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Genome-wide expression analysis detects eight genes with robust alterations specific to bipolar I disorder: relevance to neuronal network perturbation

Noriaki Nakatani[1], Eiji Hattori[1], Tetsuo Ohnishi[1], Brian Dean[3], Yoshimi Iwayama[1], Izuru Matsumoto[4], Tadafumi Kato[2], Noriko Osumi[5,6], Teruhiko Higuchi[7], Shin-ichi Niwa[8] and Takeo Yoshikawa[1,6,*]

[1]Laboratory for Molecular Psychiatry, [2]Laboratory for Molecular Dynamics of Mental Disorders, RIKEN Brain Science Institute, Saitama 351-0198, Japan, [3]The Rebecca L. Cooper Research Laboratories, Mental Health Research Institute of Victoria, Parkville Victoria 3052, Australia, [4]Department of Pathology, The University of Sydney, NSW 2006, Australia, [5]Division of Developmental Neuroscience, Center for Translational and Advanced Animal Research, Tohoku University School of Medicine, Miyagi 980-8575, Japan, [6]CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan, [7]Musashi Hospital, National Center of Neurology and Psychiatry, Tokyo 187-8551, Japan and [8]Department of Neuropsychiatry, School of Medicine, Fukushima Medical University, Fukushima 960-1295, Japan

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The limited number of genome-wide transcriptome analyses using the postmortem brains of bipolar disorder sufferers has not produced a clear consensus on the molecular pathways affected by the disorder. To expand the knowledge in this area, we examined the expression levels of more than 12 000 genes in Brodmann's Area (BA), 46 (dorsolateral prefrontal cortex) from bipolar I disorder and control samples using Affymetrix GeneChips. This analysis detected 108 differentially expressed genes in bipolar brains. Validation studies using quantitative RT–PCR on the two original diagnostic cohorts plus tissue from schizophrenic subjects, confirmed the differential expressions of eight genes (*RAP1GA1*, *SST*, *HLA-DRA*, *KATNB1*, *PURA*, *NDUFV2*, *STAR* and *PAFAH1B3*) in a bipolar-specific manner and one gene (*CCL3*) which was downregulated in both bipolar and schizophrenic brains. Of these, protein levels of RAP1GA1 (RAP1 GTPase activating protein 1) showed a trend of increase in BA46 from bipolar brains, in keeping with mRNA transcript levels. Transmission disequilibrium analysis of the nine genes using 43 single nucleotide polymorphisms (SNPs) in 229 National Institute of Mental Health bipolar trios exposed nominal SNP association and modest empirical haplotypic association ($P = 0.033$) between *SST* (somatostatin) and disease. Finally, gene network analysis using the currently obtained expression data highlighted cellular growth and nervous system development pathways as potential targets in the molecular pathophysiology of bipolar disorder.

INTRODUCTION

Bipolar disorder is characterized by mood swings with recurring phases of mania, depression and euthymia. It has a lifetime prevalence of ~1% in the general population, causing immense personal and social losses (1). It is well recognized that the disease has a partial genetic component, although the mode of inheritance and the mechanisms are complex (2). In addition to the heterogeneity of bipolar disorder in terms of genetic etiology, it is clear that therapeutic interventions cannot necessarily target the genes that confer disease risk, even if such genes could be convincingly identified. Therefore, along with classical genetic studies, investigations aimed at identifying molecular systems and networks affected

*To whom correspondence should be addressed. Tel: +81 484675968; Fax: +81 484677462; Email: takeo@brain.riken.jp

by the disease will be important for developing new drugs in the treatment of bipolar disorder.

A new strategy that could allow for the identification of molecular systems underlying bipolar disorder is comprehensive expression analysis at the transcriptome level, using DNA microarrays. To date, there are fewer reports of microarray analysis on postmortem brains from bipolar patients when compared with schizophrenics. Iwamoto *et al*. (3) detected downregulation of genes encoding receptors, channels or transporters and upregulation of genes encoding stress response proteins or molecular chaperones and proteins for transcription or translation in the frontal pole [Brodmann's Area (BA) 10]. In contrast, Tkachev *et al*. (4) reported oligodendrocyte dysfunction in the prefrontal cortex (BA9) of subjects with bipolar disorder and schizophrenia. Notably, as both groups analyzed brain samples from adjacent cortical regions using tissue from the Stanley Brain Collection, these data suggest that there are region-specific changes in gene expression in the prefrontal cortex of bipolar disorder.

Previously, we applied DNA microarray technology to a rat depression model, learned helplessness, to identify pathways involved in the molecular pathogenesis of depression and the molecular mechanisms of conventional and selective serotonin reuptake inhibitor-type antidepressants (5). Based on the hypothesis of a greater molecular distinctiveness between unipolar and bipolar-type mood disorders and because there are few suitable animal models for bipolar disorder, we have used human postmortem brains to perform a genome-wide expression study of bipolar I disorder. We have also performed a genetic association test on the expression-validated genes and bioinformatic analyses of differentially expressed transcripts to identify molecular cascades underlying bipolar pathology.

RESULTS

Expression profile from microarray analysis

We screened more than 12 000 transcripts (approximately half of the human protein-coding transcripts) using the Affymetrix GeneChip U95Av2 to identify altered expression levels in the brain tissue of subjects with bipolar I disorder when compared with controls. The transcript expression displayed good linearity in BA46 (DLPFC) from the two groups and most signal spots were clustered around the $y = x$ axis (Supplementary Material, Fig. S1). This indicated that the extracted RNA was of comparable quality in both the disease and control groups and that most transcripts showed minimal expression change between the two groups. Our stringent criteria [mean fold change (FC) ≥ 1.5 or ≤ -1.5 and non-parametric $P < 0.05$] identified 108 transcripts as differentially expressed genes (Table 1).

We classified the genes that showed altered expression levels in bipolar I disorder into functional categories, according to the biological process ontology of the Gene Ontology Consortium (http://www.geneontology.org/) (Table 1). Four genes encoding receptors were all downregulated in bipolar I disorder with FC of -1.5 to -1.9. Among them, NTRK2 (neurotrophic tyrosine kinase, receptor, type 2) is the receptor for brain-derived neurotrophic factor. The majority and in some cases, all of the genes in the categories of neurotransmission, inflammatory response, translation regulator and cell structure were downregulated, whereas the expression of the three genes belonging to the stress response category were elevated in bipolar subjects (Table 1). These tendencies are well in accordance with the previous report (3).

For transcripts relevant to oligodendrocyte function, we observed downregulation of *GSN* (gelsolin) and *S100A1* (S100 calcium binding protein A1) (Table 1). *NDUFV2* encoding a 24 kDa subunit of mitochondrial NADH dehyrogenase, is a mitochondrial complex I subunit gene, and this gene showed an increased expression in bipolar brains (Table 1).

Validation of microarray data by quantitative RT–PCR and examination of disease specificity

DNA microarray analysis measures expressional changes in thousands of transcripts, and therefore, the statistical evaluation of this data is prone to type I errors (false positive results). One way to control multiple hypotheses testing by statistics would be to apply a false discovery rate algorithm (6) to the data set. However, simulation methods have intrinsic limitations, for instance, yielding overly conservative results against small expressional changes (7). Therefore, we validated our initial microarray data by performing quantitative RT–PCR wherever possible. This involved the use of TaqMan probes and standard curve methodology, currently thought to be one of the most accurate methods of quantification.

