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ORIGINAL ARTICLE

Positional cloning, association analysis and expression studies provide convergent evidence that the cadherin gene *FAT* contains a bipolar disorder susceptibility allele

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A susceptibility locus for bipolar disorder was previously localized to chromosome 4q35 by genetic linkage analysis. We have applied a positional cloning strategy, combined with association analysis and provide evidence that a cadherin gene, *FAT*, confers susceptibility to bipolar disorder in four independent cohorts (allelic *P*-values range from 0.003 to 0.024). In two case–control cohorts, association was identified among bipolar cases with a family history of psychiatric illness, whereas in two cohorts of parent–proband trios, association was identified among bipolar cases who had exhibited psychosis. Pooled analysis of the case–control cohort data further supported association (*P* = 0.0002, summary odds ratio = 2.31, 95% CI: 1.49–3.59). We localized the bipolar-associated region of the *FAT* gene to an interval that encodes an intracellular EVH1 domain, a domain that interacts with Ena/VASP proteins, as well as putative β-catenin binding sites. Expression of *Fat*, *Catnb* (*β-catenin*), and the three genes (*Enah*, *Evl* and *Vasp*) encoding the Ena/VASP proteins, were investigated in mice following administration of the mood-stabilizing drugs, lithium and valproate. *Fat* was shown to be significantly downregulated (*P* = 0.027), and *Catnb* and *Enah* were significantly upregulated (*P* = 0.0003 and 0.005, respectively), in response to therapeutic doses of lithium. Using a protein interaction map, the expression of genes encoding murine homologs of the FAT (ft)-interacting proteins was investigated. Of 14 interacting molecules that showed expression following microarray analysis (including several members of the Wnt signaling pathway), eight showed significantly altered expression in response to therapeutic doses of lithium (binomial *P* = 0.004). Together, these data provide convergent evidence that FAT and its protein partners may be components of a molecular pathway involved in susceptibility to bipolar disorder.
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Introduction

Bipolar affective disorder is a major psychiatric illness with a population prevalence of up to 1.6%.[1] The disorder is characterized by aberrant mood swings resulting in periods of mania and depression with reversion to normal behavior between episodes. Family, twin and adoption studies strongly implicate a hereditary component in bipolar disorder, and the familial clustering of the disorder provides an opportunity to use genetic approaches to identify the predisposing genes.[2,3] An increasing number of loci have been implicated through genetic linkage studies,[4,5] with reproducible evidence for the presence of susceptibility loci reported for several regions including chromosomes 4, 12, 13, 18, 21 and 22.

Several genes have been implicated as conferring susceptibility to bipolar disorder. Prior to their association with bipolar disorder, the nested genes *G72* and *G30* were originally implicated as schizophrenia susceptibility genes, having been identified as positional candidates from a 5 Mb interval on chromosome 13q34 that had been genetically linked to schizophrenia.[6] This same chromosomal interval was also linked to bipolar disorder,[7] suggesting the

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presence of a common predisposing gene. Association analysis has subsequently provided evidence that the *G72/G30* gene locus also confers susceptibility to bipolar disorder.[8–10] Similarly, the Disrupted in Schizophrenia 1 locus (*DISC1*) was originally identified in a large Scottish family that exhibited a broad spectrum of psychiatric disorders including schizophrenia, bipolar disorder and unipolar disorder.[11] A balanced translocation (1;11)(q42.1;q14.3) in this family was shown to disrupt two genes, *DISC1* and the non-coding structural RNA gene *DISC2*.[12] Further evidence for association between bipolar disorder and the *DISC1* locus has been shown in a white North American cohort and in the broader Scottish population.[13,14] Other putative susceptibility genes have been implicated in bipolar disorder following their investigation as biological or functional candidate genes. These include *BDNF*, which showed evidence for association with bipolar disorder in two cohorts of predominantly Caucasian origin,[15,16] although this has not been replicated in several other cohorts of European and Japanese origin.[17–20] Association and functional studies have also implicated the *XBP1* gene in bipolar susceptibility,[21] but again, this has not been replicated in cohorts of European and Chinese origin.[22,23] Evidence has also been reported for association of the *GRIN1* gene with bipolar disorder in a cohort of predominantly European Caucasian origin.[24] Confirming those genes that genuinely confer susceptibility to bipolar disorder and those that represent false positives awaits replication in further large independent samples. Given the increasing number of genetic loci linked to bipolar disorder, and the mixed results from association studies among cohorts of different ethnicity, clearly other putative susceptibility genes are yet to be identified.

We previously reported a novel bipolar disorder susceptibility locus on chromosome 4q35 following linkage analysis in a large bipolar pedigree.[25] Subsequent analysis in our cohort of 55 multigenerational bipolar pedigrees significantly strengthened the evidence for linkage to chromosome 4q35, and haplotype analysis allowed us to define a candidate interval of 43 cM extending to the telomere of chromosome 4q35.[26] Other groups have now independently published support for this region. Linkage analysis by the NIMH (NIH) in 56 bipolar pedigrees identified suggestive evidence for linkage to chromosome 4q35 (NPL of 2.49).[27] Similarly, McInnis *et al*.[28] reported evidence of linkage to 4q35 (NPL of 2.43) after linkage analysis in 65 bipolar pedigrees. The Dana Consortium (Johns Hopkins University) also reported evidence of linkage to chromosome 4q35 (maximum multipoint HLOD of 2.11) following linkage analysis in 50 bipolar families,[29] although this cohort was also contained within the larger cohort studied by McInnis *et al*.[28] In addition, data from the Wellcome Trust funded UK–Irish Bipolar Sib-pair study also indicates support for a 4q35 locus.[30]

The 43 cM genetic interval that harbors a bipolar susceptibility gene on chromosome 4q35 corresponds to 4.8 Mb of DNA.[26,31] We established a comprehensive transcript map encompassing this candidate region,[31] which corresponds with the transcript maps for this region from the UCSC, Ensembl and NCBI genome annotation projects. This map provides a collection of genes for investigation for association with the disorder. We have applied a positional cloning strategy, combined with association analysis to systematically investigate candidate genes from this chromosome 4q35 interval. Here, we provide evidence that a cadherin gene, *FAT* (the homolog of the *Drosophila* tumor suppressor gene *fat*), confers susceptibility to bipolar disorder in four independent bipolar disorder cohorts. We subsequently investigated changes in gene expression in mice following administration of the mood-stabilizing drugs, and found that *Fat*, as well as genes encoding its protein partners, are significantly differentially expressed in response to therapeutic doses of lithium.

