

ORIGINAL RESEARCH ARTICLE

Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus

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Identification of the genetic bases for bipolar disorder remains a challenge for the understanding of this disease. Association between 76 candidate genes and bipolar disorder was tested by genotyping 90 single-nucleotide polymorphisms (SNPs) in these genes in 136 parent-proband trios. In this preliminary analysis, SNPs in two genes, brain-derived neurotrophic factor (BDNF) and the alpha subunit of the voltage-dependent calcium channel were associated with bipolar disorder at the $P < 0.05$ level. In view of the large number of hypotheses tested, the two nominally positive associations were then tested in independent populations of bipolar patients and only BDNF remains a potential risk gene. In the replication samples, excess transmission of the valine allele of amino acid 66 of BDNF was observed in the direction of the original result in an additional sample of 334 parent-proband trios (T/U=108/87, $P=0.066$). Resequencing of 29 kb surrounding the BDNF gene identified 44 additional SNPs. Genotyping eight common SNPs identified three additional markers transmitted to bipolar probands at the $P < 0.05$ level. Strong LD was observed across this region and all adjacent pairwise haplotypes showed excess transmission to the bipolar proband. Analysis of these haplotypes using TRANSMIT revealed a global P value of 0.03. A single haplotype was identified that is shared by both the original dataset and the replication sample that is uniquely marked by both the rare A allele of the original SNP and a novel allele 11.5 kb 3'. Therefore, this study of 76 candidate genes has identified BDNF as a potential risk allele that will require additional study to confirm.

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Introduction

Bipolar disorder is an episodic illness classically characterized by extreme disturbance in mood including mania and depression with a lifetime prevalence of approximately 1%. Family and twin studies have demonstrated a strong genetic basis for this disease,^{2,3} yet classical genetic linkage analyses have not defined the relevant loci.

Strong evidence of linkage would clearly implicate regions of the chromosome to search for disease-causing alleles. However, the many published linkage studies in bipolar disorder have only yielded suggestive evidence of linkage and none have been consistently replicated. In early studies, several regions of the gen-

ome were strongly implicated.^{4,5} However, problems with incomplete penetrance, locus heterogeneity and an insufficient number of reliable meioses likely account for the inability to replicate and extend the results. Recent large-scale genetic linkage studies of bipolar disorder using non-parametric, allele-sharing methods have identified several interesting chromosomal regions including 4p16,^{6,7} 12q23–24,^{8–11} 21q22,^{12–15} 18q21,^{16,17} 18q22^{18,19} and the centromere of 18.²⁰

Although it is premature to draw any conclusions about these reported linkages until gene(s) for bipolar disorder are identified, several general observations can be made: no finding replicates in all data sets, the effect sizes are small, statistical significance has not reached genome-wide levels, and the regions identified are large (generally >20 cM) and difficult to approach by positional cloning. These observations are consistent with the hypothesis that bipolar disorder is caused by several genes each of which exerts a modest increase in relative risk.

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Compared with linkage analysis, association studies of comparable size have the power to detect common alleles of modest effect. They have been used successfully to identify genes contributing to several complex genetic diseases including *NIDDM*,^{21,22} Alzheimer's disease^{23,24} and thrombosis.²⁵ Recently, putative risk alleles have been put forth for two additional complex trait loci, *NIDDM1* and *IBD1* (Crohn's disease), after taking a positional candidate approach.^{26–28} Success in association studies requires plausible candidate genes, available SNPs in those candidate genes, high-throughput SNP genotyping technology, as well as DNA from well-phenotyped patients.

We have adopted a systematic approach to these issues by focusing on candidate genes with functional relevance to the disease in question identified through database searches of the neurobiological and neuropsychiatric literature. In this initial survey of 76 genes, we have genotyped primarily coding-region SNPs, focusing wherever available, on missense SNPs. Furthermore, we have used family-based controls and transmission disequilibrium testing to avoid a potential for the confounding effects of population admixture. In order to rule out the possibility that our observations resulted from random fluctuations in the data given the number of genes we have tested (multiple hypothesis testing), we have re-examined any nominally positive finding in independent patient samples.

Material and methods

Subjects

Hopkins samples Pedigrees were ascertained from inpatient and outpatient clinics in Maryland and Iowa. Most of the families have been previously described.^{16,17,29,30} The ascertainment criteria were: a treated bipolar I proband, at least two affected first-degree relatives and unilineal transmission.²⁹ Proband had the following diagnoses: 106 BP1, 26 BP2 and four schizoaffective-manic (SA-M). All BP2 and SA-M probands had a BP1 sibling and both siblings were used in the analyses. Diagnoses were established using the Research Diagnostic Criteria (RDC).³¹

NIMH sample DNA was obtained from the NIMH Genetics Initiative. Pedigree ascertainment and diagnoses were as described.³² Proband had the following diagnoses: 149 BP1, one BP2 and five SA-M. Diagnoses of BP1 and SA-M were established using DSM-III-R criteria and BP2 by the Research Diagnostic Criteria (RDC).³¹

UK sample Pedigrees were ascertained as described³³ from clinics in Britain. All probands were diagnosed with BP1 and the diagnoses were established using DSM-IV criteria.

Non-BP sample The sample is comprised of 333 Scandanavian parent-proband trios where the proband has either type 2 diabetes, impaired glucose tolerance,

or impaired fasting glucose as described.²² These patients were not screened for the presence or absence of bipolar disorder.

SNP identification

SNPs were identified from genomic DNA as described previously.³⁴ For those genes where genomic sequence was not available, the gene was amplified from RNA in an ethnically diverse panel of lymphoblastoid cell lines obtained from the Coriell cell repository. Approximately 50% of genes specific to the neuropsychiatry project could not be amplified from this source of RNA. To amplify those genes, brain RNA was obtained from the Stanley Foundation. Poly A+ RNA was purified using the Oligotex Direct mRNA kit (Qiagen, CA, USA). Complementary DNA was transcribed from the polyA+ RNA using 200 ng RNA, random hexamers (750 ng) and SuperScriptII RT (Invitrogen, CA, USA) in a reaction volume of 100 μ l containing 5 \times Superscript buffer, 50 nmol dNTPs, and 1 μ mol DTT. Each gene was divided into several primary transcripts of 1 kilobase. The primary transcripts were amplified in a volume of 10 μ l. Each reaction contained 10 \times PCR buffer II, 30 nmol MgCl₂, 2 nmol dNTPs, 1 U AmpliTaq Gold (Perkin-Elmer, MA, USA), and 2.5 nmol transcript specific primers. PCR conditions were as follows: 96°C \times 10 min followed by 35 cycles of 96°C \times 30 s, 59°C \times 30 s and 72°C \times 1 min. Each primary transcript was subdivided into overlapping 500 base pair fragments that were separately amplified in a volume of 18 μ l containing 10 \times PCR buffer II, 41.25 nmol MgCl₂, 3 nmol dNTPs, 1.5 U AmpliTaq Gold and 3.5 nmol fragment specific primers and amplified using the same PCR conditions. PCR products were prepared for sequencing using solid-phase reversible immobilization (SPRI) using Bangs Estapor SuperParamagnetic Microspheres (Bangs Laboratories, IN, USA, and Seradyn Uniform Microparticles, MN, USA) as described.³⁵ Sequencing was performed using BigDye Terminator Chemistry (Perkin-Elmer) on a capillary ABI 3700. SNP detection was as described previously.³⁴ Each fragment was sequenced in 32 individual DNA samples. SNP location and surrounding sequence can be found in the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>).

