

An Integrative Genomic Study Implicates the Postsynaptic Density in the Pathogenesis of Bipolar Disorder

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Genome-wide association studies (GWAS) have identified several common variants associated with bipolar disorder (BD), but the biological meaning of these findings remains unclear. Integrative genomics—the integration of GWAS signals with gene expression data—may illuminate genes and gene networks that have key roles in the pathogenesis of BD. We applied weighted gene co-expression network analysis (WGCNA), which exploits patterns of co-expression among genes, to brain transcriptome data obtained by sequencing of poly-A RNA derived from postmortem dorsolateral prefrontal cortex from people with BD, along with age- and sex-matched controls. WGCNA identified 33 gene modules. Many of the modules corresponded closely to those previously reported in human cortex. Three modules were associated with BD, enriched for genes differentially expressed in BD, and also enriched for signals in prior GWAS of BD. Functional analysis of genes within these modules revealed significant enrichment of several functionally related sets of genes, especially those involved in the postsynaptic density (PSD). These results provide convergent support for the hypothesis that dysregulation of genes involved in the PSD is a key factor in the pathogenesis of BD. If replicated in larger samples, these findings could point toward new therapeutic targets for BD.

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INTRODUCTION

Bipolar disorder (BD) is a debilitating and highly heritable psychiatric disorder whose genetic etiology is largely unknown. Candidate gene, genome-wide association (GWAS), and gene expression studies have all implicated a variety of genes, but a coherent theory of pathogenesis has not yet emerged. Multiple variants in many genes often come together into several gene networks and fewer biological pathways. This points to the powerful strategy, known as ‘integrative genomics’ (Schadt, 2006), to address the extreme genetic heterogeneity seen in many common illnesses. This strategy has proven to be of value in interpreting the multigenic signals that have been observed in GWAS, copy number variation, and *de novo* mutation studies of many common neuropsychiatric diseases (Walsh *et al*, 2008; Choi *et al*, 2011; Fromer *et al*, 2014) but has so far been little studied in BD.

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Many of the common genetic variants identified by GWAS lie in the regulatory regions, where they can affect the expression of nearby genes (Maurano *et al*, 2012). Much of the impact of genetic variation on gene expression is tissue-dependent (Andersson *et al*, 2014). Non-protein-coding genes that produce regulatory RNAs also appear to have an important role in fine-tuning of gene expression over development and in response to environmental stressors (Barry, 2014). It has also become clear that most genes, especially those expressed in brain, produce a number of distinct messenger RNA molecules, or transcripts, owing to alternative splicing, differential exon usage, and other posttranscriptional modifications (Barry, 2014).

Thus, the integration of GWAS signals with gene expression data requires a sensitive, tissue-specific approach that can assess differences in transcript abundance, non-coding RNAs, and posttranscriptional modification. Next-generation sequencing of RNA, known as RNA sequencing (RNA-seq), addresses many of these needs better than traditional gene expression microarrays, providing a more complete picture of the ‘transcriptome’ (McGettigan, 2013).

As a first step toward an integrative genomics strategy in BD, we sequenced RNA derived from postmortem brain obtained from individuals with BD and matched controls. Our initial analysis of these data detected many differentially expressed (DE) genes in BD. These genes have important roles in neuroplasticity, circadian rhythms, and GTPase binding (Akula *et al*, 2014). When we integrated these data

with the results from previous GWAS of BD, we found that DE genes in the GTPase pathway were also enriched for single nucleotide polymorphisms (SNPs) that were associated with BD. This suggested that differential expression of these genes was not just a consequence of BD or its treatment, but also reflected inherited genetic variation associated with disease risk. However, that study was limited by analysis methods that focused on individual genes and transcripts, without regard to their correlated patterns of expression.

Here, we performed a complete re-analysis of the same RNA-seq data using methods that exploit the correlated patterns of expression among groups of genes. We used weighted gene correlation network analysis (WGCNA) (Langfelder and Horvath, 2008), a widely used method that finds modules of highly correlated genes, relates these modules to one another, and tests the influence of sample phenotypes on gene expression correlations. WGCNA has been widely used to identify co-expressed gene networks in various human brain regions (Oldham *et al*, 2008), animals (Fuller *et al*, 2007; Langfelder *et al*, 2012), and in human phenotypes, including schizophrenia (Torkamani *et al*, 2010), autism (Voineagu *et al*, 2011), cancer (Clarke *et al*, 2013), aggressive behavior (Malki *et al*, 2014), BD (Chen *et al*, 2013a), and psoriasis (Li *et al*, 2014). However, aside from one study of a few gene networks (Hong *et al*, 2013), WGCNA has not yet been applied to the complete brain transcriptome in BD as revealed by RNA-seq.

WGCNA detected a number of robust gene expression modules, several of which were enriched for GWAS signals. Functional analysis of genes within one of these modules revealed significant enrichment of several functionally related sets of genes, especially those involved in the postsynaptic density (PSD). These results provide convergent support for the hypothesis that dysregulation of genes involved in the PSD is a key factor in the pathogenesis of BD. If replicated in larger samples, these findings could point toward new therapeutic targets for BD.