Materials and methods

Cohort ascertainment

Australian cohort individuals were almost entirely of British or Irish descent. Cases were recruited as part of an ongoing bipolar genetics study via the Mood Disorders Unit, Prince of Wales Hospital/School of Psychiatry, University of New South Wales. Thirty-six percent of cases were male, and 98% were older than 40 years of age. One RDC-defined bipolar I disorder case was selected from each of 65 bipolar pedigrees, previously recruited for linkage analyses. In addition, six cases were selected from a specialized bipolar disorder clinic sample, each of whom had no known family history of the disorder. All patients were assessed using the Diagnostic Interview for Genetic Studies (DIGS). We selected the spouse of each case individual as control, each control individual being age and ethnically matched to the corresponding case. (Spouses were also all interviewed using the DIGS. If there was evidence of assortative mating, the family did not progress further in the study.) Selected cases were assessed as described previously.[26] A family history of psychiatric illness in first- or second-degree relative was present in 87% of cases. A lifetime psychotic episode was evident in 47% of cases. All Australian patients who participated in this study provided appropriate informed written consent. The Australian study was approved by the Human Research Ethics Committees of the University of New South Wales and St Vincent's Hospital, Sydney.

UK cases ($n = 669$) were all of UK Caucasian origin. These were recruited through mental health services in England and Wales and met DSM-IV criteria for bipolar I disorder; 38% male; mean age 47 years (s.d. 13 years); mean age at the onset of illness 26 years (s.d. 10 years). A family history of psychiatric illness in first- or second-degree relative was present in 59% of cases. Diagnoses were made by the consensus lifetime best-estimate method,[32] on the basis of all available information including a semistructured

interview (SCAN)[33] and review of psychiatric case records and an OPCRIT checklist was completed.[34] Key clinical variables relating to psychosis were rated using BADDS.[35] Seventy percent of UK bipolar cases had a lifetime occurrence of one or more psychotic features.

UK control individuals ($n = 679$) were all of UK Caucasian origin; 36% male; mean age 42 years (s.d. 14 years). Controls were collected from two sources: (a) The British Blood Transfusion Service ($n = 569$). This sample was not specifically screened for psychiatric illness, but individuals were not taking regular prescribed medications. (b) Family practitioner clinic ($n = 110$). Individuals were recruited from among those attending for non-psychiatric reasons. This sample was screened to exclude a personal history of mood disorder.

All UK subjects provided written informed consent to participate in genetic studies. Protocols and procedures were approved by relevant ethical review panels including the UK West Midlands Multi-centre Research Ethics Committee.

UK trios were all of UK Caucasian origin. Bulgarian trios were recruited in Bulgaria and were all of Bulgarian Caucasian origin. All affected individuals from the trio cohorts met DSM-IV criteria for bipolar I disorder. The same clinical instruments described above for UK cases were used to interview trio individuals. UK trio cases comprised 43% males; mean age 34.5 years. Bulgarian trio cases comprised 46% males; mean age 31.9 years. A family history of psychiatric illness in first- or second-degree relative was present in 60% of UK trios, and 30% of Bulgarian trios. A lifetime occurrence of one or more psychotic features was present in 46% of UK trio cases, and 42% of Bulgarian trio cases.

Genotyping

All *FAT* gene-specific single-nucleotide polymorphisms (SNPs) were selected from dbSNP (http://