BDNF resequencing Twenty-nine kb of contiguous genomic sequence were resequenced from genomic DNA as described.³⁴ Sequence was obtained from the Human Genome Browser (<http://genome.ucsc.edu/>) from human BAC AC068488.2.13. Six patients with BP1 and two control DNAs obtained from the Coriell Cell Repository were resequenced. The bipolar patients used for resequencing were not included in the association analyses. Location of SNPs are marked with respect to the ATG of BDNF.

Genotyping methodologies

Genotyping was performed by either single-base extension with fluorescent resonance energy transfer (SBE-FRET),³⁶ single-base extension with fluorescence

polarization (SBE-FP)³⁷ using a modified protocol as described previously,²² or length-multiplexed single-base extension (LM-SBE)³⁸ with the following modifications. From 16–18 individual PCR amplicons were multiplexed rather than 50 amplicons. Secondary amplification for biotinylation of the PCR products was not performed. Rather, a single amplification was performed using both sequence-specific and universal biotinylation primers simultaneously. The PCR reaction contained AmpliTAQ Gold (4 U, Perkin Elmer), dNTPs (0.5 mM), MgCl₂ (4 mM), genomic DNA (5 ng), specific locus primer mix (0.1 μM final concentration of each primer), biotinylated-T3 and biotinylated-T7 (0.125 μM) in the supplied buffer in a final volume of 20 μl using the following PCR conditions (95°C × 9 min, 37 cycles of 94°C × 30 s, 55°C × 30 s, and 72°C × 30 s followed by a final extension of 72°C × 5 min). Primers used for genotyping can be found both on our website (<http://genome-wi.mit.edu/mpg/neuropsych/>) and as supplemental material at *Molecular Psychiatry*. Genotyping of SNPs in BDNF was performed by mass spectrometry as follows. Primers were designed using SpectroDESIGNER software (Sequenom, CA, USA) to have a T_m of 56–60 degrees with a mass range between 5000 and 8000 Da as described.³⁹ PCR amplification was performed as follows. Each reaction contained AmpliTAQ Gold (0.1 U, Perkin Elmer), dNTPs (0.2 mM), MgCl₂ (1.5 mM), genomic DNA (5 ng), locus specific primers (0.2 μM final concentration of each primer), in the supplied buffer in a final volume of 6 μl using the following PCR conditions (92°C × 9 min, 46 cycles of 94°C × 20 s, 56°C × 30 s, and 72°C × 30 s followed by a final extension of 72°C × 3 min). Following the PCR reaction dNTPs were removed by shrimp alkaline phosphatase (SAP) by adding 2 μl of SAP (0.3 U) in Thermosequenase buffer and incubating at 37°C × 20 min, followed by inactivation at 85°C × 5 min. The homogeneous MassEXTEND reaction was performed by adding to the SAP-treated product 2 μl of a solution containing ddNTPs (0.50 μM each), dNTPs (0.50 μM each), MassEXTEND primers (0.6 nM), Thermosequenase buffer (Pharmacia), and Thermosequenase (0.063 U μl⁻¹). The termination mix of ddNTPs and dNTPs was predicted by the SpectroDESIGNER software and was specific for each SNP genotyped. The reactions were thermocycled under the following conditions: 94°C × 2 min, 40 cycles of 94°C × 5 s, 40°C × 5 s, 72°C × 5 s, then 72°C × 5 min. SpectroCLEAN, a proprietary ion-exchange resin, is added to remove salt. The sample plate is rotated for 4 min at RT and then centrifuged for 1 min at 1400 rpm. Using a 24-pin SpectroPOINT, 7 nl of each reaction was then loaded onto each position of a 384-well SpectroCHIP preloaded with 7 nl of matrix (3-hydroxypicolinic acid). SpectroCHIPS were analyzed in automated mode by a MassARRAY RT mass spectrometer (Bruker-Sequenom).³⁹ The resulting spectra were analyzed by SPECTROTYPYPER software (Sequenom) after baseline correction and peak identification. DNA samples for which the SPECTROTYPYPER software could not define a genotype were subjected to clustering

analysis by plotting the signal to noise ratio of the genotype-known and genotype-undetermined samples for each SNP. If the undetermined genotypes fell within clusters of known genotypes, genotypes were then obtained. The minimum acceptable signal to noise ratio was 5:1. Clusters were verified by two independent observers.

Statistical analysis

Genotyping data were assessed in the following manner. SNPs were used for TDT analysis only if they met the following criteria: (1) greater than 90% of attempted genotypes were successful; (2) parental alleles were in Hardy–Weinberg equilibrium; and (3) zero or one Mendelian inheritance error were detected. Several methods of genotyping were used for this study. For those SNPs genotyped by more than one method (23%), a consensus genotype was obtained and used for TDT analysis. Significance of single and two-marker haplotypes were analyzed using the Perm1 and Perm2 options in Genehunter 2.0. Global analysis of multimarker haplotypes was performed using TRANSMIT v2.5.2.¹ For these analyses the minimum haplotype frequency was set at 2%.

Results

Association analysis of 90 SNPs

As part of a larger ongoing study to identify genes that confer risk for neuropsychiatric disorders, we have identified a list of candidate genes covering several etiologic hypotheses of bipolar disorder. These include genes that play a role in neurochemistry, intracellular signaling, transcription, and neural development. SNP discovery from genomic DNA in a subset of these genes was performed by variant-detector arrays and denaturing high performance liquid chromatography and has been reported.³⁴ Additional genes for which genomic sequence was unavailable were amplified from RNA using RT-PCR with subsequent direct resequencing of the coding region.

We have genotyped 90 SNPs in 76 candidate genes in a sample of 136 parent-proband trios (Hopkins sample) (Table 1). The choice of SNPs for genotyping was governed by the following rationale. We reasoned that missense SNPs, because they result in amino acid changes, are those that are most likely to affect function, and thus we focused on missense SNPs when available. If these SNPs are the causal mutations, then genotyping will directly identify an association with the underlying disease or phenotype. However, because blocks of the genome are consistently inherited together in a population (linkage disequilibrium), nearby linked SNPs can also reveal an association to the underlying causative SNP. In order to take advantage of possible linkage disequilibrium, we genotyped common silent SNPs in some genes, especially when missense SNPs were unavailable. Since SNPs found in regulatory regions have the potential to control the level of gene expression, some SNPs identified in the 5' or 3' untranslated regions were also genotyped.

