

Epigenomic Profiling Reveals DNA-Methylation Changes Associated with Major Psychosis

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Epigenetic misregulation is consistent with various non-Mendelian features of schizophrenia and bipolar disorder. To date, however, few studies have investigated the role of DNA methylation in major psychosis, and none have taken a genome-wide epigenomic approach. In this study we used CpG-island microarrays to identify DNA-methylation changes in the frontal cortex and germline associated with schizophrenia and bipolar disorder. In the frontal cortex we find evidence for psychosis-associated DNA-methylation differences in numerous loci, including several involved in glutamatergic and GABAergic neurotransmission, brain development, and other processes functionally linked to disease etiology. DNA-methylation changes in a significant proportion of these loci correspond to reported changes of steady-state mRNA level associated with psychosis. Gene-ontology analysis highlighted epigenetic disruption to loci involved in mitochondrial function, brain development, and stress response. Methyloome network analysis uncovered decreased epigenetic modularity in both the brain and the germline of affected individuals, suggesting that systemic epigenetic dysfunction may be associated with major psychosis. We also report evidence for a strong correlation between DNA methylation in the *MEK1* gene promoter region and lifetime antipsychotic use in schizophrenia patients. Finally, we observe that frontal-cortex DNA methylation in the *BDNF* gene is correlated with genotype at a nearby nonsynonymous SNP that has been previously associated with major psychosis. Our data are consistent with the epigenetic theory of major psychosis and suggest that DNA-methylation changes are important to the etiology of schizophrenia and bipolar disorder.

Introduction

Schizophrenia (SZ) [MIM 181500] and bipolar disorder (BD) [MIM 125480] are etiologically related psychiatric conditions,¹ together termed “major psychosis.” Studies of major psychosis have focused primarily on the interplay between genetic and environmental risk factors. Twin and adoption studies highlight a clear inherited component to both disorders,² but whereas replicated findings exist for a number of genes, association studies are characterized by nonreplication, small effect sizes, and significant heterogeneity.³ Several epidemiological, clinical, and molecular peculiarities associated with major psychosis are difficult to explain with traditional gene- and environment-based approaches. Such peculiarities include the noncomplete concordance between monozygotic twins for both SZ (41%–65%) and BD (~60%),^{2,4} which cannot be accounted for by only environmental factors.^{3,5} Other complexities of major psychosis include a fluctuating disease course with periods of remission and relapse, sexual dimorphism, peaks of susceptibility to disease coinciding with major hormonal rearrangements, and parent-of-origin effects.³ These observations have led to speculation about the importance of epigenetic factors in mediating susceptibility to both SZ and BD.³

Epigenetics refers to the heritable, but reversible, regulation of various genetic functions, including gene expres-

sion, mediated through modifications of DNA and histones.⁶ Epigenetic processes are essential for normal cellular development and differentiation, and they allow the regulation of gene function through nonmutagenic mechanisms. The impact of DNA methylation on gene activity has been explained by two proven mechanisms. The “critical site” model puts an emphasis on the methylation of specific cytosines in transcription-factor binding sites, responsible for reducing binding affinity and thus the transcription of mRNA.⁷ The “methylation density” model suggests that the proportion of methylated cytosines across a region, rather than at any specific position, controls chromatin conformation and thus the transcriptional potential of the gene.⁷

The epigenetic model of major psychosis is based upon three general principles.³ First, like the DNA sequence, the epigenetic profile of somatic cells is mitotically inherited, but unlike the DNA sequence, epigenetic signals are dynamic. The epigenetic status of the genome is tissue-specific, developmentally regulated, and influenced by both stochastic and environmental factors. Second, because epigenetic processes regulate various genetic and genomic functions, epigenetic factors can have profound phenotypic effects. Genes, even those containing no mutations or disease-predisposing polymorphisms, can be harmful if not expressed in the appropriate amount,

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DOI 10.1016/j.ajhg.2008.01.008. ©2008 by The American Society of Human Genetics. All rights reserved.

Table 1. Demographic Data Associated with Frontal-Cortex Brain-Tissue Samples Utilized in this Study

	CTRL	SZ	BD
Number of Tissue Samples	35	35	35
Number Hybridized	28	35	32
Mean Age and Range	44.1 (31-59)	42.6 (19-59)	45.3 (19-64)
Race	35 white	35 White	33 White 1 Black 1 Native American
Sex	26M, 9F	26M, 9F	17M, 18F
Diagnosis	no axis I	27 undifferentiated 7 paranoid 1 disorganized	26 BP I 4 BP II 4 BP NOS 1 BP-SA
Psychotic Features	0	35+	20+, 11-, 4 unclear
Cause of Death	32 cardiac 3 other medical	14 cardiac 13 other medical 1 accident 7 suicide	12 cardiac 4 other medical 4 accidents 15 suicide
Postmortem Interval	Male: 26.9 (11.7) Female: 35.50 (9.6)	Male: 31.4 (17.0) Female: 30.88 (9.1)	Male: 37.8 (20.6) Female: 39.59 (17.7)
Refrigerator Interval	Male: 2.9 (1.6) Female: 2.83 (0.4)	Male: 5.67 (4.3)* Female: 6.25 (4.1)	Male: 7.80 (7.4)* Female: 13.18 (12.7)*
Brain pH	Male: 6.65 (0.26) Female: 6.45 (0.30)	Male: 6.44 (0.25)* Female: 6.61 (0.17)	Male: 6.46 (0.26)* Female: 6.44 (0.29)
Brain Weight	Male: 1486.4 (137.9) Female: 1314.2 (106.3)	Male: 1444.6 (104.8) Female: 1418.1 (128.8)	Male: 1467.6 (112.9) Female: 1316.2 (111.4)
Lifetime Alcohol Use	Male: 0.73 (0.94) Female: 1.00 (1.55)	Male: 2.36 (2.00)** Female: 1.38 (2.00)	Male: 2.86 (1.70)** Female: 2.29 (1.86)*
Lifetime Drug Use	Male: 0.27 (0.70) Female: 0.17 (0.41)	Male: 2.08 (2.00)** Female: 0.29 (0.76)	Male: 2.93 (2.05)** Female: 1.76 (1.8)*

Summary of demographic data for the brain samples obtained from the Stanley Foundation. Comparison of brain samples utilized for microarray-based epigenomic profiling. Given are the mean for each group (with SD). Affected individuals were found to have significantly lower brain pH (male SZ, male BD) and higher lifetime alcohol and drug use (male SZ, male BD, female BD) compared to unaffected controls of the same sex, reflecting data reported elsewhere. None of these demographic variables was correlated with DNA methylation.

*denotes a t test of $p < 0.05$ comparing affected group with controls.

**denotes a t test of $p < 0.001$ comparing affected group with controls.

at the correct time of the cell cycle, or in the correct compartment of the nucleus. Third, some epigenetic signals, rather than being reset and erased during gametogenesis, could be transmitted meiotically across generations.⁸ This has obvious ramifications for the identification of the molecular substrate of inherited predisposition, in which heritable phenotypic variation is assumed to result exclusively from DNA-sequence variants.

To date, few studies have investigated the role of epigenetic factors in major psychosis, and none has taken a genome-wide epigenomic approach. DNA-methylation differences have been reported in the vicinity of both catechol-O-methyltransferase (*COMT*)⁹ and reelin (*RELN*),¹⁰ although these findings were not confirmed using fully quantitative methylation-profiling methods.^{11,12} In this article we report findings from a comprehensive epigenomic study of major psychosis. Using DNA from the frontal cortex—a region previously implicated in the etiology of major psychosis¹³—derived from individuals with SZ, BD, and from matched controls (CTRL), we examined DNA methylation by utilizing two complementary approaches. First, we performed a microarray-based epigenomic scan of major psychosis using CpG-island microarrays after enrichment of the unmethylated fraction of brain DNA. Second, we performed a hypothesis-driven analysis of DNA methylation across candidate genes for which a priori evidence for a role in the etiology of major psychosis exists. In addition, to investigate whether epigenetic differences could be observed in the germline, we also used CpG-island microarrays to profile germline DNA methylation in BD patients and controls. Consistent with the epigenetic theory of major psychosis, we find

considerable evidence for epigenetic changes associated with schizophrenia and bipolar disorder.

Material and Methods

Samples

Frontal-cortex postmortem brain tissue from individuals with DSM-IV diagnosed SZ ($n = 35$), BD ($n = 35$), and matched controls ($n = 35$) was provided by the Stanley Medical Research Institute brain-array collection (courtesy of Drs. Michael B. Knable, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken). The samples consisted of frozen tissue sections, which were stored at -80°C prior to DNA extraction. Demographic data associated with these samples are summarized in Table 1. In addition, germline-DNA samples were obtained from mature spermatozoa of BD patients ($n = 20$) and unaffected controls ($n = 20$) from an ongoing study at the Centre for Addiction and Mental Health (Toronto, Canada). These individuals were matched for age (BD mean age = 44.2; CTRL mean age = 41.3) and ethnic background (all individuals of European ancestry). Extraction of all DNA was performed with a standard phenol-chloroform extraction method. The quality and quantity of DNA was assessed by spectrophotometry and agarose-gel analysis, and DNA was subsequently stored at -20°C until further use. Samples for which good-quality, nondegraded DNA was not available were excluded from subsequent analyses. The project has been fully approved by the Ethics Committee of the Centre for Addiction and Mental Health, Toronto.

Enrichment of Unmethylated DNA and Microarray Hybridization

We used our developed technology for enrichment of the unmethylated DNA fraction and for epigenetic profiling with microarrays, described in detail elsewhere.¹⁴ In brief, the

methylation-sensitive restriction enzyme *HpaII* (New England Biolabs) was used to digest 1 μ g of genomic DNA. DNA adaptors (annealing products of two primers, U-CG1A and U-CG1B [see Table S1]) were ligated to the cleaved DNA fragments, followed by treatment with *McrBC* (New England Biolabs), which digests DNA fragments containing two or more methylated cytosines, thereby further enriching the unmethylated fraction. Adaptor-PCR amplification of the ligated products, with the use of primers complementary to the adaptor sequence, consisted of 250 ng of ligated DNA, 2.5 mM $MgCl_2$, 0.2 mM aminoallyl-dNTPs (15 mM aminoallyl-2'-deoxyuridine 5'-triphosphate, 10 mM 2'-deoxythymidine 5'-triphosphate, and 25 mM each of 2'-deoxycytidine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate, and 2'-deoxyadenosine 5'-triphosphate), 200 pmol primer U-CG1B, and 5 U *Taq* polymerase (New England Biolabs) in 1 \times PCR reaction buffer (Sigma), to a final volume of 100 μ l. PCR conditions are adjusted in such a way that fragments < 1.5 kb (i.e., those that are digested, short, and thus unmethylated) will amplify preferentially. Cycling consisted of an initial cycle at 72°C for 5 min and 95°C for 1 min, 25 cycles at 95°C for 40 s and 68°C for 2 min 30 s, and a final extension at 72°C for 5 min. Given that the role of epigenetic effects in disease etiology could be sex-specific and that considerable differences are observed in the course and prognosis of major psychosis between males and females, we split our sample according to gender. For the brain samples, equal amounts of amplicons from CTRL male samples were mixed to form a male common-reference pool, and equal amounts of amplicons from CTRL female samples were mixed to form a female common-reference pool. Individual samples, including all CTRL samples, were then cohybridized with the relevant common-reference pool sample. For the germline samples, all samples were cohybridized with a common-reference pool made by combination of amplicons from all CTRL samples. Samples were hybridized on 12,192 CpG-island microarrays obtained from the University Health Network Microarray Facility in Toronto. For the brain samples, good-quality-DNA extraction, enrichment, and microarray hybridization was successful for 28 CTRL samples, 35 SZ samples, and 32 BD samples. Seven of the initial 35 CTRL samples and three of the initial 35 BD samples were excluded from the experiment on the basis of degraded DNA that could compromise efficient enrichment of the unmethylated DNA fraction. For the germline samples, good-quality-DNA extraction, enrichment, and microarray hybridization was successful for 19 CTRL samples and 20 BD samples. One CTRL sample was excluded on the basis of poor DNA quantity and quality following nucleic-acid extraction.

Microarray Data Preprocessing

Initial array-image processing and quality control was performed with GenePix Pro 6.0 (Molecular Devices). The array signals were background-corrected with NormExp and normalized with weighted block-by-block LOWESS normalization. Spots with ambiguous genome locations, including spots with no sequence or annotation, repetitive spots, and translocation hotspots were removed, leaving a total of 7834 spots.

Normality Testing

Several analyses assumed data to be drawn from a normal distribution; hence the need for normality testing. Log-intensity ratios for each spot were subjected to the Lilliefors test for normality. The resultant p values for all spots were adjusted for multiple testing by use of Benjamini and Hochberg's false-discovery rate (FDR) method.¹⁵

Microarray-Data Analysis

For analyses comparing affected individuals to unaffected individuals, affected samples were either grouped by diagnosis (i.e., SZ versus CTRL and BD versus CTRL) or, given increasing evidence for an etiological overlap between SZ and BD,^{1,16} grouped together into a "major psychosis" group (i.e., psychosis versus CTRL). Limma was used to analyze each array spot for differential methylation between affected and unaffected samples. Each spot was assigned a raw p value based on a moderated t statistic. To correct for multiple testing, the set of raw p values were converted to false-discovery rates (FDR) according to Benjamini and Hochberg.¹⁵

Gene-Ontology Analysis

A novel gene-ontological investigation approach was designed to determine if any common functional trends are associated with the genes exhibiting differences between groups. For each group interrogated, only those loci exhibiting a significance value of less than $p = 0.01$ from a spotwise t test were selected, in order to include only those loci likely to have a true DNA-methylation difference between groups. Gene IDs within 1 kb of these array loci were obtained from the microarray annotation data and cross-referenced with the April 2007 build of the Gene Ontology Database to obtain gene-ontology (GO) categories associated with each microarray locus. All loci and corresponding mean fold change values were sorted into categories on the basis of their GO classifications, and the distribution of each GO category was compared with a paired t test and the more conservative Wilcoxon Signed Rank test. In both cases, p values were adjusted with FDR to correct for multiple testing. Data were then sorted by FDR p value, revealing the most significantly different GO categories.

Network Analysis of Microarray Data

In order to investigate whether DNA methylation is coordinated across different loci, we utilized a novel network-based approach.¹⁷ For brain samples, this analysis was performed on twenty male SZ samples and on an equal number of male CTRL samples—the other diagnostic groups were not included in this analysis because of their small sample sizes. We identified the top 700 methylation-variable spots across the samples in each group. The union of these two sets, consisting of 1041 spots, was chosen for network reconstruction. To find connections between methylation at specific genomic regions (nodes), their methylation log intensities were modeled by a linear combination of the methylation log intensities at the remaining spots. After regression, the correlation between the minimized residuals was calculated for measuring the direct association between the two spots. Estimation of correlation and p value was accomplished by a regularized covariance estimator that addresses the issue of small size and large variable (20 is much smaller than 1042).¹⁸ As a control for the network analysis, in each of the 20 CTRL microarrays, we randomized the IDs of the 1041 spots and proceeded with the same estimator. A raw p value of 10^{-7} was then chosen to cut off the insignificant pairwise correlations. A connection was drawn between a pair of spots whose correlation p value survived the cut. The structure of each network was explored by calculation of the transitivity (quantification of the connectivity between a spot's neighbors) and assortativity (quantification of the tendency of attachment between high-connection spots). The modular structure of a network was detected by a partitioning algorithm¹⁷ that maximizes the within-module connection densities at the expense of between-module connection

densities. The analysis was repeated on the germline BD and CTRL samples.