Of the 108 differentially expressed transcripts detected in bipolar I disorder, 16 had no TaqMan probes available and eight transcripts failed to amplify consistently. Expression levels of the remaining 84 transcripts were examined using real-time quantitative RT–PCR. Eleven genes (*RAP1GA1*, *SST*, *HLA-DRA*, *KATNB1*, *PURA*, *H4FG*, *NDUFV2*, *CCL3*, *STAR*, *PAFAH1B3* and *RPP20*) showed significant variation with diagnosis (Table 1). Overall, the FCs for these transcripts were smaller with RT–PCR when compared with microarray analysis (Table 1; the slant of linear regression in Fig. 1 is 0.591). The FC data of the 84 transcripts from microarray analysis and real-time PCR and linear regression analysis, demonstrated a modest correlation with Pearson's correlation coefficient (R) of 0.338 and $P = 0.0018$ (Fig. 1). Notably, a cluster of transcripts (lower right quadrant of Fig. 1) were downregulated with GeneChip analysis but upregulated in quantitative RT–PCR.

In our study, 11 genes showed significant change by real-time RT–PCR. The two genes, *H4FG* that showed an expression decrease in the microarray but an increase in PCR assays (Table 1), and the *RPP20* gene with no known function were excluded from further study. To add diagnostic specificity to our studies, expression levels of the remaining nine transcripts were compared between control and schizophrenic brains. The expression of *CCL3* [chemokine (C-C motif) ligand 3] was downregulated in schizophrenia as it was in bipolar I disorder. The expression of the other eight target genes was not altered in schizophrenia (Fig. 2). In addition, we compared pooled transcripts from all schizophrenic brains and each of the control brains using GeneChips. None of the transcripts altered in schizophrenia detected by this method, overlapped with those listed in Table 1 (Supplementary Material, Table S1).

Table 1. Differentially expressed transcripts in bipolar I postmortem brains (BA46)

Functional categories and gene symbol	Gene product	Entrez ID[a]	GeneChip		qRT–PCR	
Receptor			FC[b]	*P*-value[c]	FC[b]	*P*-value[c]
NTRK2	*Neurotrophic tyrosine kinase receptor, type 2*	4 915	−1.9	0.0158	1.0	0.6991
EPHA4	*EphA4*	2 043	−1.9	0.0484	1.1	0.8182
GRIN1	*Glutamate receptor, ionotropic, N-methyl D-aspartate 1*	2 902	−1.7	0.0032	1.3	0.1797
GRIK1	*Glutamate receptor, ionotropic, kainate 1*	2 897	−1.5	0.0077	1.1	0.6991
Signal transduction						
CACNB1	**Calcium channel, voltage-dependent, beta-1 subunit**	782	1.9	0.0002	NA	
FGF7	**Fibroblast growth factor 7 (keratinocyte growth factor)**	2 252	1.8	0.0002	1.5	0.2403
PRKAR2B	**Protein kinase, cAMP-dependent, regulatory, type II, beta**	5 577	1.6	0.0158	1.4	0.0649
RAB5C	**RAB5C, member RAS oncogene family**	5 878	1.6	0.0011	−1.1	0.8182
PLCL2	**Phospholipase C-like 2**	23 228	1.5	0.0002	1.0	0.8182
RAP1GA1	**RAP1, GTPase activating protein 1**	5 909	1.5	0.0484	2.1	**0.0022**
SST	**Somatostatin**	6 750	1.5	0.0484	1.8	**0.0087**
PTPRM	**Protein tyrosine phosphatase, receptor type, M**	5 797	1.5	0.0285	1.0	1.0000
LY6E	*Lymphocyte antigen 6 complex, locus E*	4 061	−1.5	0.0077	1.0	0.8182
MYLK	*Myosin, light polypeptide kinase*	4 638	−1.5	0.0484	−1.2	0.3939
FKBP8	*FK506 binding protein 8* (38 kDa)	23 770	−1.5	0.0484	NW	
SHC3	*Neuronal Shc*	53 358	−1.5	0.0484	1.1	0.5887
MAP4K4	*Mitogen-activated protein kinase kinase kinase kinase 4*	9 448	−1.6	0.0077	1.2	0.3939
TYROBP	*TYRO protein tyrosine kinase binding protein*	7 305	−2.0	0.0032	−1.4	0.0649
CALM3	*Calmodulin 3* (*phosphorylase kinase, delta*)	808	−2.1	0.0011	1.1	0.4372
Neurotransmission						
SYBL1	**Synaptobrevin-like 1**	6 845	1.5	0.0289	1.1	0.3939
KIF5A	*Kinesin family member 5A*	3 798	−1.5	0.0484	1.0	0.6991
BAIAP3	*BAI1-associated protein 3*	8 938	−1.6	0.0158	1.3	0.3095
SYP	*Synaptophysin*	6 855	−1.6	0.0156	1.0	0.4071
SNCG	*Synuclein, gamma* (*breast cancer-specific protein 1*)	6 623	−1.6	0.0484	1.2	0.2403
CAV1	*Caveolin 1, caveolae protein*, 22 kDa	857	−2.1	0.0484	−1.1	0.9372
Stress response						
SMAD	**SMAD, mothers against DPP homolog 7** (*Drosophila*)	4 092	1.9	0.0289	−1.7	0.9372
GSTA4	**Glutathione-*S*-transferase A4**	2 941	1.6	0.0077	1.1	0.5887
DATF1	**Death-associated transcription factor 1**	11 083	1.5	0.0158	1.4	0.1797
Inflammatory response						
HLA-DRA	*Major histocompatibility complex, class II, DR alpha*	3 122	−2.0	0.0484	−1.7	**0.0411**
HLA-DRB4	*Major histocompatibility complex, class II, DR beta 4*	3 123	−2.2	0.077	NA	
CCL3	*Chemokine* (*C-C motif*) *ligand 3*	6 348	−3.5	0.032	−9.0	**0.0022**
Cell growth and maintenance						
KATNB1	**Katanin p80 (WD40-containing) subunit B1**	10 300	2.1	0.0077	1.6	**0.0260**
SPAST	**Spastin**	6 683	1.9	0.289	NW	
FSTL1	**Folistatin-like 1**	11 167	1.5	0.0484	1.1	1.0000
PCTK1	**PCTAIRE protein kinase 1**	5 127	1.5	0.0484	1.3	0.0649
PNULT2	**Peanut-like 2** (*Drosphila*)		1.5	0.0289	1.2	0.2403
PURA	**Purine-rich element binding protein A**	5 813	1.4	0.0484	1.4	**0.0260**
SNX3	**Sorting nexin 3**	8 724	1.5	0.0158	1.1	0.3939
WSB2	**WD repeat and SOCS box-containing 2**	55 884	1.6	0.0285	NA	
HPRP8BP	*U5 snRNP-specific* 40 kDa protein (*hPrp8-binding*)	9 410	−1.6	0.0158	1.1	0.3939
IGF2	*Insulin-like growth factor 2* (*somatomedin A*)	3 481	−1.6	0.0479	NW	
ATN1	*Denatonrubral-pallidoluysian atrophy* (*atrophin-1*)	1 822	−1.7	0.0158	1.3	0.1797
IGFBP4	*Insulin-like growth factor binding protein 4*	3 487	−2.0	0.0484	NW	
H4FG	*H4 histone family, member G*	8 364	−2.5	0.0484	1.4	**0.0152**
Transcription regulator						
RUNX1T1	**Runt-related factor 1**	862	2.1	0.0077	1.1	0.2403
REV3L	**REV3-like, catalytic subunit of DNA polymerase zeta (yeast)**	5 980	1.6	0.0158	1.1	0.8182