Table 1 Association study of 90 SNPs in bipolar disorder

| Whitehead ID | Gene | Location | Allele 1 trans | Allele 2 trans | chi-sq | P-value | Allele 1 | Allele 2 | AA | Allele 1 frequency | Allele 2 frequency |
|--------------|---|--------------|-------------------|-------------------|--------|---------|----------|----------|--------|-----------------------|-----------------------|
| WIAF-10293 | A2M, alpha-2-macroglobulin | 12p13.31 | 57 | 57 | 0.00 | 1.000 | G | A | V1000I | 0.68 | 0.32 |
| WIAF-12841 | ABAT, 4-aminobutyrate aminotransferase | 16p13.3 | 69 | 59 | 0.78 | 0.377 | C | A | V328V | 0.59 | 0.41 |
| WIAF-13188 | ACCN1, amiloride-sensitive cation channel 1, neuronal | 17q11.2-q12 | 46 | 42 | 0.18 | 0.670 | G | C | L94L | 0.78 | 0.22 |
| WIAF-12090 | ADAR, adenosine deaminase, RNA-specific | 1q21.1-q21.2 | 44 | 54 | 1.02 | 0.312 | A | G | K384R | 0.70 | 0.30 |
| WIAF-10095 | ANXA4, annexin A4 | 2p13.3 | 49 | 41 | 0.71 | 0.399 | C | T | T87M | 0.79 | 0.21 |
| WIAF-11938 | ASMT, acetylserotonin O- methyltransferase | Xpter-p22.32 | 20 | 20 | 0.00 | 1.000 | C | T | D287D | 0.92 | 0.08 |
| WIAF-14666 | ASTN1, astrotactin | 9q33.1 | 35 | 28 | 0.78 | 0.378 | G | C | nc | 0.83 | 0.17 |
| WIAF-10753 | BDNF, brain-derived neurotrophic factor | 11p14.1 | 53 | 34 | 4.15 | 0.042 | G | A | V66M | 0.83 | 0.17 |
| WIAF-14271 | BMP2, bone morphogenetic protein 2 | 20p13 | 44 | 48 | 0.17 | 0.677 | T | A | R190S | 0.66 | 0.34 |
| WIAF-12769 | BMP4, bone morphogenetic protein 4 | 14q22.2 | 63 | 57 | 0.30 | 0.584 | T | C | V152A | 0.51 | 0.49 |
| WIAF-12870 | BZRP, benzodiazepine receptor (peripheral) | 22q13.31 | 49 | 54 | 0.24 | 0.622 | G | A | A147T | 0.71 | 0.29 |
| WIAF-14629 | CACNA1B, calcium channel, voltage-dependent, L type, alpha 1B | 9q34.3 | 66 | 49 | 2.51 | 0.113 | T | C | F1861F | 0.71 | 0.29 |
| WIAF-13171 | CACNA1C, voltage-dependent calcium channel $\alpha 1$ subunit | 12p13 | 25 | 10 | 6.43 | 0.011 | C | T | N1735N | 0.92 | 0.08 |
| WIAF-12310 | CACNA1E, calcium channel, voltage gated, alpha-1E | 1q25-q31 | 60 | 57 | 0.08 | 0.782 | T | C | H1317H | 0.70 | 0.30 |
| WIAF-12322 | CACNB2, calcium channel, voltage-dependent, beta 2 | 10p12 | 28 | 35 | 0.78 | 0.378 | C | T | Y512Y | 0.83 | 0.17 |
| WIAF-13195 | CCKBR, cholecystokinin B receptor | 11p15.4 | 9 | 14 | 1.09 | 0.297 | G | A | V125I | 0.95 | 0.05 |
| WIAF-12119 | CHRNA1, cholinergic receptor, nicotinic, alpha polypeptide 1 | 2q24-q32 | 16 | 18 | 0.12 | 0.732 | C | T | H320H | 0.93 | 0.07 |
| WIAF-14424 | CHRNA4, cholinergic receptor, nicotinic, alpha polypeptide 4 | 20q11.23 | 20 | 21 | 0.02 | 0.876 | C | T | D213D | 0.90 | 0.10 |

(Continued)

Table 1 Continued

| Whitehead ID | Gene | Location | Allele 1 trans | Allele 2 trans | chi-sq | P-value | Allele 1 | Allele 2 | AA | Allele 1 frequency | Allele 2 frequency |
|--------------|--|-------------|-------------------|-------------------|--------|---------|----------|----------|-------|-----------------------|-----------------------|
| WIAF-12402 | CKB, creatine kinase, brain | 14q32.33 | 40 | 28 | 2.12 | 0.146 | A | G | E364E | 0.80 | 0.20 |
| WIAF-11983 | CLCN3, chloride channel 3 | 4q33 | 20 | 19 | 0.03 | 0.873 | A | G | R778R | 0.90 | 0.10 |
| WIAF-13459 | CLCN3, chloride channel 3 | 4q33 | 43 | 27 | 3.66 | 0.056 | C | T | I772I | 0.83 | 0.17 |
| WIAF-10778 | COMT, catechol-O-methyltransferase | 22q11.21 | 52 | 50 | 0.04 | 0.843 | A | G | nc | 0.60 | 0.40 |
| WIAF-10792 | COMT, catechol-O-methyltransferase | 22q11.21 | 59 | 58 | 0.01 | 0.926 | T | C | H62H | 0.51 | 0.49 |
| WIAF-12230 | CREBL1, cAMP responsive element binding protein-like 1 | 6p21.3 | 39 | 43 | 0.20 | 0.659 | C | G | S201S | 0.77 | 0.23 |
| WIAF-11953 | DDR1, discoidin domain receptor family, member 1 | 6p21.32 | 42 | 43 | 0.01 | 0.914 | T | C | S175S | 0.80 | 0.20 |
| WIAF-11996 | DDR1, discoidin domain receptor family, member 1 | 6p21.32 | 55 | 60 | 0.22 | 0.641 | T | C | V636V | 0.64 | 0.36 |
| WIAF-10805 | DRD2, dopamine receptor D2 | 11q23.2 | 48 | 47 | 0.01 | 0.918 | G | A | nc | 0.67 | 0.33 |
| WIAF-10763 | DRD2, dopamine receptor D2 | 11q23.2 | 25 | 25 | 0.00 | 1.000 | G | T | nc | 0.85 | 0.15 |
| WIAF-10762 | DRD2, dopamine receptor D2 | 11q23.2 | 50 | 49 | 0.01 | 0.920 | C | G | nc | 0.67 | 0.33 |
| WIAF-10780 | DRD3, dopamine receptor D3 | 3q13.3 | 45 | 31 | 2.58 | 0.108 | A | G | S9G | 0.70 | 0.30 |
| WIAF-12690 | EPHB1, EphB1 | 3q22.1-22.2 | 66 | 50 | 2.21 | 0.137 | C | T | Y600Y | 0.63 | 0.37 |
| WIAF-10549 | FSHR, follicle stimulating hormone receptor | 2p21-p16.3 | 50 | 44 | 0.38 | 0.536 | A | G | T307A | 0.56 | 0.44 |
| WIAF-14616 | GABRA1, gamma-aminobutyric acid (GABA) A receptor, alpha 1 | 5q34 | 41 | 36 | 0.32 | 0.569 | T | C | G52G | 0.81 | 0.19 |
| WIAF-14479 | GABRA4, gamma-aminobutyric acid (GABA) A receptor, alpha 4 | 4p12 | 46 | 55 | 0.80 | 0.371 | C | A | L26M | 0.60 | 0.40 |
| WIAF-11438 | GABRB1, gamma-aminobutyric acid (GABA) A receptor, beta 1 | 4p12 | 22 | 15 | 1.32 | 0.250 | A | G | nc | 0.90 | 0.10 |
| WIAF-14409 | GABRB2, gamma-aminobutyric acid (GABA) A receptor, beta 2 | 5q34 | 24 | 19 | 0.58 | 0.446 | C | T | A436A | 0.91 | 0.09 |
| WIAF-13729 | GBE1, glucan (1,4-alpha-), branching enzyme 1 | 3p14.1 | 12 | 9 | 0.43 | 0.513 | A | G | T507A | 0.96 | 0.04 |
| WIAF-13142 | GFRA1, GDNF family receptor alpha 1 | 10q25.3 | 15 | 9 | 1.50 | 0.221 | T | A | Y85N | 0.95 | 0.05 |
| WIAF-10554 | GNRH1, gonadotropin-releasing hormone 1 | 8p21.2 | 22 | 33 | 2.20 | 0.138 | G | C | W16S | 0.79 | 0.21 |

