Original Investigation | META-ANALYSIS

# Identification of Pathways for Bipolar Disorder
## A Meta-analysis

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**IMPORTANCE** Genome-wide investigations provide systematic information regarding the neurobiology of psychiatric disorders.

**OBJECTIVE** To identify biological pathways that contribute to risk for bipolar disorder (BP) using genes with consistent evidence for association in multiple genome-wide association studies (GWAS).

**DATA SOURCES** Four independent data sets with individual genome-wide data available in July 2011 along with all data sets contributed to the Psychiatric Genomics Consortium Bipolar Group by May 2012. A prior meta-analysis was used as a source for brain gene expression data.

**STUDY SELECTION** The 4 published GWAS were included in the initial sample. All independent BP data sets providing genome-wide data in the Psychiatric Genomics Consortium were included as a replication sample.

**DATA EXTRACTION AND SYNTHESIS** We identified 966 genes that contained 2 or more variants associated with BP at $P < .05$ in 3 of 4 GWAS data sets (n = 12 127 [5253 cases, 6874 controls]). Simulations using 10 000 replicates of these data sets corrected for gene size and allowed the calculation of an empirical $P$ value for each gene; empirically significant genes were entered into a pathway analysis. Each of these pathways was then tested in the replication sample (n = 8396 [3507 cases, 4889 controls]) using gene set enrichment analysis for single-nucleotide polymorphisms. The 226 genes were also compared with results from a meta-analysis of gene expression in the dorsolateral prefrontal cortex.

**MAIN OUTCOMES AND MEASURES** Empirically significant genes and biological pathways.

**RESULTS** Among 966 genes, 226 were empirically significant ($P < .05$). Seventeen pathways were overrepresented in analyses of the initial data set. Six of the 17 pathways were associated with BP in both the initial and replication samples: corticotropin-releasing hormone signaling, cardiac β-adrenergic signaling, phospholipase C signaling, glutamate receptor signaling, endothelin 1 signaling, and cardiac hypertrophy signaling. Among the 226 genes, 9 differed in expression in the dorsolateral prefrontal cortex in patients with BP: *CACNA1C*, *DTNA*, *FOXP1*, *GNG2*, *ITPR2*, *LSAMP*, *NPAS3*, *NCOA2*, and *NTRK3*.

**CONCLUSIONS AND RELEVANCE** Pathways involved in the genetic predisposition to BP include hormonal regulation, calcium channels, second messenger systems, and glutamate signaling. Gene expression studies implicate neuronal development pathways as well. These results tend to reinforce specific hypotheses regarding BP neurobiology and may provide clues for new approaches to treatment and prevention.

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It has been known for many decades that bipolar disorder (BP) is substantially influenced by genetic factors. Heritability of BP has been estimated at 80% to 85%.[1] Models of illness are most consistent with multifactorial inheritance. Searches for common variants of moderate effect in candidate gene studies of BP have not produced consistent results. Moreover, genome-wide association studies (GWAS) with thousands of samples have not provided evidence that such moderate effect variants exist.[2-4] However, common variants of small effect (odds ratio < 1.2) have been demonstrated and replicated.[4] Identification of individual gene variants has provided promising leads to the biology of BP, in particular the identification of genes coding for calcium channel subunits such as *CACNA1C*.[4,5] Current methods estimate that 38% of the phenotypic variance for BP may be explained by the cumulative impact of many common alleles of small effect.[6] Herein, we attempt to identify important components of that variance.

Rare variants of large effect, such as copy number variants, have been suggested to play a role in BP susceptibility.[7] Although such variants seem unlikely to be as prominent in BP as they appear to be in autism and schizophrenia, this area requires additional investigation. Genomic methods such as sequencing and methylation studies have been used in BP, but no definitive results are yet available.

In addition to specific single genes related to BP, panels of single-nucleotide polymorphisms (SNPs) that contain predictive value for BP have been identified.[8] Allele scoring methods have shown significant similarity not only between independent data sets of patients with BP but also between the genetic architectures of schizophrenia and BP.[9]

