013; Haghighi et al., 2014; Nagy et al., 2015; Schneider et al., 2015; Ju et al., 2019; Kouter et al., 2019; Policicchio et al., 2020).

We next combined these DMRs with differential expression analysis and focused only on genes that were also differentially expressed between cases and controls. This allowed for the identification of 70 DMRs that did not share any gene level overlap. Gene set enrichment analysis with these 70 genes revealed that 10 were overrepresented in, and therefore biologically relevant to, processes related to suicide (ADCY9, CRH, NFATC4, ABCC8, HMGA1, KAT2A, EPHA2, TRRAP, CD22, CBLN1). Of these 10 genes, ADCY9 appeared in the greatest number of pathways. We therefore chose to validate the differential methylation we observed in ADCY9 by using a targeted bisulfite sequencing approach.

The product of the ADCY9 gene belongs to a family of transmembrane-bound enzymes that catalyzes the formation of cyclic AMP from ATP and is implicated in intracellular signaling cascades and secondary messenger systems (Dessauer et al., 2017). The dysregulation of ADCY9 has been previously identified in the context of major depressive disorder (Fan et al., 2020). Early studies have shown that adenylate cyclase activity is altered in postmortem human brain samples from individuals with a history of mood disorders as well as those who died by suicide (Cowburn et al., 1994; Reiaich et al., 1999). In our investigation, we observed hypomethylation at the second intron of the ADCY9 gene in cases when compared to controls. This hypomethylation corresponded to a decrease in mRNA expression (Table 3). While methylation at a promoter region is typically anticorrelated with gene expression, our findings of a positive correlation between gene body methylation and expression are consistent with other studies (Ball et al., 2009; Lutz et al., 2018).

As revealed by gene enrichment analysis, genes that were both differentially methylated and expressed by suicide were found to be involved in the regulation of synaptic plasticity.

Table 2. Enrichment Analysis of Epigenomic and Transcriptomic Overlap Differences From Prefrontal Cortex of Mexican Individuals Who Died by Suicide

| Term                                      | Counts | P value  | FDR     | Network objects from active data                                      |
|-------------------------------------------|--------|----------|---------|-----------------------------------------------------------------------|
| Gene ontology process                     |        |          |         | Adenylate cyclase, CRH, Ephrin-A receptors, S100, NFATC4, CBLN1, KAT2A, NF-AT |
| Regulation of synaptic plasticity        | 8      | 1.00E-05 | 4.41E-05| Adenylate cyclase, CRH, Ephrin-A receptors, S100, NFATC4, CBLN1, KAT2A, NF-AT |
| Notochord cell differentiation            | 2      | 1.10E-05 | 4.41E-05| Ephrin-A receptors, EPHA2                                            |
| Negative regulation of maintenance of permeability of blood-brain barrier | 2 | 1.10E-05 | 4.41E-05| SUR, ABCC8                                                          |
| Negative regulation of neuroblast migration | 2      | 1.10E-05 | 4.41E-05| SUR, ABCC8                                                          |
| Regulation of maintenance of permeability of blood-brain barrier | 2 | 1.10E-05 | 4.41E-05| SUR, ABCC8                                                          |
| Cell process networks                     |        |          |         | Adenylate cyclase, CRH, Adenylate cyclase type IX, HMGA1           |
| Inflammation_MIF signaling                | 4      | .009     | 6.53E-03| Adenylate cyclase, CRH, Adenylate cyclase type IX, HMGA1           |
| Signal transduction_CREM pathway          | 3      | .019     | 6.58E-03| Adenylate cyclase, CRH, NFATC4                                      |
| Neurophysiological process_Corticotrophin signaling | 2 | .033     | 6.58E-03| Adenylate cyclase, CRH                                              |
| Transcription, Chromatin modification      | 3      | .037     | 6.58E-03| TRRAP, HMGA1, KAT2A                                                 |
| Immune response_BCR pathway               | 3      | .046     | 6.58E-03| CD22, NFATC4, NF-AT                                                  |

Abbreviations: BCR, B cell receptor; CREM, cAMP-responsive element modulator; FDR, False Discovery Rate; MIF, macrophage migration inhibitory factor.

