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Original Article

Gene expression alterations in bipolar disorder postmortem brains

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Objectives: Bipolar disorder (BD) is a mental illness of unknown neuropathology and has several genetic associations. Antipsychotics are effective for the treatment of acute mania, psychosis, or mixed states in individuals with BD. We aimed to identify gene transcripts differentially expressed in postmortem brains from antipsychotics-exposed individuals with BD (hereafter the 'exposed' group), non-exposed individuals with BD (hereafter the 'non-exposed' group), and controls.

Methods: We quantified the abundance of gene transcripts in postmortem brains from seven exposed individuals, seven non-exposed individuals, and 12 controls with the Affymetrix U133P2 GeneChip microarrays and technologies. We applied a q-value of ≤0.005 to identify statistically significant transcripts with mean abundance differences between the exposed, non-exposed and control groups.

Results: We identified 2191 unique genes with significantly altered expression levels in non-exposed brains compared to those in the control and exposed groups. The expression levels of these genes were not significantly different between exposed brains and controls, suggesting a *normalization* effect of antipsychotics on the expression of these genes. Gene ontology (GO) enrichment analysis showed significant (Bonferroni $p \leq 0.05$) clustering of subgroups of the 2191 genes under many GO terms; notably, the protein products of genes enriched are critical to the function of synapses, affecting, for example, intracellular trafficking and synaptic vesicle biogenesis, transport, release and recycling, as well as organization and stabilization of the node of Ranvier.

Conclusions: These results support a hypothesis of synaptic and intercellular communication impairment in BD. The apparent normalization of expression patterns with exposure to antipsychotic medication may represent a physiological process that relates both to etiology and improvement patterns of the disorder.

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Bipolar disorder (BD) is a chronic, severe psychiatric illness characterized by recurrent pathological swings of mood between mania and depression, often with catastrophic social, personal, medical, or vocational consequences. It is generally a lifetime diagnosis, with significant heterogeneity, that begins in late adolescence or early adulthood with variability in presentation, symptomatology and course of illness (1). Genetic predisposition to the illness and high heritability have been observed clinically in family, twin, and adoption studies (2–4), and genetic linkage and association studies have found numerous modest risk loci for BD (5), consistent with a complex mode of inheritance in BD (6, 7). The currently reported genetic variants with modest odds ratios (1.1–1.3) cannot account

for the high heritability of the disorder; additional approaches beyond population genetics will be needed to understand the etiology of BD.

Gene expression analyses have used microarray technology to identify gene transcripts differentially expressed in postmortem brains or nucleated peripheral blood cells from BD affected individuals in comparison to healthy controls (8–16). Although these studies have identified sets of genes differentially expressed between BD samples and controls (see *Supplementary Table 1*), only a few genes within each set replicate in two or more studies.

A recent meta-analysis of 12 microarray data sets was performed to address the lack of consensus between studies (17); the results suggested that inconsistency between microarray studies was due to small sample size, overly restrictive or overly broad criteria for assigning of significance, and a lack of consistent statistical adjustment for confounding effects. However, the meta-analysis did identify 375 genes with significantly altered expression patterns in BD brains compared with controls. Many genes identified in this analysis are functionally related to energy processing, supporting an energy processing dysfunction hypothesis in BD (10, 17).

Given the complex inheritance of BD and the current progress in gene mapping and expression analyses, we reason that findings from each independent study are likely to contribute to understanding the molecular basis of the illness. The integration and comparison of results from independent studies may prove helpful in the identification of genes and pathways for prioritization of deeper analyses. We compared the abundance of gene transcripts in brain samples from deceased individuals diagnosed with BD and unrelated controls, and identified transcripts with altered expression levels in BD brains (including many previously reported significant genes). When we used exposure to antipsychotic medication as a variable, we found evidence that antipsychotics normalize the expression levels of genes in BD brains to near control levels.

Materials and methods

Postmortem brain samples

Postmortem brain samples (hereafter 'brains') were obtained from the Stanley Medical Research Institute (SMRI) neuropathology consortium (18). All brains were collected using Institutional Review Board (IRB) approved protocols for postmortem tissue collection and later provided to qualified investigators for analysis. The consortium consists of brains from individuals with BD, schizophrenia and major depression (15 brains in each case) and from healthy controls (15 brains). All brains were matched by gender, age, race, postmortem interval (PMI), brain pH, and the side of the brain from which the sample was taken, and excluded neuropathological abnormalities. We analyzed the BD (n = 15) and normal control brains (n = 15) in this study (Tables 1 and 2). The tissue sample was derived from the premotor cortex (Brodmann's area 6). Due to poor RNA quality, three samples (two controls and one BD) were not processed for microarray GeneChip hybridization. Medication history indicated that, for half of the 14 BD brains included in the microarray analysis, the individual was on antipsychotics at the time of death (Tables 1 and 2).

RNA extraction

Total RNA was isolated from 200 mg of frozen tissue using the TRIzol reagent (Cat #15596-026; Invitrogen, Grand Island, NY, USA). All RNA samples were then treated with RNase-free DNase (Cat #79254; Qiagen, Valencia, CA, USA), followed by a clean-up step with the RNeasy MinElute Cleanup reagents (Cat #74204; Qiagen) according to the manufacturer's instructions. RNA quantity was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). One microgram of RNA from each sample was subjected to 1% agarose gel electrophoresis to examine RNA integrity and potential DNA contamination. The RNA samples were stored at -80°C until microarray analysis.

