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Aberrant transcriptomes and DNA methylomes define pathways that drive pathogenesis and loss of brain laterality/asymmetry in schizophrenia and bipolar disorder

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Abstract

Introduction: Although the loss or reversal of brain laterality is one of the most consistent modalities in schizophrenia (SCZ) and bipolar disorder (BD), its molecular basis remains elusive. Our limited previous studies indicated that epigenetic modifications are key to the asymmetric transcriptomes of brain hemispheres.

Methods: We used whole-genome expression microarrays to profile post-mortem brain samples from subjects with SCZ, psychotic BD [BD(+)] or non-psychotic BD [BD(-)], or matched controls (n=10/group, corresponding to different brain hemispheres) and performed whole-genome DNA methylation (DNAM) profiling of the same samples (n=3–4/group) to identify pathways associated with SCZ or BD(+) and genes/sites susceptible to epigenetic regulation. qRT-PCR and quantitative DNAM analysis were employed to validate findings in larger sample sets (n=35/group).

Results: Gene Set Enrichment Analysis (GSEA) demonstrated that BMP signaling and astrocyte and cerebral cortex development are significantly (FDR $q < 0.25$) coordinately upregulated in both SCZ and BD(+), and glutamate signaling and TGF β signaling are significantly coordinately

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upregulated in SCZ. GSEA also indicated that collagens are downregulated in right *versus* left brain of controls, but not in SCZ or BD(+) patients, and Ingenuity Pathway Analysis predicted that *TGFB2* is an upstream regulator of these genes ($p=0.0012$). While lateralized expression of *TGFB2* in controls ($p=0.017$) is associated with a corresponding change in DNAM ($p=0.023$), lateralized expression and DNAM of *TGFB2* are absent in SCZ or BD.

Conclusion: Loss or reversal of brain laterality in SCZ and BD corresponds to aberrant epigenetic regulation of *TGFB2* and changes in TGF β signaling, indicating potential avenues for disease prevention/treatment.

Keywords

Schizophrenia; Brain asymmetry; DNA methylation; *TGFB2*; *NR2E1*

INTRODUCTION

Schizophrenia (SCZ) and bipolar disorder (BD) are two interrelated life-long mental illnesses, each affecting 1% of the population worldwide. Despite several decades of extensive research, it has been challenging to define the key molecular pathways and corresponding etiologies of these diseases. During the last two decades, the use of advanced imaging techniques such as functional magnetic resonance imaging (fMRI), gamma synchrony and mapping of scalp electrical activity have helped to identify SCZ and BD endophenotypes, including atrophy of the cingulate gyrus and dorsolateral prefrontal cortex, mainly in the left brain hemisphere (Rockstroh et al., 1998; Walter et al., 2003; Weiss et al., 2004; Cullen et al., 2006; Prasad and Keshavan 2008; Venkatasubramanian et al., 2008a,b; Meda et al., 2008; Bangalore et al., 2009; Williams et al., 2009; Bleich-Cohen et al., 2012; Narayanaswamy et al., 2015; Mahon et al., 2015; Li et al., 2016; Mwansisya et al., 2017). In fact, the loss or reversal of brain asymmetry/laterality is one of the most consistent observations that have eluded an understanding in neuro-developmental diseases like SCZ and BD (Williams et al., 2009; Wang et al., 2014; Ho et al., 2017). It has been proposed that a compromised left brain's logical dominance [or right brain in left-handed individuals] is linked to the lack of insight on wishes, fears and perceptual distortions, leading to delusional thinking and hallucination in SCZ (Crow 2000). Thus, "an individual's own thoughts [originated from the right brain] are perceived as an external intruding voice" [by the left brain] (Angrilli et al, 2009).

For more than two decades, mutations in genes that are presumed to be involved in the establishment of brain laterality remained as the dominant hypothesis to explain the underlying mechanism of aberrant brain laterality in SCZ (Crow 1989 and 2000). However, more than a thousand linkage and genetic association studies, including the most recent genome-wide association studies (GWASs) involving thousands of cases and controls, have failed to identify a specific gene or group of genes with major effects responsible for the genesis of SCZ or BD (Purcell et al., 2009; Hamshere et al., 2013). On the other hand, recent research findings overwhelmingly support key roles for epigenetic modifications of genes in cell differentiation and the development of structural and functional diversity of different cells/tissues, which may include brain hemispheres. Epigenetic codes are inherited and referred to as epigenetic memory (Monk 1995; Bird 2002); however, environmental insults

such as contaminants, infectious elements, malnutrition, or ecological and social conditions can change epigenetic memory, resulting in sporadic or inherited neurodevelopmental diseases (Abdolmaleky et al., 2008 and 2015a; Szyf 2009). Additionally, a role for environmental effects arising from choline, methionine and folic acid deficiency, the gut microbiome, inflammation and oxidative stress, which are associated with the failure of the functionality of methylation machinery and the corresponding loss of epigenetic memory, has been described in major mental diseases (reviewed in Abdolmaleky et al., 2015a; Alam et al., 2017). At least 30 gene-specific and more than 10 whole genome DNA methylation analyses have reported aberrant DNA methylation of more than 100 genes in SCZ and BD (Abdolmaleky et al., 2015a).