*Top 5 significant pathways from Metacore as sorted by P values.
Table 3. List of Genes that Showed Epigenomic and Transcriptomic Overlap Differences and Enriched MetaCore Terms

| Position (build GRCh37/ hg19) | Gene annotated to DMRs | Transcript | DNA methylation | Gene expression | Number metacore term |
|-------------------------------|------------------------|------------|----------------|-----------------|---------------------|
| chr16:4102293-4103533         | ADCY9                  | Adenylate Cyclase 9 | 8.85\(^{-14}\) | 0.0059 | 4 |
| chr8:67088895-67091580        | CRH                    | Corticotrophin releasing hormone | 3.59\(^{-28}\) | 0.0006 | 4 |
| chr14:24834695-24839226       | NFATC4                 | Nuclear factor of activated T cells 4 | 6.80\(^{-21}\) | 0.0007 | 3 |
| chr11:17497693-17498952       | ABCC8                  | ATP binding cassette subfamily C member 8 | 9.23\(^{-22}\) | 0.0057 | 3 |
| chr6:34202568-34204646        | HMGA1                  | High mobility group AT-Hook 1 | 2.47\(^{-20}\) | 0.0088 | 2 |
| chr17:402790140275359         | KAT2A                  | Lysine acetyltransferase 2A | 3.80\(^{-18}\) | 0.0012 | 2 |
| chr1:16481715-16483658        | EPHA2                  | EPH receptor 2 | 1.99\(^{-27}\) | 0.0092 | 2 |
| chr7:98475615-98477438        | TRRAP                  | Transformation/transcription Domain associated protein | 3.37\(^{-47}\) | 0.0082 | 1 |
| chr19:35818807-35820181       | CD22                   | CD22 molecule | 5.89\(^{-36}\) | 0.0047 | 1 |
| chr16:49311483-49314257       | CBLN1                  | Cerebellin 1 precursor | 9.80\(^{-19}\) | 0.0058 | 1 |

Abbreviations: Chr, chromosome; DMRs, differentially methylated regions; LFC, log fold change.

Figure 4. Differential methylation between cases and controls at DMR_10219, assessed through 480 k Illumina BeadChip analysis, correlated with values assessed through targeted bisulfite sequencing. (A) Bar graphs show percentage of methylation of cases and controls detected through targeting bisulfite sequencing. (B) Bar graphs show log fold change of gene expression of cases and controls detected through HumanHT-12 v4 Expression BeadChip. (C) Scatterplots show correlation of methylation levels assessed by microarray and targeted bisulfite sequencing platforms. Abbreviations: BS, bisulfite sequencing; R, Pearson correlation coefficient.
(ADCY9, CRH, EPHA2, S100, NFATC4, CBLN1, KAT2A, NF-AT), signal transduction through CREM (ADCY9, CRH, NFATC4), and molecular pathways involved in neuropsychological processes regulated by corticotrophin-releasing hormone (ADCY9, CRH).

We were especially interested in the regulation of synaptic plasticity, since it was found as the most enriched pathway in our list of 70 genes dysregulated by suicide (Table 2). Synaptic plasticity is one of the most fundamental and important functions of the brain. The efficacy of transmission at a synapse depends on modulation of the connectivity between neurons and neuronal circuits during adaptation to the environment (Marsden et al., 2013). Previous reports have highlighted key roles of synaptic plasticity in suicide (Liu et al., 2017). For instance, Nagy et al. (2020) identified cell-type specific differentially expressed genes associated with the regulation of synaptic plasticity in the dorsolateral prefrontal cortex of male suicide cases. Our findings showed that ADCY9 and CRH were both members of the synaptic plasticity pathway and dysregulated in suicide. Accumulating evidence indicates that adenylate cyclase is an essential regulator of synaptic plasticity. For example, it regulates pathways related to long-term potentiation, such as, Ca2+/calmodulin sensitive adenylate cyclase signaling (Wang and Storm, 2003; Abel and Nguyen, 2008; Kim et al., 2011), cAMP-PKA signaling (Wang and Storm, 2003; Banko et al., 2004; Mockett et al., 2004; Abel and Nguyen, 2008; Valera et al., 2008; Kim et al., 2011). The downregulation of AMPAR and NMDAR subunits observed in postmortem brain of suicide cases may, therefore, be explained by the aforementioned mechanism (Cohen et al., 2011; Duric, 2013). We hypothesize that our findings of increased CRH expression in the prefrontal cortex of individuals who died by suicide may relate, at least in part, to the reduced expression of ADCY9 that we observed. We also provide evidence that epigenetic mechanism may be involved in the regulation of CRH expression by identifying hypermethylation in the promoter region of CRH gene (Table 3). Methylation of CpG island promoters is generally associated with the repression of gene expression (Delcuve et al., 2009; Novakovic et al., 2011). Nevertheless, non-typical gene expression and promoter methylation relationships have been described in genes with CpG-poor promoters, of which the CRH promoter falls into (Weber et al., 2007).