Microarray platforms and data acquisition

The GeneChip U133 plus 2.0 (U133P2) microarrays (Affymetrix, Santa Clara, CA, USA) were used for expression analysis of RNA from postmortem brains. Affymetrix microarray hybridization was carried out at the Johns Hopkins Medical School microarray core facilities. Image processing and scaling for normalization were carried out using GCOS1.1 software (Affymetrix). Standard Affymetrix microarray quality controls were employed, including: scaling factor, noise, background, percentage of present calls, 5′/3′ signal ratios observed in the GAPDH messenger RNA (mRNA), and the 5′/3′ signal ratios of spiking genes. There were no significant differences between BD and control groups in the variances of scaling factors, present calls, and 3′/5′ signal

Table 1. Demographics of postmortem brains from the Stanley Medical Research Institute (SMRI) neuropathology consortium[a] (see Table 2 for other medications)

ID #	Diagnosis	Gender	Age (years)[b]	PH[c]	PMI (hours)	Age of onset (years)[d]	Suicide	Psychosis	Substance abuse	Severity of substance abuse[e]	Severity of alcohol abuse[f]	Brain side	Antipsychotics
5	BD	F	25	6.4	24	19	Y	Present	Current	3	5	R	Thiothixene
14	BD	M	34	6.3	23	19	Y	Present	Past	3	5	R	Risperidone
17	BD	M	31	6.3	28	21	Y	Present	Current	5	—	R	Haloperidol
45	BD	F	48	5.8	22	16	N	Present	Current	3	5	L	Chlorprothixene
47	BD	M	30	6.1	31	22	N	Present	Never	0	3	R	Clozapine
51	BD	F	37	6.5	29	14	Y	Present	Never	4	—	R	Thioridazine
55	BD	M	50	6.2	19	17	Y	Present	Never	0	1	L	Clozapine
57	BD	M	57	6.2	19	30	N	Present	Past	0	4	L	Haloperidol
4	BD	F	50	6.1	18	34	N	Present	Current	5	3	L	
7	BD	M	48	6.1	13	27	Y	Present	Current	3	2	R	
8	BD	M	30	5.8	56	7	Y	Absent	Never	0	1	R	
24	BD	M	54	5.8	39	39	N	Absent	Past	0	4	R	
36	BD	M	30	6.3	45	14	Y	Absent	Current	3	—	L	
53	BD	F	61	6.5	60	18	Y	Present	Never	0	1	L	
54	BD	F	50	6.3	62	25	N	Absent	Never	0	0	L	
2	CON	M	59	6.4	26	—	N	Absent	Never	0	3	R	
3	CON	F	68	6.3	13	—	N	Absent	Never	0	0	L	
6	CON	M	53	6.2	28	—	N	Absent	Past	2	2	L	
9	CON	M	52	6.2	22	—	N	Absent	Current	0	3	R	
15	CON	M	52	6.5	28	—	N	Absent	Past	0	2	L	
27	CON	M	41	6.0	11	—	N	Absent	Never	0	1	R	
31	CON	M	52	6.5	8	—	N	Absent	Never	0	1	L	
35	CON	M	42	6.6	27	—	N	Absent	Never	0	0	R	
41	CON	F	57	6.0	26	—	N	Absent	Never	0	0	R	
46	CON	M	44	6.4	10	—	N	Absent	Never	0	1	L	
49	CON	F	44	6.3	25	—	N	Absent	Never	0	1	R	
50	CON	M	58	6.0	27	—	N	Absent	Never	0	1	L	
56	CON	F	29	6.2	42	—	N	Absent	Never	0	0	L	
58	CON	F	35	5.8	40	—	N	Absent	Never	0	0	L	
59	CON	F	35	6.6	23	—	N	Absent	Never	0	1	R	

BD = bipolar disorder; CON = control; F = female; ID # = SMRI-assigned patient number; L = left; M = male; N = no; PMI = postmortem interval; R = right; Y = yes.
[a]Brodmann area 6 samples were used in this study.
[b]Age at death.
[c]PH acidity–alkalinity (log scale; 7 = neutral).
[d]Age of onset of disease.
[e]0 = lowest; 1 = lower; 2 = low; 3 = high; 4 = higher; 5 = highest.
[f]0 = lowest; 1 = lower; 2 = low; 3 = high; 4 = higher; 5 = highest.

Table 2. Clinical and medical data for Stanley Medical Research Institute (SMRI) brains

ID #	Diagnosis	Mood stabilizer	Antidepressants	Anticholinergic	Microarray note
5	BD	Carbamazepine, lithium	Trazodone	—	
14	BD	Valproate	Venlafaxine	—	
17	BD	—	Trazodone	Diphenhydramine	
45	BD	Valproate, carbamazepine	Sertraline	—	Not processed
47	BD	Lithium	—	—	
51	BD	Lithium	Buproprion	—	
55	BD	Valproate	—	Benztropine	
57	BD	—	—	Diphenhydramine	
4	BD	—	—	—	
7	BD	—	—	—	
8	BD	—	—	—	
24	BD	Lithium	—	—	
36	BD	Valproate	Bupropion, fluoxetine	—	
53	BD	Carbamazepine, lithium	Fluoxetine	—	
54	BD	Valproate	Clomipiramine	—	
2	CON	—	—	—	
3	CON	—	—	—	Not processed
6	CON	—	—	—	
9	CON	—	—	—	
15	CON	—	—	—	
27	CON	—	—	—	
31	CON	—	—	—	
35	CON	—	—	—	
41	CON	—	—	—	Quality control failed
46	CON	—	—	—	
49	CON	—	—	—	
50	CON	—	—	—	
56	CON	—	—	—	
58	CON	—	—	—	Not processed
59	CON	—	—	—	