MATERIALS AND METHODS

Samples and RNA-Seq

RNA extracted from the dorsolateral prefrontal cortex of postmortem brains of 11 BD cases and 11 age- and sex-matched controls obtained from the Stanley Medical Research Institute and NIMH Brain Bank was sequenced at the National Institutes of Health Sequencing Center (NISC) using Poly-A selection. Sequencing was performed in two batches: 5 BD cases and 5 controls (NISC1), and 6 cases and 6 controls (NISC2). Owing to technical issues, one BD sample in NISC1 was excluded after initial quality control. Details about sample phenotypes, RNA-seq methods, and extensive quality control procedures are published elsewhere (Akula *et al*, 2014), and so will only be briefly summarized here. NISC1 and NISC2 cases and controls were randomized within each batch across lanes and sequenced from both ends (paired-end) on Illumina GA-IIx or HiSeq systems, respectively (Illumina Inc, San Diego, CA). Sequencing of cases and controls within each batch was performed in the same run in order to avoid batch-run effects.

TopHat (TopHat version 2.0.4; <http://tophat.cbcb.umd.edu/>) (Trapnell *et al*, 2009) was used to map the reads to the reference human genome (hg19). A total of 2.3 billion mapped reads from NISC1 and 4.3 billion mapped reads from NISC2 were included in the downstream analyses. HTSeq (<http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html>) was used to obtain the read counts per gene based on Ensemble gene annotation (http://ftp.ensembl.org/pub/release-67/gtf/homo_sapiens/).

Gene Selection for Co-Expression Analysis

A total of 17 296 genes and 16 919 genes were selected after QC in NISC1 and NISC2, respectively. Of these, 16 571 genes were common to both these datasets and were thus included in the downstream co-expression analysis. Read counts were normalized using DESeq (Anders and Huber, 2010), and the resulting variance-stabilized transformed data were used in the downstream analysis. Weighted gene co-expression network analysis (WGCNA) was used to identify the co-expression modules (sub-networks) in BD (<http://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/>). The co-expression analysis pipeline is shown in Supplementary Figure S1.

Weighted Gene Co-Expression Network Analysis (WGCNA)

In WGCNA networks, genes are represented as nodes and edges represent the correlation in expression (edge-weight) between gene pairs. The connection strength (adjacency) between two genes is calculated by raising the correlation to a specific power, β , which must be estimated with each dataset. Supplementary Figure S2 shows the relationship between the β and scale free topology fitting index in NISC1 and NISC2. At $\beta = 12$, networks created by WGCNA showed > 75% scale free topology in both datasets, so a value of 12 was used in this study. We used biweight correlation instead of the default Pearson correlation, because it is robust and resistant to outliers (Gaiteri *et al*, 2014). WGCNA identifies co-expressed genes and puts them into networks or modules. Minimum module size was set to 50 genes per module. Here, we used the terms co-expressed networks and co-expressed modules interchangeably, but we recognize that these terms are sometimes considered distinct (Dong and Horvath, 2007). Genes that did not belong to any module were assigned to a 'grey' module; this was excluded from further analysis.

First, we identified the modules that were observed in both the NISC1 and NISC2 samples. These are referred to as 'consensus modules'. Then, we tested whether the connectivity in consensus modules was preserved across both datasets (Langfelder and Horvath, 2007; Langfelder *et al*, 2011). Default parameters were used when identifying consensus and preserved modules. The R code, along with the data files used in this analysis, can be downloaded from <http://intramural.nimh.nih.gov/humangenetics/data.html>.

WGCNA calculates a module eigengene value, the first principal component of that module, for every sample. These module eigengene values were tested for correlation (Pearson correlation) with diagnosis of BD; a Student *t*-test ($df = n - 2$)

was used to estimate the statistical significance of the correlation coefficients.

Case and control samples were matched on age and sex. To test for potential impacts of unmatched factors, we used linear regression and ANOVA to test for association between module eigengene values and known biological (smoking and cause of death) and technical (RNA-integrity number and sequencing) variables.

As NISC1 and NISC2 samples were sequenced separately on different platforms, the results were calculated within each sample, and then combined by meta-analysis. Fisher's Chi-square method was used to combine the p -values of preserved modules in NISC1 and NISC2, which generated a meta- p -value under a chi-square distribution with two degrees of freedom. We used the Benjamini false discovery rate for multiple test correction (<http://www.sdmproject.com/utilities/?show=FDR>). Only preserved modules with $Z_{\text{summary}} > 10$ (Langfelder and Horvath, 2007; Langfelder *et al*, 2011) whose eigengene was correlated in the same direction with BD in both the NISC1 and NISC2 samples were used in the downstream analysis.

DE Genes in RNA-seq Data

To test which of the modules were significantly enriched with genes that are DE in BD, we compared the genes in each of the modules with the 1225 DE genes reported in our earlier study (gene-level p -value < 0.05 in Akula *et al*, 2014). A hypergeometric p -value was calculated to test the significance of overlap.