Correlation with Antipsychotics Used

Linear regression was performed on psychosis patients, with log-intensity ratios for each spot applied as dependent variables and lifetime dosages of antipsychotics applied as independent variables. Base-2 logarithms of the dosages were taken for the regression due to their wide spread. After the regression, p values based on F-statistics were gathered for all spots and converted to FDR to control for multiple testing.

Bisulfite Treatment of Genomic DNA

Bisulfite treatment was performed by use of a standard protocol. In brief, ~500 ng of genomic DNA was denatured in 0.3 M NaOH for 15 min at 37°C. After the addition of freshly prepared 3.5 M sodium metabisulfite (Sigma) and 1 mM Hydroquinone (Sigma) solution, samples were subjected to a 5 hr incubation at 55°C under exclusion of light. The samples were then purified with QIAGEN DNA-purification columns. Recovered samples were desulfonated in 0.3 M NaOH for 15 min at 37°C and neutralized. DNA was precipitated overnight in ethanol at -20°C and resuspended in 50 µl buffer EB (QIAGEN). Bisulfite-treated DNA was stored at -80°C until needed.

Bisulfite Primer Design and PCR Amplification

Primers were designed with either MethPrimer (available online) or Pyrosequencing Assay Design Software v1.0.6 (Biotage, Uppsala, Sweden). For loci nominated from microarray analyses, primers were designed, where possible, to span a region containing potentially informative HpaII sites in the vicinity of the significant clone on the CpG-island microarray. Where necessary, larger regions were covered by use of several overlapping amplicons. For selected candidate genes, the primary focus of analysis was promoter CpG islands. In some cases (e.g., *COMT* and *BDNF*), additional exonic regions in the vicinity of known genetic polymorphisms were also investigated. Where candidate genes had been previously investigated by other groups (*RELN* and *COMT*), we ensured that the same regions were adequately covered by our analyses. A full list of primer sequences and annealing temperatures for each PCR reaction can be found in Table S1. PCR amplifications were performed with a standard hot-start PCR protocol in 25 µl volume reactions containing 3 µl of sodium-bisulfite-treated DNA, 1 µM primers, and a master mix containing hot-start *Taq* polymerase (Sigma). All PCR reactions were checked on a 1.0% agarose gel to ensure successful amplification and specificity before proceeding with pyrosequencing or MS-SNuPe.

Site-Specific DNA-Methylation Analysis with Pyrosequencing and MS-SNuPe

For pyrosequencing analysis, bisulfite-PCR products were processed according to the manufacturer's standard protocol (Biotage). In brief, 4 µl of streptavidin-sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) and 40 µl of binding buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl) were mixed with 40 µl of PCR product for 10 min at room temperature. The reaction mixture was placed onto a MultiScreen-HV, Clear Plate (Millipore, Billerica, MA, USA). After application of the vacuum, the beads were treated with a denaturation solution (0.2 N NaOH) for 1 min and washed twice with washing buffer (10 mM Tris-acetate at pH 7.6). The beads were then suspended with 24 µl of annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate at pH 7.6) containing 8

pmol of sequencing primer. The template-sequencing primer mixture was transferred onto a PSQ 96 Plate (Biotage), heated to 90°C for 2 min followed by 60°C for 10 min, and finally cooled to room temperature. Sequencing reactions were performed with a PSQ 96 SNP Reagent Kit (Biotage) according to the manufacturer's instructions. The percentage methylation at each CpG site was calculated from the raw data by use of Pyro-Q-CpG Software (Biotage). MS-SNuPe analysis was performed with ABI SNaPshot reagents (Applied Biosystems) by use of a method developed in our laboratory. Extension products were separated on an ABI3100 Genetic Analyzer (Applied Biosystems). Methylation data from pyrosequencing and MS-SNuPe analysis were analyzed by use of SPSS v14 (Lead Technologies) with standard t tests and ANOVA.

Genotyping of COMT and BDNF SNPs

Nonsynonymous SNPs in *COMT* (rs4680-val108/158met) and *BDNF* (rs6265-val66met) were genotyped with the pyrosequencing assays designed to interrogate the density of methylated cytosines in these regions. In addition, genotypes were double-checked by use of the ABI TaqMan Allelic discrimination method utilizing Assay-on-Demand reagents provided by the manufacturer (Applied Biosystems) and the ABI 7900HT Sequence Detection System. DNA methylation at surrounding CpG sites was compared between samples grouped by genotype with standard t tests.

Results

Overview of Experimental Strategy

This study utilized two complimentary approaches for detection of DNA-methylation differences associated with major psychosis. Our first strategy involved enrichment of the unmethylated fraction of genomic DNA and subsequent hybridization on CpG-island microarrays. SZ and BD samples were compared to unaffected CTRL samples both separately and, given increasing evidence for an etiological overlap between the two disorders,^{1,16} as a combined major-psychosis group. Of the genomic regions showing a significant difference between affected and unaffected groups, a number of loci were of particular interest given our prior knowledge about the etiology of the disorder, and a subset of these were selected for subsequent bisulfite-based fine-mapping of methylated cytosines for verification of our microarray approach. We also employed gene-ontology analysis to uncover functional pathways epigenetically altered in major psychosis, and we utilized novel network-based analyses to investigate epigenetic modularity in affected individuals and controls. Using demographic data available from the brain samples, we investigated correlations between DNA methylation and variables including lifetime antipsychotic intake. Our second major strategy involved bisulfite-based fine-mapping across ten candidate genes previously implicated in major psychosis and principally nominated from genetic association and expression studies of both SZ and BD.

Methylomic Profiling of Brain DNA

Figure 1 illustrates raw p values for microarray signal intensity versus fold change observed for comparisons between

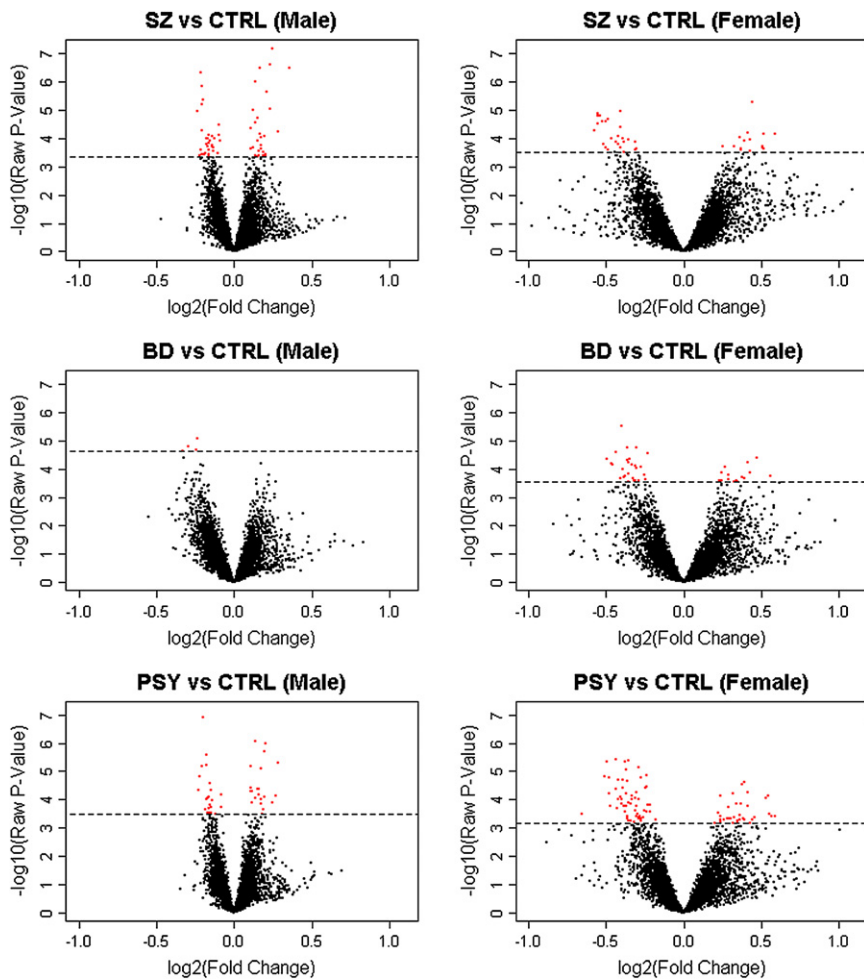


Figure 1. Frontal-Cortex DNA-Methylation Differences

Volcano plots showing raw p values for differential DNA methylation versus fold change observed when the hypomethylated fraction of brain DNA from psychosis patients was compared to that from unaffected individuals by use of 12K CpG-island microarrays. Each spot (red and black) represents an array probe averaged across all individuals in a group, with red spots denoting probes with FDR < 5%. SZ indicates schizophrenia, BD indicates bipolar disorder, and PSY indicates combined psychosis group (SZ and BD).

no significant correlation was found between males and females, suggesting that sex-specific etiological factors may play a stronger role.

Gene-expression data for the samples used in this study are available from the Stanley Medical Research Institute Online Genomics Database.²¹ Figure 3 illustrates two examples of the available expression data obtained from this database. Figure 3A highlights downregulation of *NR4A2*, a gene found to be hypermethylated in female SZ samples (FDR = 0.021). For *NR4A2*, nine out of 24

brain DNA from major-psychosis patients and unaffected controls, matched for sex. Significant (FDR < 0.05) mean differences were found for spots associated with a number of genes (Table S2 and Figure 2). Many of these loci are consistent with our knowledge about the neurobiological and genetic systems involved in major psychosis, including several glutamatergic and GABAergic genes, loci involved in neuronal development, and loci in regions highlighted in genetic linkage studies (Table S3).

Although our initial analyses separated samples by sex, we were also interested in the overlap between males and females. DNA methylation in SZ males and SZ females was significantly correlated ($r^2 = 0.13$, $p = 8.1e-26$), suggesting that there are SZ-associated epigenetic changes common to both sexes. Of particular interest was evidence for FDR-significant hypermethylation in both male and female samples in the vicinity of two genes. The first is *RPP21* (male SZ FDR = 0.025; female SZ FDR = 0.021), which encodes a component of ribonuclease P, a protein complex that generates mature tRNA molecules by cleaving their 5'-ends¹⁹. The second is *KEL* (male SZ FDR = 0.04; female SZ FDR = 0.044), encoding the Kell blood-group glycoprotein.²⁰ Interestingly, both regions are also FDR-significantly hypermethylated in female BD samples (*RPP21* FDR = 0.045; *KEL* FDR = 0.045). For BD, however,

studies on SZ samples show significantly reduced expression, with the overall analysis across all mRNA studies in the Stanley Array Collection being highly significant ($p < 0.00001$). Figure 3B highlights significant downregulation of *GLRX5*, which we found to be hypermethylated in male SZ samples (FDR = 0.04), across expression studies on SZ brains in the Stanley Array Collection ($p = 0.003$). Figure 2 summarizes the available expression data for all FDR-significant DNA-methylation changes. Interestingly, 82% of the loci found to be hypermethylated in major-psychosis samples for which expression data are available (40 out of 49) are significantly downregulated in at least one gene-expression study, with 24% (12 out of 49) showing significant downregulation of expression across all mRNA studies performed on these samples in either SZ, BD, or both. The story for hypomethylated samples is more complex, with only 34% of loci for which gene expression data are available (11 out of 32) showing significant upregulation in at least one gene-expression study.

Analysis of Demographic Data, Brain-Tissue Parameters, and Lifetime Antipsychotic Use

No FDR-significant correlations were found between any of the available demographic variables (PMI, brain weight, brain pH, lifetime alcohol use, and lifetime illicit-drug

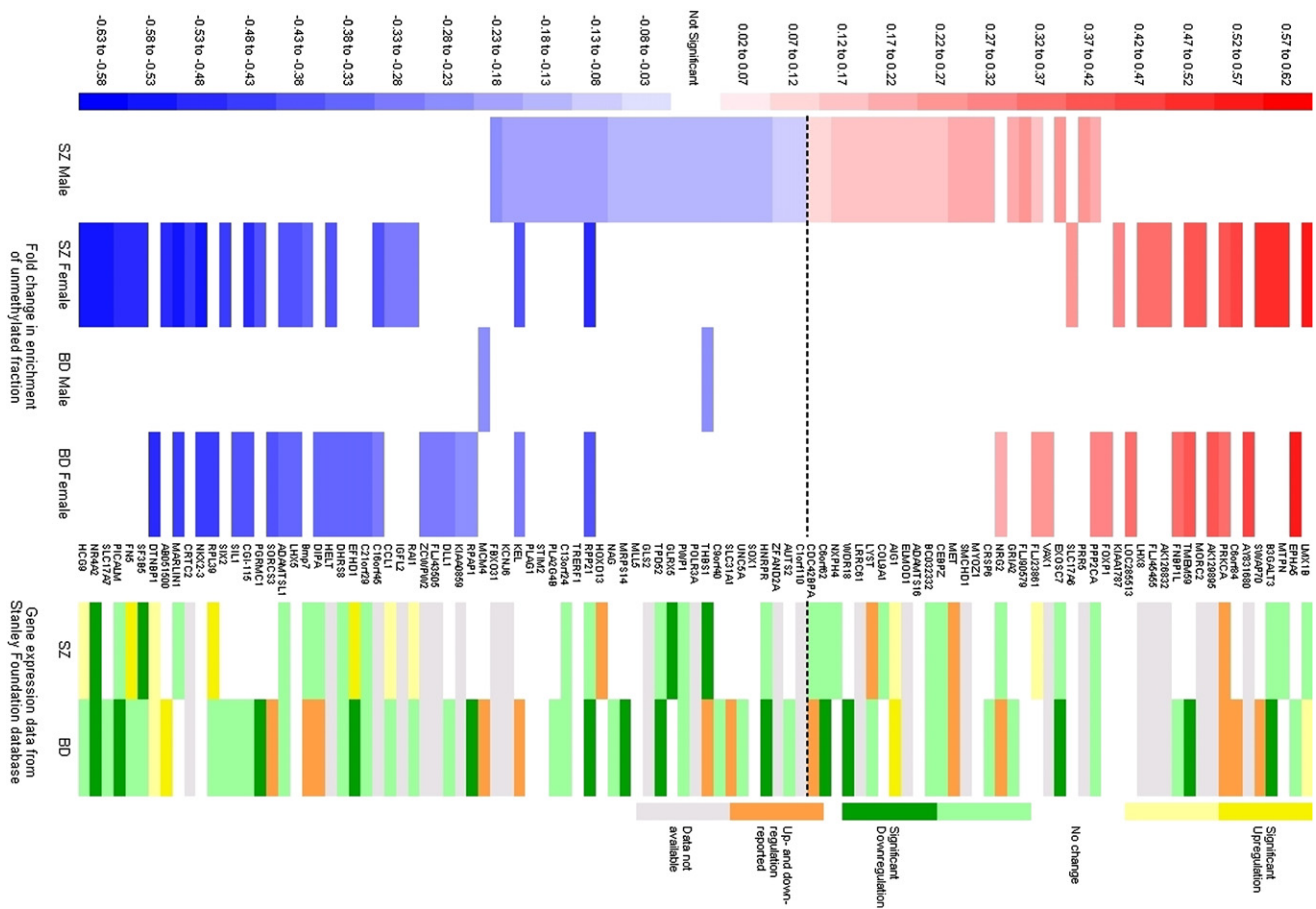


Figure 2. FDR-Significant DNA-Methylation Differences Associated with Major Psychosis

A positive fold change (red) corresponds to lower DNA methylation in the affected group, and a negative fold change (blue) corresponds to higher DNA methylation in the affected group. Also shown are gene-expression data from the same group of samples obtained from the Stanley Research Foundation database. Dark yellow/green corresponds to significant transcript up-/downregulation in a meta-analysis of all expression studies performed on these samples, and light yellow/green corresponds to evidence of significant up-/downregulation from at least one study. Orange indicates that expression has been reported as altered in both directions in different studies. Grey denotes missing data.

use) and DNA methylation. Methylation of a CpG island located ~30kb upstream of the gene-encoding mitogen-activated protein kinase kinase I (*MEK1*) was found to be significantly correlated with lifetime antipsychotic use in male SZ samples ($n = 25$), with higher lifetime antipsychotic use associated with lower DNA methylation (Figure 4A) ($r^2 = 0.6$, $p = 6.8E-06$, $FDR = 0.04$). Interestingly, a similar correlation in the same direction is also observed in female SZ samples (Figure 4B), although this does not reach FDR significance due to the small number ($n = 9$) of samples with available medication data ($r^2 = 0.5$, $p = 0.04$). No such correlation is observed in the BD samples.