(Continued)

Table 1 Continued

| Whitehead ID | Gene | Location | Allele 1 trans | Allele 2 trans | chi-sq | P-value | Allele 1 | Allele 2 | AA | Allele 1 frequency | Allele 2 frequency |
|--------------|---|---------------|-------------------|-------------------|--------|---------|----------|----------|-------|-----------------------|-----------------------|
| WIAF-15144 | GRIA2, glutamate receptor ionotropic, AMPA 2 | 4q32-q33 | 61 | 79 | 2.31 | 0.128 | C | T | H229H | 0.58 | 0.42 |
| WIAF-14603 | GRIK1, glutamate receptor, ionotropic, kainate 1 | 21q21.3 | 31 | 29 | 0.07 | 0.796 | C | T | D391D | 0.87 | 0.13 |
| WIAF-14608 | GRIK2, glutamate receptor, ionotropic, kainate 2 | 6q16.3 | 28 | 19 | 1.72 | 0.189 | C | A | I742I | 0.87 | 0.13 |
| WIAF-14477 | GRM4, glutamate receptor, metabotropic 4 | 6p21.31 | 47 | 37 | 1.19 | 0.275 | T | C | D485D | 0.77 | 0.23 |
| WIAF-13150 | GRM7, glutamate receptor, metabotropic 7 | 3p25.3 | 57 | 58 | 0.01 | 0.926 | C | T | Y732Y | 0.61 | 0.39 |
| WIAF-13211 | HLP1, Huntingtin associated protein 1-like protein | 17q21.2-q21.3 | 23 | 13 | 2.78 | 0.096 | G | C | L560F | 0.90 | 0.10 |
| WIAF-10843 | HTR1A, 5-hydroxytryptamine (serotonin) receptor 1A | 5q12.3 | 57 | 51 | 0.33 | 0.564 | C | G | nc | 0.51 | 0.49 |
| WIAF-10849 | HTR1B, 5-hydroxytryptamine (serotonin) receptor 1B | 6q14.1 | 28 | 33 | 0.41 | 0.522 | A | G | nc | 0.86 | 0.14 |
| WIAF-10850 | HTR2A, 5-hydroxytryptamine (serotonin) receptor 2A | 13q14-q21 | 55 | 54 | 0.01 | 0.924 | G | A | nc | 0.62 | 0.38 |
| WIAF-10851 | HTR2A, 5-hydroxytryptamine (serotonin) receptor 2A | 13q14-q21 | 11 | 17 | 1.29 | 0.257 | A | G | nc | 0.92 | 0.08 |
| WIAF-10898 | HTR2A, 5-hydroxytryptamine (serotonin) receptor 2A | 13q14-q21 | 22 | 22 | 0.00 | 1.000 | C | T | H452Y | 0.91 | 0.09 |
| WIAF-12590 | ICAM5, intercellular adhesion molecule 5, telencephalin | 19p13.2 | 54 | 63 | 0.69 | 0.405 | A | G | T348A | 0.54 | 0.46 |
| WIAF-12591 | ICAM5, intercellular adhesion molecule 5, telencephalin | 19p13.2 | 50 | 54 | 0.15 | 0.695 | G | A | I301V | 0.55 | 0.45 |
| WIAF-13925 | ITPKB, inositol 1,4,5-trisphosphate 3-kinase B | 1q42.13 | 43 | 57 | 1.96 | 0.162 | C | T | A158A | 0.72 | 0.28 |
| WIAF-14487 | KCNAB2, potassium voltage-gated channel, beta member 2 | 1p36.31 | 45 | 66 | 3.97 | 0.046 | G | A | S344S | 0.64 | 0.36 |
| WIAF-14552 | KLK6, kallikrein 6 (neurosin, zyme) | 19q13.33 | 12 | 20 | 2.00 | 0.157 | G | C | L162L | 0.92 | 0.08 |
| WIAF-10992 | LDLR, low density lipoprotein receptor | 10q23.32 | 63 | 66 | 0.07 | 0.792 | C | T | N591N | 0.57 | 0.43 |

(Continued)