GWAS Enrichment Analysis

Postmortem gene expression data alone cannot distinguish between genes whose expression changes are the result of an illness or its treatment and genes that have a role in etiology. As inherited DNA variation is not influenced by illness or treatment, genes that carry inherited variants associated with illness are more likely to lie within causal pathways. Thus, we performed GWAS enrichment analysis in order to help differentiate between 'causal' and 'non-causal' gene modules. (Detailed methods can be found in Akula *et al*, 2014). In short, quasi-independent ($r^2 < 0.5$) SNPs included in each of two published meta-analysis studies (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011; Chen *et al*, 2013b) were assigned to their closest genes. We calculated the total number of SNPs (N) with p -value < 0.05 in all the genes (N_{original}) in a functional category. We then randomly selected N number of SNPs 10 000 times (N_{random}). Lastly, we calculated the number of times $N_{\text{random}} \leq N_{\text{original}}$ and divided by 10 000 to obtain an empirical GWAS enrichment p -value. The minor allele frequency distributions for the test and random sets were almost identical. This approach accounts for any bias that might be introduced by variable patterns of inter-SNP linkage disequilibrium, minor allele frequency, or gene length. We further validated our GWAS enrichment results by another gene set enrichment analysis program, MAGENTA (nPermutations = 10 000) (Segre *et al*, 2010) which also accounts for gene length bias.

Functional Enrichment Analysis

WGCNA calculates an eigengene-based connectivity (kME or module membership) score for each gene in a module, which is the Pearson correlation between that gene's expression and its corresponding eigengene. Genes with consistent kME values (< 0 or > 0) in both NISC1 and NISC2 were subjected to functional enrichment analysis by use of the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al*, 2009a, b). The genes represented in the entire transcriptome dataset ($n = 16\,571$) were used as background. We used medium to high stringency. Gene ontology (GO) terms with Benjamini $q < 0.05$ were declared significant.

Co-Expression Networks from Microarray Studies

We compared our co-expression modules from RNA-seq with those generated in two previous studies that used microarray data (Oldham *et al*, 2008; Chen *et al*, 2013a). We tested the significance of gene overlap between modules by a hypergeometric test, with universe values equal to the number of genes passing QC in both studies. Universe values of 8395 and 13 514 were used for comparisons with the Oldham *et al* (2008) and Chen *et al* (2013a) studies, respectively. Comparisons with the Oldham *et al* (2008) results were limited to modules generated in human cortex. As there is no one-to-one relationship of modules between the microarray and RNA-seq studies, we did not attempt to replicate the findings in Chen *et al* (2013a), but instead report overlapping modules and their association with BD in both studies.

Cell-Type Enrichment of Co-Expression Networks

Oldham *et al* (2008) also report gene expression signatures typical of particular cell types in human brain. Their results indicate modules enriched for oligodendrocytes, astrocytes, neurons, and synapses. In order to test whether any of our 21 modules were enriched for gene expression signatures reflecting these cell types, we compared our modules to those from Oldham *et al* (2008) using a hypergeometric test.

RESULTS

Gene Co-Expression Networks

A total of 33 consensus co-expression modules were detected (Supplementary Figure S3). All 33 modules were highly preserved in both the NISC1 and NISC2 data sets ($Z_{\text{summary}} > 10$; Supplementary Figure S4). The number of genes in each module varied from 74 to 2766, with an average of 446 (Supplementary Table S1). Comparison with previously published data (Oldham *et al*, 2008) showed good agreement between these modules and those detected in microarray data from human cortex in individuals without psychiatric illness (Supplementary Table S2). Twenty-nine of 33 modules significantly overlapped with modules identified in the Oldham *et al* (2008) study. This demonstrates that WGCNA can detect robust modules of co-expressed genes across a range of data types and individuals.

Table 1 Twenty-one Gene Co-Expression Modules that Showed Consistent Correlation with Bipolar Disorder (BD)

Module	BD correlation in NISC1/NISC2	BD association <i>p</i> -value	BD association FDR	Enrichment of differentially expressed genes	GWAS enrichment
Yellow	+/+	1.94E-03	0.017	2.20E-16	ns
Light yellow	+/+	2.52E-03	0.017	ns	ns
Sky blue	+/+	2.76E-03	0.017	1.66E-05	ns
Dark turquoise	-/-	3.58E-03	0.017	ns	3.00E-04
Dark grey	+/+	3.66E-03	0.017	3.53E-09	ns
Purple	+/+	0.005	0.018	2.20E-16	9.00E-04
Dark orange	-/-	0.008	0.024	0.031	ns
Royal blue	+/+	0.012	0.032	0.024	ns
Red	-/-	0.015	0.035	2.54E-05	8.30E-03
Green	-/-	0.024	0.050	2.20E-16	5.80E-03
Turquoise	-/-	0.026	0.050	1.79E-06	ns
Light cyan	+/+	0.036	ns	ns	5.30E-03
White	+/+	0.049	ns	ns	ns
Saddle brown	+/+	ns	ns	ns	9.10E-03
Dark olive green	+/+	ns	ns	ns	ns
Salmon	-/-	ns	ns	ns	0.034
Pale turquoise	-/-	ns	ns	0.033	ns
Blue	-/-	ns	ns	ns	ns
Dark green	-/-	ns	ns	ns	0.016
Dark red	+/+	ns	ns	No overlap	ns
Midnight blue	-/-	ns	ns	ns	ns

Abbreviation: ns, not significant.