Methylomic Profiling of Germline DNA

Figure 5 illustrates raw p values for array signal intensity versus fold change observed in our comparison of germline DNA from BD patients and unaffected CTRLs. No FDR-significant differences were observed between the two groups. A comparison of the largest psychosis-associated DNA-methylation differences in the germline analysis

with those in the brain DNA analysis, taking loci with a raw $p < 0.001$, found no overlap between datasets.

Site-Specific CpG-Methylation Analysis in Selected Genes

After microarray analysis, we tested a number of loci to further verify the microarray approach. From the genes listed in Table S3, we quantitatively measured site-specific CpG methylation upstream of *DTNBP1* ($n = 30$), *GRIA2* ($n = 39$), *HCG9* ($n = 31$), *HELT* ($n = 26$), *KCNJ6* ($n = 26$), *LHX5* ($n = 24$), *MARLIN-1* ($n = 28$), *NR4A2* ($n = 24$), *RPL39* ($n = 25$), *SLC17A7* ($n = 24$), *TMEM59* ($n = 30$), and *WDR18* ($n = 29$). Given that our enrichment strategy was based on differential cleavage of *HpaII* sites, we focused primarily on these and surrounding CpG positions located in or near genomic regions corresponding to specific microarray probes.

Our site-specific CpG analyses show good agreement with data obtained from microarray analysis, although the absolute differences observed are generally small. Two examples

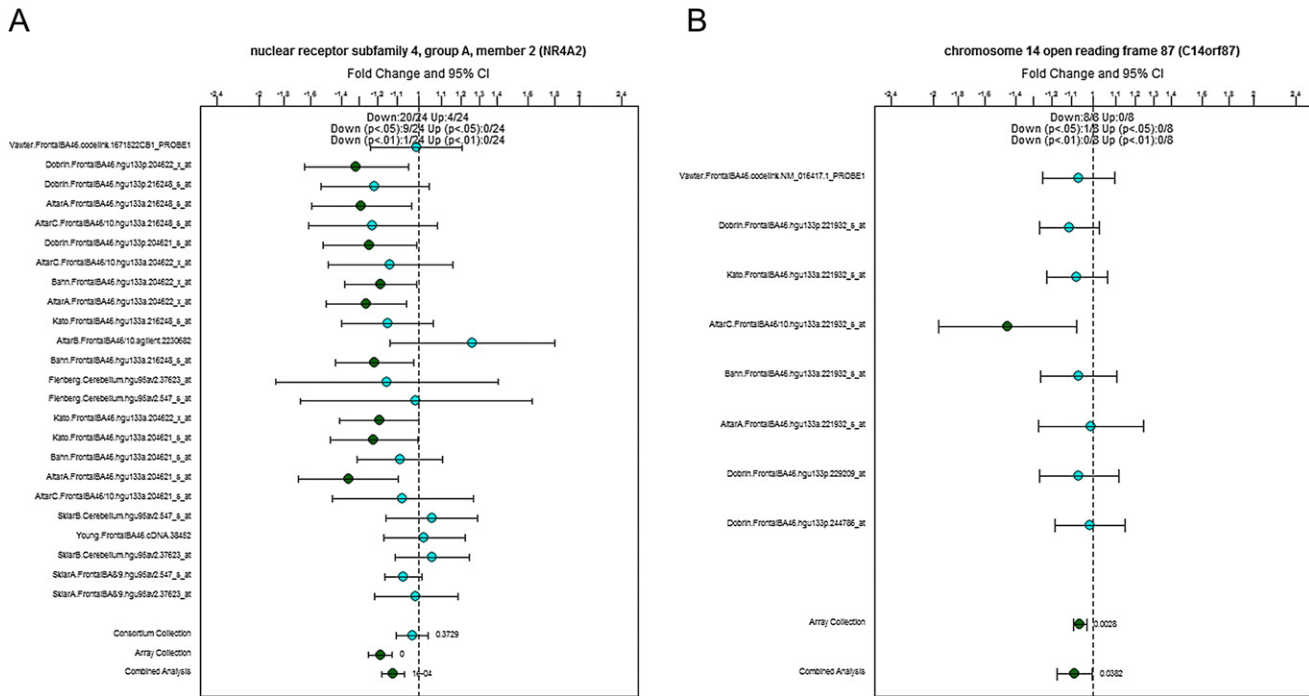


Figure 3. Examples of Data Obtained from the Stanley Research Foundation Expression Database

Shown is down-regulation of (A) *NR4A2* and (B) *GLRX5* (alternative name *C14orf87*) in SZ samples obtained from the Stanley Array Consortium. Data points refer to the relative expression fold change in the affected group relative to control samples. Scale bars denote 95% confidence intervals.

are shown in Figure 6 for regions upstream of the genes *WDR18* and *RPL39*. Microarray analysis (Figure 6A) predicted these regions to be hypomethylated in SZ male samples and hypermethylated in BD female samples, respectively. Figure 6B shows pyrosequencing data confirming *WDR18* hypomethylation in male SZ samples compared to controls ($n = 29$, average methylation 17% versus 25%, $p < 0.001$). This region contains a putative binding site for the brain-expressed transcription factor c-myc, known to be blocked by CpG methylation²²(Figure 6C). Pyrosequencing also verified *RPL39* hypermethylation in BD female samples ($n = 25$, 28% versus 22%, $p = 0.009$), especially at a CpG located within putative binding sites for several brain-expressed transcription factors, PAX-5²³ and NF- κ B,²⁴ which are known to be affected by DNA methylation (Figure 6C). Example pyro-

grams across both regions showing representative affected and unaffected individuals are shown in Figure 6D.

In addition to *WDR18* and *RPL39*, we were able to confirm significant DNA-methylation differences in *MARLIN-1*, postulated to be hypermethylated in affected female samples from microarray analysis. Average methylation of a *HpaII* site located in the genomic region spanning the microarray clone and falling in a putative binding site for a Pbx1/Meis1 heterodimer was 84% in unaffected controls compared to 93% in SZ females ($p = 0.04$) and 91% in the combined major-psychosis female group ($p = 0.03$). Interestingly, whereas only 13% of unaffected control samples were fully methylated, 71% of SZ females and 47% of the combined female major-psychosis group were fully methylated. Other significant DNA-methylation differences

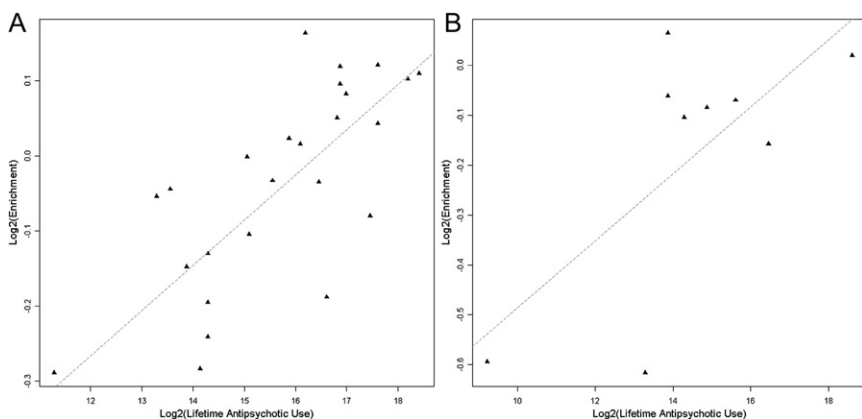


Figure 4. DNA Methylation and Lifetime Antipsychotic Use

Correlation between DNA methylation in the promoter of the mitogen-activated protein kinase I gene (*MEK1*) and lifetime antipsychotic use in (A) male schizophrenia samples ($r^2 = 0.6$, $p = 6.76E-06$) and (B) female schizophrenia samples ($r^2 = 0.5$, $p = 0.04$).

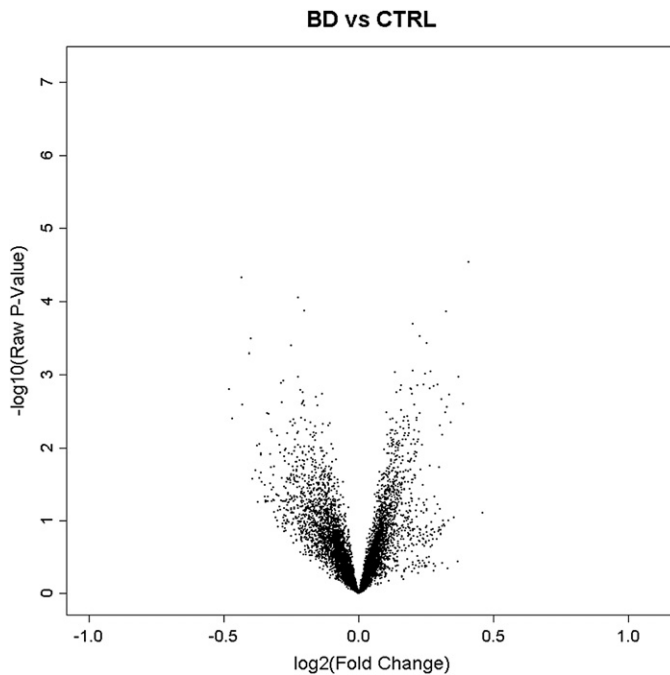


Figure 5. Germline DNA-Methylation Differences

Volcano plot showing raw p values for differential DNA methylation versus fold change observed when the hypomethylated fraction of germline DNA from BD patients was compared to that from unaffected individuals by use of 12K CpG-island microarrays. No FDR-significant DNA-methylation differences were observed.

were observed for CpG sites upstream of *DTNBP1* and *HCG9*, confirming hypermethylation in affected female samples relative to controls, as predicted by microarray data for both genes (*DTNBP1*: 62% versus 58%, $p = 0.04$; *HCG9*: 20% versus 15%, $p = 0.04$).

Overall significant DNA-methylation differences were observed in five of the 12 specific regions tested. However, even in the regions where no overall significant CpG-methylation differences were detected, changes were consistently in the direction predicted by our microarray analysis. The regions tested with bisulfite sequencing are somewhat arbitrary, and it is likely that the specific critical CpG sites causing differential enrichment (and thus microarray signal intensity) were not included in all the amplicons tested. Five confirmed loci out of 12 tested (~40%) is thus a good rate of verification, especially given that no DNA-methylation differences were observed across any of the 12 arbitrarily chosen negative-control array regions tested (*ACTL6A*, *BCL11A*, *DDX1*, *DUSP10*, *GNL1*, *GPR160*, *LGI2*, *REV3L*, *PTPNS1*, *PHGDHL1*, *STIL*, and *ZNF218*) or across any of the a priori psychosis-candidate genes, which were not selected on the basis of microarray analysis (see below). These data suggest that a substantial proportion of epigenetic differences detected in our microarray experiments are real and that the conclusions drawn from the full array dataset are likely to be based on genuine DNA-methylation changes.

Gene-Ontology Analysis of Brain Methyloomic Data

The top 60 GO categories for each diagnostic group can be seen in Figure 7, and Table S4 lists all significant GO categories with a $p < 0.01$. GO categories detected by this analysis included genes involved in the epigenetic regulation of transcription and development. Of particular interest to the etiology of psychosis were the FDR-significant associations for “response to stress” in male BD samples and

for “brain development” in both female BD samples and female SZ samples. In addition, consistent with the postulated link between mitochondrial function and psychosis,²⁵ several “mitochondrial function” GO categories showed significantly different distributions in the affected individuals compared to controls.

Modularity in DNA-Methylation Microarray Data

In the brain, the average number of connections between nodes (representing correlated methylation observed between different genomic loci) is higher in the SZ group compared to the CTRL group (2.7 versus 1.7) (Figure 8A). The large clustering coefficient (CTRL = 0.17, SZ = 0.22), and its decrease with increasing connections in both sample groups, suggests that both are hierarchically modular. The lack of clustering in a series of simulated “random” datasets suggests that this modularity is likely to be a real biological phenomenon (Figure 8B). Assortativity is higher in SZ ($k_{nn} = 9$) compared to CTRL ($k_{nn} = 6$), probably reflecting the higher number of connections between nodes in SZ (Figure 8C). Although the number of modules (CTRL = 42, SZ = 43) and median size of modules (CTRL = 10, SZ = 11) is approximately the same in both groups, the degree of modularity is higher in CTRL (0.56) than in SZ (0.44), suggesting some epigenetic dysfunction in affected individuals (Figure 9). A similar pattern is seen in the germline, with higher connectivity (3.9 versus 1.5) and assortativity (15 versus 7) in affected individuals compared to controls, a high degree of clustering in both groups (CTRL = 0.14, BD = 0.17), but higher modularity in unaffected individuals (0.42 versus 0.33).