Continued

Table 1. *Continued*

Functional categories and gene symbol	Gene product	Entrez ID[a]	GeneChip		qRT–PCR	
NFKB1	**Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)**	4 790	1.6	0.0484	1.0	0.6991
XBP1	**X-box binding protein 1**	7 494	1.5	0.0050	1.2	0.3095
TAF9	**TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor** 32 kDa	6 880	1.5	0.0077	NA	
THRA	**Thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)**	7 067	1.5	0.484	1.1	1.0000
SFPQ	**Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)**	6 421	1.5	0.0158	1.1	0.4848
ABL1	**v-abl Abelson murine leukemia viral oncogene homolog 1**	25	1.5	0.0484	1.0	0.3939
ARNTL	**Aryl hydrocarbon receptor nuclear translocator-like**	406	1.5	0.0158	1.1	0.6991
SMARCD2	*SWI/SNF related matrix-associated actin-dependent regulator of chromatin, subfamily d, member 2*	6 603	−1.6	0.0077	−1.1	0.4848
BTG2	*BTG family, member 2*	7 832	−1.6	0.0289	−1.3	0.0931
ZNF148	*Zinc finger protein 148 (pHZ-52)*	7 707	−1.6	0.0484	1.0	0.5887
HIVEP1	*Human immunodeficiency virus type 1 enhancer binding protein 1*	3 096	−1.6	0.0484	1.2	0.3939
RLF	*Rearranged L-myc fusion sequence*	6 018	−2.0	0.0213	1.4	0.0931
GATA2	*GATA binding protein 2*	2 624	−2.3	0.0002	NA	
Translation regulator						
SUPV3L1	*Suppressor of var1, 3-like 1 (S. cerevisiae)*	6 832	−1.5	0.0479	1.1	0.5887
RPL34	*Ribosomal protein L34*	6 164	−1.5	0.0289	1.0	0.8182
Cell structure						
VCL	**Vinculin**	7 414	1.6	0.0002	1.2	0.3095
ICAM2	*Intercellular adhesion molecule 2*	3 384	−1.5	0.0011	NA	
BCL74	*B-cell CLL/lymphoma 7A*	605	−1.5	0.0002	1.2	0.3095
CDH22	*Cadherin-like 22*	64 405	−1.5	0.0032	1.6	0.0931
TMOD	*Tropmodulin*	7 111	−1.7	0.0484	1.0	0.8182
LMNA	*Lamin A/C*	4 000	−1.7	0.0110	1.2	0.3939
CLDN5	*Claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)*	7 122	−1.7	0.0289	−1.2	0.2403
S100A1	*S100 calcium binding protein A1*	6 271	−1.8	0.0484	1.1	0.4848
GSN	*Gelsolin (amyloidosis, Finnish type)*	2 934	−1.9	0.0158	NA	
LU	*Lutheran blood group (Auberger b antigen included)*	2 897	−2.1	0.0032	1.2	0.3095
Metabolism						
B3GALT2	**UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypetide 2**	8 707	2.7	0.0158	NA	
ATP61P2	**ATPase, H+ transporting, lysosomal interacting protein 2**	10 159	2.0	0.0158	1.2	0.1320
PNPLA4	**Patatin-like phospholipase domain containing 4**	8 228	1.9	0.0011	NA	
NDUFV2	**NADH dehydrogenase (ubiquinone) flavoprotein 2** (24 kDa)	4 729	1.5	0.0032	1.3	**0.0411**
LDHA	**Lactate dehydrogenase A**	3 939	1.5	0.0484	NA	
STAR	**Steroidogenic acute regulatory protein**	6 770	1.5	0.0479	1.9	**0.0260**
PRPS2	**Phosphoribosyl pyrophosphate synthetase 2**	5 634	1.5	0.0484	1.0	0.6991
PAFAH1B3	**Platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit** (29 kDa)	5 050	1.5	0.0077	2.4	**0.0022**
PISD	**Phosphatidylserine decarboxylase**	23 761	1.5	0.0484	NW	
GLUD1	*Glutamate dehydrogenase 1*	2 746	−1.5	0.0213	NA	
FABP7	*Fatty acid binding protein 7, brain*	2 173	−1.5	0.0158	NA	
SORD	**Sorbitol dehydrogenase**	6 652	−1.7	0.0002	−1.1	0.6991
NPC2	**Niemann-Pick disease, type C2**	10 577	−1.7	0.0289	1.0	0.6991
GSK3B	**Glycogen synthase kinase 3 beta**	2 932	−1.7	0.0484	1.1	0.1797
Others						
YWHAZ	**Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide**	7 534	1.6	0.0289	1.0	1.0000
RNF13	**Ring finger protein 13**	11 342	1.6	0.0484	NA	
TPST2	**Tyrosylprotein sulfotransferase 2**	8 459	1.6	0.0002	NW	
MGST3	**Microsomal glutathione-*S*-transferase 3**	4 259	1.6	0.0158	1.2	0.3095

Continued

Table 1. *Continued*

Functional categories and gene symbol	Gene product	Entrez ID[a]	GeneChip		qRT–PCR	
PDE6D	**Phosphodiesterase 6D, cGMP-specific, rod, delta**	5 147	1.6	0.0484	1.2	0.3939
PCBP2	**Poly(rC) binding protein 2**	5 094	1.5	0.0484	1.1	0.8182
DDX19A	**DEAD (Asp-Glu-Ala-As) box polypeptide 19A**	55 308	1.5	0.0011	1.1	0.4848
NCOA4	**Nuclear receptor coactivator 4**	8 031	1.5	0.0289	NW	
SEC24C	**SEC24 related gene family, member C** (*S. cerevisiae*)	9 632	1.5	0.0002	1.2	0.2403
SS18L1	**Synovial sarcoma translocation gene of chromosome 18-like 1**	26 039	1.5	0.0158	1.2	0.3095
IFRD2	**Interferon-related developmental regulator 2**	7 866	1.5	0.0289	NW	
TOR1AIP1	**Torsin A interacting protein 1**	26 092	1.5	0.0484	1.0	0.9372
ISG15	*Interferon-simulated protein*, 15 kDa	9 636	−1.5	0.0077	1.0	0.8182
SUMO4	*SMT3 suppressor of mif two 3 homolog 4* (*yeast*)	38 702	−1.5	0.0002	NA	
PECAM1	*Platelet/endothelial cell adhesion molecule* (*CD31 antigen*)	5 175	−1.5	0.0158	1.1	0.8182
TXNL4A	*Thioredoxin-like protein 4A*	10 907	−1.5	0.0484	1.3	0.0649
MEL	*Mel transforming oncogene* (*derived from cell line NK14*)-*RAB8 homolog*	4 218	−1.5	0.0158	1.1	0.2403
MMP14	*Matrix metalloproteinase 14* (*membrane-inserted*)	4 323	−1.5	0.0158	1.2	0.4848
Unknown						
MMD	**Monocyte to macrophage differentiation-associated**	23 531	1.6	0.0158	1.3	0.1320
KIAA0339	**KIAA0339 gene product**	9 739	1.5	0.0077	NA	
C3orf4	**Chromosome 3 open reading frame 4**	56 650	1.5	0.0484	1.1	0.9372
KIAA0368	**KIAA0368 protein**	23 392	1.7	0.0011	NA	
RPP20	*POP7* (*processing of precursor*, *S. cerevisiae*) *homolog*	10 248	−1.7	0.0032	1.4	**0.0260**

Up-regulated genes in bipolar disorder were shown in bold face and downregulated in italic face. NA, TaqMan probes not available; NW, TaqMan probes did not give linear amplification profiles.
[a]ID for the National Center for Biotechnology Information Entrez Gene database (http://www.ncbi.nih.gov/entrez/query.fcqi?db=gene).
[b]FC was calculated between mean values of controls ($N = 7$) and bipolar brains ($N = 7$). Positive values indicate an increase, and negative values denote a decrease in gene expression in bipolar patients.
[c]*P*-value was calculated using Mann–Whitney *U* test (two-tailed). $P < 0.05$ in qRT–PCR are in bold face.