'+' and '-' represent increased and decreased gene expression in bipolar disorder, respectively.

Association with BD

Of the 33 preserved modules, 21 were selected for downstream analysis because their eigengene values were correlated with BD in the same direction in both NISC1 and NISC2 (Table 1; Supplementary Table S3 contains the module membership (kME) values for all genes in these 21 modules). The observed association with BD was not explained by differences in age or sex, because samples were matched on these variables. The observed associations were also not explained by differences in known biological (smoking and cause of death) or technical (RNA-integrity number and sequencing depth) covariates (Supplementary Table S4).

Eleven of the 21 modules were associated with BD at false discovery rate <0.05 (Table 1). The eigengene values for each of these 11 modules are depicted in Figure 1 as a heatmap. This shows that most of the genes within each of five modules (dark turquoise, green, turquoise, dark orange, and red) were downregulated in most of the BD cases we studied, compared with controls. Most of the genes in the remaining six modules (royal blue, sky blue, light yellow, dark grey, purple, and yellow) were upregulated in BD. The module assignments for all genes in the 11 BD-associated modules are depicted in Supplementary Figures S5 and S6.

Overlap with DE Genes in RNA-seq Data

To assess whether the results of the WGCNA analysis agreed with those of our previous study, we assessed overlap among

genes within each of the 21 differentially co-expressed modules identified by WGCNA with genes found to be DE in our previous RNA-seq study (Akula *et al*, 2014). There was a significant overlap in 10 modules (hypergeometric *p*-value <0.05), and only 1 module contained no genes previously identified as DE (Table 1). This shows that the WGCNA analysis largely agrees with single gene expression analysis but also identifies additional genes.

Modules Enriched with GWAS Genes (GWAS Enrichment)

In order to distinguish modules containing genes that may have a causal role in BD from those whose differential co-expression may be a consequence of BD or its treatment, we tested genes within each of the 21 modules for evidence of association with BD in previous GWAS. Eight modules were significantly enriched for GWAS-implicated genes (permutation *p*-value <0.05; Table 1), consistent with a causal role in BD. The remaining modules showed no evidence of GWAS enrichment.

As GWAS enrichment analyses can be biased by gene size, we repeated the analyses with MAGENTA (Segre *et al*, 2010), which takes gene-length bias into account. Similar results were obtained (Supplementary Table S5). The red and green modules were significantly enriched, and the purple module showed a trend toward enrichment (*p* <0.1), while the dark turquoise module was not significant in the MAGENTA analysis.

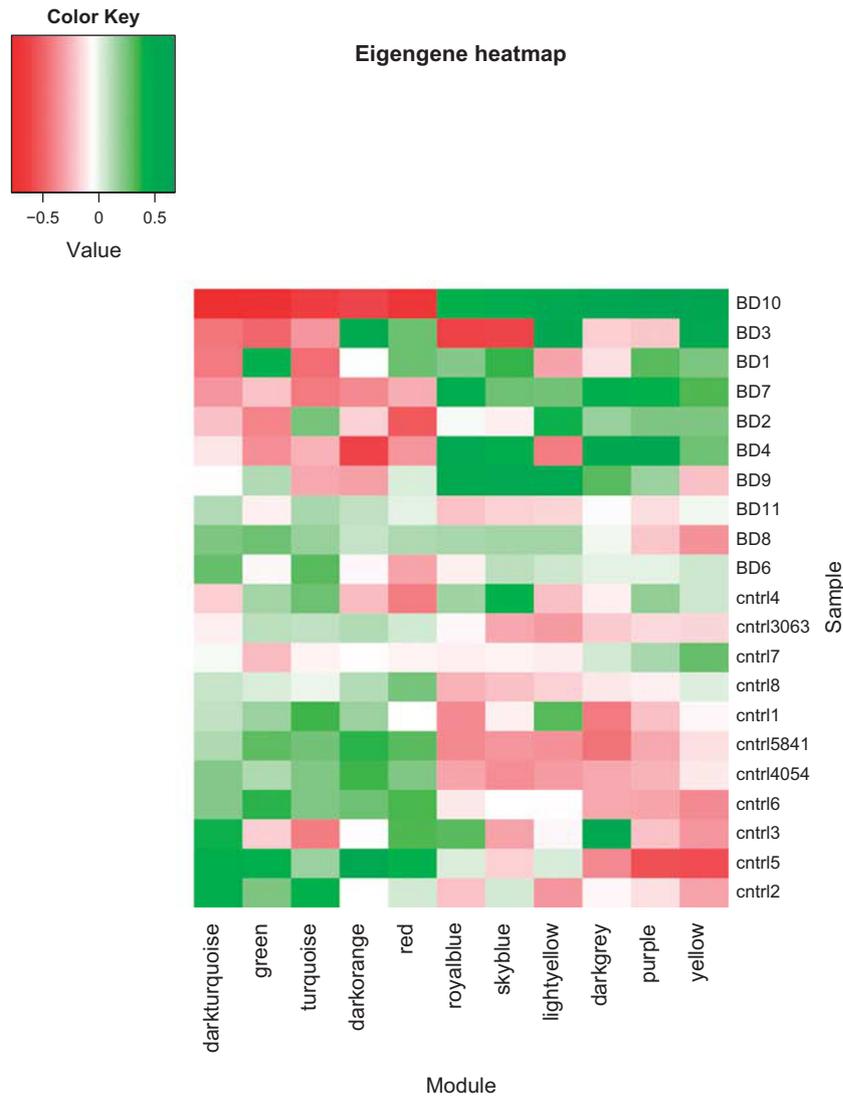


Figure 1 Eigengene heatmap. The x-axis shows the modules and the y-axis shows the samples. Red indicates negative, and green indicates positive eigengene values.