DNA-Methylation Analysis of Psychosis-Candidate Genes in Brain DNA

The second approach utilized in this study assessed DNA methylation across specific candidate-gene regions with sodium bisulfite treatment and subsequent pyrosequencing analysis to quantitatively measure the density of methylated cytosines. We found little evidence of any psychosis-associated DNA-methylation differences in any of the ten genes tested (Table S5), including the promoter regions of *COMT* and *RELN* found to be differentially methylated in previous studies. Nonsynonymous SNPs in both *COMT* (rs4680-val108/158met) and *BDNF* (rs6265-val66met) create or abolish exonic CpG sites. In *COMT*, surrounding CpG sites were highly methylated (>95%) in all samples tested, with no correlation between

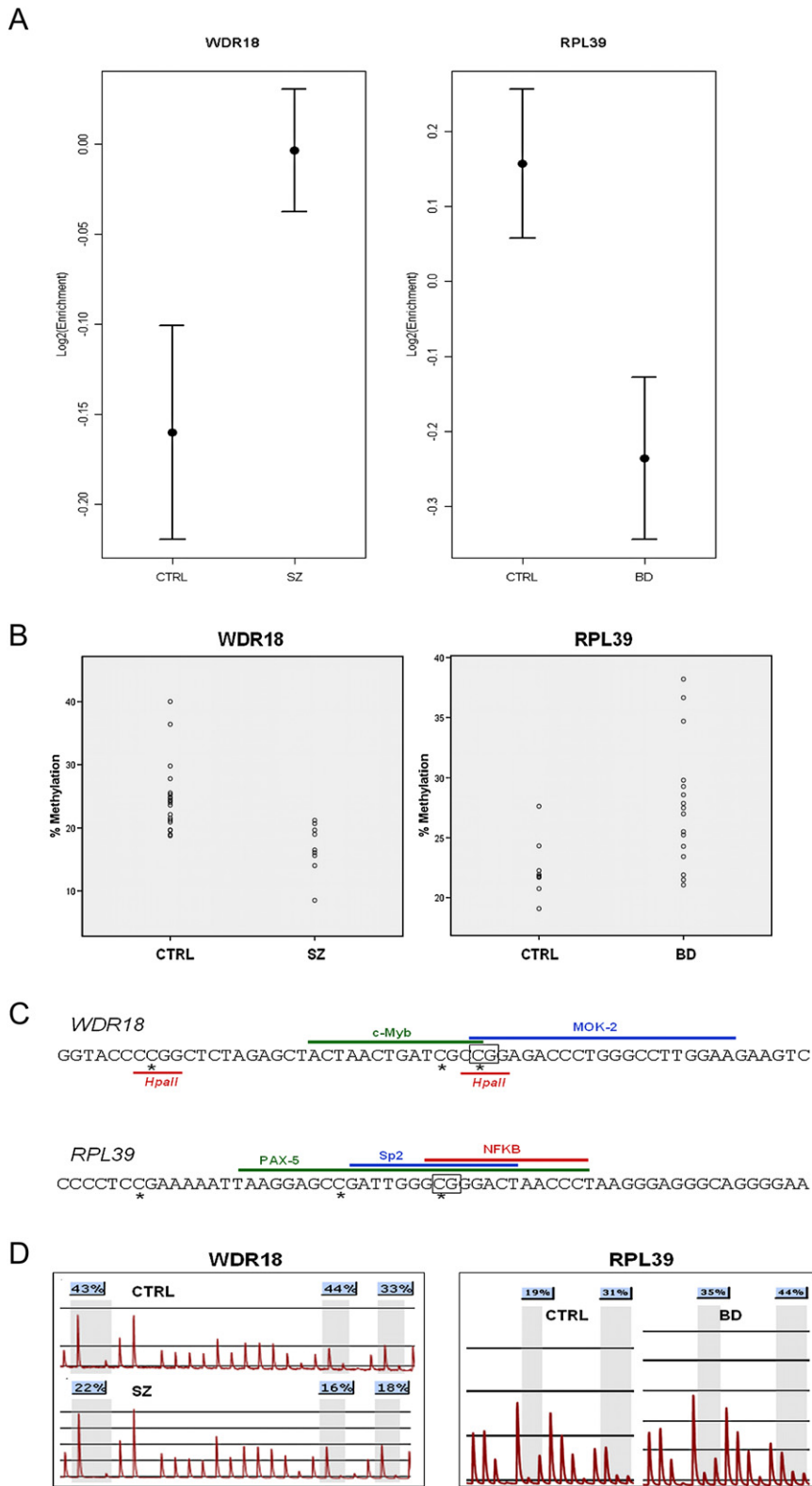


Figure 6. Bisulfite Modification and Pyrosequencing Verification of CpG-Methylation Differences in Two Genes Nominated from Microarray Analysis

(A) Microarray signal intensity for probes located in CpG islands in the promoter region of *WDR18* and *RPL39*. Scale bars denote 95% confidence intervals. For *WDR18*, male SZ samples had significantly higher intensities than did unaffected control samples (raw $p = 4.5E-05$, FDR corrected = 0.05), indicating hypomethylation in affected individuals. For *RPL39*, female BD samples had significantly lower intensities than unaffected control samples (raw $p = 4.0E-05$, FDR corrected = 0.02), indicating hypermethylation in affected individuals.

(B) Bisulfite mapping across amplicons spanning regions interrogated by CpG-island microarrays confirms DNA-methylation changes predicted by microarrays: shown are *WDR18* hypomethylation in male SZ samples ($p < 0.001$) and *RPL39* hypermethylation in female BD samples ($p = 0.009$).

(C) Predicted transcription-factor binding sites in the regions of *WDR18* and *RPL39* analyzed by pyrosequencing. Boxes indicate CpG sites with the largest DNA-methylation differences in affected individuals.

(D) Example pyrograms demonstrating relative *WDR18* hypomethylation in a SZ male sample and *RPL39* hypermethylation in a BD female sample.

quencing (average methylation = 83% versus 78%, $p = 0.02$), with CpG1 (86% versus 77%, $p = 0.01$) and CpG3 (79% versus 71%, $p = 0.03$) showing the largest differences (see Figure 10).

Discussion

In this study we performed a microarray-based epigenomic scan using CpG-island microarrays and found psychosis-associated brain-DNA-methylation differences in numerous loci, including many genes that have

been functionally linked to disease etiology. Consistent with increasing evidence for altered glutamatergic and GABAergic neurotransmission in the pathogenesis of major psychosis,^{26,27} we identified epigenetic changes in loci associated with both of these neurotransmitter pathways.

In *BDNF* there was modest evidence for an association between genotype and DNA methylation. Of the samples tested, 74% were CC (val homozygotes) and 26% were CT or TT (met carriers). Val homozygotes had significantly higher DNA methylation across the exonic region profiled with pyrosequencing.

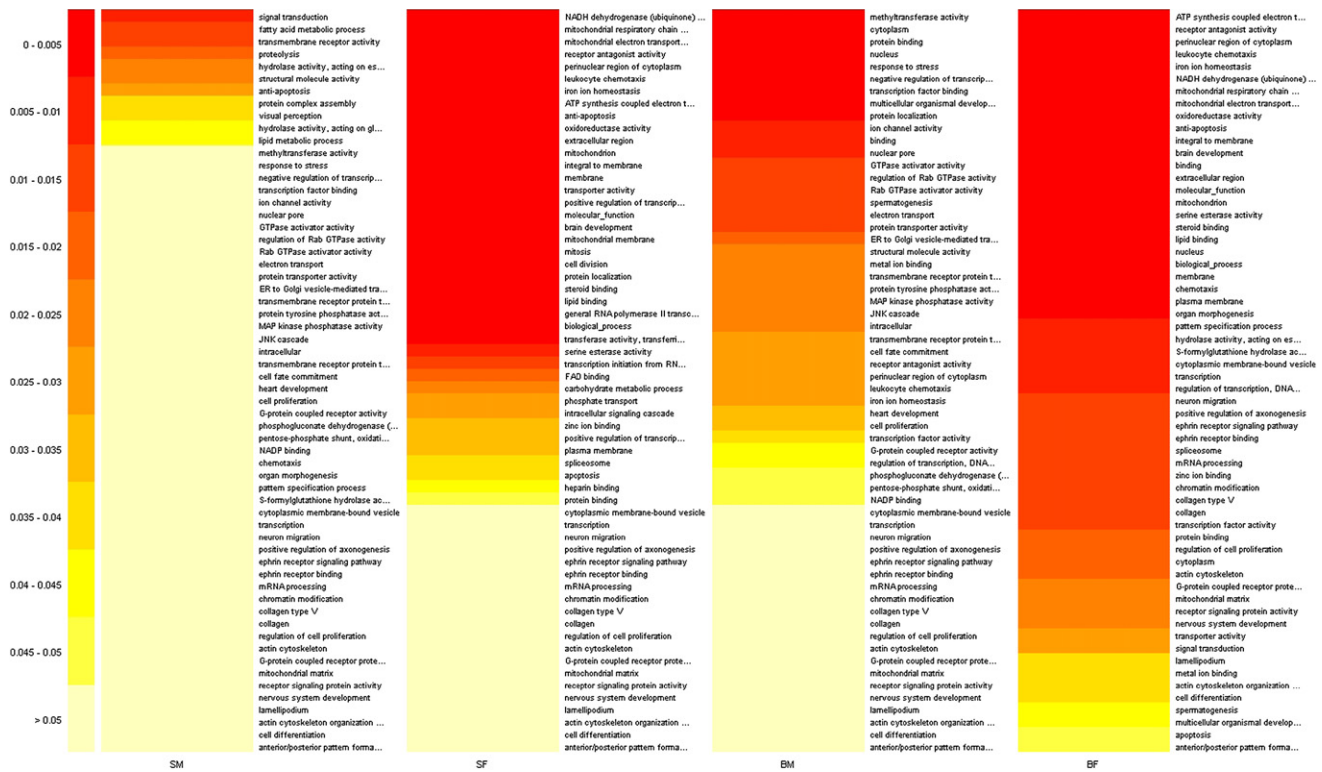


Figure 7. Gene-Ontology Analysis

The top 60 GO categories for each diagnostic group (SM = SZ male, SF = SZ female, BM = BD male, BF = BD female). Colors denote raw p values. See Table S4 for additional data.

Glutamate is the most abundant fast excitatory neurotransmitter in the mammalian nervous system, with a critical role in synaptic plasticity. Several lines of evidence link the glutamate system to psychosis, in particular the observation that glutamate-receptor agonists can cause psychotic symptoms in unaffected individuals. Probes associated

with two glutamate-receptor genes—one near *WDR18*, located ~10 kb upstream of the NMDA-receptor-subunit gene *NR3B* (also known as *GRIN3B*) and another in the promoter of the AMPA-receptor-subunit gene *GRIA2*—were found to be hypomethylated in SZ and major-psychosis males. Dysregulation of both NMDA and AMPA glutamate

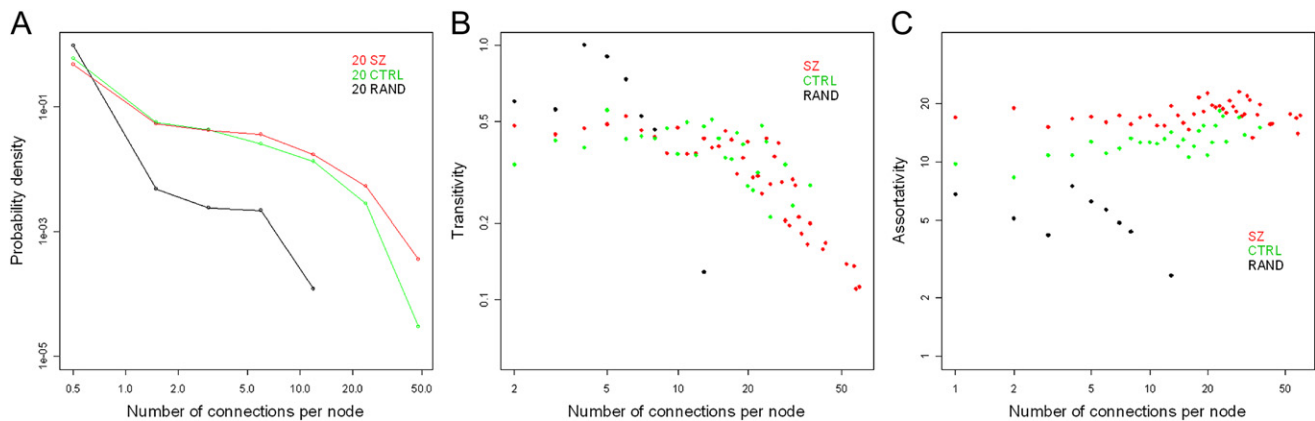


Figure 8. Network Analysis of DNA-Methylation Microarray Data

(A) The average number of connections between nodes is higher in the SZ sample group (2.7) compared to the CTRL sample group (1.7). (B) The clustering coefficient is high in both groups (CTRL = 0.17, SZ = 0.22) and decreases with increasing connections, suggesting that both groups are hierarchical to the same degree. (C) Assortativity was found to be higher in SZ (knn = 9) compared to CTRL (knn = 6), reflecting the higher number of connections between nodes in the SZ data. A simulated dataset generated by random shuffling of microarray data produced a network with a low number of connections and a low clustering coefficient.

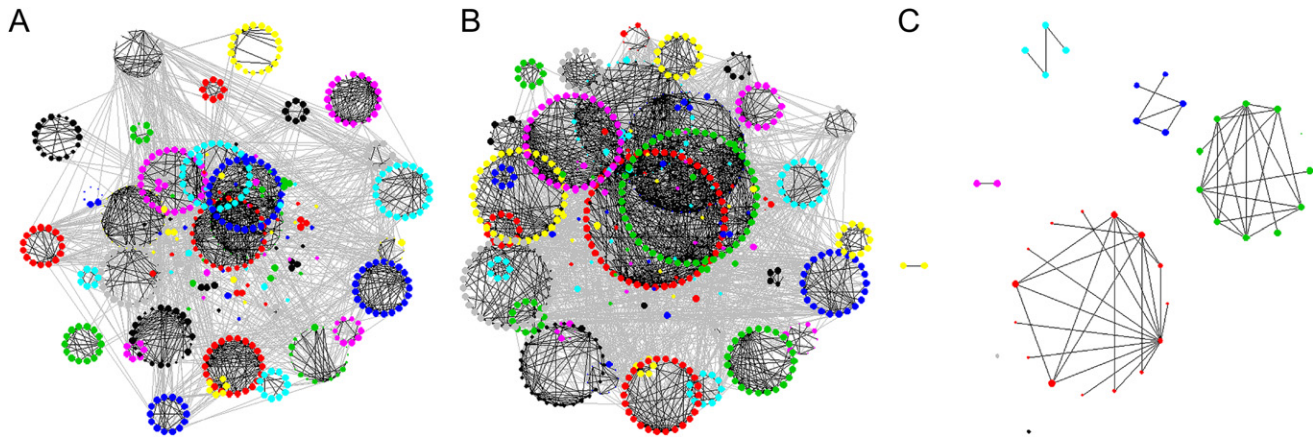


Figure 9. Reduced Epigenetic Modularity in Major Psychosis

Partial-correlation network analysis of microarray data illustrating significant connections between nodes ($p < 1e-7$) demonstrating strong hierarchical modularity for (A) male CTRL samples ($n = 20$) and (B) male SZ samples ($n = 20$) but not for (C) a randomly shuffled dataset. Although modularity is apparent in both sample groups, it is lower in the SZ group (0.44) than in the CTRL group (0.56). A similar pattern of modularity is seen in the comparison of methylation between germline male BD samples (0.33) and unaffected CTRLs (0.47).

receptors is important in the etiology of major psychosis,²⁸ and *GRIA2* expression is altered in the brains of SZ patients.²⁹

Various types of glutamate transporters are present in the plasma membranes of glial cells and neurons. Our data suggest that two vesicular glutamate transporters (VGLUTs), which pack glutamate into synaptic vesicles, are epigenetically altered in major psychosis. Given the link between DNA methylation and gene transcription, our data concur with data from gene expression studies and the observation that *VGLUT1* and *VGLUT2* are expressed in a complementary manner in cortical neurons.³⁰ *VGLUT1*, which was hypermethylated in SZ female samples, is downregulated in the brains of SZ patients.³¹ In addition, *VGLUT2*, which is upregulated in SZ patients,³² is hypomethylated in SZ females.