A number of studies have also provided evidence for epigenetic fine tuning of brain laterality affecting expression of monoaminergic genes such as *DRD2* (Popenklyte et al., 1999), *MB-COMT* (Abdolmaleky et al., 2006), *HTR2A* and *DTNBPI* in normal individuals, which is lost in SCZ and BD patients (Abdolmaleky et al., 2006, 2011 and 2015b). However, a comprehensive understanding of the identity of target genes and mechanisms underlying their dysregulation leading to partial loss or reversal of brain laterality remains unclear in SCZ and BD.

We performed gene expression profiling and DNA methylome analysis of the dorsolateral prefrontal cortex (Brodmann's area 46), one of the well characterized dysfunctional brain regions in SCZ and BD (Stanley et al., 2007; Minzenberg et al., 2009; Ragland et al., 2009; Dell'Osso et al., 2015), in post-mortem brain samples from the left and right hemispheres of SCZ and BD patients and controls to identify pathways that are affected in SCZ and BD, as well as those that lose normal brain laterality in these disease states. Our results provide evidence for shared pathway alterations in SCZ and BD, as well as aberrant brain asymmetry/laterality in SCZ and BD. We also identified epigenetic modifications that correspond to gene expression alterations of specific genes. Several key genes identified in these analyses were subjected to verification in a larger set of post-mortem brain samples using qRT-PCR analysis. Additionally, iPSC derived neurons and astrocytes were also analyzed to identify cell type origins for the expression of significant genes.

METHODS

Samples

One hundred and five RNA and DNA samples extracted from the dissects of post-mortem brains (Brodmann's area 46, dorsolateral prefrontal cortex) of patients with SCZ or BD or control subjects (n=35 per group) were obtained from the Stanley Medical Research Center (SMRC). DNA was extracted using a phenol-chloroform protocol and total RNA was isolated using TRIzol reagent (Invitrogen). An A260/A280 ratio greater than 1.7 was achieved with more than 95% of the DNA/RNA samples used in these studies. The samples used in the analysis were matched for sex, ethnicity, brain laterality, age and other demographics as described previously (Abdolmaleky et al., 2014). We also had access to other detailed information such as age of onset and duration of disease, psychotic feature, cause of death, length of stay in hospital, history/status of smoking and alcohol,

antipsychotic, mood stabilizer or antidepressant use (and the type of drugs) at the time of death, brain weight and pH, and post-mortem and refrigeration intervals.

RNA expression microarray profiling

Gene expression profiling was performed using the Affymetrix GeneChip Human Genome U133 Plus 2.0 array with 1 µg of total RNA from each sample (n=10 per group: control, SCZ and BD). All RNA samples used for microarray analysis had RIN (RNA Integrity Number) values greater than 7 as measured by Agilent Bioanalyzer. CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA) in the affy R package (version 1.36.1) and an Entrez Gene-specific probeset mapping (version 14.0.0) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan (Dai et al 2005). Array quality was assessed by computing Relative Log Expression (RLE) and Normalized Unscaled Standard Error (NUSE) using the affyPLM R package (version 1.34.0). All samples had median RLE values less than 0.1 and median NUSE values less than 1.05. Principal Component Analysis (PCA) was performed using the prcomp R function with expression values that had been normalized across all samples to a mean of zero and a standard deviation of one. Moderated (empirical Bayesian) *t* tests were performed using the implementation in the limma R package (version 3.14.4), i.e., creating simple linear models with the lmFit function, followed by empirical Bayesian adjustment with the eBayes function. Samples from subjects with BD who were not in a psychotic state at the time of death were excluded; only subjects with psychotic BD (BD(+)) were included in the analysis. The expression of each gene was modeled twice: first as a linear function of disease state (SCZ vs control, BD(+) vs control) and laterality (right vs left) (the “reduced” model), and then as a linear function of disease state, laterality, and the interaction of the two (SCZ:laterality, BD(+):laterality) (the “full” model). The significance of each disease state versus control was assessed using the reduced model, and the significance of each interaction effect was assessed using the full model. Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR). All microarray analyses were performed using the R environment for statistical computing (version 2.15.1).