BD = bipolar disorder; CON = control; ID # = SMRI-assigned patient number.

ratios of GAPDH (data not shown). One brain sample, coded UK-1.41 (SMRI sample code), had a 3′/5′ signal ratio of GAPDH 4.64, which was greater than an adequate threshold of three for brain tissue (19) and was therefore removed from the statistical analysis. Data that passed these quality control measures were then used in differential expression analyses. Thus, a total of 26 microarray profiles (14 BD and 12 controls) were included in the statistical analysis of differential gene expression.

Microarray data analysis

For analysis of the U133P2 microarray data, we processed raw data (CEL files) using the Bioconductor package 'Affy' for R (20, 21). We used the Affy Robust Multichip Average (RMA) algorithms for background adjustment, probe intensity summarization, and normalization. Log_2-transformed probe intensity RMA values were used in differential gene expression analysis.

We classified the postmortem brains into three groups (Tables 1 and 2): (i) those from BD patients who were treated with antipsychotics at the time of death and during their lifetime (the 'exposed' group; n = 7); (ii) those from BD patients who were never treated with antipsychotics (the 'non-exposed' group; n = 7); and (iii) those from normal controls (n = 12). This strategy for data analysis is different from the simple case–control comparison performed in previously published studies using the neuropathology consortium sample from SMRI (see *Supplementary Table 1*). Other medications were generally equally distributed between the two BD groups.

We used the Significant Analysis of Microarrays (SAM) software package for Microsoft Office Excel (22) for statistical analysis to identify genes that were significantly differentially expressed. We strategically fitted the multi-class algorithms of SAM with data from the three groups. We found that the exposed profiles were very similar to that of the controls; the differences among the three

groups were mostly contributed by the non-exposed group. We therefore performed a SAM two-class analysis between the non-exposed and other profiles (exposed plus controls) to identify and report differentially expressed genes.

The transcript sequences in National Center for Biotechnology Information (NCBI) UniGene, dbEST, GenBank, and Refseq databases were used to design the U133p2 GeneChip. The current annotation of these sequences is incomplete. We therefore only focused on the analysis of probe sets with NCBI's Refseq annotation.

We used SAM *q*-values [equivalent to false discovery rate (FDR)] of 0.005 as cut-off points to call significant genes. Since the correlation between fold change (FC) and functional impact empirically remains unknown, we did not apply an FC cut-off point to exclude any significant calls.

Real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) validation of microarray results

Verification of microarray results was performed with TaqMan qRT-PCR (23) using a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Two micrograms of total RNA from each sample was converted to first-strand cDNA using the Superscript first-strand cDNA synthesis system following the manufacturer's instructions (Invitrogen, Cat #18089-011; Carlsbad, CA, USA). The cDNA stocks were then diluted 50-fold with distilled deionized water. TaqMan assays were performed in 20-μL reactions in 384-well format plates, each containing 10 μL of 2 × TaqMan Master Mixture, 1 μL of 20 × primers/probe, and 9 μL of 50-fold diluted first-strand cDNA templates. The ribosomal protein S16 (RPS16) gene which did not exhibit significant variation in expression between samples was used as an endogenous control. The Assays on Demand (AOD) for seven brain-expressed genes were purchased from Applied Biosystems (Foster City, CA, USA). We used the $2^{-\Delta\Delta Ct}$ method (24) to calculate FC using the threshold cycle value (C_t) in the samples of non-exposed brains and controls after normalizing to the C_t values of the internal reference RPS16. For significance testing, two-tailed *t*-tests were employed.

Gene ontology (GO) term enrichment analysis

We used the stand-alone expression analysis systematic explorer (EASE) software for GO term enrichment and pathway analysis (25). We used the NCBI's Refseq genes as the background list. The 2022 unique genes (excluding 169 pH-related transcripts) from the significant list were used as input against the background genes for EASE to identify genes specifically enriched in the GO biological process system, molecular function system, or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (26). Significantly enriched GO terms were those with a Bonferroni p of ≤0.05.

Results

Differential gene expression in brains from individuals with BD compared with controls

A large number of genes were differentially expressed in non-exposed brains. Using the SAM multi-class algorithm (22), we identified 3376 gene transcripts differentially expressed between the non-exposed, exposed, and control groups (*q*-value ≤ 0.05, equivalent to FDR). At a *q*-value ≤ 0.01 cut-off, 1184 unique genes represented by 1419 probe sets were differentially expressed among the three groups (see *Supplementary Fig. 1*). We observed that the expression levels of these significant transcripts in the exposed brains were very similar to those of controls; the significant differences were largely contributed by the non-exposed group (see *Supplementary Fig. 1*). This observation suggests that antipsychotics have considerable effects on gene expression, and that they appear to normalize the differentially expressed genes in BD brains to the mean levels of controls.