According to our Metacore gene enrichment analysis, ADCY9 is also enriched in a pathway related to corticotrophin-releasing hormone signaling. The mechanism regulating increased CRH expression remains unclear, although methylation of CpG sites within cyclic-AMP response element (CRE) sensitive regions may be 1 explanation (Rishi et al., 2010; Pan et al., 2015). Molecular studies using CRH promoter-reporter constructs showed that transcription factor complexes bound a CRE located at CRH 200 bp upstream of the major transcription start site mediated the cAMP stimulation (Adler et al., 1990). It has been shown that methylation of the CRH CRE can increase transcription factor binding affinity and enhance CRH gene expression (Pan et al., 2015). Also, the CRH receptor is a G-protein coupled protein receptor and signals through a cAMP-dependent mechanism. Interestingly, as mentioned before, one of the most enriched pathways associated with suicide is CREM. Our observation of CRH and ADCY9 differential methylation and increased expression suggest a positive feedback among these genes in the prefrontal cortex of individuals who died by suicide. However, additional functional studies are needed for a better understanding of the role of ADCY9 in the CRH process in the context of suicide.

The present study represents an important step in identifying differentially methylated regions associated with suicide in postmortem brain of Mexicans who died by suicide. However, our results must be interpreted with caution, as there are limitations to this study. To begin with, the sample size is relatively small; however, we have fully characterized each sample with additional data, such as demographic information and detailed clinical data that were considered in our analyses. Second, a methodological limitation inherent to the methylation microarray platforms is the inability to distinguish hydroxymethylated cytosines from methylated cytosines, and it is reasonable that the differences detected are confounded by other modifications.

Another important point to consider in this study is that the robustness of our findings is limited by several factors. Firstly, due to the lack of independent data set replication, it would be desirable to replicate our results in a different cohort of Mexicans who die by suicide. Additional explanatory data such as genotype were not assessed in our study. Integrative approaches have shown that
DNA methylation is best predicted in the combination of genotype information with environmental factors (Czamara et al., 2019). Another relevant limitation for this study is the lack of technical validation in other top candidate gene regions identified as enriched in our pathways analysis; however, we have demonstrated that our findings fit with previously reported pathways, such as synaptic plasticity, which in turn supports the reliability of our results. Still, future studies are needed to replicate our findings in large independent cohorts. Nevertheless, this study represents the first, to our knowledge, to investigate a Mexican population in the context of suicide. As part of an ongoing project, which started in 2018, we expect to collect more samples and to overcome the aforementioned limitations.

In conclusion, we present new evidence that altered DNA methylation is a mechanism that affects these processes in postmortem brain of suicide cases. Overall, the available data suggest that altered methylation and expression of genes involved in synaptic plasticity, signal transduction regulated by the cAMP-responsive element modulator, and neurophysiological processes regulated by the corticotrophin-releasing hormone may serve an important role in the molecular pathology of suicide. In this study, ADCY9 gene was identified to be involved in several pathways and is, therefore, a strong candidate for future functional studies.

Supplementary Materials

Supplementary data are available at International Journal of Neuropsychopharmacology (IJNPPY) online.

Acknowledgments

Ana Luisa Romero-Pimentel is a doctoral student from the Programa de Psicología, Universidad Nacional Autónoma de México (UNAM), and was supported by Consejo Nacional de Ciencia y Tecnología (CONACyT) Scholarship #486265. This study received partial funding from the “Instituto Nacional de Medicina Genomica.”