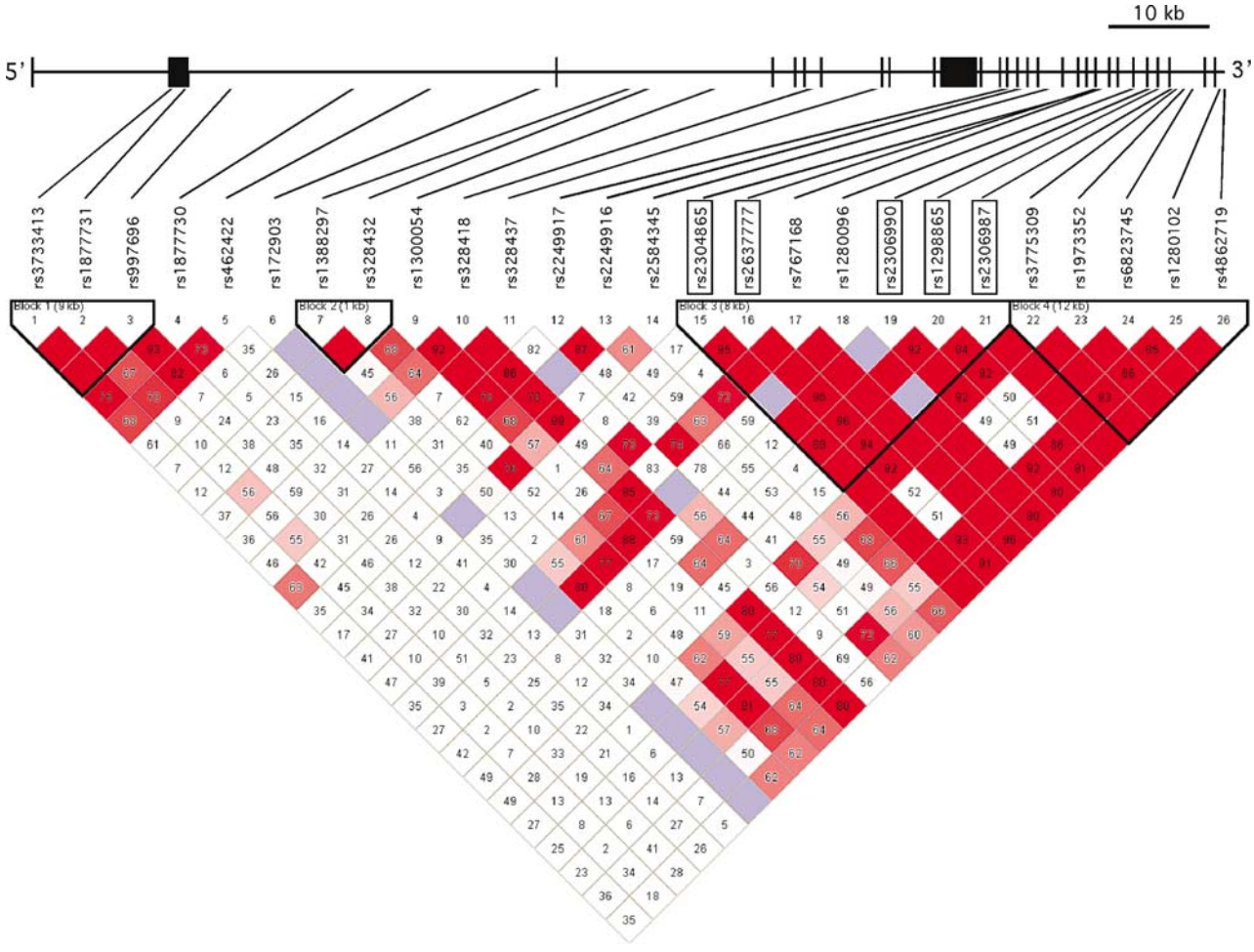

Figure 1 Genomic structure of the cadherin gene *FAT*, including analyzed SNPs and LD analysis. The location of 26 SNPs (indicated by their RefSNP ID) analyzed in the Australian case–control cohort is indicated. SNPs showing significant evidence of association with bipolar disorder are boxed. The output of LD analysis using Haploview is shown. Haplotype blocks, determined using the default confidence interval algorithm, are indicated on the LD output. The associated SNPs all fall within an 8.7 kb haplotype block (Block 3).

www.ncbi.nlm.nih.gov/SNP/). The names and positions of all SNPs tested within the *FAT* gene are provided in Figure 1 and Supplementary Table 1. Genotypes in the Australian cohort were determined by direct sequencing or by MALDI-TOF MS utilizing a Sequenom Autoflex Mass spectrometer. MALDI-TOF MS genotyping was performed by the Australian Genome Research Facility. For direct sequencing, PCR was performed following standard methods and subjected to automated sequence analysis using the ABI377 automated sequencer with Big-Dye terminator sequencing (v3.1, Applied Biosystems). Genotypes in the UK and Bulgarian cohorts were determined using the AmplifluorTM method.[36] The allele-specific products were resolved on an Analyst AD fluorescence reader (LJL Biosystems). Australian DNA samples of known genotype were used for quality control among each research group.

Statistical analyses

For association analysis, the frequencies of alleles and genotypes observed in cases and controls were compared and tested by constructing contingency tables and performing χ^2 analysis. Transmission disequilibrium was tested using the standard TDT test.[37] Hardy–Weinberg equilibrium and linkage disequilibrium (LD) analyses were performed using the Haploview v2.05 computer program (Whitehead Institute). LD-based haplotypes were determined by Haploview using the default confidence interval algorithm.[38] For haplotype analysis, haplotype frequencies were estimated by the expectation–maximization algorithm implemented in the Arlequin program (http://lgb.unige.ch/arlequin/), and compared and tested by constructing contingency tables and performing χ^2 analysis. Pooled data (meta-) analysis was performed with the Comprehensive Meta-Analysis v1.0.23 computer program (Biostat Inc.) using the Mantel–Haenszel method. Bonferroni corrections of statistical significance levels were performed using the Simple Interactive Statistical Analysis package at http://home.clara.net/sisa.

Administration of lithium and valproate

C57Bl6 mice, between the ages of 8 and 16 weeks received daily intraperitoneal injections for 7 days of either 340 mg/kg/day (8 mmol/kg/day) lithium, or 350 mg/kg/day valproate (2-propylpentanoic acid), or sterile 0.9% saline solution. Total volume of each injection did not exceed 500 μl. Lithium chloride was prepared in water, and pH adjusted to 7.5. Valproate was prepared in 0.9% saline solution. Four hours after the final injection, mice were euthanized and whole brains removed. Circulating drug levels were determined from cardiac puncture blood sample by SydPath Laboratories (St Vincent's Hospital, Sydney). Brains were selected for further analysis if serum concentrations were between 0.6 and 1.0 mmol/l lithium or 315–885 μmol/l valproate.

Expression analysis

Australian brain bank samples comprised fresh–frozen cerebellum tissue collected post mortem from 115 individuals with a mean age of 46 years (s.d. 17 years). All were considered unaffected with respect to psychiatric disease.

Total RNA was prepared from human cerebellum and whole mouse brain with TRI Reagent (Sigma-Aldrich) and reverse transcribed using Superscript™ III M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

RT-PCR was carried out on reverse-transcribed RNA derived from human cerebellum, as well as DNA from the following cDNA libraries (all from Clontech Laboratories Inc.): human brain-hypothalamus 5′ Stretch cDNA library, human hippocampus cDNA library and human brain-amygdala 5′ Stretch Plus cDNA library. RT-PCR was carried out as described previously.[31] Primer sequences and amplification conditions are available on request.