Protein expression analysis

Antibodies effective against human CNS tissue and suitable for western blotting were readily available for two of the eight proteins of interest: RAP1GA1 (RAP1, GTPase activating protein 1) and PAFAH1B3 (platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29 kDa). Antibody measurements of protein showed a trend to increased levels of RAP1GA1 protein (1.4-fold) in the BA46 (dorsolateral prefrontal cortex: DLPFC) from subjects with bipolar I disorder (Fig. 3), concurring with mRNA expression results. The expression of this protein was not altered in BA40 (parietal lobe) of subjects with bipolar I disorder. Levels of PAFAH1B3 remained unchanged in BA46 or BA40 of subjects with bipolar I disorder (Fig. 3) despite significant detectable differences in mRNA levels in BA46 using microarray and real-time PCR (Table 1, Fig. 2).

Genetic association analysis

We analyzed single nucleotide polymorphisms (SNPs) in and around the nine genes whose altered expressions in bipolar DLPFC was confirmed by quantitative RT–PCR. Of 50 SNPs, 47 were successfully genotyped by the TaqMan assay (Supplementary Material, Table S2). Only one marker, rs6797699 in *SST* (somatostatin) (Fig. 4), displayed a nominally significant association ($P = 0.0279$) in transmission disequilibrium test (TDT) analysis, but this disappeared after permutation testing ($P = 0.0655$), which serves as a gene-wide correction of multiple testing (Supplementary Material, Tables S3 and S4). In haplotype analysis using the TDTPHASE program, a two-marker haplotype in *SST* (G at rs6797699 and C at rs1365111) remained significant in permutation analysis ($P = 0.033$) (Supplementary Material, Table S5), whereas haplotypes of all other genes did not. The three genotyped markers for *SST* were located 5 kb upstream and 1.5 and 8 kb downstream of the gene and found to be in complete linkage disequilibrium with each other ($D' = 1$) (Fig. 4). According to the HapMap database, they constitute a 40 kb haplotype block (defined by a solid spine of linkage disequilibrium) encompassing the whole genomic region of *SST* along with other neighboring markers. No other genes are known to exist within this haplotype block.

Pathway analysis

Pathway analysis was applied to the differentially expressed genes to identify networks of genes that are known to interact functionally. Ingenuity pathway analysis of the 79 genes that showed a consistent direction of alteration, either up or down-regulation in both microarray and RT–PCR (Table 1) could be grouped into three major networks involving cellular growth and proliferation ($N = 14$), nervous system development

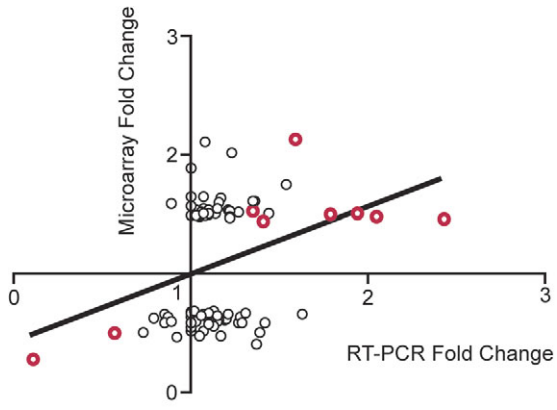

Figure 1. Correlation between the quantification of specific transcripts using microarray and real-time RT–PCR. The data represent 84 transcripts that were analyzed using both techniques, and they show the 'FC' of mRNA expression levels in tissue from the bipolar I disorder versus matched control subjects. Linear regression analysis was performed, giving Pearson's correlation coefficient, $R = 0.338$ and $P = 0.0018$. Black circles represent the transcripts whose expression changes were significant only by microarray analysis, whereas red circles indicate those being significant by both microarray and quantitative RT–PCR analyses.

($N = 13$) and cell death ($N = 11$) with all other identified networks containing fewer than four genes (data not shown). RAP1GA1, of which both mRNA and protein levels were elevated in the bipolar brains, was in one of these top three networks (network 2 in Table 2). Among other validated bipolar-specific genes, *STAR* was placed in network 2 and *HLA-DRA* in network 3 (Table 2).

DISCUSSION

We have performed a genome-wide expression study of bipolar I disorder with extensive validation by quantitative RT–PCR after the initial microarray screening, in a large postmortem brain study of mental disorders. This study demonstrated that FCs of all examined transcripts except for *CCL3* (FC = −3.5 in microarray and −9.0 in RT–PCR) were within −3 to +3, supporting previous statements that true gene-expression changes in psychiatric traits are small and that psychiatric diseases may result from cumulative subtle changes (5,8). Importantly, the present study indicates that small changes in gene expression detected by GeneChip alone need to be viewed with caution and wherever possible, validating such data with other experimental approaches is a necessity. It is known that cross-validation rates among different experimental approaches are dependent on both the magnitude and level of expressional changes (9).

Prior gene expression studies in bipolar disorder have reported a tendency for downregulation of genes encoding receptors and upregulation of genes from the stress response category in the prefrontal cortex (3), a reduction of key oligodendrocyte-related and myelin-related genes in BA9 (4), and altered expression of mitochondria-related genes (7,10). The microarray detection of downregulated *GRIK1* supports this finding (3), but it was not validated by quantitative RT–PCR. Some of the altered genes detected in the present microarray screening belong to the same functional categories [e.g. downregulation of *IGFBP6* in the study of Iwamoto *et al*. (3) and downregulation of *IGFBP4* in this study; upregulation of *YWHAE* in the prior study (3) and upregulation of *YWHAZ* in the current study]. This overlap of categories if not necessarily individual genes might suggest shared metabolic pathways between different bipolar disease populations, with the responsible molecules being heterogeneous and exerting a varying genetic impact. These results support the premise of genetic heterogeneity in bipolar disorder, but further studies are needed to resolve this issue.

Serious confounding factors that should be taken into account in postmortem brain studies include the 'quality' of brain tissues and the effects of medication. The former can be typically measured by pH. Low CNS pH causes the downregulation of large numbers of mitochondrial and energy metabolism genes (7,11). Our brain samples did not show this phenomenon. However, possible effect of sample pH would not be completely excluded. More than 70% (5/7) of bipolar subjects and all schizophrenics examined here were medicated with typical neuroleptics before death. Therefore, our study design of comparing gene expressions not only between bipolar and controls but also between schizophrenia and control samples may allow for tentative speculation on the effects of neuroleptics. In this respect, we cannot exclude the contribution of drug effects on altered *CCL3* expression in bipolar disorder, even though it showed consistent change by both methods. False positive results could arise from other factors including inter-individual variations, gender differences, chip-to-chip variability, small sample size and low transcript level changes.

Our literature survey, of the eight genes that showed confirmed expressional changes specific to bipolar disorder, the gene for somatostatin (somatotropin release-inhibiting factor) has been previously reported in the context of mental disorders. Somatostatin is a widely distributed cyclic tetradecapeptide that is an important regulator of endocrine and nervous system function (12). Sharma *et al*. (13) compared levels of cerebrospinal fluid (CSF) somatostatin in drug-free patients with different major psychiatric disorders (schizophrenia, major depressive disorder, manic disorder and schizoaffective disorder) and found that the manic patients had markedly elevated CSF somatostatin concentrations. This result is consistent with that of our mRNA expression study. However, another DNA microarray study (14) showed downregulation of *SST*. Interestingly, this is the only gene that showed genetic association with bipolar disorder in this study, although care should be taken in defining a genetic role for this gene given the number of altered genes studied. It is noteworthy that a variety of studies support a tropic role for somatostatin in the developing nervous system, evidenced as stimulation of neurite outgrowth and axonal or neuronal migration in both *in vivo* and culture models (15).