Statement of Interest

None.

References

Abel T, Nguyen PV (2008) Regulation of hippocampus-dependent memory by cyclic AMP-dependent protein kinase. Prog Brain Res 169:97–115.

Adler GK, Smas CM, Fiandaca M, Frim DM, Majzoub JA (1990) Regulated expression of the human corticotropin releasing hormone gene by cyclic AMP. Mol Cell Endocrinol 70:165–174.

Alicandro G, Malvezzi M, Gullus S, La Vecchia C, Negri E, Bertuccio P (2019) Worldwide trends in suicide mortality from 1990 to 2015 with a focus on the global recession time frame. Int J Public Health 64:785–795.

Arico M, Banko JL, Hou L, Kiann E (2004) NMDA receptor activation results in PKA- and ERK-dependent Mnk1 activation and increased eIF4E phosphorylation in hippocampal area CA1. J Neurochem 91:462–470.

Barnett Burns S, Almeida D, Turecki G (2018) The epigenetics of early life adversity: current limits and possible solutions. Prog Mol Biol Transl Sci 157:343–425.

Bengtsen CF, Bading H (2012) Nuclear calcium signaling. Adv Exp Med Biol 970:377–405.

Benito E, Valor LM, Jimenez-Minchan M, Huber W, Barco A (2011) cAMP response element-binding protein is a primary hub of activity-driven neuronal gene expression. J Neurosci 31:18237–18250.

BLUERPRINT Consortium (2016) Quantitative comparison of DNA methylation assays for biomarker development and clinical applications. Nat Biotechnol 34:726–737.

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.

Bolser DM, et al. (2012) MetaBase—the wiki-database of biological databases. Nucleic Acids Res 40:D1250–D1254.

Bruffaerts R, et al. (2010) Childhood adversities as risk factors for onset and persistence of suicidal behaviour. Br J Psychiatry 197:20–27.

Chen GG, Gross JA, Lutz PE, Vaillancourt K, Maussion G, Bramoulle A, Théroux JF, Gardini ES, Ehli U, Bourret G, Masurel A, Lepage P, Mechawar N, Turecki G, Ernst C (2017) Medium throughput bisulfite sequencing for accurate detection of 5-methylcytosine and 5-hydroxymethylcytosine. BMC Genomics 18:96.

Choi JW, Kim TH, Shin J, Han E (2019) Poverty and suicide risk in older adults: a retrospective longitudinal cohort study. Int J Geriatri Psychiatry 34:1565–1571.

Christoffel DJ, Golden SA, Russo SJ (2011) Structural and synaptic plasticity in stress-related disorders. Rev Neurosci 22:535–549.

Cohen JW, Lounueva N, Han LY, Hodes GE, Wilson RS, Bennett DA, Lucki I, Arnold SE (2011) Chronic corticosterone exposure alters postsynaptic protein levels of PSD-95, NR1, and synaptopodin in the mouse brain. Synapse 65:763–770.

Cowburn RF, Marcusson JO, Eriksson A, Wiehager B, O’Neill C (1994) Adenyl cyclase activity and G-protein subunit levels in postmortem frontal cortex of suicide victims. Brain Res 633:297–304.

Czamara D, et al.; Major Depressive Disorder Working Group of the Psychiatric Genomics Consortium (2019) Integrated analysis of environmental and genetic influences on cord blood DNA methylation in new-borns. Nat Commun 10:2548.

Delucive GP, Rastegar M, Davie JR (2009) Epigenetic control. J Cell Physiol 219:243–250.

Dessauer CW, Watts VJ, Ostrom RS, Conti M, Dove S, Seifert R (2017) International union of basic and clinical pharmacology. CII. Structures and small molecule modulators of mammalian adenylyl cyclases. Pharmacol Rev 69:93–139.

Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, Lin SM (2010) Comparison of beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics 11:587.