Quantitative real-time PCR analysis was performed in a 10 μl reaction containing 1 × Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 200 nM of each primer. Amplification conditions were as follows: 50°C for 2 min, 95°C for 2 min, followed by 35 cycles of 95°C for 5 s, annealing for 15 s and 72°C for 15 s. SYBR Green I fluorescence was measured after annealing and extension steps. Following amplification, samples were dissociated by incremental heating between 72 and 99°C, at a rate of 1°C/5 s, during which fluorescence was constantly measured. Reactions were performed in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney). PCR amplification was performed simultaneously on reverse-transcribed RNA prepared from the whole brains of seven control mice, eight mice treated with lithium and six mice treated with valproate. Quantitative PCR critical threshold values for each sample were determined using Rotor-Gene v5.0.37 computer program (Corbett Research). mRNA copy number was determined relative to DNA standards of known concentration. The relative expression for *mfat1* was expressed as a ratio of *mfat1* mRNA concentration to that of the housekeeping gene *Gapdh*. After analysis of several housekeeping genes (*β-actin*, *Hprt* and *Gapdh*), *Gapdh* was chosen as the reference gene because we found its expression remained constant across experimental conditions (control, lithium and valproate). This contrasted with the *β*-actin gene (*Actb*), which we found varied in expression between experimental conditions. The mean ratio and s.d. for control, lithium and valproate cDNAs were calculated using the StatView software v5.47 (Abacus Concepts). Unpaired Student's *t*-tests were performed, with a two-tailed α value of 0.05.

Individual total RNA samples were selected from five groups of 15 mice each, including 15 control mice; 15 mice with a lithium serum level range between 0.6 and 0.8 mmol/l (designated as low lithium); 15 mice with a lithium serum level range between 0.8 and 1.0 mmol/l (designated as high

lithium); 15 mice with a valproate serum level range between 32 and 35 mg/l (designated as low valproate); and 15 mice with a valproate serum level range between 42 and 91 mg/l (designated as high valproate). Total RNA samples from five individual mice within each group were pooled. Biotinylated copy RNA (cRNA) probes were prepared using the BioArray High Yield RNA Transcription Labeling Kit (Affymetrix, Santa Clara, USA) following the manufacturer's instructions. Prepared cRNA probes were hybridized to Affymetrix GeneChip® Murine Genome U74Av2 microarrays following the protocol outlined in the Affymetrix GeneChip® Expression Analysis Technical manual. Microarrays were washed using a semiautomated GeneChip® Fluidics Station 400 (Affymetrix) and scanned using a Agilent GeneArray Scanner (Affymetrix). The microarray fluorescent signal intensity for specific genes was determined using Affymetrix Microarray Analysis Suite software (version 5). The microarray sensitivity is a linear range of three orders of magnitude. The specificity of the microarray hybridization was predetermined using Affymetrix Test3 microarray chips for every sample before hybridization to the full microarray. As well as determining the quality of the prepared samples, these microarrays contain housekeeping genes from a large range of eukaryotic species to ensure no cross-hybridization. The specificity of individual gene hybridizations was determined by the microarray probe set for each gene. Each probe set consists of 32 oligonucleotides (16 sense and 16 antisense).

Results

The expression of mRNA from genes located within the chromosome 4q35 candidate interval (UCSC May 2004 assembly, chromosome 4 positions 186 439 570–191 259 493) was investigated by RT-PCR using cDNA derived from a range of brain tissues. In addition, tissue sources of cDNA clones from which database ESTs were derived were also inspected. Seventeen brain-expressed genes from the candidate interval were selected for association analysis. Of these 17 candidate genes, 12 showed no evidence of association in our previous studies.[39,40]

To test the remaining selected positional candidate genes for association with bipolar disorder, we identified SNPs located in exons and flanking intron sequences from the candidate genes via direct DNA sequencing in an affected individual (case) and their spouse (control) from seven bipolar kindreds previously reported by Badenhop *et al*.[26] as showing evidence for linkage to the chromosome 4q35 locus. In addition, SNPs spanning the large gene *FAT* were selected from dbSNP (http://www.ncbi.nlm.nih.gov/SNP) based on their proximity to coding sequences. In the first stage of association analysis, SNPs shown to be polymorphic among 4q35 linked pedigrees were analyzed in an Australian bipolar case–control cohort consisting of 137 individuals (71 cases and 66 controls) selected by having a strong family history of bipolar disorder (87% of cases were selected from bipolar families with illness over at least two generations, containing a minimum of three affected individuals). Genotype frequencies for each SNP were tested using χ^2 analysis and all were found to be in Hardy–Weinberg equilibrium. Statistically significant evidence for association was initially observed for SNP rs1298865, located towards the 3′-end of the *FAT* gene (Table 1, Figure 1). Consequently, further SNPs within the gene were identified from dbSNP and genotyped in the Australian case–control cohort. A further four SNPs, all located in the 3′ half of the *FAT* gene, provided statistically significant evidence for association (Table 1, Figure 1, Supplementary Table 1). Analysis of LD among all tested SNPs determined that the associated SNPs are in high LD and comprise a haplotype block that spans 8.7 kb, which is located wholly within the *FAT* gene (Figure 1). No LD was detected between *FAT* and any adjacent gene. Haplotype analysis showed that the two common haplotypes (GTGTA and CGACT) for the associated SNPs (rs2304865, rs2637777, rs2306990, rs1298865 and rs2306987) account for 92.4% of the total haplotypic diversity in this region. The GTGTA haplotype is over-represented among cases ($P = 0.045$), and the CGACT haplotype is under-represented ($P = 0.012$). A sliding window analysis of haplotypes was undertaken, which showed no greater evidence for association with any sub-region within the interval encompassing these SNPs. Analysis of the associated SNPs in cases from the seven Australian bipolar families that show evidence of linkage to chromosome 4q35 demonstrated that 86% of chromosomes constituted the GTGTA haplotype compared to 61.6% of Australian control individuals. Of all brain-expressed genes from the candidate interval, *FAT* is the only gene that showed evidence of association with bipolar disorder.