Gene Set Enrichment Analysis (GSEA)

GSEA (version 2.2.1) was used to identify biological terms, pathways and processes that are coordinately up- or down-regulated with respect to given effects in the linear models described above. The Entrez Gene identifiers of the genes interrogated by the array were ranked according to the moderated *t* statistics of the SCZ or BD(+) coefficients in the “reduced” model, or for the SCZ:laterality or BD(+):laterality coefficients of the “full” model. Each ranked list was then used to perform pre-ranked GSEA analyses (default parameters with random seed 1234) using the Entrez Gene versions of the Hallmark, BioCarta, KEGG, Reactome, and Gene Ontology (GO) gene sets obtained from the Molecular Signatures Database (MSigDB) (version 6.0).

qRT-PCR analysis

Real-time qRT-PCR analysis was performed using SYBR Green and ABI 7900 Real-Time PCR System (384-well plate) in duplicate, using cDNA synthesized from the same RNA

samples used for the microarray as well as additional samples which passed quality controls (35 samples per group). Quantification of the expression of candidate genes was performed using the C_T method normalized with the C_T (cycle threshold) of β -actin and GAPDH as previously described (Abdolmaleky et al., 2008 and 2014).

Analysis of gene expression in iPSC-derived neurons and astrocytes

Four human iPSC lines of normal controls from the Simons Foundation were cultured in StemFlex medium (Thermo Fisher catalog #A3349401). Cells were induced to differentiate to NSC and then to either astrocytes or neuronal cells using standard protocols. In brief, iPSCs were cultured in StemFlex medium for 3 passages and were induced to differentiate to NSC using neuronal induction medium (Thermo Fisher, Catalog #A1647801). NSCs were then allowed to differentiate into neurons (using neurobasal medium and B-27 supplement) or astrocytes (using DMEM, 0.5% FBS and N2 supplement). After two weeks of differentiation, acquisition of neuronal or astrocyte phenotypes were confirmed with immunohistochemical staining with corresponding primary (e.g. MAP2 and synapsin) and secondary antibodies. Cells were cultured for another two weeks, then harvested. RNA was extracted using TRIzol (ZYMO Research, Direct-zol RNA MiniPrep, catalog #R2050) and used for cDNA synthesis (Invitrogen Life Technologies, catalog# 18090050). SYBR Green based qRT-PCR was used to perform gene expression analysis.

DNA methylation microarray profiling

To identify genes that are regulated by DNA methylation in the human brain, we assessed gene-specific DNA methylation status in representative post-mortem brain samples that had been evaluated for gene expression (four samples per group) using the Illumina Infinium Human Methylation 27 (27k) BeadChip array. Following bisulfite treatment of 1 μ g of DNA using an EZ DNA Methylation kit (Zymo Research, Cat #D5001), 0.5 μ g of bisulfite-modified DNA was processed for DNA methylation analysis. The 27k array is designed to measure methylation at approximately 27,000 CpG sites, primarily at gene promoters. When treated with bisulfite, non-methylated cytosines are converted to uracil while methylated cytosines remain unchanged. Two types of beads (U and M), which are only able to hybridize to either unmethylated or methylated sites, respectively, trigger single-base extension for detection. Extensions with green and red fluorescence represent methylated and unmethylated bases, respectively, and methylation status was computed from the ratio of green (methylated) fluorescent signal to the total fluorescent intensity (beta). Raw intensities in the green and red channels of the fluorescent signal were read and converted to methylation status by the methylumi R package, with beta values calculated using the formula $\text{beta} = M/(M+U+100)$ (Du et al., 2010). Raw intensities were normalized using the normalizeMethyLumiSet function to correct dye bias and beta values were recalculated based on the corrected intensities.

Gene-specific DNA methylation analysis

The initial evaluation of CpG islands using DNA methylation microarray in representative samples enabled us to assess the overall methylation levels of potential target CpG islands for further analysis. Next, methylation specific PCR (MSP) and/or bisulfite sequencing analyses were used (Abdolmaleky et al., 2008; 2011; 2014) to confirm the presence of DNA

methylation in a selected group of candidate CpGs. The bisulfite modified placental DNA and *in vitro* methylated DNA were used as negative and positive controls for methylation, respectively (Abdolmaleky et al., 2008 and 2014). Whenever MSP was not feasible due to highly GC-rich sequences, enzymatic digestion of methylated DNA coupled with qPCR was used to evaluate 5-mc (5-methyl cytosine) as well as 5-hmC (5-hydroxymethyl cytosine) content. 5-hmC is an intermediate formed during demethylation of methylated DNA, and has been reported to be abundant in human brain and to correspond to induction of gene expression (Song et al. 2011; Nestor et al., 2012), even before its conversion to unmethylated cytosine (Wu and Zhang 2011). In order to differentiate between 5-mC and 5-hmC, we used qPCR analysis of MspI and HpaII restricted DNA treated by T4 Phage β -glucosyltransferase (T4-BGT) with uridine diphosphoglucose (UDP-Glu) using the EpiMark 5-hmC and 5-mC Analysis Kit according to the instructions of the manufacturer (New England BioLabs, Cat# E3317S). For quantification, C_T method was used and normalized with the C_T of uncut DNA.

RESULTS

Study demographics

The demographics of the subjects of this study are outlined in Table 1. Subjects with BD are divided into those with or without psychotic features, or those in whom the presence or absence of psychosis is unknown, denoted as BD(+), BD(–), and BD(unknown), respectively.