It has previously been demonstrated that additional information may be gleaned from GWAS by identifying biological pathways. Torkamani et al[10] took this approach in BP; their analysis of Wellcome Trust Case Control Consortium data using the program Metacore, which weights each gene based on the most significant SNP in that gene, suggested 5 pathways, including glutamate regulation of dopamine signaling and adrenergic mediation of cytoskeletal remodeling. Holmans et al[11] applied the method ALIGATOR (Association List Go Annotator; based on overrepresentation of pathway genes in lists of nominally significant SNPs) to Psychiatric Genomics Consortium 1 (PGC1) data on BP; using Gene Ontology (GO) categories, they identified hormonal action, transcriptional regulation, and autophagy as important in BP. O'Dushlaine et al[12] used the SNP ratio test (the ratio of nominally significant to nonsignificant SNPs in genes from a particular pathway) applied to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to identify cell adhesion molecules as likely to be involved in BP. Pedroso et al[13] used genewise analysis of 3 GWAS data sets combined with brain expression data to identify specific genes of interest; they separately identified gene sets involved in particular protein interaction subnetworks, specifying association with networks involved with neural transmission, Wnt signaling, and Notch signaling. Le-Niculescu et al[14] used convergent functional genomics, which combines GWAS data with brain expression data and animal model results. Their results emphasize growth factors, neurotransmitters, clock genes and transcription factors, neuronal metabolism, and neuronal connectivity as central mechanisms in BP. In the article by the Psychiatric GWAS Consortium Bipolar Disorder Working Group,[4] pathway analysis identified a group of calcium channel subunits as being putatively involved in BP vulnerability.

Our study differs from these in some or all of the following ways: (1) targets genes that show modest but consistent evidence of association across multiple individual data sets; (2) uses an empirical function to correct for gene size; (3) uses the extensively curated Ingenuity database of canonical pathways; (4) uses the gene set enrichment analysis (GSEA)-SNP method for pathway verification; and (5) compares results with gene expression results from an independent study of postmortem dorsolateral prefrontal cortex (DLPFC) from patients with BP. We used 4 individual GWAS data sets for our initial sample and an independent collection of samples from the PGC as a replication data set. To study brain-relevant pathophysiology of BP, we integrated a meta-analysis of gene expression studies from the Stanley Medical Research Institute (SMRI) microarray collection[15] with the results of pathway analysis.

## Methods

### Association Studies

#### Ascertainment and Assessment

Each of the 4 individual data sets has been previously described and GWAS results have been published.[2,3,16,17] Recruitment and screening of participants have been described for the Genetic Association Information Network,[16] the Systematic Treatment and Enhancement Program for Bipolar Disorder,[17] the German Genome Research Network,[2] and the Wellcome Trust Case Control Consortium.[3] Some of the control samples were used in both the Genetic Association Information Network and Systematic Treatment and Enhancement Program for Bipolar Disorder original analyses, and this overlap was modeled in the simulations described later.

Participants in the replication sample have been described previously.[4] Briefly, patients with BP type I (or a small fraction of patients with BP type II) mood disorder were ascertained through treatment facilities and evaluated with a structured interview (Structured Clinical Interview for *DSM-IV*, Diagnostic Interview for Genetic Studies, or Schedule for Affective Disorders and Schizophrenia). Studies at each site were approved by local institutional review boards, and participants gave written informed consent for genetic studies and for data sharing. Characteristics of the initial and replication samples are shown in **Table 1**.

#### Molecular Methods

Participants in the 4 initial studies were genotyped using Affymetrix or Illumina microarrays, testing 500 000 to 1 million SNPs across the genome. A search strategy was used to identify and count SNPs of interest within genes in each of the 4 sets of GWAS results, with gene boundaries defined by the National Center for Biotechnology Information Gene database and extended by 10 kilobases (kb) in the 5′ and 3′

directions. For subsequent consideration, genes were required to have at least 2 SNPs with $P < .05$ in at least 3 of the 4 initial GWAS.

### Simulations

We performed simulation studies to predict the global empirical false-positive error rate of this approach as a function of the number of SNPs typed in that gene, which is highly correlated with gene size. Genes containing more genotyped SNPs have a higher false-positive error rate unless corrected. Twenty-five genes of various lengths were selected to represent the range of gene size, with overrepresentation of genes of 5 to 200 kb to improve precision for that range (eTable in Supplement). We simulated genotype data for the 4 GWAS samples for these 25 genes using the software Hap-Sample.[19] Under the null hypothesis of no association, HapMap CEU samples were selected to mimic the sample size of case and control participants for each GWAS. The resampling approach takes into account the linkage disequilibrium structure of each gene. We also mimicked shared control samples between the Genetic Association Information Network and Systematic Treatment and Enhancement Program for Bipolar Disorder GWAS. Based on 10 000 replicates, we predicted the required number of SNPs at $P < .05$ for each of the 25 genes to satisfy a gene-specific false-positive error rate of 5%. We then fit a linear regression model to predict the mean SNP number per gene needed for empirical significance for each of the 966 genes as a function of the maximum number of SNPs for that gene in the 4 studies (eFigure 1 in Supplement). For example, a larger gene might require 15 to 20 SNPs at $P < .05$ in 3 of 4 data sets to reach empirical significance.