Several other glutamatergic genes showed evidence of epigenetic dysregulation in major psychosis. *GLS2*, which encodes a glutaminase enzyme that catalyzes the hydroly-

sis of glutamine to glutamate, was hypermethylated in SZ male samples. Previous studies report that glutaminase expression is altered in the pathology of SZ.³³ The gene encoding Secretogranin II (*SCG2*), a secretory protein located in neuronal vesicles that is known to stimulate the release of glutamate, was hypomethylated in major-psychosis females relative to unaffected controls. *SCG2* expression is known to be modulated by both chronic PCP exposure, which mimics symptoms of major psychosis,³⁴ and lithium treatment.³⁵

Unlike glutamate, which is a strong excitatory neurotransmitter, GABA acts as a potent inhibitory neurotransmitter. Hypofunctioning GABAergic interneurons appear to be important in the etiology of major psychosis.²⁷ Our data suggest that *MARLIN-1*, a RNA-binding protein that is widely expressed in the brain and regulates the production of functional GABA(B) receptors,³⁶ is hypermethylated in SZ, BD, and major-psychosis female

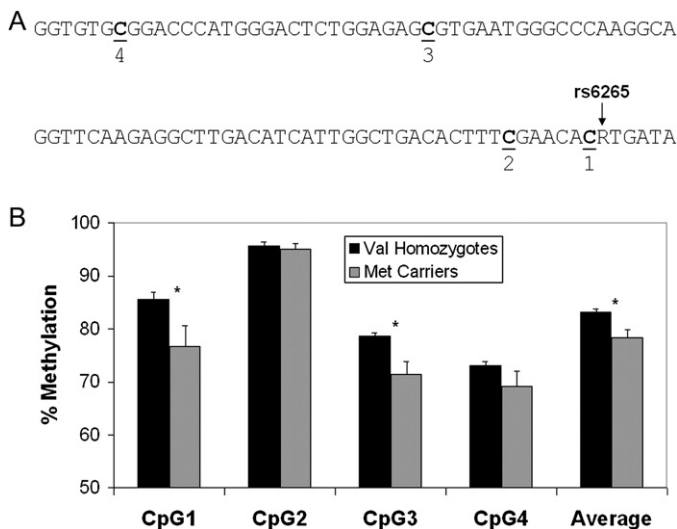


Figure 10. BDNF genotype and DNA Methylation

Association of *BDNF* genotype with DNA methylation at nearby exonic CpG sites in DNA obtained from frontal-cortex brain tissue.

(A) the exonic region of *BDNF* covered by out bisulfite mapping indicating the four CpG sites tested.

(B) DNA methylation at the four CpG sites in the vicinity of rs6265. Asterisk denotes t test of $p < 0.05$, bars denote mean standard error.

samples. In addition, *KCNJ6*, a G protein-coupled inwardly rectifying potassium channel that has been linked to the regulation of GABA neurotransmission,³⁷ was found to be hypermethylated in SZ and major-psychosis males. Increasing evidence suggests that both the glutamate and GABA systems are synergistically involved in major psychosis,²⁶ supporting our observation of increased *HELT*-promoter methylation in SZ and BD female samples. *HELT* is known to determine GABAergic over glutamatergic neuronal fate in the developing mesencephalon.³⁸

We observed evidence for epigenetic dysregulation near several genes involved in neuronal development in the brain. *WNT1*, an integral part of the Wnt signaling pathway that is critical for neurodevelopment, which is differentially expressed in SZ brains,³⁹ was significantly hypermethylated in major-psychosis females relative to controls. The transcriptionally inducible nuclear-receptor *NR4A2*, downregulated in both SZ and BD (see Figure 3A and⁴⁰), was found to be hypermethylated in SZ females. *FOSB*, which encodes a protein controlling cell proliferation in the brain known to be expressed following chronic antipsychotic treatment,⁴¹ was hypomethylated in major-psychosis females relative to controls. Finally, the LIM homeobox transcription factors *LMX1B* and *LHX5*, linked to normal learning and motor functions,⁴² also showed significant methylation changes in female psychosis samples, with *LMX1B* demonstrating putative hypomethylation and *LHX5* demonstrating putative hypermethylation.

Several other genes with links to major psychosis were found to be epigenetically altered. Given that phospholipid metabolism is disturbed in SZ,⁴³ it is noteworthy that the phospholipase gene *PLA2G4B* was hypermethylated in SZ male, major-psychosis male, and major-psychosis female samples. *RAI1*, hypermethylated in SZ female samples, is located in an unstable genomic region encoding a polymorphic polyglutamine tract associated with SZ and response to antipsychotic medication.⁴⁴ *AUTS2*, hypermethylated in SZ male samples, spans a translocation breakpoint associated with mental retardation and autism.⁴⁵ Finally, a probe located ~90 kb upstream of one of our pre-nominated "psychosis-candidate genes," *DTNBP1*, was hypermethylated in affected females.

There is considerable clinical, epidemiological, genetic, and neurochemical evidence to support a role for sex-specific factors in the etiology of both SZ⁴⁶ and BD.⁴⁷ For these reasons, our initial analyses considered males and females separately. It can be hypothesized that sex-specific differences represent underlying differences in etiology that may be mediated by epigenetic processes.⁴⁸ For example, although sex hormones cannot change DNA sequence, it is known they can be potent modifiers of epigenetic status and gene expression. There are several reports of the female sex hormone estrogen, for example, altering the chromatin-configuration and DNA-methylation profile of specific loci in the genome,^{49,50} potentially controlling gene expression in a sex-specific manner. We

were also interested in the overlap between data from male and female psychosis samples. Methyloomic array data from SZ males and SZ females were significantly correlated, signifying an overlap in the genomic regions epigenetically altered in both sexes. Of particular interest was evidence for FDR-significant hypermethylation in both male and female samples in the vicinity of two genes. The first is *RPP21*, which encodes a component of ribonuclease P, a protein complex that generates mature tRNA molecules by cleaving their 5'-ends¹⁹. The second is *KEL*, encoding the Kell blood-group glycoprotein.²⁰ Interestingly, abnormal expression of Kell antigens is one cause of McLeod Syndrome [MIM 314850], which is known to manifest itself in symptoms of schizophrenia.⁵¹ Of note, both *RPP21* and *KEL* are also FDR-significantly hypermethylated in female BD samples. For BD, however, no significant overall correlation was found between males and females, suggesting that sex-specific etiological factors could play a stronger role in BD than SZ. Taken together, these data reinforce the benefits of performing epigenetic studies separated by sex, but they also indicate that there is significant overlap between males and females for DNA-methylation profiles associated with SZ.

No correlation was found between any demographic variables or postmortem brain parameters and DNA methylation. Given the dynamic nature of the epigenome, however, and evidence linking drug exposure to DNA methylation, we also examined the epigenetic effect of antipsychotic treatment. Methylation of a CpG island located upstream of *MEK1* was found to be strongly correlated with antipsychotic use in SZ, as measured by haloperidol intake. This correlation was particularly strong in male SZ samples, but it was also present in female SZ samples. No correlation was seen in BD samples. The link between *MEK1* and antipsychotic exposure in SZ is striking given the involvement of mitogen-activated protein-kinase (MAPK) signaling pathways in mediation of intraneuronal signaling and the observation that clozapine, a widely used medication in the treatment of SZ, selectively activates this pathway via an interaction with *MEK1*.⁵²

GO analysis allows the investigation of functionally linked biological pathways in microarray datasets.⁵³ Several interesting GO categories are highlighted by our analysis, including several involved in various epigenetic processes, transcription, and development. In addition, we find an association with genes involved in brain development in both female BD and SZ samples and in response to stress in male BD samples, consistent with the popular diathesis-stress hypothesis of psychosis susceptibility. In addition, given the postulated link between mitochondrial dysfunction, oxidative stress, and psychosis,²⁵ it is interesting that a number of mitochondrial GO categories show significantly different distributions in affected individuals. Our methylome results are in close agreement with a parallel microarray-based transcriptomics, proteomics, and metabolomics study, also performed on brain tissue obtained from the Stanley Foundation, in which genes

and proteins associated with mitochondrial function and oxidative stress responses were the most altered group.²⁵

Traditional etiological studies of complex disease, both genetic and epigenetic, have tended to investigate discrete regions of DNA in isolation. It is plausible, however, that the epigenome, like many other biological systems, comprises a complex network of interacting processes and that DNA methylation in different genomic regions is interdependent. Understanding the system-level features of biological organization across the epigenome is an important aspect of elucidating the epigenetic changes associated with disease. In order to investigate whether DNA methylation is coordinated across different loci, we utilized a novel network-based approach to test the modularity of our methylome data. In this way, a network comprises distinct clusters of elements, termed "modules," which are highly connected within themselves but have fewer connections with the rest of the network.¹⁷ The study of interaction networks has proven fruitful in many areas of biological research, highlighting distinct modularity in metabolic networks,⁵⁴ cellular networks,⁵⁵ and protein-interaction networks.⁵⁶ Although such an approach has not been previously applied to the epigenome, recent evidence suggests the involvement of coordinated epigenetic silencing across large genomic regions in cancer.⁵⁷

The goal of our network analysis was twofold: first, to see whether there is modularity in the methylome; second, if such epigenetic modularity exists, to see whether there are any differences between affected and unaffected groups. For both brain and germline DNA, we found evidence for significant epigenetic modularity in both groups analyzed. No modules were observed in a series of simulated "random" datasets, suggesting that the modular structure of the methylome is a real biological phenomenon and that the epigenome can be split into distinct groups of correlated loci, potentially corresponding to distinct functional pathways and/or physical regions. Although DNA methylation in both affected and unaffected groups is clearly modular, the number of interconnections between specific genomic regions is higher in the affected group compared to the CTRL group, resulting in more between-module interference, in both brain and germline DNA. Given that modules within such biological networks are likely to have specific functional tasks separate to those of other modules,¹⁷ the lower degree of DNA-methylation modularity observed in the major-psychosis samples points to some degree of systemic epigenetic dysfunction associated with major psychosis.

In addition to the microarray-based screening for epigenetic changes, our second approach utilized in this study focused on DNA methylation in the vicinity of genes with a priori evidence for an etiological role in major psychosis. These regions were profiled directly with bisulfite modification and pyrosequencing, with assays designed to span CpG-rich promoter regions, along with some exonic and intronic regions for several genes. Little

evidence was found to suggest that DNA methylation in these genes is associated with either SZ or BD. Our analyses included the promoter regions of both *COMT* and *RELN* that have been previously shown to be epigenetically altered in psychosis in previous studies.^{9,10,58} Unlike these studies that report *COMT* hypomethylation and *RELN* hypermethylation in SZ samples, we found no evidence for DNA-methylation changes in these genes associated with either SZ or BD. Our data are in agreement with a previous study on *COMT* reporting no association between promoter methylation and major psychosis¹² and a recent study reporting very low levels of methylation across the *RELN* region and no association with major psychosis.¹¹ It should be noted that some of the methods used in previous studies of these genes, for example methylation-specific PCR, can lead to biased assessment of methylated cytosines and are not able to assess epigenetic changes in a truly quantitative manner as is possible with the pyrosequencing methodology utilized in this study.

The observation of an association between genotype at a nonsynonymous SNP (rs6265) in *BDNF* and DNA methylation at surrounding CpG sites in DNA from frontal-cortex brain tissue adds to the increasing evidence that DNA sequences can influence epigenetic profiles (e.g.,^{59,60}). Although DNA alleles and haplotypes can be subject to differential epigenetic modification, it appears that epigenetic status cannot be unequivocally deduced from DNA-sequence data alone. The notion that epigenetic changes might be associated with DNA-sequence variation is relevant to the inconsistent genetic-association studies in complex diseases and suggests that a comprehensive epigenetic analysis of candidate SNPs and haplotypes is warranted.

Our tandem use of two complementary approaches allowed us to test both a priori hypotheses and identify novel regions of the genome that may be epigenetically dysfunctional in major psychosis. The unbiased microarray approach was far more productive in identification of differentially methylated loci than was the candidate-gene approach; this has implications for the design of future epigenetic studies of complex disease. Of note is the observation that a high proportion of the microarray-nominated loci can be considered good functional and/or positional candidates. Given the relatively large number of differences observed between affected and unaffected individuals in our microarray screen and the laborious nature of current bisulfite-based mapping approaches, it was unfeasible to further investigate each nominated gene at the level of specific CpG nucleotides in the course of this study. Our analyses were stringently controlled for multiple testing by use of the FDR statistic, but as with all microarray-based experiments, it is possible that some of the genes uncovered are false-positives; more in-depth screening of specific gene regions will be needed to verify the specific DNA-methylation changes involved. In general, the actual DNA-methylation differences observed between major-psychosis and CTRL groups appear quite

subtle but consistent across affected individuals. This pattern of findings reflects that seen for other etiological approaches in major psychosis (e.g., gene-association studies, gene-expression studies, and brain-imaging studies) and is as expected given the highly complex and heterogeneous nature of the phenotypes being studied.

It is plausible that differences in the cellular composition of our brain-tissue samples can actually account for some of the differences we observed. Conversely, however, we cannot exclude the possibility that actual epigenetic defects are more substantial than reported here but only present in a specific cell type in the brain. This point is highlighted by a recent study identifying epigenetic changes occurring specifically in cortical-interneuron cells, suggesting that different cell populations within a single tissue type can have quite distinct epigenetic profiles.⁶¹ Of course, access to postmortem brain tissue from affected individuals is difficult, and at present it would probably be very difficult, logistically and financially, to perform a microarray-based study focused on various types of cell populations. However, with the rapid technological advances currently taking place, a future line of research could be the search for cell-type-specific epimutations by use of laser-capture microdissection technology, which enables the isolation of single cells from whole tissue, thus avoiding the confounding effects of cell-type variation.