Duric V, Banasr M, Stockmeier CA, Simen AA, Newton SS, Overholser JC, Jurus GJ, Dieter L, Duman RS (2013) Altered expression of synapase and glutamate related genes in post-mortem hippocampus of depressed subjects. Int J Neuropsychopharmacol 16:69–82.
Dwivedi Y, Rao JS, Rizavi HS, Kotowski J, Conley RR, Roberts RC, Tamminga CA, Pandey GN (2003) Abnormal expression and functional characteristics of cyclic adenosine monophosphate response element binding protein in postmortem brain of suicide subjects. Arch Gen Psychiatry 60:273–282.

Ernst C, Chen ES, Turecki G (2009) Histone methylation and decreased expression of TrkB.T1 in orbital frontal cortex of suicide completers. Mol Psychiatry 14:820–832.

Fan T, Hu Y, Xin J, Zhao M, Wang J (2020) Analyzing the genes and pathways related to major depressive disorder via a systems biology approach. Brain Behav 10:e01502.

Fiori LM, Turecki G (2011) Epigenetic regulation of spermidine/spermine N1-acetyltransferase (SAT1) in suicide. J Psychiatr Res 45:1229–1235.

Guintivano J, Aryee MJ, Kaminsky ZA (2013) A cell epigenotype specific model for the correction of brain cellular heterogeneity bias and its application to age, brain region and major depression. Epigenetics 8:290–302.

Haghighi F, Xin Y, Chantion B, O’Donnell AG, He Y, Dwork AJ, Arango V, Mann JJ (2014) Increased DNA methylation in the suicide brain. Dialogues Clin Neurosci 16:430–438.

Haines DE (2000) Neuroanatomy: an atlas of structures, sections, and systems. Philadelphia, PA: Lippincott Williams & Wilkins.

Heim C, Mletzko T, Purselle D, Musselman DL, Nemeroff CB (2008a) The dexamethasone/corticotropin-releasing factor test in men with major depression: role of childhood trauma. Biol Psychiatry 63:398–405.

Heim C, Newport DJ, Mletzko T, Miller AH, Nemeroff CB (2008b) The link between childhood trauma and depression: insights from HPA axis studies in humans. Psychoneuroendocrinology 33:693–710.

Hiroi N, Wong ML, Licinio J, Park C, Young M, Gold PW, Åsberg M, Schiöth HB (2018) Epigenetic changes in the CRH receptor type I and type II mRNA in suicide victims and controls. Mol Psychiatry 6:540–546.

Hill MJ, Szyf M, Turecki G, Meaney MJ, Palkovits M, Giedd JN (2001) Increased expression of TrkB.T1 in orbital frontal cortex of suicide completers. Arch Gen Psychiatry 58:364–371.

Hiroi N, Wong ML, Licinio J, Park C, Young M, Gold PW, Åsberg M, Schiöth HB (2018) Epigenetic changes in the CRH receptor type I and type II mRNA in suicide victims and controls. Mol Psychiatry 6:540–546.

Himeno I, Zarrilli F, Tomaiuolo R, Carli V, Keller S, et al. (2010) Increased BDNF promoter methylation in suicide victims revealing impact on gene expression. J Affect Disord 117:409–414.

H.Low EH, Beijersbergen RL, van den Berg LT, Dijkman SP, Meijer DH, et al. (2006) Genome-wide DNA methylation differences mediate the association between obesity and depression. Nat Genet 38:514–520.

Hiroi N, Wong ML, Licinio J, Park C, Young M, Gold PW, Åsberg M, Schiöth HB (2018) Epigenetic changes in the CRH receptor type I and type II mRNA in suicide victims and controls. Mol Psychiatry 6:540–546.

Himeno I, Zarrilli F, Tomaiuolo R, Carli V, Keller S, et al. (2010) Increased BDNF promoter methylation in suicide victims revealing impact on gene expression. J Affect Disord 117:409–414.

Hiroi N, Wong ML, Licinio J, Park C, Young M, Gold PW, Åsberg M, Schiöth HB (2018) Epigenetic changes in the CRH receptor type I and type II mRNA in suicide victims and controls. Mol Psychiatry 6:540–546.
Merali Z, Du L, Hrdina P, Palkovits M, Faludi G, Poulter MO, Anisman H (2004) Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and GABA(A) receptor subunits in frontal cortical brain region. J Neurosci 24:1478–1485.