The current DNA microarray and quantitative RT–PCR analyses revealed the upregulation of *RAP1GA1* transcripts in bipolar I disorder with a corresponding trend of increase for the protein. RAP1GA1 has the GAP (GTPase activating protein) activity towards RAP1, a RAS-related small G protein (16). RAP1 was first identified as a molecule capable

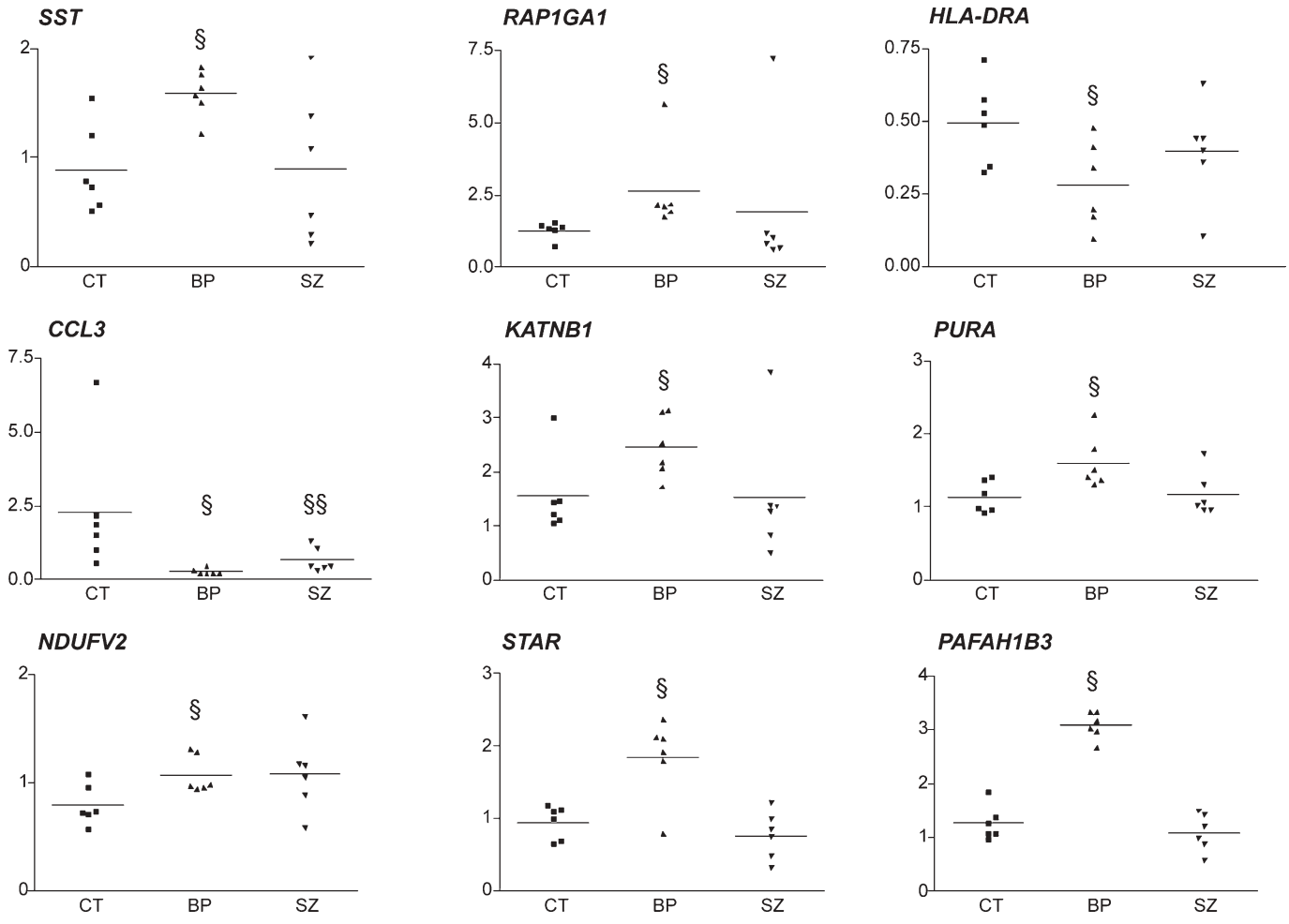

Figure 2. Messenger RNA levels for nine differentially expressed genes in BA46 from control (CT, $N = 7$), bipolar I disorder (BP, $N = 7$) and schizophrenic (SZ, $N = 7$) subjects quantified using RT–PCR. Each data point is expressed as a ratio of the internal control (β2-microglobulin) for each sample. Horizontal bars indicate the mean value. (Section indicate) Significant difference between bipolar I and control levels ($P < 0.05$ by the two-tailed Mann–Whitney U test). (Double-section indicate) Significant difference between schizophrenia and control levels ($P < 0.05$ by the two-tailed Mann–Whitney U test).

of reverting the oncogenic phenotype of RAS-transformed fibroblasts (17). In rat pheochromocytoma PC12 cells, nerve growth factor (NGF) and fibroblast growth factor (FGF) cause neurite outgrowth and induce neuronal differentiation (18,19). These processes are thought to be RAP1-dependent, at least with the help of the Src homology 2 domain adaptor protein Shb (20). Transient expression of RAP1GA1 to block the RAP1 pathway reduced the NGF-dependent neurite outgrowth in Shb-overexpressing PC12 cells. Meng and Casey (21) also reported that recruitment of RAP1GA1 to the membrane by activated Gzα can effectively downregulate RAP1 signaling and attenuate PC12 differentiation induced by NGF treatment. These data suggest that upregulation of RAP1GA1 in bipolar brains may hamper neurite outgrowth or axon extension leading to the disturbance of neuronal network integrity, a pathology highlighted by the bioinformatics pathway analysis. The fact that the expression of RAP1GA1was not altered in BA40 (parietal lobe) may support the hypothesis that changes in gene expression show anatomical specificity (5) along with diagnostic specificity. However, a systematic validation of this hypothesis remains to be performed.

The evident upregulation of *PAFAH1B3* mRNA fits with the presently proposed disturbed neuronal networks for bipolar pathophysiology. The precise reason for the discrepancies between mRNA and protein expressional changes remains unknown. One explanation may be the ability of cells to maintain normal protein levels despite changes in gene expression rates (22). PAFAH1B3 is the catalytic gamma subunit of type I platelet-activating factor acetylhydrolase, isoform 1B (PAFAH1B), a phospholipase, that inactivates platelet-activating factor (PAF) by removing the acetyl moiety at the *sn*-2 position of the glycerol backbone (23). Isoform 1B of PAFAH consists of three subunits, alpha (PAFAH1B1), beta (PAFAH1B2) and gamma (PAFAH1B3). The catalytic activity of the enzyme resides in the beta and gamma subunits, whereas the alpha subunit has regulatory activity. The alpha subunit is also known as LIS1 (lissencephaly 1 protein). LIS1, a causal protein of classical lissencephaly-1 (a brain malformation characterized by 'smooth brain', a brain