Several strategies exist for enriching DNA prior to microarray hybridization, dependent upon DNA methylation status. While each method has distinct advantages and disadvantages, we utilized a method based on cleavage with methylation-sensitive restriction enzymes.¹⁴ We have previously verified this method, and shown it to be both sensitive and reproducible.^{14,59} Because the majority of CpG sites in the genome are methylated, it has been shown that enrichment of the unmethylated DNA fraction is more effective at detecting small DNA methylation changes than alternative methods based on enrichment of the methylated DNA fraction, such as methylated DNA immunoprecipitation (MeDIP).⁶² Given the small DNA methylation changes likely associated with complex diseases such as psychosis, in part verified by the absolute changes we detected in our study, such sensitivity is likely to be a major advantage of our approach. One potential limitation to *all* enrichment strategies is that they can be affected by the presence of SNPs and copy number variation (CNV). Until the full extent (and location) of CNV in the genome is ascertained, it will be hard to fully address this issue. Our bisulfite-mapping results suggest that neither SNPs nor CNVs are systemically influencing our microarray data, but both should be taken into consideration when investigating further the epigenetic differences reported here.

Proving a direct causal link between epigenetic factors and disease is not straightforward. It is possible that the epigenetic differences observed actually result from psychosis-induced changes in the brain. Tissues that are not the disease site or directly affected by antipsychotic

medications, could thus actually be very useful in elucidating epigenetic changes associated directly with major psychosis. It has been proposed that germline epimutations may be important in disease etiology,⁶³ and potentially transmitted between generations. A recent study from our group reported considerable intra- and inter-individual epigenetic variation in male germ cells.⁵⁹ Our microarray analysis of a BD germline DNA sample, however, did not detect any FDR-significant DNA methylation differences associated with BD (Figure 3). The reason for discrepant brain and the germline results could be numerous. For example, epigenetic changes predisposing one to or causing major psychosis might be tissue-specific and restricted to the main sites of disease manifestation (e.g., the brain). In addition, previous studies of germline epimutations have found that aberrantly methylated cytosines may be present at very low frequencies (<1%),⁶³ and such small changes cannot be accurately detected with current microarray technology. Recent technological advances in DNA methylation analysis, such as the deep-sequencing of bisulfite-treated DNA, may make the detection of such minute DNA methylation differences more easily detectable.

To conclude, consistent with the epigenetic theory of major psychosis, a number of loci were found to be epigenetically altered in the brain of SZ and BD patients relative to unaffected controls. This study clearly demonstrates that epigenomic studies can be done cost-effectively with current technologies. Microarray technology is advancing at a tremendous pace, and it will soon be economically feasible to perform similar experiments on very high-density tiling microarrays covering the entire genome. Future studies can also be broadened to include other epigenetic processes such as histone modifications and small non-coding RNA molecules.

Supplemental Data

Five Supplemental tables are available at <http://www.ajhg.org/>.

Acknowledgments

Postmortem brains were donated by The Stanley Medical Research Institute's Brain Collection courtesy of Michael B. Knable, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken. This project was supported by the Ontario Mental Health Foundation (OHMF), Canadian Institutes for Health and Research (CIHR), the National Alliance for Research on Schizophrenia and Depression, and the Stanley Foundation. Development of some analytical tools used in this study was funded by the National Institute of Mental Health (R01 MH074127-01). A.P. is an OMHF Senior Fellow, and J.M. was supported by a CIHR postdoctoral fellowship.

Received: September 12, 2007

Revised: November 12, 2007

Accepted: January 4, 2008

Web Resources

The URLs for data presented herein are as follows:

Raw microarray data, www.epigenomics.ca
Stanley Foundation Brain Collection, http://www.stanleyresearch.org/programs/brain_collection.asp
Gene-expression data for the Stanley Brain samples, <https://www.stanleygenomics.org/>
MethPrimer, <http://www.urogene.org/methprimer/index1.html>
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>
Gene Ontology Database, www.geneontology.org

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Supplemental Data

Epigenomic Profiling Reveals DNA-Methylation Changes

Associated with Major Psychosis

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TABLE S1. Primer Sequences Utilized in this Study

Primer Name	Oligo Sequence
ARVCF-INT3-F	BIOTIN-AAGAGGAGGGTTAAATTGTTA
ARVCF-INT3-PYRO	TCAAACATAAACCACCTTA
ARVCF-INT3-R	ATTAACCTAAAAAACCCCTAAC
BDNF-EXON-F	BIOTIN-TGTTTTTATGAAAGAAGTAAATATT
BDNF-EXON-PYRO	AATCCTCATCCAACAA
BDNF-EXON-R	TCCTTATTATTTCTTCATTAAAC
BDNF-INT1A-F	GATGTTTTATTGAGTTTAGGTT
BDNF-INT1A-PYRO	TTGGGAGTAGAAGGTTT
BDNF-INT1A-R	BIOTIN-AACTAATTAATAACTCTATCCAA
BDNF-INT1B-F	BIOTIN-GGGTTAGATATTATTAGTTT
BDNF-INT1B-PYRO	AAAAATAAAAAACAAACCC
BDNF-INT1B-R	ACTAAAACTAAAACTAAAAACAC
BDNF-PRM-F	TAGGGTTTTTTGGGAGAGTT
BDNF-PRM-PYRO	TTATTTTAGTTTTGGTTTT
BDNF-PRM-R	BIOTIN-ATTACCCACAAAAACCTATATAAA
COMT-EXON4-F	BIOTIN-GTGTTTGGGGATTTAAGTTT
COMT-EXON4-PYRO	TCAAACATAACACACCTTA
COMT-EXON4-R	ACCCTTTTTCCAAATCTAAC
COMT-PRM-F	TTGAGTAAGATTAGATTAAGAGGT
COMT-PRM-PYRO	GGGATATTTGGTTAT
COMT-PRM-R	BIOTIN-ACAACCCTAACTACCCCAAAAAC
DRD4-F	GGTAGAGTTTGGTTTAGGTT
DRD4-PYRO	TAGATATTAGGTGGAT
DRD4-R	BIOTIN-ACCAAACCAAACCCTAAAAAC
DTNBP1-ARRAY-F	BIOTIN-TTGGGAAGTGTGGTTTGTAGGAA
DTNBP1-ARRAY-F	BIOTIN-TTGGGAAGTGTGGTTTGTAGGAA
DTNBP1-ARRAY-PYRO	CACCTTTAAACCTCCTATT
DTNBP1-ARRAY-PYRO	CACCTTTAAACCTCCTATT
DTNBP1-ARRAY-R	ACCTCCAAATATAACCACCATCTC
DTNBP1-ARRAY-R	ACCTCCAAATATAACCACCATCTC
DTNBP1-INT1-F	TTTTTTTTGTTTAGGAGTTTTTTT
DTNBP1-INT1-PYRO	GGTAAAGGTAGAGAAAGGA
DTNBP1-INT1-R	BIOTIN-CTAAAACTAAACCAACCACCCTC
DTNBP1-PRM-F	BIOTIN-GAAGGGTTTTTAGTATTGT
DTNBP1-PRM-PYRO	AAAAAACTAAAATTAC
DTNBP1-PRM-R	AAAAACTACTAACCCCTCTC
GAD1-INT1-F	BIOTIN-GTTAGGTATTTGTAGAGGAGTT
GAD1-INT1-PYRO	CTAATTCCTCTC
GAD1-INT1-R	TCACCTCCAACCTACTTCTC

GAD1-INT3-F	BIOTIN-TAGTTGAGTGATTTTGGTTGAAT
GAD1-INT3-PYRO	TACAAAAAACACCCAAA
GAD1-INT3-R	CTCTACTCTAACTACAACTA
GAD1-PRM-F	BIOTIN-GAAGGTATGAAGAGGTAAGT
GAD1-PRM-PYRO	AAATTCACCAAAAA
GAD1-PRM-R	AAAATTCTCCCTTTACAATATTTAA
GRIA2-F	AAGATAGTAGGGTTTGGTGAGAGG
GRIA2-PYRO	ATAATTAGTAATTAGGTTTTTATAT
GRIA2-R	BIOTIN-TCTCTTCTCCCTCTCTCCTCTCT
GRIA2-SS1	ATACAACAAAATAATCTCC
GRIA2-SS2	GAGTTGTGTTTTTTTAG
GRIN2B-F	GGTTTGTGTTGAATGGGTTT
GRIN2B-PYRO1	TGAATGGGTTTTGAT
GRIN2B-PYRO2	GGGTTTTATTTGTAA
GRIN2B-R	BIOTIN-TCATCCCTTCACCTAACAAAA
HCG9-F	BIOTIN-GGATTTTAGGGAGAGGATAGGG
HCG9-PYRO	CTAAACTATTCCTATAAATAACATT
HCG9-R	CCCCACCCCTACACTTT
HELT-PRM-F	BIOTIN-AGTGTGTATGGAATGAAATGTGGT
HELT-PRM-PYRO	CCCACTCCCATTTTTA
HELT-PRM-R	CCCTCCCAAATTACTCTACCA
KCNJ6-F	TTTTAGTTTTAGAAATAAAATAGAAA
KCNJ6-R	ATAATCTCTTACTCAACAAAAACTC
KCNJ6-SS	GGAGAGTTGAATTTAGAGAGT
LHX5-F	TTATAAATTTAGGAGGTGTAGGGATTT
LHX5-R	CCCAAACTCAACAAAAAAAATAAAT
LHX5-SS1	TGGGGTTTTGAAGGATTGA
LHX5-SS2	ATTTTGTATTAGGTATT
MARLIN1-F	TAAGGTTTTAGTGTGGGGTGGTTT
MARLIN1-R	AAACAAATATAATCCCCACCTTCA
MARLIN1-SS	AGTTATTTTGTGAATGT
MTHFR-PRMA-F	GTAAAGTATGGGATATTAAGTT
MTHFR-PRMA-PYRO	GATTTTTAGAAAGGTTT
MTHFR-PRMA-R	BIOTIN-ATAACTCAATAACCTAATAACTAA
MTHFR-PRMB-F	TAGTTATTGGGAGTTATATTAATT
MTHFR-PRMB-PYRO	GGGAGGTTGTTTGT
MTHFR-PRMB-R	BIOTIN-CTCCAACAACCTAACACCTA
NR4A2-F	TGTGGGGAGGGTGTAAATAAAAGTA
NR4A2-PYRO	GGGGAGGGTGTAAATAAA
NR4A2-R	BIOTIN-CACTCCCATTCCCTTTCAAATA
NRG1-INTRON1-F	BIOTIN-GAGTGGGATTTGGGTTATAGGAGT
NRG1-INTRON1-PYRO	CTTACCCTATACCCAAA
NRG1-INTRON1-R	ACACAAAATAAATCAAATAAAACC
NRG1-PRM-F	GGAGATTTTGTGGGGTAT
NRG1-PRM-PYRO	GAGTAGTTTTTTTAGG
NRG1-PRM-R	BIOTIN-CAACCCCTTTTCCTCCC
RELN-PRMA-F	BIOTIN-ACTCCCAAATTACTTTAAACC
RELN-PRMA-PYRO	TTTTAAGAAGGTGTGGAG
RELN-PRMA-R	GGGGTTTTAAGAAGGTGTG
RELN-PRMB-F	GTTTGAAGGGGAAGGTTAGTT
RELN-PRMB-PYRO	AGGGAAGGAGAGG
RELN-PRMB-R	BIOTIN-AAAATCCTCTACAAATAAACTCTA
RELN-PRMC-F	GGTTGTTATGGTTTTTTGTTTTTAAG
RELN-PRMC-PYRO	AAGGGATGAGAAAGGTG
RELN-PRMC-R	BIOTIN-AAATACTCATTTCCTACATATTAC

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RPL39-F	BIOTIN-GGTAGTGTGTTAGGGGTATTTTGT
RPL39-PYRO	CCTCTAAAAAATAACACTTACTC
RPL39-R	ACTATCCCTTCCCACACCTC
SLC17A7-F	AGGAGGGTGATTTTTTTTTTATTA
SLC17A7-PYRO	GGGTGGGAGGAGTAGA
SLC17A7-R	BIOTIN-AAACCCAAAAACACAACCAATC
TMEM59-F	BIOTIN-GGGTTATTAATTAATTATTGTGG
TMEM59-PYRO	AAATTTATCCTACACTACCCT
TMEM59-R	ACTCCTATTTTCCCTCCCTAATCC
U-CG1A	CGTGGGAGACTGACTACCAGAT
U-CG1B	AGTTACATCTGGTAGTCAGTCTCCA
WDR18-F	TTGGGAGGATTATTTGAGTTTAGG
WDR18-PYRO	AAATGTTTAGGAGGAAAAG
WDR18-R	BIOTIN-ACTTCTTCCAAAACCCAAAA

TABLE S2. Closest Genes Associated with all FDR-Significant Regions Nominated from Microarray Analysis

Rank	Closest Gene	Location	FDR Value	Fold Change
<i>Schizophrenia Males</i>				
1	EXOSC7	3p21.31	0.0005	0.2468
2	GRIA2	4q31.1	0.0006	0.2301
3	ELMOD1	11q22.3	0.0006	0.1658
4	KCNJ6	21q22.13	0.0007	-0.2132
5	WDR18	19p13.3	0.0011	0.1380
6	PLA2G4B	15q15.1	0.0014	-0.2045
7	PPP2CA	5q31.1	0.0020	0.2089
8	C13orf24	13q22.1	0.0032	-0.1995
9	FLJ90579	12q21.31	0.0057	0.2340
10	LRRC61	7q36.1	0.0092	0.1496
11	NXPH4	12q13.3	0.0129	0.1368
12	ZFAND2A	7p22.3	0.0142	-0.0956
13	STIM2	4p15.2	0.0196	-0.2059
14	PRR5	22q13.31	0.0216	0.2842
15	ADAMTS16	5p15.32	0.0232	0.1685
16	AUTS2	7q11.22	0.0232	-0.0948
17	TPD52	8q21.13	0.0232	-0.1617
18	MYOZ1	10q22.2	0.0232	0.1990
19	MRPS14	1q25.1	0.0250	-0.1671
20	RPP21	6p21.33	0.0250	-0.1804
21	HNRPR	1p36.12	0.0250	-0.1288
22	THBS1	15q14	0.0261	-0.1564
23	FLJ23861	2q34	0.0261	0.1517
24	C1orf110	1q23.3	0.0261	-0.0929
25	MLL5	7q22.2	0.0301	-0.1629
26	GLS2	12q13.2	0.0301	-0.1618
27	HOXD13	2q31.1	0.0328	-0.1796
28	BC032332	20q13.33	0.0339	0.1755
29	SLC31A1	9q32	0.0341	-0.1390
30	C9orf40	9q21.13	0.0357	-0.1421
31	NAG	2p24.3	0.0360	-0.1696
32	UNC5A	5q35.2	0.0360	-0.1356
33	C6orf62	6p22.2	0.0361	0.1213
34	CDC42BPA	1q42.13	0.0369	0.1101
35	CEBPZ	2p22.2	0.0369	0.1755
36	KEL	7q34	0.0394	-0.2105
37	GLRX5	14q32.13	0.0395	-0.1615
38	AIG1	6q24.2	0.0416	0.1583
39	SOX1	13q34	0.0416	-0.1343
40	MET	7q31.2	0.0452	0.1906
41	GLRX5	14q32.13	0.0460	-0.1051
42	PLAG1	8q12.1	0.0470	-0.2080
43	CRSP6	11q21	0.0470	0.2048
44	POLR3A	10q22.3	0.0470	-0.1598
45	TRERF1	6p21.1	0.0471	-0.1955