Since the exposed profiles were very similar to, and highly correlated with, those of controls (see *Supplementary Fig. 1*), we combined those groups and then compared them with the non-exposed group using the SAM two-class algorithms. We identified 2191 unique transcripts (2818 probe sets) differentially expressed in the non-exposed brains ($q \leq 0.005$) (Fig. 1). Among these 2191 transcripts, 116 showed an increase and 2075 a decrease in mean abundance level in the non-exposed group relative to the combined group (see *Supplementary Table 2*). There was high concordance between the multi-class and two-class analyses, with only 1.96% of the 2191 significant genes not identified by SAM multi-class analysis (data not shown). The two-class analysis achieved a much lower *q*-value (≤0.005) for the 2191 significant genes.

We also observed that most of the 2191 significant genes identified showed modest mean expression level changes (< 2-fold). This is consistent with published data (see *Supplementary Table 1*). The minimum exposed versus control FC was

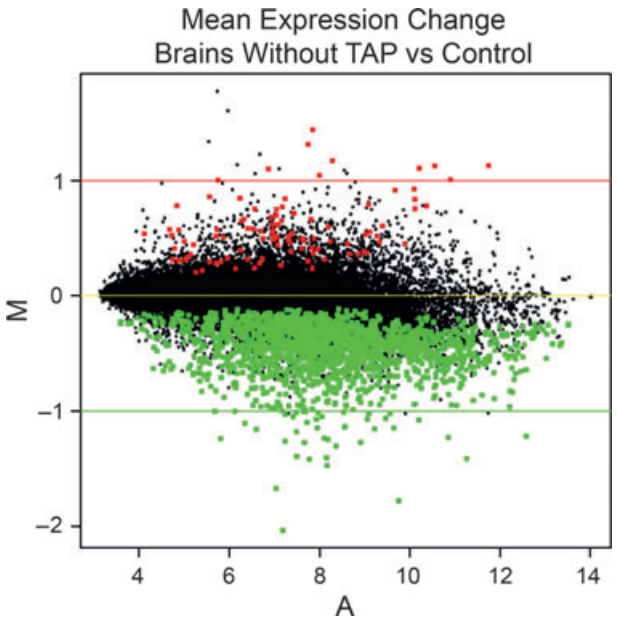

Fig. 1. Scatter plot illustrating the mean difference in $\log_2$ ratio (M) versus the average expression level in $\log_2$ average (A) for each of the genes analyzed. The yellow line indicates no difference in mean expression levels. A 2-fold mean difference ($\log_2$ ratio) is indicated by a red line (up-regulated) or a green line (down-regulated). The points shown in red or green are the altered expression levels of the 2191 genes in non-exposed samples versus controls.

−1.09-fold, and the maximum was −4.5-fold. We also identified multiple significant transcript variants from the same gene detected by corresponding probe sets (see *Supplementary Table 2*).

Antipsychotic treatment normalizes gene expression in BD brains

We found that the mean levels of the significant transcripts were similar between the exposed brains and controls (see *Supplementary Fig. 1*). For instance, in the non-exposed brains the mean abundance of regulator of G protein signaling 4 (*RGS4*), BCL 2 associated athanogene 3 (*BAG3*), and synapsin 2 (*SYN2*) transcripts showed greater than 2-fold change compared to that of the combined samples, but there was no significant difference between the exposed group and controls (Fig. 2). This is of potential relevance as *RGS4*, *BAG3*, and *SYN2* have been implicated in psychiatric illnesses (27, 28) and in changes in the central nervous system in response to antipsychotic treatment (29–31).

Real-time qRT-PCR validation

Real-time qRT-PCR was employed to validate seven randomly selected significant genes using cDNAs derived from total RNA extracted from the non-exposed brains and controls. The TaqMan results confirmed our microarray findings for all seven genes (Table 3). The changes were not correlated with age, gender, brain pH, or PMI.

Gene ontology term enrichment analysis revealed functional themes

EASE analysis excluded 169 unique genes known to be related to low brain pH (10, 15, 32). There was significant enrichment of genes in one KEGG pathway, as well as 18 biological process terms and 11 terms of molecular function (Bonferroni $p \leq 0.05$) (see *Supplementary Table 3*). Significant GO terms of biological processes presented a theme of protein metabolic process, transport, localization, post-translational protein modification, protein folding, and synaptic transmission. Significant GO terms of molecular function identified protein and nucleotide categories including ATP binding, ligase, and catalytic activities. Interestingly, long-term potentiation was the one significant KEGG pathway identified in this analysis.

We found that multiple members of gene families were often enriched under corresponding GO terms. For example, 13 genes encoding multiple subunits of adaptor-related protein (AP) complexes (AP1M1, AP1S1, AP1S2, AP2A1, AP2A2, AP2B1, AP2M1, AP2S1, AP3B2, AP3M2, AP4B1, AP4M1, and AP4S1) were enriched under the term *intracellular transport*; and six genes encoding eukaryotic translation initiation factors (EIF2B3, EIF2B5, EIF2S1, EIF4E, EIF4G2, and EIF5) were enriched under the GO term *protein metabolic process*. Although no direct evidence relates any of the AP subunits to BD, AP complexes are critical proteins in synaptic vesicle formation and transport (33). Multiple BD risk factors have been proposed to interrupt protein synthesis via the translation initiation complex EIF2B (34). Our data implicate the functionally related genes encoding proteins involved in neurotransmitter biosynthesis, or protein synthesis and modification; however, postmortem data such as these cannot determine if there are causal or consequential relationships between disease, pharmacological intervention, and observed gene expression changes. This will require cellular models that can be studied prospectively.