The evidence of association between *FAT* genotype and bipolar disorder observed in the initial screening of case–control cohort could have occurred by chance. To test this, putatively associated SNPs were analyzed for association in an independently ascertained bipolar disorder case–control cohort comprising 1348 UK individuals (669 cases and 679 controls). Given the high LD between putatively associated SNPs observed in the Australian case–control cohort, representative tagSNPs were selected for analysis among the UK cohort (Table 1, Supplementary Table 2). In addition to total cohort analysis, association was also tested in the presence of a known family history of psychiatric illness (in first- or second-degree relative), as this constituted the largest common group across both case–control cohorts (87 and 59% of the Australian and UK case–control cohorts, respectively). Statistically significant evidence for association with bipolar disorder was observed in the UK case–control cohort for those cases with a family history of psychiatric illness (rs2304865 allelic $P = 0.007$, genotypic $P = 0.025$). This

Table 1 Association analysis (*P*-values) of chromosome 4q35 tagSNPs[a]

	rs2304865		*rs1298865*		*rs2306987*	
	Allele	*Genotype*	*Allele*	*Genotype*	*Allele*	*Genotype*
Overall						
Aust case–control	0.007	0.009	0.072	0.044	0.020	0.005
UK case–control	0.274	0.542	0.838	0.147	0.463	0.439
UK trios					0.084	
Bulgarian trios					>0.5	
Family history[b] *positive*						
Aust case–control	0.007	0.006	0.010	0.007	0.010	0.004
UK case–control	0.007	0.025	0.038	0.078	0.365	0.592
UK trios					0.060	
Bulgarian trios					0.128	
Pooled analysis[c] (odds ratio)	0.0002 (2.31)		0.003 (1.44)		0.083 (1.19)	
Family history negative						
Aust case–control	0.310	0.528	0.980	0.639	0.384	0.228
UK case–control	0.841	0.975	0.082	0.020	0.050	0.022
UK trios					NA	
Bulgarian trios					NA	
Lifetime psychotic episode (during manic or depressive episode)						
Aust case–control	0.092	0.176	0.197	0.382	0.178	0.261
UK case–control	0.466	0.487	0.512	0.194	0.329	0.484
UK trios					0.003	
Bulgarian trios					0.024	
No psychotic episode						
Aust case–control	0.012	0.011	0.029	0.009	0.013	0.005
UK case–control	0.085	0.079	0.729	0.272	0.947	0.298
UK trios					>0.5	
Bulgarian trios					0.058	

Aust = Australian; NA = not applicable; UK = United Kingdom.
[a]Allele and genotype frequencies are provided online as Supplementary Table 2.
[b]Family history of psychiatric illness.
[c]Pooled (meta) analysis of case–control cohort data. Empty cells indicate that the marker was not tested.

Table 2 Comparison of bipolar disorder-associated alleles/haplotypes of chromosome 4q35 tagSNPs

	rs2304865	*rs1298865*	*rs2306987*
Australian	G	T	A
UK-family history positive	G	T	
UK-family history negative		C	T
UK trios			A
Bulgarian trios			A

result is consistent with that observed for the Australian case–control cohort, with the same haplotype over represented (Table 2).

Pooled data (meta-) analysis (Mantel–Haenszel method) was performed using tagSNP allelic data from the Australian and UK case–control cohorts. To establish whether pooled data analysis was appropriate, we first compared the cohorts for differences between control allele or genotype frequencies. χ^2 analysis showed that there were no significant differences between control allele or genotype frequencies for any marker analyzed, and as such, pooled data analysis could be performed. Pooled data analysis was performed using the largest common group of bipolar disorder cases, those with a family history of psychiatric illness (Table 1). SNP rs2304865 showed significant evidence for association ($P = 0.0002$) with a summary odds ratio (OR) of 2.31 (95% CI: 1.49–3.59). SNP rs1298865 also showed significant evidence for association ($P = 0.003$) with a summary OR of 1.44 (95% CI: 1.13–1.83). SNP rs2306987 showed weak evidence for association ($P = 0.06$) with a summary OR of 1.27 (95% CI: 0.99–1.64). The statistics for rs2304865 and rs1298865 remained significant after applying a Bonferroni correction for multiple testing (corrected $\alpha = 0.017$).

Given the statistically significant evidence for association, we subsequently undertook association analysis of sub-phenotype categories of these case–control cohorts (Table 1, Supplementary Table 2). These categories included those cases without a family history of mental illness, and those cases that exhibited the presence or absence of psychosis during manic or depressive episodes. Statistically significant evidence for association was observed in the UK case–control cohort for those cases without a family history of mental illness (rs2306987 allelic $P=0.050$, genotypic $P=0.020$). However, the observed bias was for the alternate haplotype to that over-represented among cases with a family history. No case–control cohort showed evidence for association among psychosis or non-psychosis subsets.

One associated tagSNP (rs2306987) was subsequently analyzed for association in two additional independently ascertained bipolar disorder cohorts comprising 90 UK parent–proband trios, and 173 Bulgarian parent–proband trios (Table 1). Neither the UK trios nor the Bulgarian trios showed evidence for association among family history or non-family history subsets. However, both trio cohorts did show statistically significant evidence for association among those bipolar cases who had exhibited psychosis (UK trios $P=0.003$, Bulgarian trios $P=0.024$; allele transmissions/non-transmissions were 38/16 and 37/20, respectively). Combined with the case–control cohort data described above, this provides evidence for association between *FAT* genotype and bipolar disorder among unrelated individuals from four independent cohorts.

To determine whether the bipolar disorder-associated *FAT* genotype is correlated with *FAT* mRNA levels *in vivo*, we undertook quantitative real-time RT-PCR in brain tissue. No correlation between *FAT* genotype (bipolar disorder-associated SNP genotype) and mRNA levels was observed among 115 post-mortem cerebellum samples. We next tested whether mood-stabilizing drugs such as lithium and valproate modulate *FAT* mRNA expression in mouse brain tissues. Mice received daily administration of lithium, or valproate, or saline vehicle for a period of 7 days. Circulating drug concentrations were assayed to ensure levels equivalent to those used to obtain high and low human therapeutic ranges.[41] The mRNA from whole brain was analyzed by quantitative real-time PCR to determine the steady-state mRNA level of the *mfat1* gene (murine *FAT* orthologue) and a control gene, *Gapdh*. *Gapdh* was chosen as the reference transcript because we found its expression remained constant across different experimental conditions (control, lithium and valproate). In addition, *Gapdh* has been previously used in several studies that investigated the effects of lithium and valproate on gene expression in rats and mice.[42–44] We found the relative mRNA expression levels for *mfat1* were reduced following administration of either drug (Figure 2a), with administration of lithium significantly reducing *mfat1* expression by 2.7-fold ($P=0.027$).