Functional Gene Set Enrichment Analysis

A critical question in this study concerns the potential functional relationships among the implicated genes. In order to explore this question, we performed gene set enrichment analysis in each of the eight gene modules that were consistent with causal involvement in BD. Three of the eight 'causal' modules (green, red, and salmon) yielded several significantly enriched GO terms, including: cell-cell signaling, PSD, ion transport, synapse, regulation of transcription, and passive transmembrane transporter activity (Table 2). A few specific GO terms, such as PSD, synapse, cation channel activity, and ribosomal subunit were strikingly (>2.5-fold) enriched, whereas most of the other GO terms showed ~twofold enrichment. (Gene significance and module membership values for all the genes in the green module are shown in Supplementary Figures S7 and S8.) Of the remaining 13 modules that were not enriched for GWAS signals, 7 modules (blue, dark olive green, dark red, pale turquoise, royal blue, turquoise, and yellow) yielded

significant enrichment for particular GO terms. These included zinc ion binding, defense response, immune system development, response to wounding, proteolysis, and carboxylic acid binding, among others. Most of the immune-related GO terms showed > fivefold enrichment. The enriched GO terms, along with the genes and their respective *p*-values, are given in Supplementary Table S6. The top 25 genes in each of the modules that yielded significant functional enrichment results are shown in Figure 2.

RNA-seq Co-Expression Networks Agree with Those Implicated in a Published Microarray-Based WGCNA Analysis

Chen *et al* (2013a) found 23 co-expression modules that were associated with BD in multiple microarray datasets. We calculated the extent of overlap between these modules and those we found in the RNA-seq data. There was a highly significant overlap between the co-expressed modules in

Table 2 Functional Enrichment of Genes in Co-Expressed Modules^a

Category	Module	Term	Fold enrichment	P value	Benjamini Q value
Causal	Green	GO:0014069 ~ postsynaptic density	4.6	1.09E-04	3.30E-03
		GO:0045211 ~ postsynaptic membrane	3.9	1.27E-05	4.83E-04
		GO:0045202 ~ synapse	3.1	5.61E-09	8.49E-07
		GO:0030425 ~ dendrite	2.8	8.05E-04	2.01E-02
		GO:0022803 ~ passive transmembrane transporter activity	2.8	1.84E-06	4.94E-04
		GO:0005261 ~ cation channel activity	2.6	3.87E-04	1.87E-02
	Red	GO:0006396 ~ RNA processing	2.9	9.68E-06	1.02E-02
	Salmon	GO:0045449 ~ regulation of transcription	2.3	1.31E-05	9.09E-03
Consequential	Blue	GO:0003735 ~ structural constituent of ribosome	2.8	9.97E-06	8.14E-03
		GO:0045449 ~ regulation of transcription	3.6	9.45E-04	2.18E-02
		GO:0008270 ~ zinc ion binding	3.2	6.38E-03	4.99E-02
	Dark olive green	GO:0033279 ~ ribosomal subunit	16.5	1.41E-03	4.06E-02
		GO:0044445 ~ cytosolic part	7.7	2.53E-04	1.31E-02
		GO:0030529 ~ ribonucleoprotein complex	3.8	2.22E-04	1.72E-02
	Dark red	GO:0002684 ~ positive regulation of immune system process	17.0	5.11E-10	1.36E-07
		GO:0045321 ~ leukocyte activation	16.4	9.17E-12	4.88E-09
		GO:0001817 ~ regulation of cytokines	12.9	1.34E-05	6.48E-04
		GO:0002520 ~ immune system development	10.1	4.08E-07	3.62E-05
		GO:0006952 ~ defense response	8.0	1.58E-07	1.69E-05
		GO:0009611 ~ response to wounding	6.5	1.56E-05	7.24E-04
	Pale turquoise	GO:0009967 ~ positive regulation of signal transduction	6.4	6.65E-04	2.12E-02
		GO:0010033 ~ response to organic substance	4.0	2.81E-04	1.03E-02
		GO:0043167 ~ ion binding	2.1	5.66E-05	6.66E-03
	Royal blue	GO:0016879 ~ ligase activity, forming carbon-nitrogen bonds	2.0	6.99E-06	3.73E-03
	Turquoise	GO:0046395 ~ carboxylic acid catabolic process	3.7	8.19E-05	2.66E-02
		GO:0016042 ~ lipid catabolic process	3.3	5.02E-05	2.29E-02
GO:0031406 ~ carboxylic acid binding		3.1	1.90E-04	3.33E-02	
GO:0006631 ~ fatty acid metabolic process		3.0	3.56E-05	4.02E-02	
Yellow					

^aFor modules with multiple GO terms, only those with fold change >2.5 are presented in this table. See Supplementary Table S6 for full details.

both studies (Supplementary Table S7). Several modules had corrected hypergeometric p -values < 0.05, indicating the high reproducibility of the co-expressed gene network structure, even though fewer genes can be detected by microarray.