### Pathway Identification

The final list of 226 genes was run on a standard Ingenuity Pathway Analysis (Ingenuity Systems Inc). All canonical pathways with a nominal significance of $P < .05$ were included in subsequent analyses. Genes driving the pathway results are those in the list of 226 that overlap with the predesignated list of genes in each Ingenuity canonical pathway.

### Pathway Testing

Canonical pathways identified in the Ingenuity Pathway Analysis were tested in an independent replication data set composed of samples in PGC2 (all PGC2 data sets with complete GWAS information reported by the Psychiatric GWAS Consortium Bipolar Disorder Working Group[4] plus the Thematically Organized Psychosis Sample 7 data set). Imputation to generate a common SNP map for the PGC2 studies was performed using Impute[20] and the Markov Chain Haplotyping (MACH) algorithm[21] to provide a final data set of 2.5 million SNPs. For computational tractability, a subset of these SNPs corresponding to the Affymetrix version 6.0 platform was used for further analyses. Pathway testing was performed using the GSEA-SNP method,[22] which compares the rank order of the set of SNPs in the genes of the pathway among all SNPs in the PGC2 GWAS results with the null rank order distribution. A significant shift in the rank order pattern was considered evidence of association at the pathway level. The false discovery rate (FDR) was estimated against a general pathway set including pathways from GO, KEGG, and other sources. In each case, the individual Ingenuity pathway of interest was included in the general pathway set to calculate the q value.

**Table 1. Genome-Wide Association Studies in Bipolar Disorder Included in Pathway Analysis**

| Sample | Cases, No. (n = 8760) | Controls, No. (n = 11 763) |
|---|---|---|
| Initial sample | 5253 | 6874 |
| GAIN EA[a] | 1152 | 1152 |
| GGRF | 772 | 876 |
| STEP-BD/UCL[a] | 1461 | 2008 |
| WTCCC | 1868 | 2838 |
| Replication sample | 3507 | 4889 |
| Fran | 451 | 1631 |
| Ftg2[b] | 1914 | 1114 |
| Sw34 | 825 | 2084 |
| TOP7[c] | 317 | 60 |

Abbreviations: Fran, French National Sample[4]; GAIN EA, Genetic Analysis Information Network European-American sample[15]; GGRF, German Genome Research Foundation[2]; STEP-BD/UCL, Systematic Treatment Enhancement Program for Bipolar Disorder/University College London[17]; Sw34, Swedish National Samples 3 and 4[4]; TOP7, Thematically Organized Psychosis Sample 7; WTCCC, Wellcome Trust Case Control Consortium.[3]

[a] There is overlap between the GAIN EA control sample and the STEP-BD control sample (both used the National Institute of Mental Health Genetics Initiative control sample described by Sanders et al[18]; the overlap between the 2 groups was modeled in the simulations carried out for this study).

[b] Combination of Fast STEP2 (a second sample from the Systematic Treatment Enhancement Program for Bipolar Disorder) and TGEN (translational genomics sample from the Bipolar Genome Study), with both samples using the same control group.[4]

[c] Sample is from Norway and was submitted by Ole A. Andreassen, MD, PhD, to the Psychiatric Genomics Consortium Bipolar Group.

## Gene Expression in DLPFC of Patients With BP

A prior meta-analysis[15] of DLPFC (Brodmann area 46) samples using the SMRI microarray collection was queried for expression differences in BP samples as compared with controls (https://www.stanleygenomics.org/). The SMRI microarray collection was conducted using the same 105 DLPFC RNA samples (extracted at SMRI) and then processed at 6 independent laboratories by microarray analysis. The demographic and sample characteristics for respective BP cases and controls are as follows: n = 34 and 35; 16 (47.1%) and 26 (74.3%) male; mean (SD) age, 44.1 (7.7) and 45.4 (10.7) years; mean (SD) postmortem interval, 38.6 (18.2) and 29.4 (12.9) hours; and mean (SD) pH, 6.4 (0.3) and 6.6 (0.3).