Miller AB, Esposito-Smythers C, Weismoore JT, Renshaw KD (2013) The relation between child maltreatment and adolescent suicidal behavior: a systematic review and critical examination of the literature. Clin Child Fam Psychol Rev 16:146–172.

Mockett BG, Brooks WM, Tate WP, Abraham WC (2004) Dopamine D1/D5 receptor activation fails to initiate an activity-independent late-phase LTP in rat hippocampus. Brain Res 1021:92–100.

Moore LD, Le T, Fan G (2013) DNA methylation and its basic function. Neuropsychopharmacology 38:23–38.

Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK, Beck S (2014) ChAMP: 450k chip analysis methylation pipeline. Bioinformatics 30:428–430.

Murphy TM, Crawford B, Dempster EL, Hannon E, Burrage J, Turecki G, Kaminsky Z, Mill J (2017) Methylation profiling of cortex samples from completed suicide cases implicates a role for PSORS1C3 in major depression and suicide. Transl Psychiatry 7:e989.

Musazzi L, Racagni G, Popoli M (2011) Stress, glucocorticoids and glutamate release: effects of antidepressant drugs. Neurochem Int 59:138–149.

Nagy C, Suderman M, Yang J, Szyf M, Mechawar N, Ernst C, Turecki G (2015) Astrocortical abnormalities and global DNA methylation patterns in depression and suicide. Mol Psychiatry 20:320–328.

Nagy C, Maitra M, Tanti A, Suderman M, Théroux JF, Davoli MA, Perlman K, Yerko V, Wang YC, Tripathy SJ, Pavlidis P, Mechawar N, Ragoussis J, Turecki G (2020) Single-nucleus transcriptomics of the prefrontal cortex in major depressive disorder implicates oligodendrocyte precursor cells and excitatory neurons. Nat Neurosci 23:771–781.

National Code of Criminal Procedures (2016) Diario Oficial de la Federación. Mexico: Camara de Diputados de H. Congreso de la Union.

Nemeroff CB, Owens MJ, Bissette G, Andorn AC, Stanley M (1988) Reduced corticotropin releasing factor binding sites in the frontal cortex of suicide victims. Arch Gen Psychiatry 45:577–579.

Nolte J (2002) The human brain: an introduction to its functional neuroanatomy. St Louis, MO: Mosby.

Novakovic B, Gordon I, Wong NC, Moffett A, Manuelpillai U, Craig JM, Sharkey A, Safery R (2011) Wide-ranging DNA methylation differences of primary trophoblast cell populations and derived cell lines: implications and opportunities for understanding trophoblast function. Mol Hum Reprod 17:344–353.

Oyesanya M, Lopez-Morinigo J, Dutta R (2015) Systematic review of suicide in economic recession. World J Psychiatry 5:243–254.

Pan X, Bowman M, Scott RJ, Fitter J, Nicholson RC, Smith R, Zakar T (2015) Methylation of the corticotropin releasing hormone gene promoter in BeWo cells: relationship to gene activity. Int J Endocrinol 2015:861302.

Pandey GN, Dwivedi Y, Ren X, Rizavi HS, Roberts RC, Conley RR (2007) Cyclic AMP response element-binding protein in post-mortem brain of teenage suicide victims: specific decrease in the prefrontal cortex but not the hippocampus. Int J Neuropsychopharmacol 10:621–629.

Pandey GN, Rizavi HS, Bhaumik R, Ren X (2019) Increased protein and mRNA expression of corticotropin-releasing factor (CRF), decreased CRF receptors and CRF binding protein in specific postmortem brain areas of teenage suicide subjects. Psychoneuroendocrinology 106:233–243.

Peters TJ, Buckley MJ, Statham AL, Pidgley R, Samaras K, V Lord R, Clark SJ, Molloy PL (2015) De novo identification of differentially methylated regions in the human genome. Epigenetics Chromatin 8:6.

Petronis A (2010) Epigenetics as a unifying principle in the aetiology of complex traits and diseases. Nature 465:721–727.