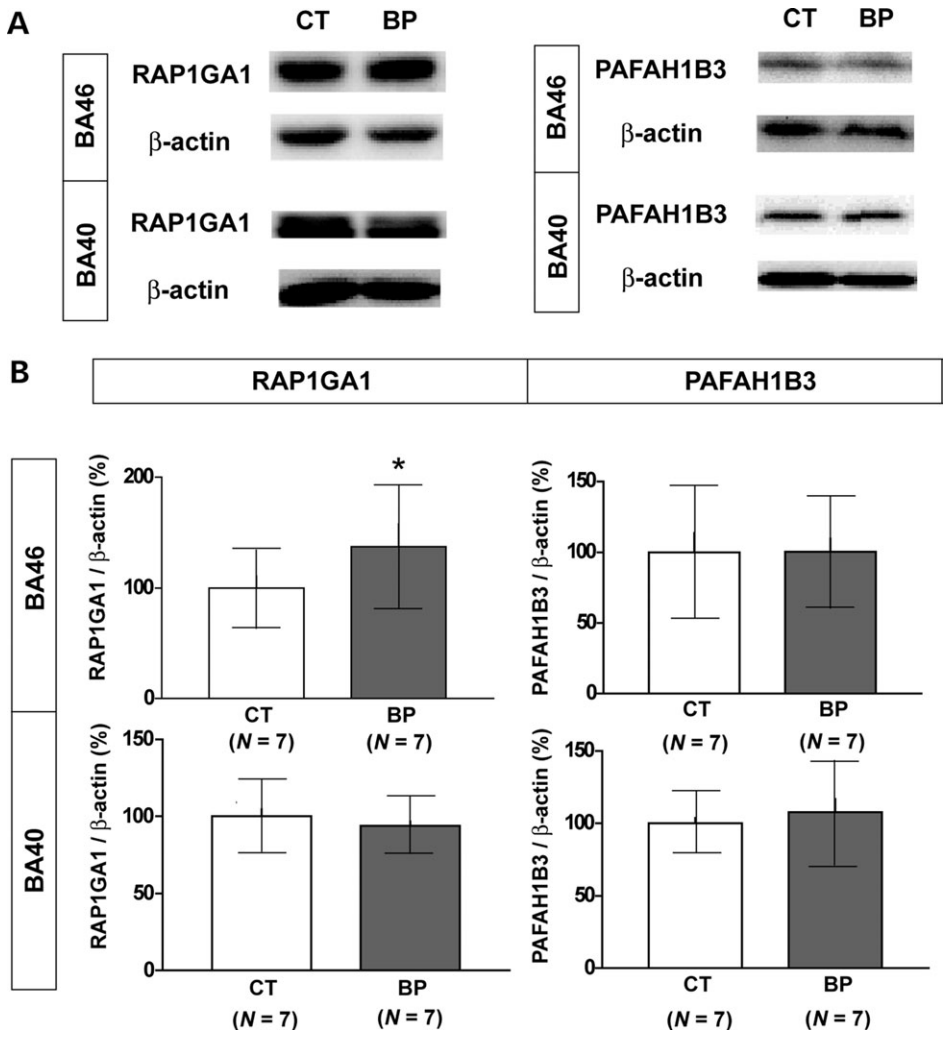

Figure 3. Quantitation (mean ± SD) of RAP1GA1 and PAFAH1B3 protein in BA46 from control and bipolar I disorder using western blot analysis. (**A**) A representation of a typical western blot. CT, control; BP, bipolar disorder. (**B**) Levels of RAP1GA1 and PAFAH1B3 protein, expressed as a ratio of β-actin in BA46 and BA40 from subjects with bipolar I disorder (BP) and control subjects (CT). $^{*}P < 0.05$ by the two-tailed Mann–Whitney *U* test.

without convolutions or gyri, and by a disorganization of the clear neuronal lamination of normal six-layered cortex), can interact with a number of proteins, including tubulin (24), cytoplasmic dynein (25,26) and NUDEL (27–29) that in turn interacts with DISC1 (30). Through interaction with these proteins, LIS1 plays important roles in the regulation of neuronal functions such as division of neuronal progenitor cells in the developing brain, dynamic neurite extension, axon growth and neuronal migration, necessary processes for the formation and maintenance of neuronal networks.

There are numerous human leukocyte antigen (HLA) studies in schizophrenia with mixed results. For bipolar disorder, a preliminary genetic study failed to demonstrate association between HLA alleles and disease (31). Our results showed that *HLA-DRA* was decreased in bipolar patients in both microarray and quantitative RT–PCR analyses. The expression of *HLA-DRA* is probably controlled by X-box binding protein 1 (XBP1) binding to the *HLA-DRA* promoter (32). Interestingly, *XBP1* is a component of network 2 (nervous system development and function; Table 2) by the pathway analysis, and a genetic association between *XBP1* and bipolar disorder has been reported (33). *STAR* (a gene for steroidogenic acute regulatory protein) is also mapped to network 2 (Table 2). STAR appears to mediate the rapid increase in pregrenolone synthesis stimulated by tropic hormones. *STAR* homozygous mutation carriers showed a high level of serum ACTH (34).

Purine-rich element-binding protein A (PURA) is a single-stranded DNA-binding protein with specific affinity for the purine-rich element configuration of $(GGN)_n$, present in several initiation zones of eukaryotic DNA replication (35). PURA is implicated in the transcriptional control of a number of cellular genes, including myelin basic protein, amyloid beta A4 precursor protein-binding family A member 1 and neuronal acetylcholine receptor protein, beta-2 subunit precursor (35). PURA is also involved in the control of cell growth and interacts with the hypophosphorylated form of the retinoblastoma tumor suppressor gene

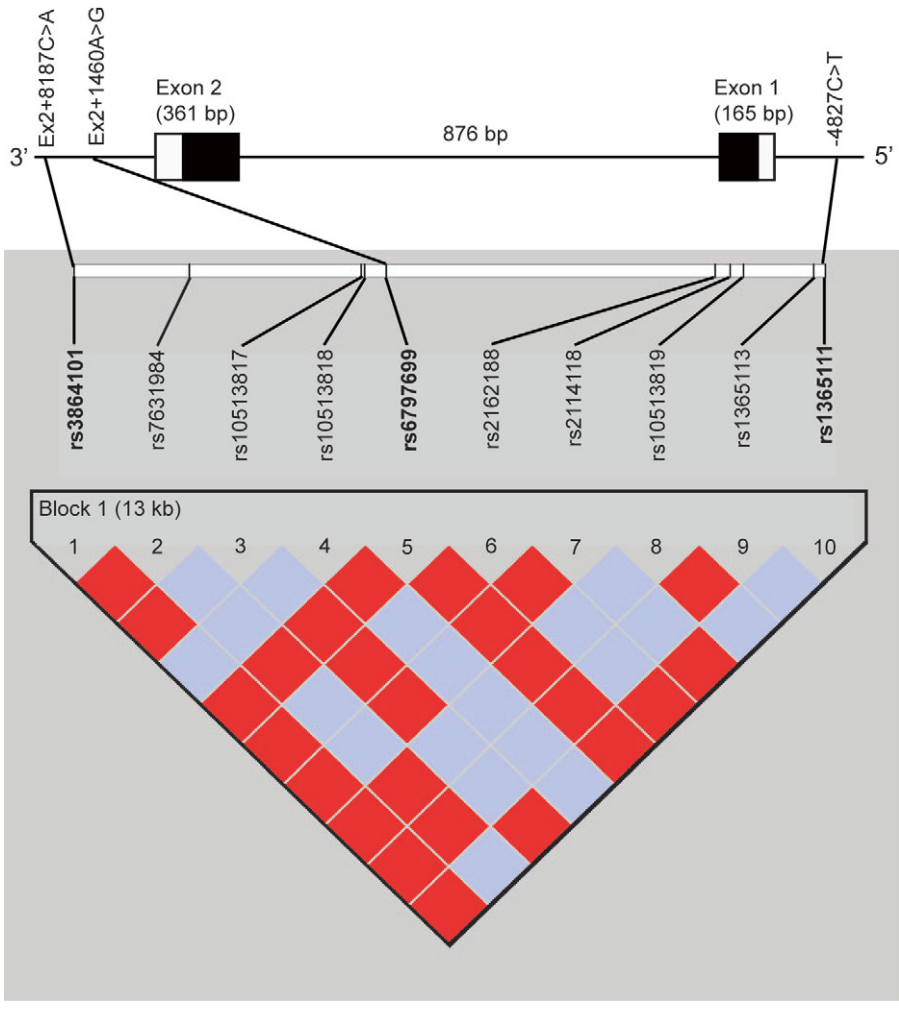

Figure 4. Genomic structure and location of genotyped polymorphic sites (tagSNPs) for *SST* (upper panel) and haplotype organization of *SST* (lower panel). The haplotype block pattern was constructed using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) and the genotype data of CEPH pedigrees from the HapMap database (http://www.hapmap.org/index.html.ja). Each cell color is graduated relative to the strength of linkage disequilibrium between markers, which is defined by both the D' value and confidence bounds on D'. $D'=1$ for red.

product (35). KATNB1 is an 80 kDa subunit of the microtubule-severing protein katanin, responsible for regulating microtubule disassembly (36), a basic biological process underlying neuronal migration, extension and shape maintenance. The upregulation of *NDUFV2* seen here was previously noticed in the frontal cortex of our learned helplessness rat, an animal model of depression (5), but not detected in a prior bipolar brain study (37). This gene is located on human chromosome 18p11, a bipolar linkage locus (38). Washizuka *et al*. (39) reported a transcription enhancing effect of a *NDUFV2* promoter polymorphism (-602G>A) that was associated with bipolar disorder in a Japanese population but not in a subset of National Institute of Mental Health (NIMH) samples which was smaller in size than in the present study. We combined genotyping data from Washizuka *et al*. (39) and the current study, and by applying a method of de Bakker *et al*. (40), found that -602G>A is in strong linkage disequilibrium ($r^2 = 0.738$) with a three-SNP haplotype (T at rs2377961-T at rs906807-A at rs4148967). No evidence of association between this haplotype and bipolar disorder was found in the present data set ($\chi^2 = 2.32$, Permutation $P = 0.6204$). Thus, effect of this single SNP on the development of the illness, if any, may substantially differ between Japanese and Caucasian populations.