Before meta-analysis of individual genes, quality-control filters based on Affymetrix metrics and principal component analysis were used to remove statistical outliers. Both within- and cross-study normalizations were used such that the median expression value for each study was equal to 100. In the meta-analysis, expression was compared between BP and controls using linear regression models on a gene-by-gene basis adjusting for the demographic terms that met the criteria for significance for that gene (pH, sex, age, RNA quality, postmortem interval, disease severity, drug use, smoking, and other

**Box. List of 226 Genes or Loci Associated With Bipolar Disorder at Empirical *P*<.05**

Gene names in bold are those genes driving the pathway results; gene names underlined are those genes also significant in the gene expression studies.

*A2BP1, AAA1, ABLIM1, ACSS3, ADAMTS12, ADAMTS19, ADAMTS2, AGBL1, AGBL4,* ***AKAP12****, ANK3, ANKFN1, ANKS1B, ANO3,* ***ARHGEF3****, ARL15, ASAP2, ASB18, ATXN1, AUTS2, B3GALTL, BAI3, BNC2, C10orf11, C14orf101, C21orf34, C6orf105, C8orf34, CA10,* ***CACNA1C****, CACNA2D3, CADM2, CAMTA1, CDH18, CDKAL1, CGNL1, CHST11, CMIP, CMYA5, CNBD1, CNTN3, CNTN6, CNTNAP2, COL13A1, COL1A2, COL28A1,* ***CREBBP****, CSMD1, CTNNA3, CYP4Z1, CYTSB, DCC, DGKB, DGKH, DGKI, DIS3L2, DLG2, DOCK2, DPP10, DTNA, DYRK1A, ELMO1, ELOVL2, EML1, ENPP2, EYS, FAM107B, FAM171A1, FAM184A, FBN2, FBXL17, FBXO18, FGF12, FHOD3, FLJ30838, FLJ41278, FLJ46010, FOXN3, FOXP1, FRMD6, FRY, FUT9, FYB, GFRA1,* ***GLI3****,* ***GNG2****, GNPTAB, GOLSYN, GPC5, GPC6, GPR1, GREM2,* ***GRM1****,* ***GRM7****, HHAT, HTR2A, IQGAP2, ISPD, ITGBL1, ITIH5,* ***ITPR2****, KCND2, KCNMA1, KIAA1797, KIFAP3, KIRREL3, LAMC2, LASS6, LGMN, LHPP, LMBR1, LOC100129633, LOC100132891, LOC100288428, LOC100505875, LOC100506027, LOC100506072, LOC100506380, LOC100506403, LOC100507421, LOC400794, LOC642924, LOC643542, LRP2, LRRC16A, LRRC3B, LRRTM4, LSAMP, MAD1L1, MAML3, MAN1C1, MAST4, ME1,* ***MEF2C****, MGC27382, MKL1, MPP7, MTHFD1L, MYBPC1, MYO5B, MYOM2, NALCN, NAV2, NCAM1, NCOA2, NOS1AP, NPAS3, NPSR1, NRXN3, NT5DC1, NTRK3, ODZ4, OTUD7A, PAK7, PALLD, PAN3, PCGF5, PCNXL2, PCSK2,* ***PDE7B****, PDZRN4, PHACTR1, PHF21A, PHF21B,* ***PLA2R1****,* ***PLCE1****,* ***PLCG2****,* ***PLD1****, PLEKHG1, POU2F1, PPFIBP1, PPP1R1C,* ***PPP2R5E****, PTGIS, PTPRR, PTPRT, RALGAPA2, RBPMS, RFX2, RGS6, RGS7, ROR1, RP1-210I8.1, RP3-398D13.1, SATB2, SEMA3C, SEMA5A, SEZ6L, SGCG, SGMS1, SHANK2, SIAH3, SIK3, SLC10A7, SLC2A13, SLIT3, SMG6, SNTB1, SNX29, SORCS2, SPATS2L, SPTLC3, SRGAP3, ST7, STK24, STK4, SYN3, SYNE1, SYT14, tcag7.1213, TCF7L1, THSD4, THSD7A, TMEM108, TRDN, TRPV3, TSPAN18, TULP4, ULK4, VATIL, VAV3, WDFY4, WDR72, ZCCHC17, ZNF236, ZNF385B*

covariates [up to 23 demographic effects]). The fold change and *P* values for each gene were condensed, when appropriate, from multiple probe sets on the microarray platforms by using all probes from each microarray study independently. The consensus fold changes and associated 95% confidence intervals and *P* values were reported as a weighted combination of the individual fold changes and standard errors of the 6 studies.

From this differential expression meta-analysis of the 6 gene expression studies, there were 919 genes nominally significant in BP compared with controls after adjustment for covariates.