Discussion

We examined the gene expression profiles of a postmortem brain sample well matched by gender,

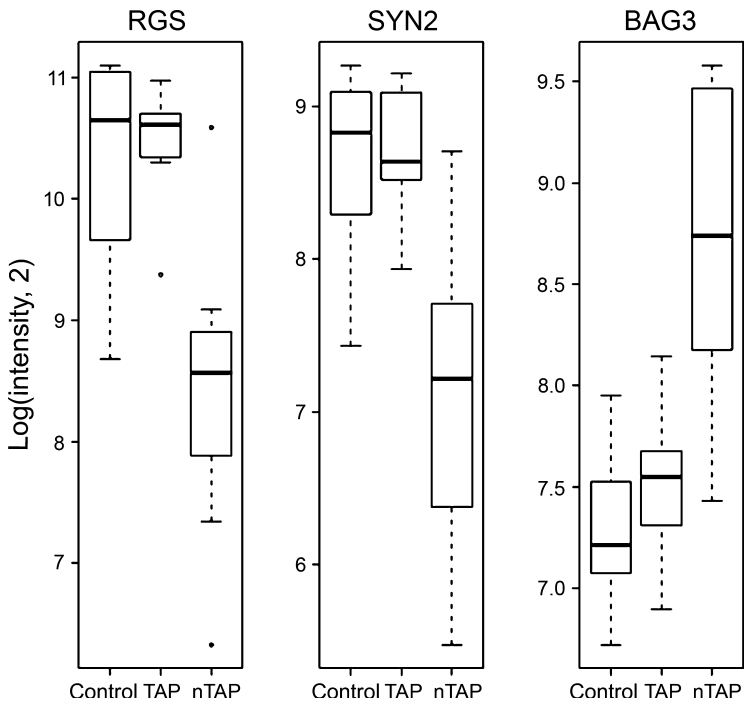

Fig. 2. A boxplot showing three messenger RNA (mRNA) species encoding BCL 2 associated athanogene 3 (BAG3), regulator of G protein signaling 4 (RGS4), and synapsin 2 (SYN2) which were differentially expressed between exposed samples, controls, and non-exposed samples. Shown on the *y*-axis are the measured expression levels of the genes, given as $\log_2$ values, and on the *x*-axis are the three groups of brain samples.

Table 3. TaqMan analysis of differential gene expression between non-exposed brains and controls

	TaqMan assay			Microarray	
Gene symbol	*t*-test	Fold change[a]	Internal control gene	SAM *q*-value	Fold change
FKBP5	1.23E-03	1.88	RPS16	≤0.005	1.88
BAG3	9.90E-03	2.50	RPS16	≤0.005	2.52
ALDH1L1	5.72E-04	1.70	RPS16	≤0.005	1.72
CALM1	6.80E-04	−1.80	RPS16	≤0.005	−1.80
DUSP3	8.96E-04	−1.60	RPS16	≤0.005	−1.70
SYN2	1.21E-04	−3.10	RPS16	≤0.005	−3.05
KCNK1	8.47E-04	−1.92	RPS16	≤0.005	−1.96

ALDH1L1 = aldehyde dehydrogenase 1 family, member L1; BAG3 = BCL 2 associated athanogene 3; CALM1 = calmodulin 1; DUSP3 = dual specificity protein phosphatase 3; FKBP5 = FK506 binding protein 5; KCNK1 = potassium channel subfamily K member 1; SAM = Significant Analysis of Microarrays; SYN2 = synapsin 2.
[a]TaqMan fold changes are derived from $2^{(-\Delta\Delta Ct)}$,−ΔΔCt = (mean exposed ΔCt − mean control ΔCt).

age, PMI, pH, side of the brain, and race (18). We identified 2191 unique genes (FDR ≤ 0.005) differentially expressed in the premotor cortex of individuals with BD who were not exposed to antipsychotics at the time of death or during their lifetime compared to exposed brains and controls. We observed that in exposed brains the expression levels of these genes were similar to those of controls. This suggests that antipsychotics may 'normalize' (or at least suppress differences in) the expression levels of several genes relevant to the molecular pathophysiology of BD. This broad effect on gene expression of the antipsychotics may be one of several factors influencing the heterogeneity of the illness.

The normalization effect of antipsychotics is supported by a recent report using induced pluripotent stem cell (iPSC)-derived neurons to model schizophrenia (35). This study identified signaling pathways that were abnormal in cells derived from individuals with schizophrenia, which could be ameliorated by treatment with a typical antipsychotic medication, loxapine (35). While the outcome of gene expression modulation through antipsychotic medication is common to the current study and an iPSC-derived model of schizophrenia,

there are clearly many confounds from the environment that affect the adult postmortem brain. Medication is one of many tools to alter the environment at defined stages, the results of which may have causal relevance to either the illness or the neuropharmacology of the medication.

Using their method of *convergent functional genomics*, Le-Niculescu and colleagues (36) generated a list of 1529 genes implicated in BD or depression. In a study of genomic variation for schizophrenia, Lee and colleagues (37) reported 2725 genes containing common single nucleotide polymorphisms (SNPs) that captured a significant proportion of the variance in liability to schizophrenia. In other common conditions, many genes are implicated: for example, thousands of genes were found to change expression levels in response to viral infection in peripheral blood mononuclear cells using integrative personal 'omics' profiling (38).