In summary, we performed a rigorous genome-wide examination of transcripts with expressional changes that are specifically associated with bipolar I disorder and in some cases, in a region-specific manner. Our aim was to identify surrogate biomarkers that aid the understanding of disease mechanism and the development of novel therapeutic interventions. The main outcome is the proposal of neuronal network perturbation as a molecular pathology underlying bipolar disorder, although the primary defects are unaccounted for in this study. However, this hypothesis supports the growing appreciation that mood stabilizers possess neurotrophic effects and could, therefore, target cellular plasticity and resilience in critical neuronal circuits (41). The current findings are highly worthy of further investigation.

Table 2. Gene networks identified by pathway analysis

Network ID	Genes in network[a]	Score[b]	Number of focus genes[a]	Principal categories
1	*ACP1, ADRB3*, ***ARNTL***, *ASC2*, ***CAV1***, *CAV2*, ***CBFA2T1, CCL3***, *CEACAM6, CLOCK, DIO1, EP300*, ***GATA2***, *GJA1*, ***GSN***, *H1F1A*, ***IGF2***, *IGFBP2*, ***IGFBP4***, *JUN, LEP*, ***NCOA4***, *NCOR2*, ***NFKB1***, *NR2F2, PPARG, PRNP, PTGS2, SCD*, ***SMAD7***, *SNAP25, SRC*, ***SYBL1, SYP, THRA***	21	14	Cellular growth and proliferation
2	*ACTB, ATF1, BRAF, CAMK4, CCNA2, CDH1, CDH2, CREB1, CREM, FOS*, ***FSTL1***, *ICAM1*, ***ICAM2***, *IL2, KRAS2*, ***LDHA, PDE6D***, *PRKAR2A*, ***PRKAR2B***, *PRKCM*, ***PTPRM, RAP1GA1***, *R1N1, RPS6KA2, RPS6KA4, SMARCDI*, ***SMARCD2, SNX3, STAR***, *TSC1*, ***VCL***, *VIL2*, ***XBP1***, *YWNAQ*, ***YWHAZ***	19	13	Nervous system development and function
3	***ABL1***, *ARL61P*, ***BTG2***, *CABLES1, CDK5, CKS2, E2F4, EEF2, GPI, H2AFZ, HAS2*, ***HLA-DRA, HLA-DRB3***, *HLA-DRB4, HMGN2, HMMR, IFI202B*, ***MGST3***, *MYC*, ***MYLK***, *NDEL1, NDN, PAK2*, ***PCTK1***, *PLSCR1, PSAT1, RAD54L*, ***SFPQ***, *SMARCA4*, ***SUMO2, TAF9***, *TAGLN2, TMSB4X, TP53*, ***ZNF148***	15	11	Cell death

[a]Included in the pathway analysis are the 79 genes that were consistently altered in both microarray and RT–PCR experiments. Among the 79 genes, the detected genes in the network are in bold face and counted.
[b]Score is calculated taking the number of focus genes and the size of the networks.

MATERIALS AND METHODS

Collection of human brain tissues

Following approval from the North-Western Healthcare Network Human Ethics Committee, two different cortical tissues, BA46 (DLPFC) and BA40 (parietal cortex) were collected by the Victorian Institute of Forensic Medicine, affiliated with the State Coroner's Office in Australia. At autopsy, brains were removed and 1 cm coronal slices from the left hemisphere were rapidly frozen to −80°C. The postmortem diagnoses were confirmed according to DSM-IV criteria by a psychiatrist and a senior psychologist following an extensive case history review using the Diagnostic Instrument for Brain Studies (42). Notable to this study, the inclusion criteria limited subjects to those with bipolar I disorder.

During the case history reviews prior to final diagnosis it was determined that five of the seven bipolar I subjects and all of the subjects with schizophrenia had a history of treatment with typical antipsychotic dugs (Table 3). In Australia, severe mania is treated with antipsychotic drugs and some of the bipolar I subjects manifested this condition. The sex distribution, mean age, mean postmortem interval (PMI) and pH for the tissues were not significantly different among control ($N = 7$), bipolar ($N = 7$) and schizophrenia ($N = 7$) groups (Table 3). The control subjects had no known history of major psychiatric illness. We were unable to ascertain the subjects' exact ethnicity.

RNA preparation and array hybridization

Total RNA was extracted from brain tissues using an acid guanidium thiocyanate/phenol chloroform extraction method (ISOGEN, NIPPON Gene, Toyama, Japan). RNA quality was assessed by high-resolution electrophoresis (Agilent Technologies, Palo Alto, CA, USA) (43). Double-stranded cDNA was synthesized from 10 μg of total RNA using the SuperScript Choice System (Invitrogen, Carlsbad, CA, USA) and a primer containing poly (dT) and T7 RNA polymerase promoter sequences (Geneset, La Jolla, CA, USA). Biotin-labeled cRNA was synthesized from cDNA using an Enzo BioArray High Yield RNA Transcript Labeling kit (Enzo Diagnostics, Santa Clara, CA, USA). After fragmentation, 15 μg of cRNA was hybridized for 16 h at 45°C to a U95Av2 chip (Affymetrix, Santa Clara, CA, USA), which contained probes for more than 12 000 transcripts including ∼10 000 full-length genes (http://www.affymetrix.com/products/arrays/specific/hgu95.affx). A different DNA chip was used for each brain sample. After hybridization, arrays were washed automatically and stained with streptavidin–phycoerythrin using the fluidics system. The hybridization signal on the chip was scanned using an HP GeneArray scanner (Hewlett-Packard, Palo Alto, CA, USA) and was processed by GeneSuite software (Affymetrix).

Analysis of microarray data

The microarray raw data were processed by Microarray Suite 5.0 (MAS5) software (Affymetrix) and imported into GeneSpring 6.1 software (Silicon Genetics, Redwood, CA, USA). All arrays were scaled to a target intensity of 100, using MAS5. Quality control measures including 3′/5′ ratios for *GAPDH* and β-actin, scaling factors, background and *Q*-values were within acceptable limits. The calculation of the ratio between perfect match (PM) to mismatch (MM) (PM/MM ratio) was used to define transcripts as present (P), marginal (M) or absent (A). We used the default settings provided by Affymetrix for this determination. Only transcripts that gave an 'absolute call' of 'P' in at least four of seven controls or seven patients with bipolar were considered for further analysis.