Principal Component Analysis (PCA)

Gene expression microarrays were used to profile gene expression in the left or right dorsolateral prefrontal cortex (Brodmann's area 46) of post-mortem brain samples obtained from patients with SCZ or BD or from matched controls (n=10 per group). We used Principal Component Analysis (PCA) to cluster these 30 samples with regard to the expression of all ~19,000 genes interrogated by the microarray platform (Supplementary Figure 1). Although there was no clear division among samples with respect to disease state, brain hemisphere, or sex, there was also no indication of any outliers that separated them from the other samples used in the study.

Identification of differentially regulated pathways in SCZ and BD(+)

A linear modeling approach was then used to model gene expression as a function of disease state (after correcting for brain laterality) and to perform a *t* test on the coefficient of the SCZ and BD(+) model terms to assign significance to each disease state. BD(–) subjects were excluded from analysis in order to reduce confounding variables and because BD(+) has previously been shown to be more similar to SCZ than BD(–) (Choi et al., 2008). The full set of results from this statistical analysis is provided in Additional File 1. Only one gene passed FDR correction at a reasonable threshold ($q < 0.25$) for the SCZ term, and none passed FDR correction for the BD(+) term, indicating that the experiment is not powered to discriminate true and false positives at the level of individual genes.

To improve statistical power, all genes interrogated by the array were ranked according to the t statistics computed from the SCZ or BD(+) terms of the model and Gene Set Enrichment Analysis (GSEA) was used to identify pathways that are coordinately regulated in either disease state with respect to controls. The full set of GSEA results are provided in Additional File 2. We found that gene sets related to BMP signaling, astrocyte differentiation, and cerebral cortex development are significantly (FDR $q < 0.25$) coordinately upregulated in both SCZ and BD(+); the leading edges of these gene sets are shown in Figure 1. These include the Reactome BMP signaling pathway (SCZ FDR $q = 0.024$, BD(+) FDR $q = 0.014$) and the Gene Ontology (GO) term “response to BMP” (SCZ FDR $q = 0.18$, BD(+) FDR $q = 0.06$), which includes the TGF β receptor gene *TGFBR3* and several Smad genes (Figure 1A); the GO terms “astrocyte differentiation” (SCZ FDR $q = 0.016$, BD(+) FDR $q = 0.15$), which includes the transcription factor *SOX9*, and “regulation of astrocyte differentiation” (SCZ FDR $q = 0.18$, BD(+) FDR $q = 0.24$), which includes the transcription factor *NR2E1* (Figure 1B); and the GO term “cerebral cortex development” (SCZ FDR $q = 0.13$, BD(+) FDR $q = 0.17$), which includes the transcription factors *PAX6*, *LHX2* and *NR2E1* (Figure 1C).

We also found that pathways related to glutamate signaling and TGF β signaling showed significant coordinate upregulation in SCZ with weak or no association with BD(+). These include the GO terms “glutamate metabolic process” (SCZ $p = 0.0045$, FDR $q = 0.065$; BD(+) $p = 0.044$, FDR $q = 0.33$) and “glutamate secretion” (SCZ $p = 0.016$, FDR $q = 0.16$; BD(+) $p = 0.63$, FDR $q = 0.92$), indicating the involvement of glutamate/glucose transporters *SLC1A2* and *SLC1A3* (Figure 1D); and the KEGG (SCZ $p < 0.001$, FDR $q = 0.086$; BD $p = 0.021$, FDR $q = 0.32$) and BioCarta (SCZ $p = 0.046$, FDR $q = 0.24$; BD $p = 0.82$, FDR $q = 0.98$) highlighting the role of TGF β pathways (Figure 1E). As the TGF β ligand genes *TGFB1* and *TGFB3* were poorly expressed in the dorsolateral prefrontal cortex (called Present in only 10% or 13% of samples, respectively) and are not significantly associated with SCZ ($p > 0.5$), and *TGFB2* was called Present in all samples and is strongly associated with both SCZ ($p = 0.007$) and BD(+) ($p = 0.0097$), TGF β 2 appears to be the ligand driving the TGF β pathway GSEA results.

Loss of lateralized/asymmetric gene expression in the brain of SCZ and BD(+) patients

In the same manner, a linear modeling approach was then used to assign statistical significance to each gene with respect to the interaction of each disease state (SCZ or BD(+)) and brain laterality. The full set of results from this statistical analysis are provided in Additional File 1. As no genes passed FDR correction for these interaction coefficients, GSEA was used to identify pathways that are regulated in a disease-dependent manner with regard to brain laterality (full set of results provided in Additional File 2). Sets of genes annotated with Gene Ontology (GO) terms associated with collagen deposition are significantly associated with the interaction of either or both of SCZ and BD(+) with laterality (“complex of collagen trimers”: SCZ FDR $q = 0.022$, BD(+) FDR $q = 0.058$; “collagen trimer”: SCZ FDR $q = 0.11$), and show a pattern of coordinate downregulation in the right versus left brain of controls and upregulated or unchanged expression in the right versus left brain of SCZ or BD(+) patients (Figure 2A). These include the gene *COL3A1*

(SCZ:laterality $p = 0.0052$; BD(+):laterality $p = 0.11$; control: 1.5-fold lower in right brain; SCZ: 1.26-fold higher in right brain; BD(+): 1.01-fold lower in right brain).