When our significant genes were compared with the 375 significant genes identified in the meta-analysis of 12 microarray data sets (17), 185 genes were found to be common to both lists (Fisher's exact test $p \leq 0.015$), and 99% of these common changes were in the same direction (mostly down-regulated) (see *Supplementary Table 4*). The significant overlap suggests high concordance between this current study and the meta-analysis. It also implies that there may be common aspects of pathophysiology in some brain regions, since the current study was performed on tissue from the BA6 region, while the meta-analysis (17) combined profiles from different brain regions (mainly BA6, 9, 10, and 46) from 12 studies, including our microarray raw data.

A recent microarray analysis of the thalamic transcriptome performed on material from the SMRI neuropathology consortium identified 72 genes with highly significantly altered expression levels in bipolar brains (15). Of these, we found that 22 genes were common to our study (Fisher's exact test $p = 2.14 \times 10^{-6}$) and all common changes were in the same directions (two increased and 20 decreased) (see *Supplementary Table 5*). This comparison is reasonably sound since both experiments were performed on the U133P2 Genechips and tested similar numbers of probe sets (around 22000).

It remains a challenge to identify causative alterations in gene expression using microarray data from postmortem tissues. We observed 86 genes that showed a 2-fold or greater difference in mean expression levels between BD brains and the combined group; for example, *RGS4*, *BAG3*, and *SYN2* showed 2-fold or greater changes (see Fig. 2). *RGS4* (regulator of G protein signaling 4) has been associated with schizophrenia (39) and implicated in BD (40). Though *BAG3* has not been previously implicated in BD, it has been identified with relevant expression changes in response to antipsychotic treatment in animal brains (29). *SYN2*, a gene involved in synaptic function, has been associated with BD in a functional analysis of extant data (41). Given the assumption that larger FCs have a greater functional impact, priority may be given to the genes with higher magnitude changes for further detailed analysis.

Functional GO term enrichment analysis identified significant clusters of genes under several biological process terms, including protein metabolic process, protein transport, macromolecule localization, protein localization, intracellular protein transport, establishment of protein localization, post-translational protein modification, protein folding, molecular function, vesicle-mediated transport, synaptic transmission, and intracellular transport; or GO terms of molecular function relating to protein binding, nucleotide/ribonucleotide ATP binding, ligase and catalytic activities. These data and neuropathological studies of BD brains suggest that, as in neurodegenerative conditions, protein processing and transport may be significantly altered.

The enriched genes under specific GO terms often included multiple members of gene families. For example, five members of the disintegrin and metalloprotease (ADAM) gene family, *ADAM10*, *ADAM11*, *ADAM15*, *ADAM17*, and *ADAM23*, were in enriched under the GO term *protein metabolic process*. The ADAM genes encode membrane-anchored proteins that have been shown to play important roles in the development of the nervous system, including regulation of the proliferation, migration, differentiation and survival of various cells, as well as axonal growth and myelination (42). Enriched under the GO term *protein transport* were 13 members of adaptor-related protein complex (AP) gene families: *AP1M1*, *AP1S1*, *AP1S2*, *AP2A1*, *AP2A2*, *AP2B1*, *AP2M1*, *AP2S1*, *AP3B2*, *AP3M2*, *AP4B1*, *AP4M1*, and *AP4S1*. AP complexes, AP-1, AP-2, AP-3, and AP-4, play important roles in synaptic vesicle formation and endocytosis (33). The expression levels of these genes were significantly lower in non-exposed brains compared to controls, suggesting a hypothesis of synapse impairment in BD. How these genes may be involved in BD is currently unknown, and awaits analysis in relevant cellular models.

A number of 'high profile' genes previously implicated in BD also stand out in our study. For

example, the mRNA levels of the glycogen synthase kinase 3 beta (*GSK3B*), FK506 binding protein 5 (*FKBP5*), and Ankyrin 3 (*ANK3*) genes were altered in the non-antipsychotic medication brains. GSK3β is a known target of lithium, and has been hypothesized to be the molecular basis of lithium treatment of BD (43). FK506 binding protein 51, the protein product of the *FKBP5* gene, forms part of a complex with the glucocorticoid receptor and can modulate cortisol-binding affinity (44). Variations in FKBP5 have been reported to be associated with BD (45).

The *ANK3* gene product, Ankyrin-G, is present at the axonal initial segment and at nodes of Ranvier. Ankyrin-G plays key roles in node formation and function in the central and peripheral nervous systems. Genome-wide association analysis identified SNPs at the *ANK3* locus associated with BD (46), and cis-acting variations in the *ANK3* locus were shown to affect its expression (47). In addition to *ANK3*, several genes encoding key components of the node of Ranvier or paranodal region, such as neuronal cell adhesion molecule (*NRCAM*), sodium channel, voltage gated type 8 alpha (*SCN8A*), potassium voltage-gated channel 2 (*KCNQ2*), spectrin, beta, non-erythrocytic 4 (*SPTBN4*), erythrocyte membrane protein 4.1-like 3 (*EPB41L3*), and *ANK2*, are among the list of genes identified in this study (see *Supplementary Table 2*). These observations suggest node impairment may be a neural mechanism of BD.