A step-by-step summary of the methods is included in the eBox in the Supplement.

## Results

From the 4 data sets in our initial sample (Table 1), we identified 966 genes with at least 2 SNPs at $P < .05$ in at least 3 of 4 data sets. Simulation was performed on 25 individual genes using simulated data sets representing each of these 4. Results of the simulation are shown in the eTable in the Supplement, and the resulting regression function is shown in eFigure 1 in the Supplement. Using this regression model, 226 empirically significant genes were detected and chosen for the pathway gene set analysis (**Box**). By application of the binomial theorem (and an estimate of 20 000 genes in the human genome), we would expect that 11 of these 226 genes are likely to be false positives (a proportion of 0.048) and the others are likely to be true positives.

The pathway analysis identified 17 nominally significant canonical pathways (**Table 2** and **Table 3**). These were tested in a replication data set by GSEA along with the total number of genes in each of the pathways of interest. Of the 17 pathways, 6 showed significant association: corticotropin-releasing hormone (CRH) signaling, cardiac β-adrenergic signaling, phospholipase C (PLC) signaling, glutamate receptor signaling, endothelin 1 signaling, and cardiac hypertrophy signaling. The 6 significant pathways are shown in Table 2 and in eFigures 2-7 in the Supplement, with the identified genes highlighted.

It was observed that the 6 significant pathways were tagged in the Ingenuity data set by only 16 of the 226 genes (Box and **Table 4**): *ARHGEF3*, *CREBBP*, *GNG2*, *ITPR2*, *PLCG2*, *PLD1*, *AKAP12*, *CACNA1C*, *PDE7B*, *PPP2R5E*, *GRM1*, *GRM7*, *GLI3*, *MEF2C*, *PLA2R1*, and *PLCE1*. Many of these genes were present in multiple pathways. Because each of these pathways was tagged by multiple genes and because every SNP in every gene in each pathway was tested by GSEA in an independent data set, we do not expect that any of the 6 pathways is a false positive.

The list of 226 genes was compared with the list of 919 genes that differed in expression between DLPFC samples from patients with BP and controls. Nine genes were found to overlap: *CACNA1C*, *DTNA*, *FOXP1*, *GNG2*, *ITPR2*, *LSAMP*, *NPAS3*, *NCOA2*, and *NTRK3* (Box). We note that 3 of these genes are also in the list of 16 genes driving the pathway results described earlier (*GNG2*, *CACNA1C*, and *ITPR2*). The concordance of 3 genes in the 2 lists is greater than would be expected by chance (odds ratio observed/expected > 4.43, goodness-of-fit $\chi^2$ $P < .005$). If we restrict the list of 919 brain-dysregulated genes to those meeting FDR criteria (n = 26) and then test for overlap with the 226 genes from GWAS, the single gene in the 2 lists is *CACNA1C*.

## Discussion

Using GWAS results from a large sample of patients with BP with full genome-wide SNP data and imputation (a total of 8760 independent cases and 11 763 independent controls), we identified 6 significantly overrepresented pathways: glutamate receptor signaling, PLC signaling, CRH signaling, endothelin 1 signaling, cardiac β-adrenergic signaling, and cardiac hypertrophy signaling (Table 2). All of these pathways involve signaling, including G proteins and second messenger systems, and all except 1 (cardiac β-adrenergic signaling) involve nuclear gene regulation. The pathway with the strongest evidence statistically is the CRH signaling pathway. The GSEA-SNP analysis also showed the highest proportion of pathway genes significantly overrepresented in GWAS results in this pathway (61.5%).

Table 2. Biological Pathways Significant in Both Initial and Replication Data Sets and Their Association With Bipolar Disorder

| Pathway | *P* Value in Initial Data Set | Genes Driving Pathway Results | *P* Value in Replication Data Set | FDR | Genes at FDR <0.05, No./Total Genes Tested, No.[a] |
|---|---|---|---|---|---|
| Phospholipase C signaling | .005 | *PLCE1*, *ITPR2*, *PLCG2*, *CREBBP*, *MEF2C*, *ARHGEF3*, *GNG2*, *PLD1* | .005 | 0.029 | 63/154 |
| Cardiac β-adrenergic signaling | .02 | *AKAP12*, *PDE7B*, *CACNA1C*, *GNG2*, *PPP2R5E* | .001 | 0.009 | 49/99 |
| Glutamate receptor signaling | .03 | *GRM7*, *GRM1*, *GNG2* | .01 | 0.035 | 27/49 |
| Corticotropin -releasing hormone signaling | .04 | *GLI3*, *ITPR2*, *PLCG2*, *MEF2C* | .001 | 0.007 | 40/65 |
| Endothelin 1 signaling | .04 | *PLCE1*, *ITPR2*, *PLCG2*, *PLA2R1*, *PLD1* | .02 | 0.037 | 66/155 |
| Cardiac hypertrophy signaling | .045 | *PLCE1*, *PLCG2*, *CREBBP*, *CACNA1C*, *MEF2C*, *GNG2* | .02 | 0.040 | 78/195 |

Abbreviation: FDR, false discovery rate.