Prediction of *TGFB2* as an upstream regulator of loss of brain laterality in SCZ

Ingenuity Pathway Analysis (IPA) was then used to identify upstream regulators of the loss of laterality of gene expression observed in SCZ relative to controls. In a Core Analysis performed using the 265 genes with SCZ:laterality $p < 0.05$ and called Present in at least 3 samples (i.e., the size of the smallest experimental group in the analysis), *TGFB2* was predicted to be a likely upstream regulator of these changes in gene expression ($p = 0.0012$). A mechanistic network including *TGFB2* and the genes that support this prediction is shown in Figure 2B.

Assessment of cell-type-specific *TGFB2* expression

To determine which cell types contribute to increased TGF β signaling in these samples, we used qRT-PCR to examine the expression of *TGFB2* in human iPSC-derived astrocytes and neurons. This analysis indicates that the expression of *TGFB2* is significantly higher in astrocytes than in neurons ($p = 0.029$) (Supplementary Figure 2), suggesting that astrocytes are the cell type responsible for this effect.

Validation of *NR2E1* and *TGFB2* expression

We then performed qRT-PCR analysis to validate the differential expression of *NR2E1* and *TGFB2* (primers listed in Table 2) using a larger set of post-mortem brain samples ($n=35$ per diagnosis). The expression of these genes by qPCR was highly concordant with the microarray data (*NR2E1* Pearson $r = 9.6 \times 10^{-5}$; *TGFB2* Pearson $r = 0.0041$) (Supplementary Figure 3). The expression of *NR2E1* was significantly increased in both SCZ (1.26-fold, $p=0.038$) and BD(+) (1.64-fold, $p=0.016$) (Figure 3A), whereas *TGFB2* expression was significantly increased in SCZ (1.22-fold, $p=0.026$) but only trended in that direction in BD(+) (1.32-fold, $p=0.11$) (Figure 3B). Surprisingly, the expression of *TGFB2* with respect to laterality (1.26-fold higher in the right brain of controls, $p = 0.017$) was the opposite of that predicted by IPA from the expression of collagens and other downstream genes (which were more highly expressed in the left brain of controls).

Assessment of methylation at *TGFB2* locus

Enzymatic digestion of methylated DNA followed by qPCR was then used to measure methylation within two sites: 1) an amplicon encompassing a CpG island in the 5' UTR of the *TGFB2* gene locus, and 2) a 170-base amplicon approximately 3.4 kb upstream of the *TGFB2* transcriptional start site (Figure 4A; primers listed in Table 3). As described in the Methods, we measured total methylation as well as 5-hmC at each site. Because we found that the 5-hmC:5-mC ratio varies substantially with respect to disease state (Supplementary Figure 4), we subtracted 5-hmC signal from total methylation signal in order to obtain a pure measurement of 5-mC at each site. The amount of 5-mC at site 1 (Figure 4B) was significantly lower in the right brain of controls than in the left brain of controls (1.59-fold, $p = 6.8 \times 10^{-3}$), and was unchanged with respect to brain laterality in SCZ ($p = 0.76$) or BD(+) ($p = 0.77$). A similar but less significant pattern was observed at site 2 (Figure 4C) (1.2-fold

lower in right brain of controls, $p = 0.023$; SCZ right vs. left $p = 0.84$; BD(+) right vs. left $p = 0.91$). This is the inverse of the pattern of lateralized *TGFB2* expression observed by qPCR, indicating that the laterality of this gene is regulated at least in part by DNA methylation. However, in contrast to the *TGFB2* expression qPCR, there was no statistically significant change in 5-mC with regard to disease state at either site (SCZ vs. control $p = 0.47$ and 0.64 and BD(+) vs. control $p = 0.92$ and 0.11 for sites 1 and 2, respectively).

Genome-wide assessment of DNA methylation

Illumina 27k DNA methylation microarrays were also used to profile 3–4 of the same samples from each disease group (control, SCZ, and BD). The annotation for each probe and beta values (fraction of methylated CpG residues) are provided in Additional File 3.

To identify genes that might be regulated by methylation, we matched the Illumina 27k data with the HG-U133 Plus 2.0 data using the annotated Entrez Gene ID (generating a set of 25,972 CpG-gene pairs, representing 13,601 unique genes) and performed Spearman correlation tests for each pair. The complete set of these results are provided in Additional File 4. We identified the *CCND1* locus as of particular interest, as it was interrogated by a large number of probes, its expression was significantly downregulated in SCZ ($p = 0.00044$) and BD(+) ($p = 0.044$), and its methylation was generally anti-correlated with its expression (11/18 probes with Spearman $\rho < 0$). Methylation was increased to a greater extent in BD (up to 39%) than in SCZ (up to 14%) in probes interrogating a CpG island extending from upstream of *CCND1* through its second intron; however, methylation was generally increased in both SCZ and BD (up to 17%) in and around a CpG island contained in its third intron.