Table 1 Continued

| Whitehead ID | Gene | Location | Allele 1 trans | Allele 2 trans | chi-sq | P-value | Allele 1 | Allele 2 | AA | Allele 1 frequency | Allele 2 frequency |
|--------------|---|-------------|----------------|----------------|--------|---------|----------|----------|-------|--------------------|--------------------|
| WIAF-12625 | LIMK2, LIM domain kinase 2 | 22q12.2 | 31 | 39 | 0.91 | 0.339 | G | C | L403L | 0.73 | 0.27 |
| WIAF-10497 | LIPA, lipase A, lysosomal acid, cholesterol esterase | 10q23.31 | 21 | 30 | 1.59 | 0.208 | G | A | G23R | 0.88 | 0.12 |
| WIAF-14673 | MAP1A, microtubule-associated protein 1A | 15q15.3 | 9 | 4 | 1.92 | 0.166 | C | G | P221A | 0.98 | 0.02 |
| WIAF-12650 | MAP1B, microtubule-associated protein 1B | 5q13.2 | 30 | 34 | 0.25 | 0.617 | G | A | V594I | 0.86 | 0.14 |
| WIAF-13219 | MOG, myelin oligodendrocyte glycoprotein | 6p21.33 | 31 | 29 | 0.07 | 0.796 | G | C | L171V | 0.84 | 0.16 |
| WIAF-13220 | MOG, myelin oligodendrocyte glycoprotein | 6p21.33 | 25 | 22 | 0.19 | 0.662 | G | A | V174I | 0.89 | 0.11 |
| WIAF-10769 | NGFB, nerve growth factor, beta polypeptide | 1p13.2 | 67 | 64 | 0.07 | 0.793 | A | G | nc | 0.50 | 0.50 |
| WIAF-10770 | NGFB, nerve growth factor, beta polypeptide | 1p13.2 | 51 | 50 | 0.01 | 0.921 | G | T | nc | 0.63 | 0.37 |
| WIAF-10066 | NOS2A, nitric oxide synthase 2A | 17cen-q11.2 | 38 | 37 | 0.01 | 0.908 | C | T | D385D | 0.77 | 0.23 |
| WIAF-10276 | NOS3, nitric oxide synthase 3 | 10q21.3 | 35 | 44 | 1.03 | 0.311 | C | G | A666A | 0.76 | 0.24 |
| WIAF-13214 | NPY, neuropeptide Y | 7p15.3 | 53 | 53 | 0.00 | 1.000 | G | A | S50S | 0.57 | 0.43 |
| WIAF-13215 | NPY, neuropeptide Y | 7p15.3 | 56 | 64 | 0.53 | 0.465 | C | T | S68S | 0.58 | 0.42 |
| WIAF-13457 | NRP1, neuropilin 1 | 10p11.22 | 20 | 29 | 1.65 | 0.199 | G | A | V73I | 0.89 | 0.11 |
| WIAF-11982 | NRP2, neuropilin 2 | 2q33.3 | 31 | 22 | 1.53 | 0.216 | T | G | P558P | 0.89 | 0.11 |
| WIAF-11986 | NRP2, neuropilin 2 | 2q33.3 | 65 | 59 | 0.29 | 0.590 | T | C | Y357Y | 0.54 | 0.46 |
| WIAF-12713 | PEPD, peptidase D | 19q13.12 | 51 | 40 | 1.33 | 0.249 | T | C | L435F | 0.77 | 0.23 |
| WIAF-10140 | PLCG1, phospholipase C, gamma 1 (formerly subtype 148) | 20q12 | 64 | 70 | 0.27 | 0.604 | T | C | I813T | 0.59 | 0.41 |
| WIAF-10163 | PLCG1, phospholipase C, gamma 1 (formerly subtype 148) | 20q12 | 44 | 40 | 0.19 | 0.663 | A | G | S279G | 0.80 | 0.20 |
| WIAF-16172 | PLCG2, phospholipase C, gamma 2 (phosphatidylinositol-specific) | 16q24.1 | 52 | 65 | 1.44 | 0.229 | C | T | D383D | 0.62 | 0.38 |
| | PPARG, peroxisome proliferator-activated receptor-gamma | 3p25 | 12 | 16 | 0.57 | 0.450 | C | G | P12A | 0.93 | 0.07 |

(Continued)

Table 1 Continued

| Whitehead ID | Gene | Location | Allele 1 trans | Allele 2 trans | chi-sq | P-value | Allele 1 | Allele 2 | AA | Allele 1 frequency | Allele 2 frequency |
|--------------|--|----------|-------------------|-------------------|--------|---------|----------|----------|-------|-----------------------|-----------------------|
| WIAF-12103 | PPP2R3, protein phosphatase PP2A | 3q22.3 | 56 | 58 | 0.04 | 0.851 | A | G | D67G | 0.64 | 0.36 |
| WIAF-12397 | PRKCZ, protein kinase C, zeta | 1p36.33 | 37 | 46 | 0.98 | 0.323 | A | G | P106P | 0.80 | 0.20 |
| WIAF-12398 | PRKCZ, protein kinase C, zeta | 1p36.33 | 49 | 55 | 0.35 | 0.556 | T | C | D88D | 0.65 | 0.35 |
| WIAF-12805 | PSMA4, proteasome subunit, alpha type 4 | 15q25.1 | 59 | 58 | 0.01 | 0.926 | C | T | H240H | 0.57 | 0.43 |
| WIAF-14444 | SLC12A3, sodium/chloride transporters | 16q12.1 | 27 | 27 | 0.00 | 1.000 | G | A | S628S | 0.86 | 0.14 |
| WIAF-15440 | SLC1A3, glial high affinity glutamate transporter | 5p13.2 | 50 | 50 | 0.00 | 1.000 | G | A | nc | 0.75 | 0.25 |
| WIAF-14604 | SLC1A6, high affinity aspartate/glutamate transporter | 19p13.13 | 21 | 18 | 0.23 | 0.631 | T | C | I494I | 0.89 | 0.11 |
| WIAF-10857 | SLC6A1, neurotransmitter transporter, GABA | 3p25.2 | 61 | 49 | 1.31 | 0.253 | A | G | nc | 0.52 | 0.48 |
| WIAF-10864 | SLC6A4, neurotransmitter transporter, serotonin | 17q11.2 | 23 | 26 | 0.18 | 0.668 | A | C | nc | 0.85 | 0.15 |
| WIAF-11729 | SREBF2, sterol regulatory element binding transcription factor 2 | 22q13.2 | 16 | 18 | 0.12 | 0.732 | G | C | R860S | 0.92 | 0.08 |
| WIAF-10865 | TH, tyrosine hydroxylase | 11p15.5 | 46 | 52 | 0.37 | 0.544 | G | A | V81M | 0.57 | 0.43 |
| WIAF-10017 | THBS1, thrombospondin 1 | 15q14 | 34 | 28 | 0.58 | 0.446 | A | G | N700S | 0.86 | 0.14 |
| WIAF-12833 | TLE3, transducin-like enhancer of split 3 | 15q33.33 | 31 | 34 | 0.14 | 0.710 | G | C | S205S | 0.77 | 0.23 |
| WIAF-13210 | VAMP1, vesicle-associated membrane protein 1 (synaptobrevin 1) | 12p13.31 | 54 | 68 | 1.61 | 0.205 | C | A | A84A | 0.69 | 0.31 |

Whitehead ID can be found in db SNP (<http://www.ncbi.nlm.nih.gov/SNP/>). Gene abbreviations are from LocusLink (<http://www.ncbi.nlm.nih.gov/genome/guide/human/>). Chromosomal locations are from the Human Genome Browser (<http://genome.ucsc.edu/goldenPath/hgTracks.html>) if available or Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov/Omim/searchomim.html>).

Allele 1 trans = transmission of allele 1 from heterozygous parents to bipolar offspring. P-values are two-tailed. AA = amino acid substitution, nc = non-coding. Allele frequencies are calculated from the parental chromosomes only.

Given the heterogeneous nature of bipolar disorder, we anticipated that any effects we were likely to find would be weak. In order to be efficient, and yet retain power to detect modest effects, we designed our study as follows: an initial set of trios was used as a screening sample; any nominally positive results were genotyped in additional, larger independent sample populations. This is analogous to performing a genome scan in a subset of patients and then following up only suggestive linkage peaks in a larger independent sample for confirmation.

In our initial screening set of SNPs, two genes displayed a nominal association to bipolar disorder ($P < 0.05$): (1) a valine to methionine polymorphism (V66M) in brain-derived neurotrophic factor (BDNF); and (2) a silent C/T polymorphism in the alpha subunit of the neuronal type sodium channel (CACNA1C). BDNF is synthesized from a larger precursor peptide. While the valine to methionine SNP is located in the portion of the molecule thought to be cleaved from the mature peptide, this region is conserved across many species. TDT analysis in parent-proband trios from the Hopkins sample revealed that the common valine variant is transmitted significantly more often from parents to their bipolar probands than expected by chance ($T/U = 53/34$, $P = 0.04$).