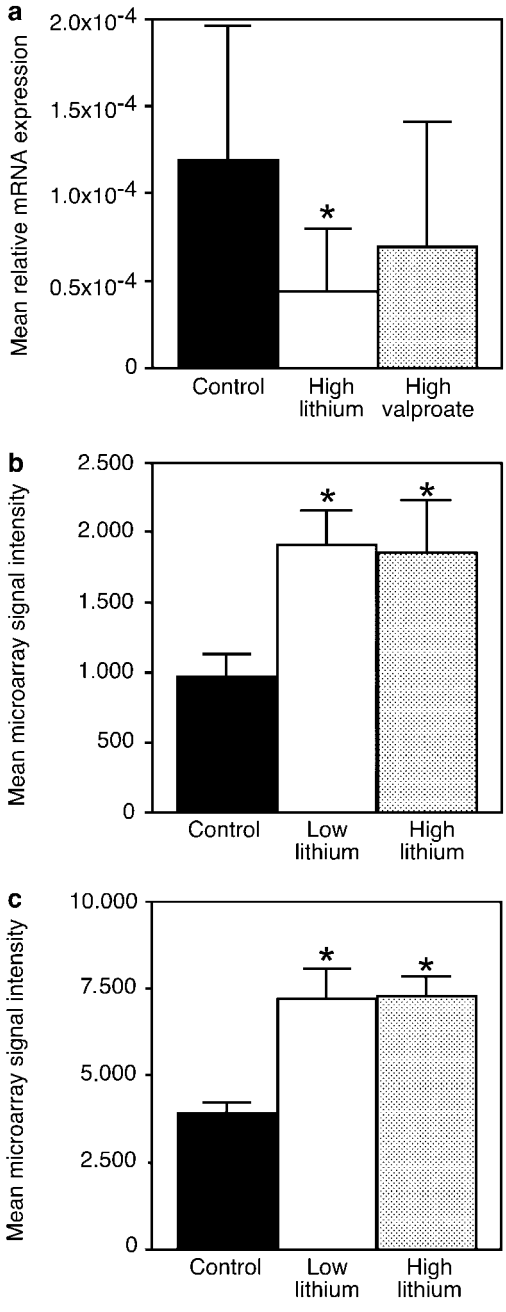

Figure 2 Relative mRNA expression levels in mice for *mfat1*, *Enah* and *Catnb* (*β-catenin*) following administration of bipolar therapeutic drugs. (**a**) *mfat1* expression was significantly decreased 2.7-fold ($P=0.027$) in response to high serum levels of lithium. (**b**) *Enah* expression was significantly increased 2.0-fold ($P=0.005$) and 1.9-fold ($P=0.017$) in response to low and high serum levels of lithium, respectively. (**c**) *Catnb* expression was significantly increased 1.8-fold ($P=0.0003$) and 1.9-fold ($P=0.003$) in response to low and high serum levels of lithium, respectively.

The FAT protein regulates cell–cell contact and polarity via a process that is mediated via interaction

with Ena/VASP proteins (ENAH, homolog of *Drosophila* enabled [ena]; EVL, Ena-vasodilator-stimulated phosphoprotein; and VASP, vasodilator-stimulated phosphoprotein).[45,46] The Ena/VASP proteins are thought to play redundant roles in regulating actin cytoskeletal dynamics (reviewed by Renfranz and Beckerle[47]). Interaction between FAT and Ena/VASP proteins is mediated via an EVH1 domain. We observed that the 8.7 kb LD block associated with bipolar disorder encodes the EVH1 domain of the FAT protein. Expression of the three genes (*Enah*, *Evl* and *Vasp*) that encode the Ena/VASP proteins were therefore investigated in the mice treated with lithium and valproate, via microarray analysis with Affymetrix gene chip arrays. *Enah* was significantly increased 2.0-fold ($P = 0.005$) and 1.9-fold ($P = 0.017$), in response to low and high therapeutic doses of lithium, respectively (Figure 2b). No change in *Evl* and *Vasp* expression was detected.

The structural organization and function of many cadherins is mediated by the binding of β-catenin, which through the cadherin–catenin complex links cadherins to the actin cytoskeleton. The 8.7 kb LD block within *FAT* that is associated with bipolar disorder also encodes the potential β-catenin binding regions, FC1 and FC2.[48] Cox *et al.*[49] reported that mouse mfat1 did indeed bind β-catenin through these regions in two-hybrid and co-immunoprecipitation assays. Expression of the murine gene encoding β-catenin, *Catnb*, was also investigated in the mice treated with lithium and valproate, via microarray analysis. *Catnb* was significantly increased 1.8-fold ($P = 0.0003$) and 1.9-fold ($P = 0.003$), in response to low and high therapeutic doses of lithium, respectively (Figure 2c, Table 3).

To investigate whether other potential upstream or downstream effectors of FAT show altered expression in response to bipolar therapeutic drugs, we identified potential effectors by searching protein–protein interaction databases. As FAT was not present in the mammalian PPID database, we used FlyBase GRID[50] to establish a *Drosophila melanogaster* protein interaction map, encompassing ft, the homolog of the human FAT protein (Figure 3). *D. melanogaster* is a proven model system for many aspects of mammalian biology. Genes that encode the murine homologs of these *Drosophila* proteins (Table 3) were investigated in the mice treated with lithium and valproate, via microarray analysis. The expression of 12 of these interacting protein homologs was detected on microarray chips, and seven of these showed significantly altered expression in response to therapeutic doses of lithium (Table 3). The binomial probability of this result is $P = 0.007$. Combining this with the data for the Ena/VASP proteins described above, we have found significantly altered expression in response to therapeutic doses of lithium, for eight of 14 interacting genes that were detected on microarray chips ($P = 0.004$).