DISCUSSION

Schizophrenia (SCZ) and bipolar disorder (BD) are two severe mental disorders that share some common clinical phenotypes and hundreds of SNPs and epigenetic alterations underlying their disease pathology. Although numerous studies have attempted to define molecular pathways corresponding to the pathology to devise effective therapeutics, the etiology of their pathogenesis remains elusive. At present, the treatment of these diseases relies predominantly on antipsychotic drugs and mood stabilizers that were serendipitously found to improve some of the disease symptoms. However, these drugs have little effect on cognitive dysfunction to improve the functional capabilities of patients, particularly those with SCZ. While one of the overlooked phenotypes in SCZ and to some extent in BD is the loss of brain laterality, its molecular basis has remained elusive and its elucidation may provide leads for implementing novel therapeutic approaches. Our limited previous studies provided evidence for epigenetic modifications as the key to asymmetric transcription of genes in the brain and corresponding functional diversity of brain hemispheres (Abdolmaleky et al., 2006, 2011 and 2014). For the first time to our knowledge, here we report the examination of gene expression profiles of post-mortem brain samples in the left versus right brain to identify differentially activated molecular pathways, and to correlate altered gene expression to differential DNA methylation patterns in order to decipher the

molecular basis and underlying mechanisms for the loss or reversal of brain laterality in these interlinked diseases.

Based on our data, *TGFB2* and *NR2E1* appear to be key drivers for dysregulation of many genes in SCZ and BD. These findings are consistent with a previous meta-analysis which concluded that $TGF\beta$ is a disease state marker in SCZ, as its expression increased in acutely relapsed patients and those exhibiting first-episode psychosis, and returned to normal levels following antipsychotic treatment (Miller et al., 2011). *TGFB2* is downregulated in the retina of *NR2E1* knockout mice (Zhang et al 2006) and there are 20 binding sites for NR2E1 (AAGGTCA/TGACCTT) in the body of the *TGFB2* gene, explaining the direct correlation between *NR2E1* and *TGFB2* expression. *NR2E1* (TLX) is an orphan nuclear receptor (Benod et al., 2014) expressed exclusively in the brain (Yu et al., 1994) and has been shown to either act as a transcriptional repressor (when bound to the *p21*, *PTEN*, *GFAP* or *S100b* promoters) or an activator (if it binds to other specific proteins) (Benod et al., 2014). During the development and adulthood of vertebrates, *NR2E1* is expressed in the forebrain, specifically in the neurogenic regions as well as in the retina, where it regulates multiple pathways involved in DNA replication, cell cycle and cell adhesion via repression or activation of target genes. *NR2E1* is also “a master regulator of neural stem cell maintenance and neurogenesis” (Islam and Zhang, 2014), preventing senescence (O’Loughlen et al., 2015), mitigating the impacts of IL-1 β on hippocampal neurogenesis (Ó’Léime et al., 2018) and involved in aggressive behavior (Abrahams et al., 2005). Furthermore, genetic mutations in *NR2E1* have been linked to BD pathogenesis (Kumar et al., 2008).

A recent study combining serial analysis of gene expression (SAGE) and transcription factor binding site prediction identified 64 novel candidate target genes of *NR2E1* involved in neocortical development (Schmuth et al., 2015). These include *LHX2*, which is involved in “post-mitotic regulation of sensory area patterning in the mammalian neocortex” (Zembrzycki et al., 2015) and its cofactor *SOX9*. It is also known that coordination and cooperation between NR2E1 and PAX6 is required for brain patterning during development (Stenman et al., 2003). Our gene expression microarray analysis indicated that these three genes are upregulated in SCZ and in BD, and contribute to the coordinate upregulation of gene sets related to cerebral cortex development or astrocyte differentiation in the corresponding disease states. NR2E1 also controls cyclin D expression via PTEN by binding to the *PTEN* promoter to suppress its activity during retinogenesis (Zhang et al., 2006). Interestingly, the expression of *CCND1* was decreased in SCZ and BD, and its DNA methylation was increased in SCZ and BD, indicating that DNA hypermethylation may also partly explain this effect.