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46	PWP1	12q23.3	0.0484	-0.1601
47	COL9A1	6q13	0.0484	0.1563
48	RPP21	6p21.33	0.0484	-0.1608
49	SMCHD1	18p11.32	0.0484	0.1918
50	FBXO31	16q24.1	0.0484	-0.2240
51	LYST	1q42.3	0.0484	0.1498
<i>Schizophrenia Females</i>				
1	C6orf84	6q14.3	0.0209	0.4436
2	HCG9	6p21.33	0.0209	-0.5535
3	SLC17A7	19q13.33	0.0209	-0.5397
4	NR4A2	2q24.1	0.0209	-0.5515
5	AB051500	18q12.1	0.0214	-0.4895
6	RPP21	6p21.33	0.0214	-0.5238
7	FN5	11q21	0.0214	-0.5033
8	NKX2-3	10q24.2	0.0222	-0.5524
9	ADAMTSL1	9p22.2	0.0275	-0.4097
10	MARLIN1	4p16.1	0.0321	-0.5787
11	TMEM59	1p32.3	0.0330	0.4103
12	MTPN	7q33	0.0330	0.5117
13	LMX1B	9q33.3	0.0330	0.5869
14	HELT	4q35.1	0.0352	-0.4203
15	LHX8	1P31.1	0.0352	0.3597
16	LHX5	12q24.13	0.0352	-0.3929
17	CRTC2	1q21.3	0.0352	-0.4635
18	PGRMC1	xq24	0.0352	-0.3961
19	MORC2	22q12.2	0.0352	0.4233
20	Bmp7	20q13.31	0.0352	-0.3563
21	SIX2	2p21	0.0366	-0.4339
22	CCL1	17q12	0.0366	-0.3109
23	C16orf45	16p13.11	0.0366	-0.3878
24	LHX5	12q24.13	0.0403	-0.4214
25	PICALM	11q14.2	0.0406	-0.5156
26	KIAA1787	17p13.1	0.0430	0.3267
27	SLC17A6	11p14.3	0.0430	0.2542
28	SF3B5	6q24.2	0.0435	-0.5020
29	SWAP70	11p15.4	0.0435	0.5038
30	KEL	7q34	0.0435	-0.4062
31	B3GALT3	3q26.1	0.0435	0.5117
32	FLJ45455	17p13.1	0.0436	0.3658
33	RAI1	17p11.2	0.0443	-0.3083
34	AK126832	2p21	0.0443	0.3683
35	IGFL2	19q13.32	0.0443	-0.3096
36	CGI-115	1q41	0.0447	-0.4902
37	PRKCA	17q24.2	0.0453	0.4270
<i>Bipolar Males</i>				
1	THBS1	15q14	0.0403	-0.2361
2	MCM4	8q11.21	0.0403	-0.2426
<i>Bipolar Females</i>				
1	CGI-115	1q41	0.0200	-0.4032

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2	RPL39	xq24	0.0341	-0.4401
3	CGI-115	1q41	0.0341	-0.2332
4	AY831680	3q13.12	0.0341	0.4692
5	DHRS8	4q22.1	0.0341	-0.3460
6	DTNBP1	6p22.3	0.0341	-0.4966
7	ADAMTSL1	9p22.2	0.0341	-0.3663
8	HELT	4q35.1	0.0341	-0.3538
9	MARLIN1	4p16.1	0.0341	-0.4673
10	NKX2-3	10q24.2	0.0341	-0.4604
11	EFHD1	2q37.1	0.0341	-0.3370
12	DLL1	6q27	0.0341	-0.2755
13	ZCWPW2	3p24.1	0.0341	-0.3065
14	SORCS3	10q25.1	0.0354	-0.3901
15	VAX1	10q25.3	0.0424	0.2414
16	AK129895	10p11.23	0.0424	0.4238
17	DIPA	11q13.1	0.0454	-0.3539
18	KIAA0859	1q24.3	0.0454	-0.2527
19	PPP2CA	5q31.1	0.0454	0.2903
20	EPHA5	4q13.1	0.0454	0.5550
21	RPP21	6p21.33	0.0454	-0.3775
22	FNBPI1L	1p22.1	0.0454	0.3731
23	C21orf29	21q22.3	0.0454	-0.3262
24	TMEM59	1p32.3	0.0454	0.3886
25	SIL1	5q31.2	0.0454	-0.4085
26	RPAP1	15q15.1	0.0454	-0.2490
27	C16orf45	16p13.11	0.0454	-0.3231
28	NRG2	5q31.3	0.0454	0.2295
29	KEL	7q34	0.0454	-0.3094
30	LOC285513	4q22.1	0.0454	0.3411
31	FLJ23861	2q34	0.0454	0.2402
32	FLJ43505	1q41	0.0454	-0.2876
33	FOXP1	3p14.1	0.0454	0.3213
34	PRKCA	17q24.2	0.0454	0.3429
35	LHX5	12q24.13	0.0460	-0.3573
<i>Psychosis Males</i>				
1	KCNJ6	21q22.13	0.0008	-0.2027
2	ELMOD1	11q22.3	0.0022	0.1388
3	EXOSC7	3p21.31	0.0022	0.2067
4	GRIA2	4q32.1	0.0034	0.1948
5	C13orf24	13q22.1	0.0035	-0.1788
6	THBS1	15q14	0.0047	-0.1782
7	WDR18	19p13.3	0.0047	0.1117
8	PPP2CA	5q31.1	0.0054	0.1728
9	STIM2	4p15.2	0.0087	-0.2180
10	C9orf40	9q21.13	0.0145	-0.1537
11	ADAMTS16	5p15.32	0.0173	0.1605
12	LYST	1q42.3	0.0173	0.1500
13	MRPS14	1q25.1	0.0173	-0.1516
14	FBXO31	16q24.1	0.0173	-0.2262

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15	CDC42BPA	1q42.13	0.0183	0.1149
16	C6orf62	6p22.2	0.0183	0.1100
17	ZFAND2A	7p22.3	0.0206	-0.0848
18	BC032332	20q13.33	0.0206	0.1601
19	PRR5	22q13.31	0.0221	0.2722
20	FLJ90579	12q21.31	0.0223	0.1968
21	FLJ23861	2q34	0.0223	0.1361
22	PLA2G4B	15q15.1	0.0226	-0.1705
23	MYOZ1	10q22.2	0.0233	0.1751
24	IKIP	12q23.1	0.0237	-0.1802
25	TPD52	8q21.13	0.0240	-0.1442
26	GGN	19q13.2	0.0269	0.2440
27	LRRC61	7q36.1	0.0270	0.1186
28	CEBPZ	2p22.2	0.0292	0.1741
29	MLL5	7q22.2	0.0309	-0.1581
30	HSD17B7	1q23.3	0.0350	-0.0794
31	VPS33B	15q26.1	0.0350	-0.1462
32	FLJ41423	11p11.2	0.0382	-0.1648
33	ZNF195	11p15.4	0.0382	0.1879
34	NAG	2p24.3	0.0478	-0.1622
35	C16orf45	16p13.11	0.0482	-0.1702
36	POLR3A	10q22.3	0.0486	-0.1455
37	CRSP6	11q21	0.0496	0.1825

Psychosis Females

1	CGI-115	1q41	0.0079	-0.4380
2	DHRS8	4q22.1	0.0079	-0.3533
3	ADAMTSL1	9p22.2	0.0079	-0.3802
4	DTNBP1	6p22.3	0.0079	-0.4971
5	CGI-115	1q24.2	0.0107	-0.2389
6	MARLIN1	4p16.1	0.0107	-0.5081
7	NKX2-3	10q41	0.0107	-0.4801
8	DLL1	6q27	0.0107	-0.2728
9	RPP21	6p21.33	0.0107	-0.4191
10	HELT	4q35.1	0.0107	-0.3663
11	TMEM59	1p32.3	0.0115	0.3861
12	PRKCA	17q24.2	0.0131	0.3767
13	RPAP1	15q15.1	0.0140	-0.2389
14	ISL2	15q24.3	0.0140	-0.2466
15	SORCS3	10q25.1	0.0140	-0.3916
16	RPL39	xq24	0.0149	-0.4359
17	AK129895	10q37.1	0.0175	0.4103
18	EFHD1	2p11.23	0.0175	-0.3065
19	LHX5	12q24.13	0.0175	-0.3701
20	SLC17A7	19q13.33	0.0175	-0.4788
21	FOXP1	3p14.1	0.0176	0.3151
22	C16orf45	16p13.11	0.0186	-0.3356
23	EPHA5	4q13.1	0.0186	0.5457
24	VAX1	10q25.3	0.0186	0.2354
25	SMUG1	12q13.13	0.0190	-0.4064

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26	ZNF582	19q13.43	0.0195	-0.3132
27	MTPN	7q33	0.0195	0.5284
28	KEL	7q34	0.0195	-0.3375
29	FLJ43505	1q41	0.0195	-0.2912
30	RPP21	6p21.33	0.0195	-0.3981
31	GLRX5	14q32.13	0.0233	-0.3571
32	AK127494	19p13.2	0.0241	-0.3386
33	FNBP1L	1p22.1	0.0241	0.3647
34	FLJ45455	17p13.1	0.0241	0.3359
35	CBX8	17q25.3	0.0241	-0.4036
36	ATF7IP	12p13.1	0.0241	-0.2177
37	PSIP1	9p22.3	0.0241	-0.2317
38	SIL1	5q31.2	0.0249	-0.3742
39	SWAP70	11p15.4	0.0249	0.4102
40	ATOX8	2p11.2	0.0249	-0.3876
41	UXS1	2q12.2	0.0249	-0.4706
42	PPP2CA	5q31.1	0.0266	0.2886
43	HCG9	6p21.33	0.0290	-0.4265
44	C9orf40	9q21.13	0.0327	-0.3146
45	COQ5	12q24.31	0.0327	-0.2538
46	FN5	11q21	0.0339	-0.3808
47	KIAA0859	1q24.3	0.0343	-0.2402
48	TCF7L2	10q25.2	0.0375	0.2205
49	GATAD2A	19p13.11	0.0378	-0.6567
50	FLJ20643	19q13.33	0.0382	-0.2953
51	CLK2	1q22	0.0382	0.5518
52	LMX1B	9q33.3	0.0415	0.5844
53	PLA2G4B	15q15.1	0.0415	-0.3618
54	PSMB7	9q33.3	0.0415	0.5638
55	NRG2	5q31.3	0.0415	0.2370
56	LHX8	1p31.1	0.0418	0.3255
57	B3GALT3	3q26.1	0.0426	0.4555
58	WNT1	12q13.12	0.0426	-0.2894
59	EBPL	13q14.3	0.0426	0.2948
60	ZCWPW2	3p24.1	0.0426	-0.2747
61	HLX1	1q41	0.0426	-0.2878
62	FOSB	19q13.32	0.0426	0.3011
63	CYB5R4	6q14.2	0.0428	0.3911
64	MORC2	22q12.2	0.0428	0.3570
65	C14orf138	14q22.1	0.0428	-0.1832
66	TYMS	18p11.32	0.0428	-0.1812
67	AY831680	3q13.12	0.0428	0.4401
68	CNTN5	11q22.1	0.0438	0.3437
69	TRPS1	8q23.3	0.0438	-0.2733
70	PGRMC1	xq24	0.0438	-0.3393
71	OCIAD1	4p12	0.0465	-0.3200
72	RAB38	11q14.2	0.0475	0.2384
73	SCG2	2q36.1	0.0497	0.3157
74	KIAA1787	17p13.1	0.0497	0.3435

TABLE S3. FDR-Significant Microarray Differences for Regions Located Near Genes that Can Be Considered Good Functional and/or Positional Candidates for a Role in Major Psychosis

Gene Name and Symbol	Probe Location	FDR-Significant Methylation Change*	Link to Major Psychosis
Autism Susceptibility Candidate 2 (<i>AUTS2</i> or <i>KIAA0442</i>)	7q11.22	SZ MALE ↑	Spans a translocation breakpoint associated with mental retardation and autism; highly expressed in frontal cortex of brain ¹
Dysbindin (<i>DTNBP1</i>) [^]	6p22.3	BD FEMALE ↑ PSY FEMALE ↑	A compelling candidate gene for PSY (see also Table S5) nominated by both association and linkage studies ^{2,3}
Fbj Murine Osteosarcoma Viral Oncogene Homolog B (<i>FOSB</i>)	19q13.32	PSY FEMALE ↓	FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. <i>FOSB</i> is expressed following chronic antipsychotic drug treatment ⁴
Glutamate Receptor, Ionotropic, AmpA 2 (<i>GRIA2</i>)	4q31.1	SZ MALE ↓ PSY MALE ↓	One of four ionotropic glutamate receptor subunits, found to be differentially expressed in the brains of SZ patients ^{5,6}
Glutaminase 2 (<i>GLS2</i>)	12q13.2	SZ MALE ↑	Catalyzes the hydrolysis of glutamine to glutamate; found to be elevated in brains of SZ patients ⁷
Homo sapiens Hey-like transcriptional repressor (<i>HELT</i>)	4q35.1	SZ FEMALE ↑ BD FEMALE ↑ PSY FEMALE ↑	<i>HELT</i> determines GABAergic over glutamatergic neuronal fate in the developing mesencephalon ⁸
HLA Complex Group 9 (<i>HCG9</i>)	6p21.33	SZ FEMALE ↑ PSY FEMALE ↑	Located within the MHC class I region on chromosome 6p implicated in a genome-scan meta-analysis of schizophrenia ³ . The function of the encoded protein has not been determined, but immune-system disruption reported in SZ.
Islet 2 Transcription Factor, Lim/Homeodomain (<i>ISL2</i>)	15q24.3	PSY FEMALE ↑	A transcriptional factor that defines subclasses of motoneurons in the nervous system. 15q24.3 falls within a region implicated in a genome-scan meta-analysis of schizophrenia ³
Lim Homeobox Protein 5 (<i>LHX5</i>)	12q24.13	SZ FEMALE ↑ BD FEMALE ↑ PSY FEMALE ↑	A transcriptional regulator involved in the control of differentiation and development of the forebrain and knockout mice show learning impairments and motor dysfunction ⁹
Lim Homeobox Transcription Factor 1, Beta (<i>LMX1B</i>)	9q33.3	SZ FEMALE ↓ PSY FEMALE ↓	A transcription factor important for the development of dopaminergic neurons in the brain.
Multiple Alpha Helices and RNA-Linker protein-1 (<i>MARLIN-1</i> or <i>JAKMIP1</i>)	4p16.1	SZ FEMALE ↑ BD FEMALE ↑ PSY FEMALE ↑	A RNA-binding protein widely expressed in the brain that associates with GABA(B) receptors ¹⁰ . The 4p16.1 region has been linked to both BD and SZ ¹¹