BD-Associated Co-Expression Networks do not Show Expression Signatures for Specific Cell Types

Comparison with the Oldham *et al* (2008) data found evidence of significant gene overlap between several modules and genes characteristic of oligodendrocytes, astrocytes, microglia, neurons, glutamatergic neurons, and synaptic proteins (Supplementary Table S2). The strongest gene overlaps (> 50%) were observed for modules characteristic of oligodendrocytes.

DISCUSSION

To our knowledge, this is the first study to perform WCGNA analysis on RNA-seq data of the complete brain transcriptome in BD cases. These results provide a high-resolution account of

the interacting gene networks in brain that are involved in BD. By incorporating the GWAS signals, we have attempted to distinguish gene modules that have a causal role from those that appear to be a consequence of BD or its treatment. The preserved, differentially co-expressed, GWAS-enriched modules point toward a number of biological pathways as important factors in the pathogenesis of BD. Three modules were associated with BD and enriched for DE genes and BD GWAS signals. Of these, the green module showed a striking (4.6-fold) enrichment for genes involved in the PSD (Figure 3). This finding is noteworthy in light of the several prior studies that have implicated the PSD in BD and other neuropsychiatric disorders (el-Mallakh and Wyatt, 1995; Kristiansen and Meador-Woodruff, 2005; Beneyto and Meador-Woodruff, 2008; Pennington *et al*, 2008; Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). Our results provide independent support for those findings and suggest that genes involved in the PSD are a key factor in the pathogenesis of BD. If replicated in larger samples, our results could point toward new therapeutic targets for BD among the numerous proteins active in the PSD (Feng and Zhang, 2009).

This study has several limitations. The sample size was relatively small compared with the samples used in previous