Some other genes known to be linked to psychiatric diseases also exhibited dysregulation in patients with SCZ and BD in our expression profiling studies. For example, the genes *NOTCH2*, which is a key player in neurodevelopment and SCZ pathogenesis (Sundararajan, Manzardo and Butler 2018), and *SELENBP1*, which exhibits increased expression in the frontal cortex and blood cells in SCZ (Glatt et al., 2005), are also up-regulated in SCZ and BD as evident from our microarray analysis. The expression of the glutamate/glucose transporter *SLC1A2* (Koeglspenger et al., 2013), which is linked to lithium response in BD, and is associated with the AMPA2 glutamate receptor network in human brain, was also

highly upregulated in SCZ, consistent with findings related to prefrontal cortex of SCZ patients (Matute et al., 2005). In GWAS studies *SLC1A2* has an effect size of 0.3 in disease pathogenesis with an allelic frequency of 0.4 (Higgins, Allyn-Feuer & Athey, 2015). Interestingly, it has been shown that in mouse astrocytes, *TGFβ* induces the expression of *SLC1A2* as well as the glutamate/glucose transporter *SLC1A3* and an increase in astroglial glutamate uptake (Koeglspenger et al., 2013). In addition to *SLC1A2*, *SLC1A3* also exhibited a trend for increased expression in SCZ and BD in our analysis, suggesting that *TGFβ*-mediated astrocyte dysfunction may be involved in the pathogenesis of these major mental diseases. Our examination of iPSC-derived astrocytes and neurons also found that astrocytes express *TGFB2* more than in neurons supporting this notion.

According to our array data, several collagen gene products downstream of *TGFβ* signaling emerged as likely mediators for the establishment of brain lateralization in normal controls that is lost in SCZ and BD. Among them, *COL3A1* is known to regulate cortical development and is the major ligand for GPR56 in the developing brain. Its binding to GPR56 inhibits neuronal migration and activates the RhoA pathway by coupling GPR56 to GNA13 and possibly to GNA12. It also appears that the expression of *TGFB2*, which appears to encode the active *TGFβ* isoform in the dorsolateral prefrontal cortex, is partially regulated by methylation. However, it is unclear why the transcription of *TGFB2* is discordant with that of the collagen products with respect to laterality in control subjects. As it has been previously reported that the transcription and translation of *TGFβ* are regulated by distinct pathways (Xiao et al., 2008), it may be that translational and post-translational regulation of *TGFβ* signaling explains this discrepancy. This will need to be examined in further studies.

In summary, our results indicate that key genes of the *TGFβ* signaling pathway are upregulated in SCZ and BD, and the expression of collagen genes is associated with left/right brain asymmetry in controls but not in SCZ and BD.

CONCLUSIONS

Evidence from developmental biology research indicates that several *TGFβ* superfamily signaling proteins (e.g., lefty-1, lefty-2, nodal, ACVR2B, Smad2 and Smad5) are involved in left-right axis determination (Meno et al., 1996 and 1998; Nomura and Li 1998; Hamada et al., 2002; Chang et al., 2000; Smith et al., 2011). Despite the limited availability of post-mortem brain samples, our observations are consistent with and extend the previous findings of developmental biologists (Vonica et al., 2011) in supporting that the brain laterality could also be regulated by *TGFβ* superfamily signaling pathways. Furthermore, the association between the loss or reversal of brain laterality in SCZ and BD due to aberrant epigenetic regulation of the key affected genes suggests potential avenues for disease prevention at onset and/or during progression. For example, *TGFB2* itself, as well as collagens or other genes downstream of *TGFB2* exhibiting aberrant lateralized expression might be appropriate targets for designing novel therapeutics for the SCZ and BD patients. It is noteworthy that the currently available drugs affect both brain hemispheres and can only render partial improvement of symptoms and most of the patients remain dysfunctional throughout their life. Therefore, elucidating the hemisphere-specific molecular basis of SCZ and BD

pathogenesis as described in this report is a good beginning for the quest to design novel therapeutic strategies (such as unilateral transcranial magnetic stimulation, or ECT) for these major psychiatric disorders with implications to other mental diseases (e.g. obsessive compulsive disorder).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Leading edges of gene sets in pathways coordinately upregulated by GSEA in SCZ and/or BD(+).

Linear modeling was performed to assign t statistics to each gene with respect to SCZ or BD(+), and GSEA was performed to identify Gene Ontology (GO) terms or BioCarta, KEGG or Reactome pathways with significant coordinate regulation in either or both disease states. Groups of significant gene sets representing several pathways or processes were selected, and the expression of the genes in any of the "leading edges" of each set (i.e., the genes that contributed the most to the significance of the result) is shown across all subjects (including non-psychotic BD, which was not included in the analysis). Rows and columns correspond to genes and samples, respectively. Expression values were scaled by z -normalizing expression values to a mean of zero and standard deviation of one across all samples in each row, with red and blue indicating z -scores $+2$ or -2 , respectively. Genes are arranged in descending order from top to bottom by the magnitude of the t statistic used to rank genes for GSEA. Gene sets are grouped according to theme: (A) BMP signaling; (B) astrocyte differentiation; (C) glutamate metabolism and secretion; (D) cerebral cortex development; (E) TGFβ signaling. The membership of each gene in the leading edge of each

gene set for each disease state (SCZ or BD(+)) in each analysis is denoted in a separate color (orange or yellow) for each gene set in each panel.