Piacentini M (2014) Measuring income inequality and poverty at the regional level in OECD countries. OECD Statistics Working Papers. OECD Publishing. https://www.oecd-ilibrary.org/economics/measuring-income-inequality-and-poverty-at-the-regional-level-in-oecd-countries_5jzxf5khtg9t-en

Pidgley R, Y Wong CC, Volta M, Lunnon K, Mill J, Schlunkwyc LC (2013) A data-driven approach to preprocessing Illumina 450K methylation array data. BMC Genomics 14:293.

Policichio S, Washr S, Vianna J, Iatrou A, Burraghe J, Hannon E, Turecki G, Kaminsky Z, Mill J, Dempster EL, Murphy TM (2020) Genome-wide DNA methylation meta-analysis in the brains of suicide completers. Transl Psychiatry 10:69.

Popoli M, Yan Z, McEwen BS, Sanacora G (2012) The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. Nat Rev Neurosci 13:22–37.

Poulter MO, Du L, Weaver IC, Palkovits M, Faludi G, Merali Z, Szyf M, Anisman H (2008) GABAA receptor promoter hypermethylation in suicide brain: implications for the involvement of epigenetic processes. Biol Psychiatry 64:645–652.

Read J, Agar K, Barker-Collo S, Davies E, Moskowitz A (2001) Assessing suicidality in adults: integrating childhood trauma as a major risk factor. ProfessPsychol Res Pract 32:367–372.

Reiach JS, Li PP, Warsh JK, Kish SJ, Young LT (1999) Reduced adenylyl cyclase immunolabeling and activity in postmortem temporal cortex of depressed suicide victims. J Affect Disorder 56:141–151.

Rishi V, Bhattacharya P, Chatterjee R, Rozenberg J, Zhao J, Glass K, Fitzgerald P, Vinson C (2010) CpG methylation of half-CRE sequences creates CREBalpha binding sites that activate some tissue-specific genes. Proc Natl Acad Sci U S A 107:20311–20316.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47.

Romero-Pimentel AL, Mendoza-Morales RC, Frenan A, Garcia-Dolores F, Gonzalez-Saenz EE, Morales-Marín ME, Nicolini H, Borges G (2018) Demographic and clinical characteristics of completed suicides in Mexico City 2014-2015. Front Psychiatry 9:402.

Roy B, Dwivedi Y (2017) Understanding epigenetic architecture of suicide neurobiology: a critical perspective. Neurosci Biobehav Rev 72:10–27.

Sandi C (2011) Glucocorticoids act on glutamatergic pathways to affect memory processes. Trends Neurosci 34:165–176.

Schneider E, El Hajj N, Müller F, Navarro B, Haaf T (2015) Epigenetic dysregulation in the prefrontal cortex of suicide completers. Cytogeten Genome Res 146:19–27.

Shen L (2020) GeneOverlap: test and visualize gene overlaps. R package version 1.26.0 (computer program).

Valera E, Sánchez-Martín FJ, Ferrer-Montiel AV, Meseguer A, Merino JM (2008) NMDA-induced neuroprotection in hippocampal neurons is mediated through the protein
kinase A and CREB (cAMP-response element-binding protein) pathway. Neurochem Int 53:148–154.
Vijayakumar L, John S, Pirkis J, Whiteford H (2005) Suicide in developing countries (2): risk factors. Crisis 26:112–119.
Wang H, Storm DR (2003) Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. Mol Pharmacol 63:463–468.
Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, Schübeler D (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39:457–466.
World Health Organization (2014) Preventive suicide. A global imperative. https://www.who.int/publications/i/item/preventing-suicide-a-global-imperative. Accessed January 20, 2020.
World Health Organization (2018) Suicide. https://www.who.int/news-room/fact-sheets/detail/suicide. Accessed January 20, 2020.
Yamada S, Yamamoto M, Ozawa H, Riederer P, Saito T (2003) Reduced phosphorylation of cyclic AMP-responsive element binding protein in the postmortem orbitofrontal cortex of patients with major depressive disorder. J Neural Transm 110:671–680.
Zhao J, Qi XR, Gao SF, Lu J, van Wamelen DJ, Kamphuis W, Bao AM, Swaab DF (2015) Different stress-related gene expression in depression and suicide. J Psychiatr Res 68:176–185.