[a] All genes with adequate single-nucleotide polymorphism coverage in the genome-wide association studies were tested in the gene set enrichment analysis. Genes with a single-nucleotide polymorphism among the top 5% of the gene set enrichment analysis results were designated as significant.

Table 3. Biological Pathways Significant in the Initial Data Set Only and Their Association With Bipolar Disorder

| Pathway | *P* Value in Initial Data Set | Genes Driving Pathway Results |
|---|---|---|
| Phospholipid degradation | <.001 | *DGKH*, *PLCE1*, *PLCG2*, *PLA2R1*, *DGKB*, *DGKI*, *PLD1* |
| Glycerophospholipid metabolism | <.001 | *DGKH*, *PLCE1*, *PLCG2*, *PLA2R1*, *ELOVL2*, *DGKB*, *DGKI*, *PLD1* |
| Protein kinase A signaling | <.001 | *AKAP12*, *HHAT*, *PLCE1*, *PDE7B*, *GLI3*, *ITPR2*, *PLCG2*, *CREBBP*, *DCC*, *TCF7L1*, *GNG2* |
| CREB signaling in neurons | .004 | *GRM7*, *PLCE1*, *GRM1*, *ITPR2*, *PLCG2*, *CREBBP*, *GNG2* |
| Axonal guidance signaling | .006 | *SLIT3*, *SRGAP3*, *GLI3*, *NTRK3*, *SEMA5A*, *DCC*, *PAK7*, *SEMA3C*, *GNG2*, *SHANK2*, *ABLIM1* |
| Neuropathic pain signaling in dorsal horn neurons | .006 | *GRM7*, *PLCE1*, *GRM1*, *ITPR2*, *PLCG2* |
| Synaptic long-term depression | .02 | *GRM7*, *GRM1*, *ITPR2*, *PLA2R1*, *PPP2R5E* |
| Fcγ receptor–mediated phagocytosis in macrophages and monocytes | .03 | *VAV3*, *DGKB*, *FYB*, *PLD1* |
| Sonic hedgehog signaling | .04 | *GLI3*, *DYRK1A* |
| Glycine, serine, and threonine metabolism | .047 | *PLCE1*, *PLCG2*, *ELOVL2* |

Abbreviation: CREB, cyclic adenosine monophosphate response element–binding protein.

Table 4. Function of Genes Driving the Pathway Results

| Gene | Function |
|---|---|
| *CREBBP* | Transcription factor |
| *GNG2* | Guanine nucleotide binding protein |
| *ITPR2* | IP3 receptor |
| *PLCG2* | Production of IP3 and DAG |
| *PLD1* | Phospholipase producing choline |
| *AKAP12* | Anchor/scaffold protein, binds PKA |
| *CACNA1C* | Calcium channel subunit |
| *PDE7B* | Downregulation of cAMP and cGMP |
| *PPP2R5E* | Protein phosphatase |
| *GRM1* | Glutamate receptor involved in activation of PLC |
| *GRM7* | Glutamate receptor involved in inhibition of cAMP |
| *GLI3* | Zinc finger transcription factor |
| *MEF2C* | Myocyte enhancer |
| *PLA2R1* | PLA2 receptor involved in clearance |
| *PLCE1* | Phospholipase that produces IP3 and DAG |
| *ARHGEF3* | Activates 2 Rho GTPases, brain expressed in UniGene |

Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; GTPases, guanosine triphosphatases; IP3, inositol triphosphate; PKA, protein kinase A; PLA2, phospholipase A2; PLC, phospholipase C.

These canonical pathways provide independently defined, functionally clustered sets of genes. We have highlighted (1) genes driving the pathway results and (2) genes implicated in studies of brain gene expression.