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Figure 2: Collagen gene sets are coordinately regulated downstream of TGF-beta with respect to brain laterality in a disease-dependent manner.

(A) Leading edges of collagen-related gene sets with significant interaction between SCZ or BD(+) with laterality. Linear modeling was performed to assign t statistics to each gene with respect to the interaction of SCZ or BD(+) with laterality, and GSEA was performed to identify Gene Ontology (GO) terms or BioCarta, KEGG or Reactome pathways with coordinate regulation in either or both disease states with respect to laterality that differs significantly from that in controls. The expression of the genes in any of the "leading edges" of each set (i.e., the genes that contributed the most to the significance of the result) is shown across all subjects (including non-psychotic BD, which was not included in the analysis). Rows and columns correspond to genes and samples, respectively. Expression values were scaled by z -normalizing expression values to a mean of zero and standard deviation of one across all samples in each row, with red and blue indicating z -scores ≥ 2 or ≤ -2 , respectively. Genes are arranged in descending order from top to bottom by the magnitude of the t statistic used to rank genes for GSEA. The membership of each gene in the leading edge of each gene set for each disease state (SCZ or BD(+)) in each analysis is denoted in a separate color (orange or yellow) for each gene set. (B) Mechanistic network of TGF β 2-driven regulation of genes with significant SCZ:laterality interaction. Ingenuity

Pathway Analysis was used to predict upstream regulators of the 265 genes with SCZ:laterality $p < 0.05$ and called Present in 3 samples. TGFB2 and SMAD3 were predicted to be activated, and SMAD7 was predicted to be inhibited, leading to coordinate downregulation of genes such as collagens in the right brain of controls only.

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Figure 3: Validation of *NR2E1* and *TGFB2* gene expression by qPCR.

Relative expression of (A) *NR2E1* (n=100) and (B) *TGFB2* (n=102), normalized to beta-actin, divided by disease state and brain laterality. The mean of each group is indicated by a heavy gray line. Filled circles indicate samples that were profiled by gene expression microarray.

Figure 4: Measurement of *TGFβ2* DNA methylation by qPCR.

(A) Location of two qPCR amplicons (site 1 and site 2) in the *TGFβ2* locus. (B, C) Measurement of 5-methylcytosine (5-mC) at each site (site 1: n=103 samples total; site 2: n=90 samples total), divided by disease state and brain laterality. The mean of each group is indicated by a heavy gray line. Filled circles indicate samples that were profiled by gene expression microarray.

Table 1.

Demographics of samples in study. The sex and age of the subjects is shown, divided by disease state and brain laterality.

Used in both qPCR and microarray analysis (n=105)										
Disease state	control		SCZ		BD(-)		BD(+)		BD (unknown)	
Laterality	left	right	left	right	left	right	left	right	left	right
n	16	19	17	18	8	4	11	10	1	1
Sex (% male)	88%	63%	71%	78%	63%	75%	27%	50%	0%	100%
Age (mean $\pm$ sd)	45.6 $\pm$ 6.6	43.1 $\pm$ 8.4	41.3 $\pm$ 8.1	43.8 $\pm$ 8.8	43.8 $\pm$ 11.0	45.2 $\pm$ 18.9	43.7 $\pm$ 9.8	48.3 $\pm$ 9.1	48	42
Used in gene expression microarray analysis only (n=30)										
Disease state	control		SCZ		BD(-)		BD(+)		BD (unknown)	
Laterality	left	right	left	right	left	right	left	right	left	right
n	5	5	5	5	4	-	3	3	-	-
Sex (% male)	80%	60%	40%	80%	75%	-	0%	100%	-	-
Age (mean $\pm$ sd)	45.0 $\pm$ 8.6	45.2 $\pm$ 10.3	43.2 $\pm$ 4.2	41.6 $\pm$ 6.9	37.8 $\pm$ 8.5	-	47.7 $\pm$ 16.2	41.3 $\pm$ 8.5	-	-

Table 2.

Primers for gene expression qRT-PCR analysis. The primers for NR2E1 and TGFB2 were obtained from the Harvard primer bank.

Gene	Forward (5'–3')	Reverse (5'–3')
<i>ACTB</i>	CGAGCACAGAGCCTCGCCTTTGCC	TGTCGACGACGAGCGGGCGATAT
<i>NR2E1</i>	CAAACGGAGCATCCGAAGGAA	GTCCACGGAAGTAGAGGGC
<i>TGFB2</i>	AGAGTGCCTGAACAACGGATT	CCATTGCCTTCTGCTCTT

Table 3.

Primers for methylation qRT-PCR analysis.

Amplicon	Forward (5'–3')	Reverse (5'–3')
<i>TGFB2</i> site 1	CGTGGTTCAGAGAGAACTTATAAATCTCC	TCTGTCTTTCTCTTGTGAGGAGC
<i>TGFB2</i> site 2	CCTTTTACCATGAAGACTGTAGAGAC	TCAAATGATCAGTTCTTTGAAGACCT

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