We also inspected microarray data to determine whether any of the remaining brain-expressed genes from the chromosome 4q35 bipolar candidate interval (i.e. other than *FAT*) showed expression changes in response to therapeutic doses of mood-stabilizing drugs. Of these 16 genes, four showed no significant change in expression level, five showed no expression (below detection level), with the remaining genes not present on analyzed chips.

Discussion

The data from three complementary strategies provide convergent evidence that the cadherin gene *FAT* confers susceptibility to bipolar disorder. We first used a positional cloning strategy to localize a bipolar susceptibility gene to a gene poor interval on chromosome 4q35. Secondly, association analysis provided significant evidence that *FAT* is associated with the disorder in four independent bipolar disorder cohorts. Finally, microarray and real-time PCR analysis of transcripts in mice treated with mood-stabilizing drugs provided evidence that *Fat*, as well as genes encoding its protein partners, are differentially expressed in response to therapeutic doses of lithium. Together, these data provide convergent evidence that FAT and its protein partners are components of a molecular pathway involved in susceptibility to bipolar disorder.

Table 3 Microarray analysis of mRNA expression response for genes encoding murine homologs of *Drosophila*-interacting proteins, following treatment with bipolar therapeutic drugs

Fly	*Mouse*	*Lithium-fold Δ (P-value)*		*Valproate-fold Δ (P-value)*	
		Low	*High*	*Low*	*High*
Apc	*Apcl*	2.4 ↓ (0.001)	2.5 ↓ (0.0005)	No change	No change
arm	*Catnb*	1.8 ↑ (0.0003)	1.9 ↑ (0.003)	1.3 ↓ (0.010)	No change
brm	*Smarca4*	2.5 ↓ (0.0005)	2.9 ↓ (<0.0001)	1.2 ↓ (0.020)	No change
cbp	*Crebbp*	1.9 ↑ (0.006)	1.8 ↑ (0.001)	No change	No change
fz	*Fz4*	1.6 ↑ (0.024)	1.6 ↑ (0.013)	No change	No change
N	*Notch*	2.5 ↑ (0.008)	No expression	No expression	No expression
pka-c1	*prkaca*	2.7 ↓ (0.008)	2.8 ↓ (0.024)	No change	No change

↓ = downregulated; ↑ = upregulated.

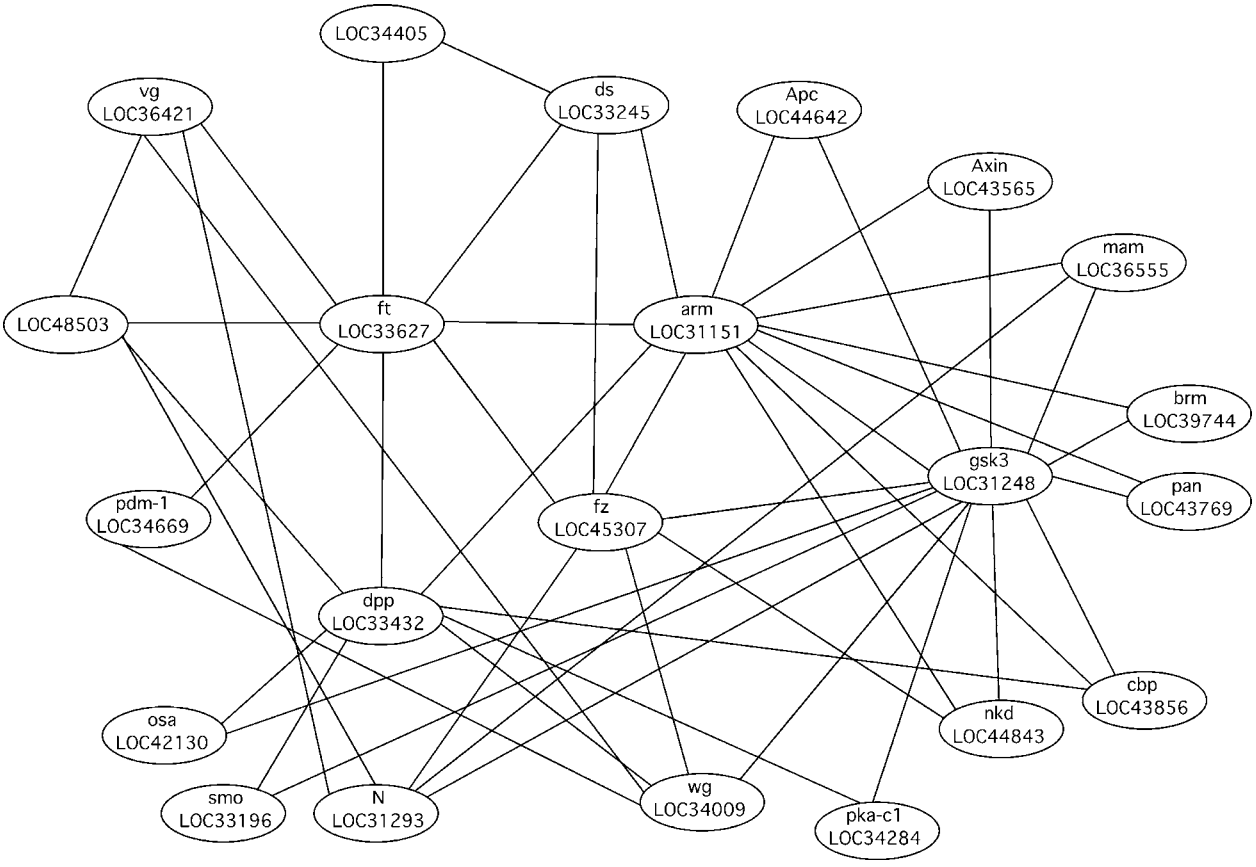

Figure 3 *Drosophila* protein interaction map encompassing ft (fat), the homolog of the human FAT protein.