The genes driving the pathway results (n = 16) are listed in Table 4. They include 3 genes with dysregulated expression in BP frontal cortex: *CACNA1C*, *GNG2*, and *ITPR2*. They also include *DTNA* (dystrobrevin, part of the dystrophin complex), *FOXP1* (forkhead box transcription factor, involved in language impairment), *LSAMP* (limbic system-associated membrane protein, involved in cell adhesion), *NPAS3* (transcription factor involved in neurogenesis), *NCOA2* (activates nuclear hormone receptors), and *NTRK3* (neurotrophic tyrosine receptor kinase, involved in neuron migration).

The CRH signaling pathway is an index of the hypothalamic-pituitary-adrenal system (eFigure 2 in Supplement). This stress-responsive system is consistently dysregulated in studies of mood disorder. Well-described hypothalamic-pituitary-adrenal abnormalities associated with mood disorders include increased secretion of cortisol over 24 hours[23] and decreased suppression of cortisol by dexamethasone in about 50% of patients with major depressive disorder[24] and specifically in BP.[25] Increased plasma cortisol responses to the dexamethasone/CRH test have been associated with euthymic BP.[26] Molecules driving this pathway include PLC γ, inositol trisphosphate (IP3) receptor, and myocyte enhancer factor 2 as well as the transcription factor GLi3, which has notable influence on development and is also involved in corticotropin synthesis.

The cardiac β-adrenergic signaling pathway (eFigure 3 in Supplement) and the cardiac hypertrophy signaling pathway (eFigure 7 in Supplement) are both driven by the presence of an L-type calcium channel along with G-protein–coupled second messenger systems. L-type calcium channels have been

clearly implicated in meta-analysis of the BP GWAS data,[4] and their prominence in our analysis provides validation of our methods. Carman and Wyatt[27] summarized data showing that pharmacological or dietary manipulation of calcium alters mood in patients with BP. Dubovsky and Franks[28] described early data on the treatment of mania with calcium antagonists; more recent data have been summarized by Casamassima et al.[29] Grunze et al[30] suggested that lamotrigine may act through alteration of calcium currents in the cell. Variants in the calcium channel protein TRPM2 have been associated with BP.[31] Variants in *P2RX7*, which codes for a calcium-stimulated adenosine triphosphatase, are also associated with BP.[32] Differences in subcortical morphology have been reported in patients with BP carrying the *CACNA1C* risk variant compared with controls, and there is also evidence for reduced corticolimbic connectivity in carriers.[33] Calcium channel dysregulation in BP was also supported by the convergent functional genomics method.[14] The involvement of calcium channels in the genetic predisposition to BP is the single most consistent finding in BP genetic studies to date.

Molecules driving the association with the CRH signaling pathway are also driving association with the PLC signaling pathway (eFigure 4 in Supplement), including PLC γ, IP3 receptor, and myocyte enhancer factor 2. In addition, PLC may produce diacylglycerol and IP3. Lithium blocks the conversion of inositol phosphate to inositol and leads to the accumulation of IP3. The IP3 system has been related to lithium therapeutic activity.[34] Williams et al[35] proposed that inositol was involved in the therapeutic actions of valproic acid and carbamazepine as well as lithium. The endothelin 1 signaling pathway (eFigure 6 in Supplement) includes several phospholipases with major activity on second messenger systems and intracellular metabolism in addition to PLC. These are phospholipase A2, which activates the arachidonic acid cascade, and phospholipase D, which hydrolyzes phosphatidylcholine to produce phosphatidic acid and choline, the precursor for acetylcholine. These phospholipases are important not only in endothelial cells but also in neuronal cells. They may play a role in inflammatory responses,[36] and inflammation has been implicated in the pathophysiology of BP in numerous studies.[37]

As the major excitatory neurotransmitter in the mammalian central nervous system, glutamate signals are relayed by ionotropic and metabotropic (G-protein–coupled) receptors from the cell membrane through various second messenger systems (eFigure 5 in Supplement). One of the strongest candidate gene findings in BP has been the association with D-amino acid oxidase activator (DAOA, also known as G72), which deactivates *N*-methyl-D-aspartate receptors by degrading serine.[38] A functional variant in this gene has been associated with reduced cortical thickness in schizophrenia.[39] Riluzole, a glutamate-modulating agent, has shown preliminary antidepressant results in an open-label study in combination with lithium in patients with BP in the depressed state.[40]

We believe that our method of pathway identification has certain advantages. We consider evidence from multiple GWAS and deliberately focus on associations that are modest but consistent, a strategy that has proven successful in prior studies.[4,8] We correct for gene size using a permutation procedure to calculate empirical *P* values. We also incorporate FDR into our pathway assessment procedure. Our results are readily interpretable at the gene and pathway levels. To our knowledge, our data set, which includes most of the PGC1 and PGC2 BP data sets, incorporates and expands on all GWAS data sets used in previously published BP pathway studies, except for the PGC article itself.[4] We note an emphasis on glutamate mechanisms,[10,14] hormonal action,[11] and calcium signaling[4] in our results as well as others. One area not emphasized in our results but noted in others[12,14] is that of mechanisms of neuronal growth and cell adhesion molecules. This area is prominent in the genes that overlap with gene expression results (see earlier) in this study.