The limitations of the current analysis relate primarily to the relatively small number of individuals included in the study of postmortem samples. It would be useful to replicate these findings in additional samples and studies; however, there is considerable overlap in the genes identified in the current study with those from previous studies (15, 17). There are many confounding factors that essentially limit the utility of postmortem gene expression analyses, which include sex-dependent expression differences, death and agonal factors including the PMI and tissue pH. The postmortem brain most likely reflects the end-stage organ disease state and may not reflect the initial etiological mechanisms of the disorder. There are several options for improving the study design and include: (i) the ascertainment and procurement of additional postmortem brains for study and (ii) the establishment of cellular models that are derived from individuals with BD for whom there are considerable phenotypic and longitudinal data that may be factored into the analyses. Clearly, the second option is the ideal option. Individuals with BD who have common phenotypic features may be ascertained and sampled, their cell lines grown under controlled conditions of exposure to environmental perturbations (medications and other biological variations) and measured consistently. Cellular models range from iPSC-derived neurons to cells derived from B lymphocytes transformed with Epstein Barr Virus (EBV), the consistent feature being cellular tissue derived from an individual with the disorder. The limitations of the cellular models are the lack of ability to study the complex circuitry of the human brain; however, complex biochemical pathways could be studied at a sophisticated biological level to determine interactive correlates between pathways.

In summary, we identified a large number of genes with altered expression in brains from individuals with BD who had not been exposed to antipsychotics. In brains of individuals with BD treated with antipsychotics, expression of these genes was normalized to the levels found in healthy controls. Functional GO terms analysis suggests that these gene products are involved in neuronal communication that may be impaired in BD brains. Although our current results are concordant with previous findings, caution must be exercised in interpreting data generated from postmortem brains that are further confounded by agonal status, tissue pH, PMI, medication, etc. Neuropsychiatric and neurological diseases are increasingly thought to be developmental in nature, and the fact that disease-specific changes are unlikely to be distinguished from confounders in postmortem brains suggests that disease-specific live neurons are critically required to study the molecular basis of BD and to assemble a palette of disease-causing genes. This goal will be achieved in the systematic study of live neurons derived from iPSCs (48) or from differentiated easily accessible tissue of non-neural origin (35, 49).

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Disclosures

The authors of this paper declare no potential conflicts of interest in connection with this manuscript.

References

1. Goodwin FK, Jamison KR. Manic Depressive Illness. New York: Oxford University Press, 1990.
2. Gershon ES, Hamovit J, Guroff JJ et al. A family study of schizoaffective, bipolar I, bipolar II, unipolar, and normal control probands. Arch Gen Psychiatry 1982; 39: 1157–1167.
3. Bertelsen A, Harvald B, Hauge M. A Danish twin study of manic-depressive disorders. Br J Psychiatry 1977; 130: 330–351.
4. Mendlewicz J, Rainer JD. Adoption study supporting genetic transmission in manic–depressive illness. Nature 1977; 268: 327–329.
5. Serretti A, Mandelli L. The genetics of bipolar disorder: genome 'hot regions,' genes, new potential candidates and future directions. Mol Psychiatry 2008; 13: 742–771.
6. Craddock N, Khodel V, Van EP, Reich T. Mathematical limits of multilocus models: the genetic transmission of bipolar disorder. Am J Hum Genet 1995; 57: 690–702.
7. Crow TJ. How and why genetic linkage has not solved the problem of psychosis: review and hypothesis. Am J Psychiatry 2007; 164: 13–21.
8. Iwamoto K, Kakiuchi C, Bundo M, Ikeda K, Kato T. Molecular characterization of bipolar disorder by comparing gene expression profiles of postmortem brains of major mental disorders. Mol Psychiatry 2004; 9: 406–416.
9. Jurata LW, Bukhman YV, Charles V et al. Comparison of microarray-based mRNA profiling technologies for identification of psychiatric disease and drug signatures. J Neurosci Methods 2004; 138: 173–188.
10. Iwamoto K, Bundo M, Kato T. Altered expression of mitochondria-related genes in postmortem brains of patients with bipolar disorder or schizophrenia, as revealed by large-scale DNA microarray analysis. Hum Mol Genet 2005; 14: 241–253.
11. Tsuang MT, Nossova N, Yager T et al. Assessing the validity of blood-based gene expression profiles for the classification of schizophrenia and bipolar disorder: a preliminary report. Am J Med Genet B Neuropsychiatr Genet 2005; 133: 1–5.
12. Ryan MM, Lockstone HE, Huffaker SJ, Wayland MT, Webster MJ, Bahn S. Gene expression analysis of bipolar disorder reveals downregulation of the ubiquitin cycle and alterations in synaptic genes. Mol Psychiatry 2006; 11: 965–978.
13. Choudary PV, Molnar M, Evans SJ et al. Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. Proc Natl Acad Sci USA 2005; 102: 15653–15658.
14. Bousman CA, Chana G, Glatt SJ et al. Preliminary evidence of ubiquitin proteasome system dysregulation in schizophrenia and bipolar disorder: convergent pathway analysis findings from two independent samples. Am J Med Genet B Neuropsychiatr Genet 2010; 153B: 494–502.
15. Chu TT, Liu Y, Kemether E. Thalamic transcriptome screening in three psychiatric states. J Hum Genet 2009; 54: 665–675.
16. Choi KH, Higgs BW, Wendland JR, Song J, McMahon FJ, Webster MJ. Gene expression and genetic variation data implicate PCLO in bipolar disorder. Biol Psychiatry 2011; 69: 353–359.
17. Elashoff M, Higgs BW, Yolken RH et al. Meta-analysis of 12 genomic studies in bipolar disorder. J Mol Neurosci 2007; 31: 221–243.
18. Torrey EF, Webster M, Knable M, Johnston N, Yolken RH. The Stanley Foundation Brain Collection and Neuropathology Consortium. Schizophr Res 2000; 44: 151–155.
19. Popova T, Mennerich D, Weith A, Quast K. Effect of RNA quality on transcript intensity levels in microarray analysis of human post-mortem brain tissues. BMC Genomics 2008; 9: 91.
20. Irizarry RA, Hobbs B, Collin F et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003; 4: 249–264.
21. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy—analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 2004; 20: 307–315.
22. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 2001; 98: 5116–5121.
23. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'—3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci USA 1991; 88: 7276–7280.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402–408.
25. Hosack DA, Dennis G Jr, Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. Genome Biol 2003; 4: R70.
26. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000; 28: 27–30.
27. Prasad KM, Almasy L, Gur RC et al. RGS4 polymorphisms associated with variability of cognitive performance in a family-based schizophrenia sample. Schizophr Bull 2010; 36: 983–990.
28. Dean B, Boer S, Gibbons A, Money T, Scarr E. Recent advances in postmortem pathology and neurochemistry in schizophrenia. Curr Opin Psychiatry 2009; 22: 154–160.
29. Ikeda M, Tomita Y, Mouri A et al. Identification of novel candidate genes for treatment response to risperidone and susceptibility for schizophrenia: integrated analysis among pharmacogenomics, mouse expression, and genetic case-control association approaches. Biol Psychiatry 2010; 67: 263–269.
30. Lane HY, Liu YC, Huang CL et al. RGS4 polymorphisms predict clinical manifestations and responses to risperidone treatment in patients with schizophrenia. J Clin Psychopharmacol 2008; 28: 64–68.
31. Thomas EA. Molecular profiling of antipsychotic drug function: convergent mechanisms in the pathology and treatment of psychiatric disorders. Mol Neurobiol 2006; 34: 109–128.