We then attempted to replicate the finding by genotyping a second sample of 189 trios obtained from the NIMH Genetics Initiative, and a third sample of 145 trios obtained from the UK collaboration between the University of Wales College of Medicine in Cardiff and the University of Birmingham in Birmingham. In both datasets the excess transmission of the valine allele was as in the original dataset (Table 2). The combined dataset of both replication samples shows excess transmission of the valine allele ($T/U = 108/87$, $P = 0.066$). In a sample of 333 non-BP patients tested for the same SNP no excess transmission was observed ($T/U = 71/78$). This indicates that the observed association is not the result of transmission ratio distortion.

Examination of the silent C/T SNP in the neuronal sodium channel in the Hopkins trios revealed a ratio of transmitted alleles to non-transmitted alleles of 25/10 ($P = 0.01$, $T/U = 2.5$, 95% CI 0.58–5.8). Examination of this SNP in the NIMH dataset did not demonstrate the same finding (12/18, $P = 0.68$, $T/U = 0.67$, 95% CI 0.36–0.59).

Since neighboring SNPs, if sufficiently close, will be inherited with a disease causing SNP, the inheritance of a haplotype can be used to uniquely tag a region of the genome for closer study. Haplotypes were constructed from the SNPs in the 11 genes that were genotyped for two or more SNPs. TDT analysis of multimarker haplotypes (data not shown) revealed a single nominally positive haplotype in the gene inositol 1,4,5 triphosphate 3 kinase with a transmission ratio of 35/56 ($P = 0.028$, $T/U = 0.63$, 95% CI 0.42–0.96). Examination of these SNPs in the NIMH dataset does not demonstrate the same finding (49/45, $P = 0.28$, $T/U = 1.09$, 95% CI 0.73–1.66).

Haplotype structure of BDNF

The initial genotyping strategy relied on genotyping single SNPs within BDNF. In order to identify haplotypes with significant transmission disequilibrium to map and identify a true susceptibility allele, we proceeded to obtain additional SNPs for genotyping. The coding sequence for BDNF is quite small and had previously been resequenced using variant detector arrays in 60 individuals, however no flanking sequence was interrogated.³⁴ We examined approximately 29 kilobases of contiguous genomic sequence found in the human genome browser, including approximately 8 kb of 5' UTR. This was resequenced in DNA from six patients affected with BP1 disorder and two control DNAs.

We have identified 44 SNPs. Twenty-three SNPs were chosen for genotyping based on allele frequency estimates from the resequencing. Genotyping has been successful for 10 (two are rare-minor allele frequency $\leq 1\%$, four are monomorphic in our patient samples, and nine failed in our genotyping format). The data for the eight informative SNPs are presented in Table 3 and their relative positions indicated in Figure 1. To reconstruct accurate multi-marker haplotypes, a screen was performed to catch genotyping problems so that markers used for further analyses are in Hardy–Weinberg equilibrium ($P < 0.05$) and have no Mendelian inheritance errors. Genotypes were obtained for an average of 96% of DNA samples tested. The Hopkins sample includes 109 trios where the proband has BP1 or SA-M disorder (80%) and 27 trios where the proband has BP2 (20%). In this sample, an allele of four of the eight common SNPs tested was associated with

Table 2 Association between G allele (Val66) of BDNF (BDNF a39) and bipolar disorder

| | T | U | χ^2 | P value | Ratio (T/U) | 95% CI | No. trios |
|---------------------------------|-----|----|----------|---------|-------------|-----------|-----------|
| Hopkins | 53 | 34 | 4.10 | 0.042 | 1.560 | 1.05–2.47 | 136 |
| NIMH | 70 | 55 | 1.80 | 0.090 | 1.270 | 0.92–1.84 | 189 |
| UK | 38 | 32 | 0.51 | 0.237 | 1.190 | 0.77–1.96 | 145 |
| Replication samples (NIMH + UK) | 108 | 87 | 2.26 | 0.066 | 1.240 | 0.95–1.66 | 334 |

T = number of times G allele transmitted from heterozygous parents to affected proband. U = number of times G allele not transmitted. P value is two-tailed for the Hopkins sample and one-tailed for the replication samples. Ratio = transmission ratio (T/U). Under a multiplicative model the transmission ratio is an estimator of the genotype relative risk. 95% CI = 95% confidence interval around the transmission ratio.

Table 3 Genotyping of eight BDNF SNPs in family-based samples

| SNP | Location | Bases | A | F | Hopkins <i>n</i> = 136 | | | | NIMH <i>n</i> = 189 | | | | UK <i>n</i> = 145 | | | |
|-----|----------|-------|---|------|---------------------------|----|----------|----------|------------------------|----|----------|----------|----------------------|----|----------|----------|
| | | | | | T | U | χ^2 | <i>P</i> | T | U | χ^2 | <i>P</i> | T | U | χ^2 | <i>P</i> |
| a44 | -1480 | C/G | C | 0.75 | 29 | 33 | 0.26 | 0.6115 | 53 | 40 | 1.82 | 0.178 | | | | |
| a40 | -633 | T/A | T | 0.57 | 38 | 67 | 8.00 | 0.0047 | 89 | 83 | 0.21 | 0.647 | 65 | 62 | 0.07 | 0.79 |
| a39 | 196 | G/A | G | 0.78 | 53 | 34 | 4.10 | 0.0416 | 70 | 55 | 1.80 | 0.090 | 38 | 32 | 0.51 | 0.24 |
| a30 | 3071 | G/A | G | 0.30 | 44 | 42 | 0.05 | 0.8292 | 82 | 64 | 2.22 | 0.136 | | | | |
| a22 | 9202 | G/A | G | 0.45 | 58 | 40 | 3.31 | 0.0690 | 56 | 63 | 0.41 | 0.521 | | | | |
| a20 | 11757 | C/G | C | 0.76 | 59 | 30 | 9.45 | 0.0021 | 70 | 56 | 1.56 | 0.106 | | | | |
| a15 | 12910 | C/A | C | 0.70 | 38 | 38 | 0.00 | 1.0000 | 51 | 67 | 2.17 | 0.141 | | | | |
| a13 | 14569 | G/A | G | 0.44 | 67 | 41 | 6.26 | 0.0124 | 73 | 89 | 1.58 | 0.209 | | | | |

SNP location is calculated from the ATG for BDNF. A = transmitted base. F = Allele frequency for the transmitted allele calculated from the untransmitted parental chromosomes in the Hopkins dataset. T = transmissions from heterozygous parent to affected offspring, U = non-transmissions, *P* = *P* value, two-tailed nominal *P* values are reported for the Hopkins sample, one-tailed nominal *P* values are reported for trends in the same direction in the NIMH and UK sample.