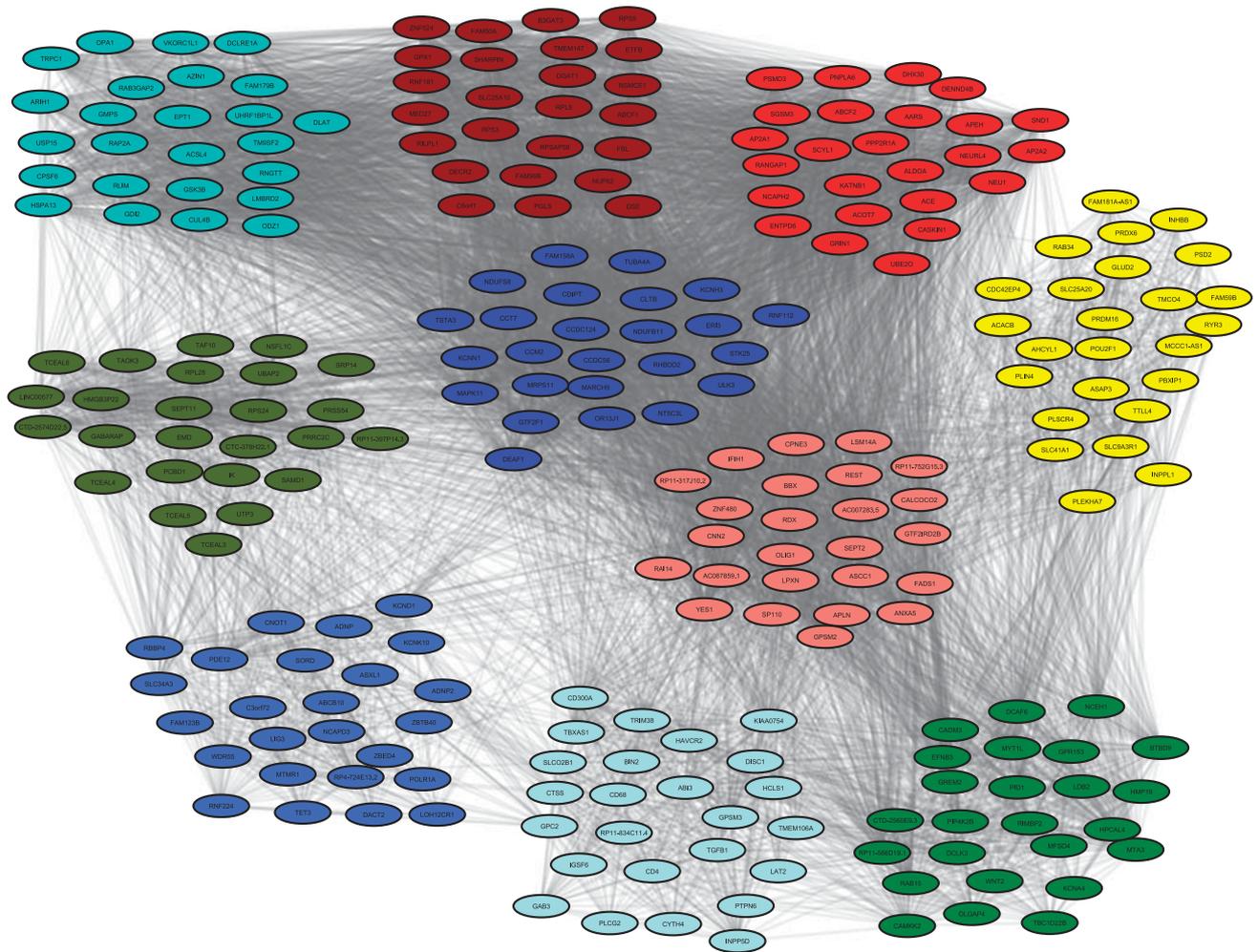


Figure 2 Gene co-expression networks in bipolar disorder. The top 25 genes in each of the 10 modules correlated with bipolar disorder are illustrated. Thickness of the grey lines are proportional to (absolute) magnitude of the observed gene-gene correlations. Colors correspond to those in Table 2.

microarray studies. Nevertheless, we were able to successfully replicate many of the published findings and extend those findings to non-coding genes and previously undiscovered functional gene networks. This reflects the precision and wide dynamic range of high-depth, RNA-seq-based transcriptome data (Iancu *et al*, 2012, 2014; Zhang *et al*, 2014). Given the small sample size, subtle biological or technical biases cannot be ruled out. As is inherent in 'omics' studies where the number of variables far exceeds the number of subjects, the results should be cautiously interpreted until replicated in a larger dataset (Bild *et al*, 2014). Some of the gene modules that were associated with BD in this study did not reveal recognized functional pathways. This may reflect the limitations inherent in analyses that depend on known relationships between genes in the published literature. This limitation will diminish as more empirical gene-gene relationships are revealed. Other limitations of this study include the focus on only one brain region and lack of cellular resolution. However, a comparison with published cell-specific gene expression signatures (Oldham *et al*, 2008) suggests that several cell types contribute to these results.

The major strength of this study is the ability to integrate expression data in groups of genes with risk allele data from GWAS. We identified 11 modules that were associated with BD, 4 of which were significantly enriched with GWAS variants. The results for GWAS enrichment were further validated using MAGENTA (Segre *et al*, 2010), which supported significant GWAS enrichment among genes in the green and red modules. A significant enrichment of miR-137 targets among genes in the red module was observed. A potential causal role for the remaining modules cannot be ruled out, however, because genes in those modules might harbor rare single nucleotide or copy number variants that would not be detectable by GWAS.

As this study relies on the observed co-expression of both protein-coding and non-protein-coding genes in the brain, rather than on genetic relationships apparent in the published literature, it offers a more unbiased account of genetic relationships in the brain. For example, the analysis was able to detect several non-protein-coding genes, such as lncRNAs, that so far are not well understood in the context of brain function but seem to have a key role in tying together otherwise disparate sets of genes involved in BD. Recent research has shown that lncRNAs are highly