Although association between *FAT* and bipolar disorder was replicated in four independent cohorts, this association was not achieved with a single phenotypic category. It is possible that the multiple statistically significant *P*-values arose by chance; however, our pooled data (meta-) analysis suggested that these are unlikely to represent false-positive associations ($P = 0.0002$, summary OR = 2.31, 95% CI: 1.49–3.59). It is also possible that population stratification between the two case–control cohorts may have led to false-positive associations. We did not undertake any genomic analysis of the two case–control cohorts to test for population stratification; however, careful attention to the standard principles of epidemiological study design was applied in ascertaining these cohorts. Each case–control cohort was carefully controlled for ethnicity, and controls were selected to ensure that they are representative of the source population of cases. This approach significantly reduces the potential for population stratification.

Multiple testing is an issue among association studies, with greater numbers of statistical tests increasing the likelihood of obtaining false-positive results. In our screen for association within the chromosome 4q35 candidate region, we tested 65 SNPs across all brain-expressed genes before identifying association with SNPs in the *FAT* gene. Applying a Bonferroni correction for this number of tests provides a corrected statistical significance level of 0.0008. If this corrected significance level is accepted, no *P*-value obtained in this study reached significance. However the testing of multiple SNPs in a linked region and in defined LD blocks represents tests that are not independent. It should be appreciated that this corrected significance level is highly conservative, and few association studies of complex traits, such as bipolar disorder, would ever meet this threshold.

The issue of multiple testing also applies to the microarray analysis, where a Bonferroni correction provides a corrected statistical significance level of 0.002. Only four of the seven genes in Table 3 still meet this conservative threshold. However, the degree of false-positive discovery is likely to be very low, because of the specificity and sensitivity of these commercially available microarrays.

Significant evidence for association was observed in the UK case–control cohort for both those cases with, and those without, a family history of mental illness. However, the observed bias in each case was for the alternate haplotype. It is possible that one or both of these are false-positive results. Alternatively, it is possible that this is an example of molecular

heterosis, a common phenomenon that may be seen in as many as 50% of association studies.[51] A more complete assessment of this and the genetic associations that were observed among other cohorts await further investigation in the patient cohorts of other investigators, to establish whether there is wider support for *FAT* as a putative susceptibility gene.

The FAT protein regulates cell–cell contact and polarity, probably through regulation of actin cytoskeletal organization at cell peripheries, which is mediated via interaction with Ena/VASP proteins.[45,46] This interaction is mediated via an EVH1 domain. All bipolar disorder-associated SNPs fall within an 8.7 kb LD block of *FAT*, which encodes the EVH1 domain of the FAT protein. The Ena/VASP protein ENAH has been implicated as a downstream effector of FAT[46] and was found in our analysis to be significantly differentially regulated in response to bipolar disorder therapeutic drugs. *ENAH* maps to chromosome 1q42, a region that has been strongly implicated in linkage studies as harboring a bipolar disorder susceptibility gene.[52–54] *ENAH* can now be investigated as a positional candidate gene.

The mood-stabilizing drugs used to treat bipolar disorder are believed to provide their therapeutic action via transcriptional mechanisms. This is because the efficacy of the drugs is typically realized after chronic administration. In the case of lithium, the antimanic effect occurs within 5–10 days, but there is a delay of 6–8 weeks before it exerts an antidepressant effect.[55] We treated mice with therapeutic drugs for 7 days prior to investigation of differential gene expression. This length of time may be sufficient for the antimanic effects to be observed, but insufficient for the antidepressant effects to manifest. Maintaining a human therapeutic range of circulating lithium concentration in the mice for periods longer than seven days led to toxicity. Hence, we were unable to investigate the effect of longer chronic drug administration on gene expression. However, other groups have successfully maintained chronic administration of mood-stabilizing drugs in rats,[56,57] and as such, this may be a better therapeutic model for the future assessment of the differential expression of *FAT* and genes encoding its protein partners.

Of the seven genes from our protein interaction map that showed differential expression following lithium administration, only two were also altered by valproate (Table 3). This is not necessarily surprising, as these two agents are structurally highly dissimilar, and with the exception of GSK3 (glycogen synthase kinase 3), they have generally been found to target different molecules (reviewed by Gould *et al*.[58]). Unfortunately, *GSK3* was not present on the Affymetrix gene chip arrays that were analyzed in this study, and as such, any common differential expression of this molecule could not be investigated.

A challenge for bipolar disorder pharmacogenomics is to differentiate between those lithium-responsive genes that are involved in the therapeutic action of lithium, and those that are involved in the side effect or non-therapeutic action. We have identified *FAT* as a gene that shows evidence of association with bipolar disorder, and is responsive to lithium at therapeutically relevant concentrations in mice. In addition, the mammalian homologs of two ft (fat)-interacting proteins, GSK3 and β-catenin (arm), have previously been shown to also respond to lithium, as well as valproate treatment.[59] GSK3 is a direct target of lithium[60–62] and is a key regulator of the Wnt signaling pathway[63] (Figure 3). GSK3 phosphorylates β-catenin leading to ubiquitin-dependent degradation. Inhibition of GSK3 therefore leads to accumulation of cytoplasmic β-catenin, which becomes available for translocation to the nucleus where it binds TCF/LEF transcription factors, inducing target gene expression. The two cellular pools of β-catenin, the cytoplasmic pool and the cadherin-bound pool are not mutually exclusive. Evidence suggests that the cadherin-bound pool of β-catenin may be released to the cytoplasm and made available for signaling.[64] Other studies have suggested that cadherins may act as negative regulators of β-catenin-mediated signaling by sequestering and binding β-catenin at the cell surface (reviewed by Nelson and Nusse[64]). There is increasing evidence of a convergence of the cadherin, β-catenin and Wnt pathways,[64] and the data presented here further support these inter-relations. Our investigation of the mammalian homologs of ft (fat)-interacting proteins demonstrated that several members of the Wnt signaling pathway are also responsive to lithium (Table 3). Together, these data provide evidence for a lithium-responsive gene network. Work can now commence to determine how these molecules and pathways may be involved in pathogenesis of bipolar disorder. As such, this approach[65] offers the opportunity of identifying novel targets with enhanced efficacy for the long-term treatment of bipolar disorder.

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