32. Li JZ, Vawter MP, Walsh DM et al. Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. Hum Mol Genet 2004; 13: 609–616.
33. Danglot L, Galli T. What is the function of neuronal AP-3? Biol Cell 2007; 99: 349–361.
34. Carter CJ. eIF2B and oligodendrocyte survival: where nature and nurture meet in bipolar disorder and schizophrenia? Schizophr Bull 2007; 33: 1343–1353.
35. Brennand KJ, Simone A, Jou J et al. Modelling schizophrenia using human induced pluripotent stem cells. Nature 2011; 473: 221–225.
36. Le-Niculescu H, Patel SD, Bhat M et al. Convergent functional genomics of genome-wide association data for bipolar disorder: comprehensive identification of candidate genes, pathways and mechanisms. Am J Med Genet B Neuropsychiatr Genet 2009; 150B: 155–181.
37. Lee SH, DeCandia TR, Ripke S et al. Estimating the proportion of variation in susceptibility to schizophrenia captured by common SNPs. Nat Genet 2012; 44: 247–250.
38. Chen R, Mias GI, Li-Pook-Than J et al. Personal omics profiling reveals dynamic molecular and medical phenotypes. Cell 2012; 148: 1293–1307.
39. Craddock N, O'Donovan MC, Owen MJ. The genetics of schizophrenia and bipolar disorder: dissecting psychosis. J Med Genet 2005; 42: 193–204.
40. Li Y, Zhao Q, Zhang Z et al. Association study between RGS4 and bipolar disorder in the Chinese Han population. Psychiatr Genet 2010; 20: 130–132.
41. Le-Niculescu H, Balaraman Y, Patel S et al. Towards understanding the schizophrenia code: an expanded convergent functional genomics approach. Am J Med Genet B Neuropsychiatr Genet 2007; 144B: 129–158.
42. Yang P, Baker KA, Hagg T. The ADAMs family: coordinators of nervous system development, plasticity and repair. Prog Neurobiol 2006; 79: 73–94.
43. Jope RS. Anti-bipolar therapy: mechanism of action of lithium. Mol Psychiatry 1999; 4: 117–128.
44. Binder EB. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology 2009; 34 (Suppl. 1): S186–S195.
45. Willour VL, Chen H, Toolan J et al. Family-based association of FKBP5 in bipolar disorder. Mol Psychiatry 2009; 14: 261–268.
46. Ferreira MA, O'Donovan MC, Meng YA et al. Collaborative genome-wide association analysis supports a role for ANK3 and CACNA1C in bipolar disorder. Nat Genet 2008; 40: 1056–1058.
47. Quinn EM, Hill M, Anney R, Gill M, Corvin AP, Morris DW. Evidence for cis-acting regulation of ANK3 and CACNA1C gene expression. Bipolar Disord 2010; 12: 440–445.
48. Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131: 861–872.
49. Pang ZP, Yang N, Vierbuchen T et al. Induction of human neuronal cells by defined transcription factors. Nature 2011; 476: 220–223.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Scatter plot of the mean expression levels in exposed versus control samples (top), non-exposed versus control samples (middle), and non-exposed samples versus control and exposed samples combined (bottom). The red dash-dotted line is the correlation fit line.

Table S1. Published microarray studies with corresponding references listed.

Table S2. List of the 2191 genes with altered expression in the non-exposed brains (q-value < 0.005).

Table S3. Gene ontology (GO) term enrichment analysis results with Bonferroni $p \leq 0.05$. Shown are the GO terms significantly enriched in the 2191 genes altered in non-exposed brains.

Table S4. Common significant genes between this current analysis and the 12-study meta-analysis (meta12).

Table S5. Significant common genes between our analysis and findings reported by Chu et al. (2009).