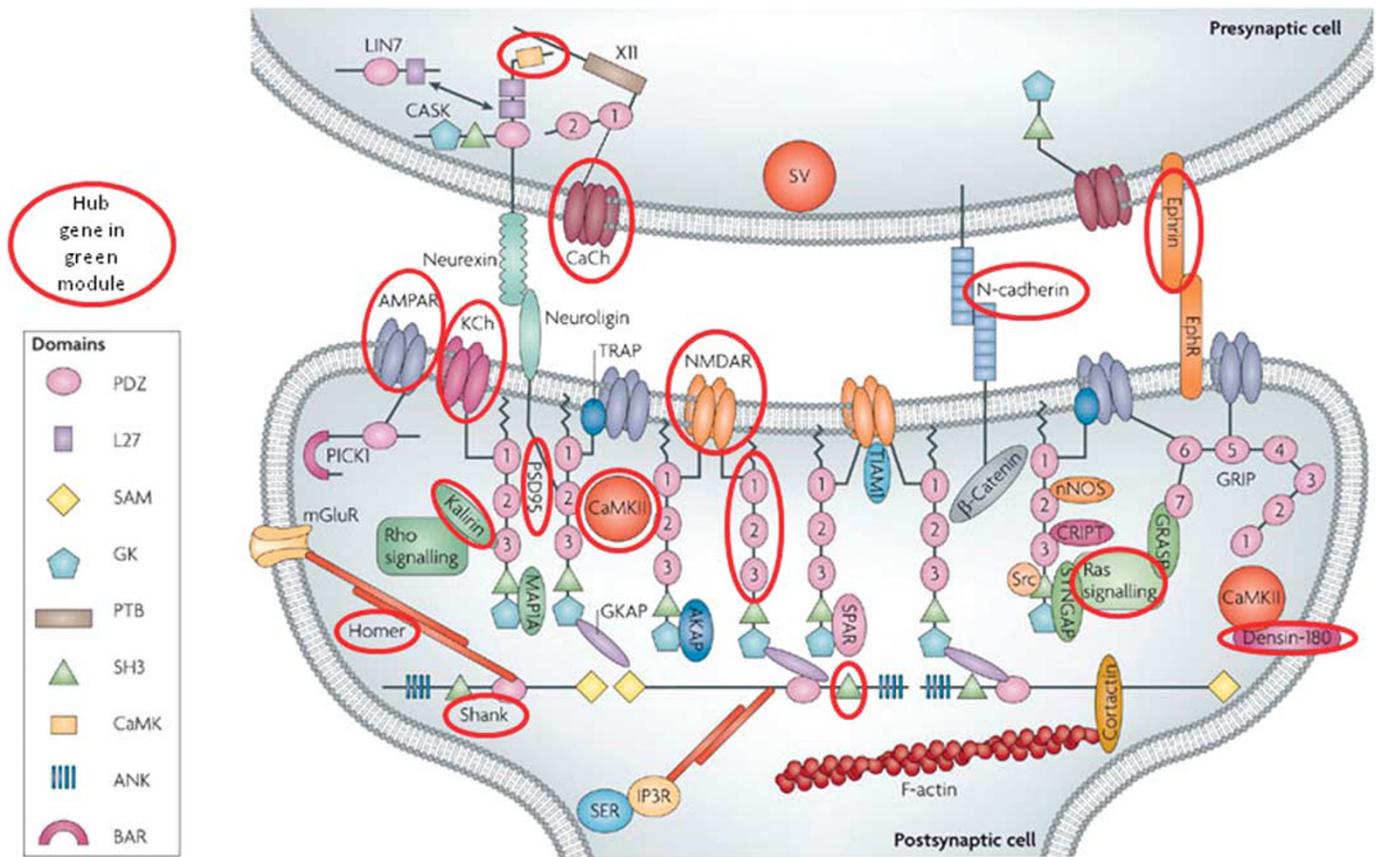


Figure 3 Post-synaptic and ion channel genes in the green module. The postsynaptic density figure has been adapted from Feng and Zhang (2009). Proteins enclosed in red circles are encoded by one or more genes assigned to the 'green' module in the present study. CaCh: CACNA1E, CACNA1G, CACNB1, CACNG3; CaMK: CAMK2A, CAMK4; N-cadherin: CDH12, CDH8; PSD95: DLGAP3, DLGAP4; Ephrin: EFNB3, EPHA4, EPHA6; AMPAR: GRIA1, GRIA2, GRIA3; NMDAR: GRIN2B; Homer: HOMER1; Kalirin: KALRN; KCh: KCNA4, KCNB2, KCNC3, KCNG1, KCNH5, KCNIP3, KCNQ2, KCNQ4, KCTD1; Densin-180: LRRC7; PDZ: PDZD2; Ras signaling: RAP2B; SH3: SH3KBPI, SH3PXD2A; Shank: SHANK1, SHANK2.

conserved and have an essential role in synapse formation (Bernard *et al*, 2010; He *et al*, 2014) and other key aspects of brain development.

The 'salmon' module deserves special mention because it contains three replicated GWAS hits for BD: *TRANK1*, *SYNE1*, and *CACNA1C* (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011; Chen *et al*, 2013b; Muhleisen *et al*, 2014). Contingency table analysis (detailed in Supplementary Table S8) shows that it is highly unlikely that these three genes would fall into the same module by chance (Fisher exact $p=0.01$). As WGCNA relies on observed co-expression, rather than reports in the literature, it was able to pull together genes like *CACNA1C*, that are relatively well-studied, with the other genes that are still relatively understudied in the published literature. However, we did not detect differential co-expression of the 'salmon' module in BD in this sample. This might reflect limited statistical power, the particular anatomical brain region we chose to study, or reliance of the GWAS enrichment strategy on existing studies with limited statistical power.

Several hub genes present in the 11 BD-associated modules overlap with those implicated in other neuropsychiatric disorders. For example, over 100 genes overlap with those within the 108 loci implicated in a recent GWAS of schizophrenia (Supplementary Table S9) (Schizophrenia

Working Group of the Psychiatric Genomics Consortium, 2014). Eighteen genes overlap with those implicated in autism (Poultney *et al*, 2014; Supplementary Table S10). BD, schizophrenia, and autism are all brain disorders, so overlapping findings are perhaps not surprising, but they reaffirm the considerable genetic overlap among these clinically distinct neuropsychiatric disorders.

The strong evidence of immunological enrichment among genes in the modules showing no GWAS enrichment points toward immunological events as a consequence of BD or its treatment. The results suggest that the most genes in the immunologically enriched modules are downregulated in BD, in contrast to the finding of increased expression of immune-related genes reported in an earlier WGCNA analysis in autism (Voineagu *et al*, 2011). However, the results resonate broadly with a recent report of abnormal activation of peripheral blood monocytes and lymphocytes in BD (Gumieiro *et al*, 2010).

This study has produced results that are overall consistent with the prior findings, but also implicate novel genes and biological pathways that may contribute to the risk for BD. The integration of RNA-seq-based gene expression data with GWAS data highlights potentially important differences in gene co-expression networks that contain genes harboring risk alleles and those that do not. The current findings may

also be valuable for helping to interpret the results of future studies of rare variation in BD. Integration of GWAS with gene co-expression data is a promising approach to better understand the mechanisms of highly genetically heterogeneous neuropsychiatric disorders.

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