Figure 1 Location of BDNF SNPs. Seventeen kb of genomic sequence are shown. The box represents the BDNF coding region. The arrow marks the amino terminus of the mature BDNF peptide. Positions of SNPs are indicated. Note that while a39 is not in the mature peptide, it is in a region highly conserved across species.

transmission to bipolar patients (Table 3). Transmission disequilibrium was calculated for pairwise haplotypes for adjacent markers using the TDT2 implementation in GENEHUNTER2.0. All adjacent pairwise markers displayed significant transmission of one allele to the probands with chi-squares between 8 and 12. To assess the significance of these results, permutation tests were performed in which the genotype data were held constant but the transmission status of each chromosome (transmitted vs untransmitted) was assigned at random. In one hundred thousand permutations of the entire data set of eight markers, a single-allele chi-square value > 9.45 (the best observed in our data) was observed only 1416 times (corresponding to a gene-wide empirical *P*-value < 0.01) and only 1135 simulations had two markers with chi-squares > 11.52 (the best observed in our data, corresponding to a gene-wide empirical *P*-value < 0.001). Thus, we have identified four markers within a 17 kb region associated with bipolar disorder.

These eight SNPs were also genotyped in a sample of 189 NIMH trios in which the proband had BP1 disorder (*n* = 176, 93%) or BP2 disorder (*n* = 13, 7%). No individual SNPs were significant at the *P* = 0.05 level. Although not statistically significant, two markers revealed excess transmission of a single allele (a39 and a20) in the direction observed in the original Hopkins dataset. Transmission disequilibrium was calculated for pairwise markers with two of seven comparisons with *P* values < 0.05. However, the overtransmitted haplotypes in the NIMH dataset differed from those

observed in the Hopkins set. Because of the obvious difference between the percentages of BP2 in the two datasets, a single subanalysis of the BP1 and SA-M samples together was performed to evaluate the role that phenotypic differences between the samples might play. Despite the decrease in sample size, the significance of the observation in the Hopkins trios improves slightly, while no statistically significant improvement is detected in the NIMH samples, or change in two marker haplotypes was observed (data not shown).

Strong linkage disequilibrium was observed between all eight SNPs. The extent of LD between adjacent SNPs was determined by calculating the statistic *D'*.⁴⁰ An absolute value of *D'* of 1 indicates complete LD, while 0 corresponds to no LD. All pairwise combinations of SNPs tested across this region in both the Hopkins and NIMH dataset are in nearly complete LD (Hopkins *D'* ≥ 0.90; NIMH *D'* ≥ 0.88).

Multiple haplotype analysis was performed using TRANSMIT v2.5.2 and results are shown in Table 4. The program estimates the association from probabilities of a haplotype transmission to affected offspring even when there are uncertain marker haplotype assignments. In the Hopkins dataset, 13 haplotypes were observed, of these only six were present with probabilities greater than 2% and these accounted for the vast majority of the haplotype diversity (96.5%). The global *P* value for these six haplotypes is 0.034 (χ^2 = 13.6, 6 df). There are three common major haplotypes, 3, 5 and 6 with rarer haplotypes 2 and 4 differing by only a single marker from 3 and 5 respectively. Of

Table 4 Estimated haplotype probabilities and chi-squared test of multimarker haplotypes using TRANSMIT

| Haplotype | a44 | a40 | a39 | a30 | a22 | a20 | a15 | a13 | Hopkins | | O | E | Var(O-E) | Chi-sq | P value |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----------------------|--|--------|--------|----------|--------|---------|
| | | | | | | | | | Estimated probability | | | | | | |
| 1 | G | T | G | A | A | C | C | A | 0.025 | | 5.00 | 7.23 | 2.35 | 2.12 | 0.14532 |
| 2 | G | T | G | A | A | G | C | A | 0.021 | | 3.00 | 5.46 | 2.47 | 2.46 | 0.11705 |
| 3 | G | T | A | A | A | C | C | A | 0.196 | | 41.94 | 51.45 | 19.22 | 4.70 | 0.03013 |
| 4 | G | T | G | G | A | C | A | A | 0.046 | | 12.32 | 11.91 | 4.42 | 0.04 | 0.84655 |
| 5 | C | T | G | G | A | C | A | A | 0.245 | | 59.68 | 62.53 | 19.64 | 0.41 | 0.52099 |
| 6 | G | A | G | A | G | C | C | G | 0.433 | | 134.00 | 117.00 | 26.50 | 10.91 | 0.00096 |
| NIMH | | | | | | | | | | | | | | | |
| 1 | G | T | G | A | A | C | C | A | 0.021 | | 10.00 | 7.50 | 3.75 | 1.67 | 0.1967 |
| 3 | G | T | A | A | A | C | C | A | 0.182 | | 66.00 | 72.97 | 30.97 | 1.57 | 0.2104 |
| 4 | G | T | G | G | A | C | A | A | 0.085 | | 31.65 | 30.95 | 13.62 | 0.04 | 0.8494 |
| 5 | C | T | G | G | A | C | A | A | 0.192 | | 81.16 | 72.34 | 27.46 | 2.84 | 0.0922 |
| 6 | G | A | G | A | G | C | C | G | 0.481 | | 174.00 | 178.00 | 45.00 | 0.36 | 0.5510 |

Markers are shown in order. TRANSMIT v2.5.2 used to analyze data. O = observed transmissions of haplotype to affected offspring, E = expected transmission under Mendelian inheritance, Var (O-E) = variance of (O-E), P value calculated for 1 df. Haplotypes were omitted from analysis if the estimated haplotype probabilities were less than 2%.

these, haplotype 6 is significantly overtransmitted to the bipolar probands and haplotype 3 is undertransmitted. In the NIMH dataset the global P value is not significant ($\chi^2 = 5.3$, 5 df). Furthermore, the overtransmission of haplotype 6 is not observed, while haplotype 5 shows some excess transmission and haplotype 3 is undertransmitted. Of note, both datasets share undertransmission of haplotype 3 which is uniquely marked by the rare A allele of the originally positive SNP, a39, and by a newly identified SNP, a20, that shows a more significant association to the phenotype in the Hopkins samples (T/U = 59/30, $P = 0.0021$). In conclusion, BDNF has limited haplotype diversity in both samples which is nearly fully characterized by the SNPs genotyped. A single undertransmitted haplotype is shared by these samples characterized by alleles of SNPs a39 and a20 that may mark a protective haplotype for bipolar disorder.

Discussion

We have identified a potential association between the valine allele of the gene BDNF and susceptibility to bipolar disorder. These data were obtained in an association study of 76 genes in bipolar disorder. In our screening phase, we obtained two nominally positive results, approximately the number expected by chance. We then attempted to replicate these results in larger independent collections. For the sodium channel, the replication sample conclusively ruled out this SNP as a risk allele. For BDNF, we were unable to rule out an effect of the valine allele, finding a strongly suggestive trend in the same direction in our replication samples. The fact that the replication did not achieve statistical significance does not mean that the valine allele is not associated with bipolar disorder. Our data demonstrate that there is a bias in transmissions from heterozygous parents that is consistent both with the original finding

and with the valine allele having an effect. Furthermore, if true, its importance to bipolar disorder is supported by previous linkage data and the biochemistry and physiology of BDNF. Genotyping of further SNPs in BDNF revealed an additional three markers that are associated with increased risk of bipolar disorder and analysis of multimarker haplotypes revealed a significant overall transmission disequilibrium for this locus, as well as identifying an undertransmitted haplotype marked by the original SNP and a second novel SNP in the 3'UTR.