Our study has limitations. Our method identifies genes with abnormalities in *cis* regulation (within the gene or promoter) in BP but not genes with abnormalities in *trans* regulation (regulatory changes at distant sites). Future studies should be able to use methods and databases that will include *trans*-regulated abnormalities. We have chosen a threshold (≥2 SNPs at $P < .05$ in 3 of 4 data sets) that minimizes false-positive results and includes a sufficient number of true-positive results to support pathway detection. This threshold provides better power to detect larger genes than smaller genes (<25-50 kb), which are underrepresented in the list of 226 we have used for tagging pathways. The pathways themselves, however, include small and medium-sized genes as well as larger genes. Canonical pathways, while having the advantage of providing a predetermined gene list, are based on the existing neurobiological literature as curated by Ingenuity Systems Inc. As data sets expand and other pathway sources (KEGG, GO) are used, we expect additional biological mechanisms to be discovered and present results to be refined. It is possible that some associated variants may be markers for true susceptibility variants in nearby genes. We have minimized (but not eliminated) this possibility by using GSEA-SNP, which tests all the SNPs in each selected gene, not just those that passed the threshold for gene identification. Functional studies and insights from systems biology will be required to determine which genes and pathways are truly relevant to BP and what mechanisms are involved. The comparison between results from GWAS and gene expression studies using postmortem brain samples must be interpreted carefully because of differences in subject ascertainment and probable nongenetic causes of some gene expression changes.

## Conclusions

In summary, 17 pathways were identified in an initial screen of GWAS data from 4 samples totaling 5253 BP cases and 6874 controls, of which 6 pathways were verified in an independent set of 3507 cases and 4889 controls. The identification of these pathways was driven by genes involved in hormone regulation, calcium channel genes, genes involved in second messenger systems, and glutamate receptor genes. Nine genes involved in the pathway identification were also dysregulated in brain samples from patients with BP. In addition to the functions described earlier, these included genes that are involved in neuronal development.

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**Additional Information:** Genotype data from this article for the 10 257 samples can be obtained from the Center for Collaborative Genetic Studies on Mental Disorders in accordance with National Institute of Mental Health data release policies (https://zork5.wustl.edu//nimh/). Genotype data from the Wellcome Trust Case Control Consortium sample can be obtained from http://www.wtccc.org.uk/info/access_to_data_samples.html. Genotype data from the BOMA-Bipolar Study can be obtained by contacting Sven Cichon, PhD (sven.cichon@uni-bonn.de). Data and biomaterials from the National Institute of Mental Health Genetics Initiative Molecular Genetics of Schizophrenia control sample were collected by NorthShore University HealthSystem, Evanston, Illinois (supported by grant R01 MH59571 from the National Institute of Mental Health to Pablo V. Gejman, MD, collaboration coordinator, principal investigator) as part of a collaborative R01 application comprising 10 sites. From 2003 to 2006, the principal investigators and coinvestigators were the following: *NorthShore University HealthSystem, Evanston, Illinois* (grant R01 MH59571): Pablo V. Gejman, MD (collaboration coordinator, principal investigator), Alan R. Sanders, MD; *Emory University School of Medicine, Atlanta, Georgia* (grant R01 MH59587): Farooq Amin, MD (principal investigator); *University of California, San Francisco* (grant R01 MH60870): William F. Byerley, MD (principal investigator); *University of Iowa, Iowa City* (grant R01 MH59566): Donald W. Black, MD (principal investigator), Raymond R. Crowe, MD; *Washington University, St Louis, Missouri* (grant R01 MH60879): C. Robert Cloninger, MD (principal investigator); *University of Colorado, Denver* (grant R01 MH59565): Robert Freedman, MD (principal investigator), Ann Olincy, MD; *Stanford University, Palo Alto, California* (grant R01 MH61675): Douglas F. Levinson, MD (principal investigator); *Louisiana State University, New Orleans* (grant R01

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