Before statistical comparisons, the expression level of each transcript was converted into a logarithmic value and normalized

Table 3. Demographic and treatment data for the bipolar I, schizophrenia and control brains (BA46 and BA40)

Subject ID	Age (year)	Sex	PMI (h)	pH	DOI (year)	Cause of death	FRAD	FAPDD
Bipolar I patients								
1	74	F	45.0	6.26	35	Combined drug toxicity	Fluphenazine	100
2	58	F	41.0	5.68	10	Ischemic heart disease	Nil	
3	59	M	34.0	6.46	24	Ruptured aorta (natural)	Nil	
4	66	M	17.0	6.41	Unknown	Apnea (food aspiration)	Modecate	166
5	55	F	52.0	6.46	14	Unascertained	Chlorpromazine	300
6	60	F	50.0	6.08	23	Cardiomegaly	Stalazine—ceased 8 weeks prior to death	?
7	61	M	58.0	6.44	40	Myocardial infarction	Flupenthixol	550
Mean ± SD	61.9 ± 6.3		42.4 ± 13.6	6.26 ± 0.29				
Schizophrenia patients								
1	48	F	52.5	6.21	22	Pulmonary thromboembolism	Flupenthixol Chlopromazine	700
2	65	M	41.0	6.57	35	Ischemic heart disease	Fluphenazine decanoate	150
3	65	F	50.0	6.35	18	Rupture of abdominal aortic aneurysm	Fluphenazine decanoate Haloperidol	550
4	57	M	24.0	6.06	28	Coronay artery atheroma	Fluphenazine decanoate	150
5	69	M	48.0	6.44	6	Co poising (suicide)	Haloperidol	650
6	47	F	50.0	6.31	20	Pneumonia	Risperidone	600
7	79	F	26	6.27	Unknown	Unascertained	Fluphenazine deconate	330
Mean ± SD	61.4 ± 11.5		41.6 ± 11.9	6.32 ± 0.16				
Control subjects								
1	59	F	20.5	6.58		Congestive cardiac failure		
2	62	F	40.0	6.45		Ischemic heart disease		
3	57	M	27.0	6.43		Ischemic heart disease		
4	68	M	41.0	6.06		Aortic stenosis		
5	68	M	69.0	6.59		Coronary artery atheroma		
6	39	F	65.0	6.38		Mitral valve prolapse		
7	77	F	17.0	6.32		Hypertensive heart disorder		
Mean ± SD	61.4 ± 12.0		39.9 ± 20.6	6.40 ± 0.18				

DOI, duration of illness; FRAD, final recorded antipsychotic drug; FAPDD, final recorded antipsychotic drug dose (milligrams of chlorpromazine equivalents/day); M, male; F, female.

to itself by making a synthetic positive control and dividing all measurements by this control, assuming that the control value was at least 0.01 (5). A synthetic control is the median of the transcript's expression values over all the samples. Differentially expressed probes between bipolar and control samples were selected using the following criteria: (i) $P < 0.05$ by the two-tailed Mann–Whitney U test; and (ii) FC of expression levels ≥ 1.5 (increased in bipolar samples compared with controls) or ≤ -1.5 (decreased in bipolar brains).

Real-time quantitative PCR

We evaluated the differential gene expressions detected in microarray analysis, by performing real-time quantitative RT–PCR, using TaqMan universal PCR mastermix, transcript-specific minor groove binding probes (Assays-on-Demand, Applied Biosystems, Foster City, CA, USA) and an ABI 7900 sequence detection system (Applied Biosystems), according to the manufacturer's instructions. The β2-microgloblin gene was chosen as a control from the TaqMan Human Endogenous Control Plate (Applied Biosystems) after evaluation of 11 available internal control probes (43). The PCR assay was performed simultaneously with test and standard samples and no template controls in the same plate. A standard curve plotting the cycle of threshold values against input quantity (log scale) was constructed for both the β2-microgloblin gene and the target molecules for each PCR assay. All real-time quantitative PCR data were captured using the SDS v2.2 (Applied Biosystems). The ratio of the relative concentration of the target molecule to β2-microgloblin gene (target molecule/β2-microgloblin gene) was calculated. We used the Mann–Whitney U test (two-tailed) to detect significant changes in expression for each gene. Pearson's correlation between expressional data obtained by RT–PCR and microarray analyses were calculated.

Western blotting

Frozen tissue blocks of BA46 and BA40 from the subjects used in the microarray analysis, were homogenized in 10 mM Tris–HCl containing 1% w/v SDS and 1 mM Na_2VO_4. Protein concentrations in the homogenate were determined by the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) and then frozen at −80°C until use. Samples were denatured by heating at 95°C for 5 min in electrophoresis buffer (25 mM Tris–HCl pH 6.5, 5% w/v glycerol, 0.05% w/v bromophenol blue, 5% v/v 2-β-mercaptoethaol and 2% w/v SDS). Equal amounts of protein were subjected to SDS–PAGE, and blotted onto a PVDF membrane (Millipore, Billerica, MA, USA) at a constant current (2 mA/cm^2) with a transfer buffer (25 mM Tris

base, 200 mM glycine, 20% v/v methanol) and using the Trans-Blot cell apparatus (Bio-Rad Laboratories). Each signal was detected with the indicated antibody, developed using the ECL system (Amersham Bioscience, Piscataway, NJ, USA), and quantified by the bioimage analyzer BAS1000 (Fuji Film, Tokyo, Japan). The intensity of each protein was normalized to that of β-actin. The specific antibodies for RAP1 GTPase activating protein 1 (RAP1GA1) and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the antibody for platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit (PAFAH1B3) was kindly given from Prof. Hiroyuki Arai (Graduate School of Pharmaceutical Sciences, University of Tokyo).

Genotyping and association analysis

We used 229 independent bipolar trio samples of Caucasian origin, collected by the NIMH Genetics Initiative group (version 2.0) for a family-based genetic association study. Of these, 228 probands had bipolar I, and one suffered from schizoaffective disorder, bipolar-type. The detailed ascertainment information on these samples has been published elsewhere (44).

We analyzed nine genes (*RAP1GA1*, *SST*, *HLA-DRA*, *KATNB1*, *PURA*, *NDUFV2*, *STAR*, *PAFAH1B3* and *CCL3*), whose expressional changes were confirmed by quantitative RT–PCR. A set of SNPs were selected for genotyping by applying the 'greedy algorithm' implemented in LDSelect program (45) to genotype data of the CEPH pedigrees from the HapMap database (release 16c.1) (http://www.hapmap.org/index.html.ja) (46). This program selects a set of SNPs (tagSNPs), which maximally capture information on genomic variations based on r^2, a measure of linkage disequilibrium between pairs of markers. We used a relatively stringent threshold of $r^2 = 0.85$. The range of this SNP tagging included at least 10 kb up and downstream of each gene. In addition, all the non-synonymous variations in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) were chosen. By these criteria, the total number of SNPs analyzed totaled 50.

For SNP genotyping, we used the TaqMan assay (Applied Biosystems): probes and primers were designed using Assays-by-Design™ SNP genotyping system (Applied Biosystems) and fluorescence was determined using the ABI 7900 sequence detector single point measurement and SDS v2.2 software (Applied Biosystems). Detailed information on primer sequences and PCR conditions are available upon request. Association of individual SNPs and association of haplotypes of all possible lengths in each gene were evaluated by either 'exhaustive allelic TDT' (EATDT) (47) (for genes with ≥ 5 SNPs) or TDTPHASE (48) (for genes with < 5 SNPs) programs, each employing permutation testing (10 000 times) to compute gene-wide empirical *P*-values.

Pathway analysis

In an attempt to identify aberrant neuronal pathways in bipolar I disorder brains, genes were tested if they matched any gene network that could potentially form a biological unit, and analysis was completed with Ingenuity Pathways Analysis system (Ingenuity Systems, Mountain View, CA, USA; https://analysis.ingenuity.com/pa). This system, a web-based interface, provides computational algorithms to identify and generate gene networks that are particularly enriched with genes of interest. The analysis is performed using the company's knowledge base, which contains gene–gene interaction findings collated from the literature. It also ranks networks by a score that takes into account the number of focus genes and the size of the networks.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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