BDNF and bipolar disorder

BDNF maps to chromosome 11p13-15. Cytogenetic and linkage data have implicated chromosome 11 in bipolar disorder. Egeland⁵ reported the first evidence for linkage to the TH-HRAS region in a large Old Order Amish pedigree at 11p15 (maximum lod 4.08). Reanalysis of the same data with updated psychiatric diagnoses did not provide strong evidence for linkage but continued to provide modest supportive evidence with a maximum two-point lod score of 1.0. Furthermore, several subsequent genome scans provide modest evidence of linkage slightly distal to the region of HRAS reported in the Amish study with maximum lod scores obtained at approximately 31-35 cM.^{30,41} Thus it is possible that a bipolar disease susceptibility gene of modest effect lies in this region. This region of chromosome 11 also contains several other obvious functional candidates for bipolar disorder including tyrosine hydroxylase (TH) at 10 cM and tryptophan hydroxylase (TPH) at 24 cM from pter. However, previous association studies with both TH and TPH have been unable to identify consistent associations with bipolar disorder.⁴²⁻⁵¹ The patient samples used in this study are not fully contiguous with patient samples tested in the genome scans previously published.^{30,52} For the Hopkins samples, single-point and multipoint

lod scores were less than 1.0. The NIMH dataset showed no significant excess allele sharing ($P < 0.05$) for any consecutive pair of markers on the chromosome 11.⁵²

BDNF is a member of the neurotrophin superfamily, interacting with the trkB receptor tyrosine kinase,⁵³ causing many neuronal populations to respond to its growth promoting activity. BDNF is made as part of a larger precursor molecule and both the precursor and mature molecule exhibit an unusual amount of cross-species conservation at the amino acid level.⁵⁴ The mature peptide is conserved 100% and 91% at the amino acid level in mouse and zebrafish, respectively. The precursor sequence is conserved 93% and 52% at the amino acid level in mouse and zebrafish, respectively. Despite the cross species conservation of the precursor portion of BDNF, there are no published studies investigating whether it might be functional on its own, or involved in regulating the expression of mature BDNF, although full length proBDNF has been demonstrated to be functional in assays of trkB autophosphorylation.⁵⁵ Additional evidence that BDNF is of interest comes from targeted disruption of the gene in mice showing that it is essential for development of sensory ganglia and the cerebral cortex, hippocampus and striatum.⁵⁶ In a recent study, overexpression of BDNF in the mouse brain has been shown to lead to precocious visual acuity and a premature termination of the critical period for the formation of ocular dominance columns.⁵⁷ Perhaps most intriguing are reports that intracerebral administration of BDNF to animals may have antidepressant properties^{58,59} and that antidepressants can correct some of the behavioral abnormalities observed in BDNF knock-out mice.⁶⁰ Consistent with our data is the finding that the rare A allele (Met66) of BDNF was recently associated with lower overall Neuroticism scores in the general population (S Sen, R Nesse and M Burmeister, personal communication). Several facets of Neuroticism, as measured by the NEO Personality Inventory may be trait markers of vulnerability to major depression and bipolar disorder.^{61,62}

Errors in brain development have been long postulated to be associated with schizophrenia. Because BDNF is required for neuronal survival, previous genotyping of BDNF has focused exclusively on schizophrenia using a dinucleotide microsatellite marker 1 kb from the BDNF coding sequence. Using this microsatellite, several groups were unable^{63–65} to detect an association with schizophrenia. In a sample of schizophrenic patients, Andreasen provides preliminary but interesting evidence of an association of parietal lobe volume with a particular allele of this microsatellite.⁶⁵ Recent brain imaging studies suggest subtle abnormalities in brain structure suggestive of developmental defects may also be found among patients with bipolar disorder.^{66–68} Volumetric brain imaging suggests that there is reduced gray matter volume in the prefrontal cortex in major depressive disorder and post-mortem studies suggest regional decreases in volume, cell number and cell body size in

both bipolar disorder and major depression.^{69–73} The mood stabilizing treatments for bipolar disorder, lithium and valproate have recently been shown to have neuroprotective effects both *in vitro* and *in vivo* and are postulated to accomplish this by increasing the levels of the neuroprotective protein bcl-2 in the CNS.⁷⁴ This is consistent with the hypothesis that neurotrophins promote cell survival by suppressing cellular apoptotic machinery.⁷⁵

Haplotype analysis of BDNF

Although we saw quite suggestive evidence in our combined replication sample for association with the valine allele at amino acid 66 (BDNFA39), our inability to achieve statistical significance most likely results from the overall effect of the allele being modest with the effect overestimated in the original data. The more modest effect will require larger datasets to confirm conclusively. It is formally possible that the LD relationships may vary from sample to sample, however, this is unlikely to be the case since all of our samples are Euro-Caucasians, and we have shown that there is nearly complete LD in both samples in which extensively haplotyping has been undertaken. The haplotype based analyses rule out the possibility that genotyping bias or transmission distortion accounted for our original positive finding with SNP BDNF a39.

Analysis using the program TRANSMIT for multi-marker haplotype analysis in the Hopkins samples indicated global transmission disequilibrium at this locus. While independent corroboration of these results in replication samples has not yet been achieved, excess transmission of the risk allele was observed for both SNPs that mark the undertransmitted risk haplotype (a39 and a20). Our data are most consistent with there being a risk haplotype for BDNF and our lack of statistical significance in the individual replication samples likely results from an inadequate sample size.

Our results do not definitively implicate alleles of BDNF in bipolar disorder. Since the SNPs tested across 16 kb are in complete LD, genotyping of additional SNPs both upstream and downstream will be needed to fully delimit the extent of the risk haplotype. Although complete sequence is as yet unavailable in this region, there are several EST clusters and a single gene, the human homolog of the mouse gene *lin-7c*, may be as close as ~20 kb. This is an interesting candidate as it is a gene of unknown function with sequence homology to human PSD-95 that is involved in binding to the NMDA receptor and may be involved in its synaptic localization.⁷⁶

In summary, we have conducted a screen of 76 candidate genes in a family based sample of patients with bipolar disorder. We identified two potential risk alleles, one of which was subsequently ruled out by genotyping in additional samples. The other, BDNF, remains a putative risk locus for bipolar disorder in both genotyping of individual SNPs and haplotype analyses. Surrounding this gene, we have identified an undertransmitted haplotype that is associated with

bipolar disorder and is uniquely marked by a missense mutation in a highly conserved portion of the coding sequence that may have functional consequences and a mutation in the 3'UTR. Although replication has not been definitively confirmed, BDNF is a strong candidate gene based on previous molecular biological, pharmacologic studies and linkage studies. Replication of these findings in large data sets will be needed to establish their public health significance for bipolar disorder.

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