Neuregulin 2 (<i>NRG2</i>)	5q31.3	BD FEMALE ↓ PSY FEMALE ↓	Neuregulins are a family of growth and differentiation factors that interact with ERBB receptors to induce the growth and differentiation of epithelial, neuronal, and glial cells. The gene for another neuregulin, <i>NRG1</i> , has been widely implicated in SZ ¹² . 5q31.1 falls within a region implicated in a genome-scan meta-analysis of SZ ³
Nuclear Receptor Subfamily 4, Group A, Member 2 (<i>NR4A2</i> or <i>NURRI</i>)	2q24.1	SZ FEMALE ↑	Plays a critical role in the development of midbrain dopaminergic neurons. Reduced expression observed in BD and SZ brains ¹³ .
Phospholipase A2, Group 4B (<i>PLA2G4B</i>)	15q15.1	SZ MALE ↑ PSY MALE ↑ PSY FEMALE ↑	Phospholipid metabolism shown to be disturbed in SZ; the phospholipid structure of neuronal membranes is essential for normal functioning ¹⁴ .
Potassium Channel, Inwardly Rectifying, Subfamily J, Member 6 (<i>KCNJ6</i> or <i>GIRK2</i>)	21q22.13	SZ MALE ↑ PSY MALE ↑	G protein-coupled inwardly rectifying potassium channels (GIRKs) link numerous neurotransmitter receptors to the regulation of synaptic transmission in the brain
Retinoic Acid Inducible-1 (<i>RAI1</i>)	17p11.2	SZ FEMALE ↑	Located in a very unstable genomic region containing a polyglutamine tract associated with SZ ¹⁵ . Highly suggestive evidence of linkage to this region with SZ has been reported ¹⁶
Ribosomal Protein L39 (<i>RPL39</i>)	Xq24	BD FEMALE ↑ PSY FEMALE ↑	Located at Xq24 in a region on the X-chromosome found to be linked to BD in several studies ^{17, 18}
Secretogranin II (<i>SCG2</i>)	2q36.1	PSY FEMALE ↓	A secretory protein located in the vesicles of many endocrine cells and neurons that has been shown to stimulate neurotransmitter release ¹⁹ . Chronic PCP exposure, which produces signs of persistently altered frontal brain activity and related behaviors that are also seen in patients with SZ, modulates <i>SCG2</i> expression ²⁰ . Expression is also altered following lithium treatment, a common medication for BD ²¹
Solute Carrier Family 17, Member 6 (<i>SLC17A6</i> or <i>VGLUT2</i>)	11p14.3	SZ FEMALE ↓	Encodes a vesicular glutamate transporter (VGLUT), co-expressed with <i>VGLUT1</i>
Solute Carrier Family 17, Member 7 (<i>SLC17A7</i> or <i>VGLUT1</i>)	19q13.33	SZ FEMALE ↑ PSY FEMALE ↑	Encodes a vesicular glutamate transporter (VGLUT) found to be downregulated in SZ brains ²² . Region implicated in a genome-scan meta-analysis of BD ²³
Thymic dendritic cell-derived factor 1 (<i>TMEM59</i>)	1p32	BD FEMALE ↓	1p32 falls within a region of strong suggestive linkage to BD ²³ .
Vacuolar Protein Sorting 33B (<i>VPS33B</i>)	15q26.1	PSY MALE ↑	<i>VPS33B</i> plays an important role in vesicular transport in numerous tissues. 15q26.1 falls within a region implicated in a genome-scan meta-analysis of SZ ³
WD Repeat Domain 18	19p13.3	SZ MALE ↓	Probe located 10kb upstream of NMDA receptor subunit gene (<i>NR3B</i>), postulated to

(<i>WDR18</i>)		PSY MALE ↓	be involved in schizophrenia ^{24, 25}
Wingless-Type Mmtv Integration Site Family, Member 1 (<i>WNT1</i>)	12q13.12	PSY FEMALE ↑	The Wnt pathway is critical for neurodevelopment and regulates cell adhesion, synaptic rearrangement, and plasticity; found to be over-expressed in the brains of SZ patients ²⁶

SZ denotes schizophrenia, BD denotes bipolar disorder, and PSY denotes combined-psychosis group.

* Downward arrows (↓) indicate hypomethylation in affected group; upward arrows (↑) indicate hypermethylation in affected group.

^ The probe found to be differently enriched is located ~80 KB upstream of the transcription start site and does not overlap with the region investigated in our candidate-gene approach.

TABLE S4. Gene-Ontology Analysis of Microarray Data

GO Category	N	Mean Difference	p Value	Description
<i>Male Bipolar Disorder</i>				
GO:0008168	4	0.098	0.00006*	methyltransferase activity
GO:0005737	50	-0.080	0.00007*	cytoplasm
GO:0005515	139	-0.047	0.00031*	protein binding
GO:0005634	158	-0.037	0.00074*	nucleus^
GO:0006950	5	-0.171	0.00168*	response to stress
GO:0016481	5	-0.156	0.00309	negative regulation of transcription
GO:0008134	5	-0.153	0.00401	transcription factor binding
GO:0007275	37	-0.073	0.00418	multicellular organismal development
GO:0008104	3	0.136	0.00480	protein localization^
GO:0005216	3	0.092	0.00564	ion channel activity
GO:0005488	10	-0.116	0.00766	binding^
GO:0005643	6	-0.159	0.00845	nuclear pore
<i>Female Bipolar Disorder</i>				
GO:0042773	9	0.940	0.00000*	ATP synthesis coupled electron transport^
GO:0006879	11	0.817	0.00000*	iron ion homeostasis^
GO:0030595	11	0.817	0.00000*	leukocyte chemotaxis^
GO:0048019	11	0.817	0.00000*	receptor antagonist activity^
GO:0048471	11	0.817	0.00000*	perinuclear region of cytoplasm^
GO:0005747	13	0.730	0.00002*	mitochondrial respiratory chain complex I^
GO:0006120	13	0.730	0.00002*	mitochondrial electron transport, NADH to ubiquinone^
GO:0008137	13	0.730	0.00002*	NADH dehydrogenase (ubiquinone) activity^
GO:0016491	23	0.487	0.00003*	oxidoreductase activity^
GO:0006916	17	0.576	0.00006*	anti-apoptosis^
GO:0016021	72	0.194	0.00013*	integral to membrane^
GO:0007420	6	0.297	0.00017*	brain development^
GO:0005488	5	-0.142	0.00039*	binding^
GO:0005576	20	0.459	0.00051*	extracellular region^
GO:0003674	15	-0.201	0.00055*	molecular_function^

GO:0005739	33	0.302	0.00069*	mitochondrion^
GO:0004759	4	-0.191	0.00107*	serine esterase activity^
GO:0005496	3	-0.306	0.00112*	steroid binding^
GO:0008289	3	-0.306	0.00112*	lipid binding^
GO:0005634	153	0.069	0.00133*	nucleus^
GO:0008150	13	-0.200	0.00163*	biological_process^
GO:0016020	108	0.113	0.00199*	membrane^
GO:0006935	4	0.493	0.00278*	chemotaxis
GO:0005886	16	0.166	0.00360*	plasma membrane
GO:0009887	9	0.264	0.00446*	organ morphogenesis
GO:0007389	3	-0.173	0.00749	pattern specification process
GO:0016023	3	-0.199	0.00768	cytoplasmic membrane-bound vesicle
GO:0016788	3	-0.199	0.00768	hydrolase activity, acting on ester bonds
GO:0018738	3	-0.199	0.00768	S-formylglutathione hydrolase activity
GO:0006350	44	0.103	0.00923	transcription
GO:0006355	72	0.084	0.00966	regulation of transcription, DNA-dependent
<i>Male Schizophrenia</i>				
GO:0007165	33	0.059	0.00865	signal transduction
<i>Female Schizophrenia</i>				
GO:0005747	20	0.712	0.00000*	mitochondrial respiratory chain complex I^
GO:0006120	20	0.712	0.00000*	mitochondrial electron transport, NADH to ubiquinone^
GO:0008137	20	0.712	0.00000*	NADH dehydrogenase (ubiquinone) activity^
GO:0006879	18	0.737	0.00000*	iron ion homeostasis^
GO:0030595	18	0.737	0.00000*	leukocyte chemotaxis^
GO:0048019	18	0.737	0.00000*	receptor antagonist activity^
GO:0048471	18	0.737	0.00000*	perinuclear region of cytoplasm^
GO:0042773	13	0.754	0.00000*	ATP synthesis coupled electron transport^
GO:0006916	25	0.577	0.00000*	anti-apoptosis^
GO:0016491	30	0.490	0.00000*	oxidoreductase activity^
GO:0005576	29	0.490	0.00000*	extracellular region^
GO:0005739	40	0.351	0.00001*	mitochondrion^
GO:0016021	89	0.179	0.00002*	integral to membrane^
GO:0016020	130	0.137	0.00003*	membrane^

GO:0005215	7	0.266	0.00012*	transporter activity
GO:0045944	7	-0.273	0.00013*	positive regulation of transcription from RNA polymerase II promoter
GO:0003674	15	-0.241	0.00066*	molecular_function^
GO:0007420	5	0.262	0.00110*	brain development^
GO:0031966	5	0.690	0.00137*	mitochondrial membrane
GO:0007067	4	-0.247	0.00151*	mitosis
GO:0051301	4	-0.247	0.00151*	cell division
GO:0008104	3	-0.178	0.00220*	protein localization^
GO:0005496	3	-0.371	0.00241*	steroid binding^
GO:0008289	3	-0.371	0.00241*	lipid binding^
GO:0016251	5	0.165	0.00244*	general RNA polymerase II transcription factor activity
GO:0008150	13	-0.233	0.00270*	biological_process^
GO:0016757	4	0.292	0.00288*	transferase activity, transferring glycosyl groups
GO:0004759	3	-0.172	0.00524*	serine esterase activity^

Shown are all GO categories with a $p < 0.01$. A positive mean difference suggests hypomethylation in the affected group relative to unaffected controls.

* indicates categories with FDR value < 0.05

^ indicates categories present in more than one diagnostic group

TABLE S5. Average Percentage of CpG Methylation Assessed with Bisulfite Modification and Pyrosequencing across Candidate-Gene Regions

Gene (Location)	Link to Major Psychosis	Amplicon	Amplicon Location*	Assay CGs	Mean Amplicon Methylation (SD)		
					SZ	BD	CTRL
<i>ARVCF</i> <i>22q11</i>	Located next to <i>COMT</i> in a region deleted in velocardiofacial syndrome	Intron 3	22:18354314-18354584	10	4.53 (0.78)	5.02 (0.95)	4.69 (0.53)
<i>BDNF</i> <i>11p13</i>	A prosurvival factor necessary for survival of striatal neurons in the brain	Exon ^	11:27636431-27636634	4	82.48 (2.03)	81.14 (5.24)	82.11 (2.72)
		Intron 1(a)	11:27697459-27697652	10	4.19 (0.72)	2.93 (1.57)	4.43 (3.35)
		Intron 1(b)	11:27678599-27678889	12	6.40 (1.39)	5.97 (0.48)	6.40 (0.96)
		Promoter	11:27700523-27700854	15	2.64 (0.72)	2.95 (0.58)	2.52 (0.36)
<i>COMT</i> <i>22q11</i>	Catalyzes the transfer of a methyl group to catecholamine neurotransmitters including dopamine, epinephrine, and norepinephrine.	Exon 4	22:18331021-18331322	9	74.4 (1.65)	74.05 (3.21)	75.17 (2.56)
		Promoter	22:18309077-18309496	12	2.35 (1.53)	2.79 (2.71)	3.12 (3.44)
<i>DRD4</i> <i>11p15</i>	Shows high affinity for the antipsychotic clozapine.	Promoter	11:626685-626839	12	12.23 (4.61)	12.46 (2.96)	13.67 (2.51)
<i>DTNBP1</i> <i>6p22</i>	A ubiquitously expressed protein that binds to alpha- and beta-dystrobrevins, and has been widely associated with schizophrenia.	Intron 1 #	6:15770400-15770612	12	5.02 (3.46)	3.87 (1.76)	4.50 (2.15)
		Promoter #	6:15771064-15771358	15	5.92 (1.98)	5.24 (1.84)	5.11 (1.80)
<i>GAD1</i> <i>2q31</i>	Catalyzes the conversion of glutamic acid to gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the vertebral central nervous system.	Intron 1	2:171381495-171381705	10	3.13 (0.54)	3.49 (1.24)	3.57 (0.78)
		Intron 3	2:171387778-171388116	13	11.05 (1.82)	11.21 (1.69)	11.56 (3.18)
		Promoter	2:171380534-171380762	12	6.00 (0.47)	7.22 (1.98)	5.97 (0.88)
<i>GRIN2B</i> <i>12p12</i>	A critical structural and functional subunit of the NMDA glutamate receptor that has been widely implicated in psychosis	Promoter	12:14025024-14025307	10	5.60 (1.89)	7.19 (2.00)	5.97 (1.49)

<i>MTHFR</i> <i>1p36</i>	Polymorphisms in the 5,10-@Methylenetetrahydrofolate Reductase gene have been associated with both bipolar disorder and schizophrenia.	Promoter (a)	1:11788017 - 11788228	12	1.19 (0.78)	1.12 (0.31)	1.03 (0.21)
		Promoter (b)	1:11788356-11788642	18	1.27 (0.15)	1.29 (0.23)	1.48 (0.18)
<i>NRG1</i> <i>8p22-11</i>	One of four neuregulin growth factor genes that signal through the ERBB receptor kinase pathways, and is known to be involved in neuronal migration and cellular differentiation in the developing brain.	Intron 1	8:32525871-32526230	8	5.16 (1.26)	4.63 (0.70)	3.99 (1.56)
		Promoter	8:32524919-32525183	14	2.54 (0.39)	3.98 (2.52)	2.41 (0.47)
<i>RELN</i> <i>7q22</i>	Thought to control cell-cell interactions critical for cell positioning in the brain. Hypermethylation of the promoter region of <i>RELN</i> has been found in some schizophrenic brain samples	Promoter (a)	7:103417221-103417716	11	3.51 (0.98)	3.98 (0.67)	3.15 (0.34)
		Promoter (b)	7:103417669-103417968	11	1.89 (0.47)	2.74 (1.28)	2.31 (0.56)
		Promoter (c)	7:103416649-103416879	12	3.64 (1.59)	4.60 (1.70)	3.69 (1.85)

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^ *BDNF* DNA methylation around SNP rs6265 associated with genotype (see main text and Figure 10)

A region further upstream of *DTNBP1* was found to be significantly hypermethylated in affected females relative to controls by microarray analysis (see Table S2).

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