Examination of the clock gene *Cryptochrome 1* in bipolar disorder: mutational analysis and absence of evidence for linkage or association

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Bipolar disorder is associated with malfunctions of the circadian system, which regulates individual circadian rhythms and which enables the adaptation to a daily 24-h cycle and seasonal change. One of the human circadian clock genes, *cryptochrome 1* (*Cry1*) (located on 12q23-q24.1) was analyzed because of its close correspondence to a linkage hotspot for bipolar disorder.

We found no evidence for linkage of 52 bipolar families to two *Cry1* flanking microsatellites under several parametric and non-parametric models. In order to employ association for a more sensitive test, 25 affected subjects selected from families with positive LOD scores were screened for mutations by sequencing 9.5% of the *Cry1* gene. A total of 16 single nucleotide polymorphisms (SNPs) and a 3 base pair insertion were identified. However, no mutations with probable functional impact were found. These novel SNPs and data on allele frequency and linkage disequilibrium structure will be useful for future association analyses. Nine SNPs have been analyzed in a set of 159 parent proband triads. Linkage disequilibrium analyses using single SNPs and haplotypes showed no association to bipolar disease.

Additional, more powerful, studies involving *Cry1* and other circadian clock genes need to be tested before an association of circadian abnormalities with bipolar disorder can be excluded. *Psychiatr Genet* 15:45–52 © 2005 Lippincott Williams & Wilkins.

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Introduction

Bipolar disorder is a recurring illness characterized by the occurrence of mania, most frequently combined with spells of depression (American Psychiatric Association, 1994; Angst and Sellaro, 2000). The lifetime prevalence is often estimated at 0.5–1.5%, but subthreshold symptoms such as excessive happiness, excitement, or talkativeness may affect as many as 6.4% (Judd and Akiskal, 2003). Bipolar disorder appears to arise largely from genetic susceptibilities (Kelsoe, 2003).

Several analyses have suggested that bipolar disorder might be caused by genetic variations altering the function of the circadian clock (Kripke *et al.*, 1978; Bunney and Bunney, 2000; Gould and Manji, 2002). The hypothesis is supported by circadian abnormalities observed in a number of bipolar patients (Wehr *et al.*, 1982; Wehr and Goodwin, 1983), but these observations have been sporadic. Likewise, studies of the circadian hormone melatonin have yielded inconsistent abnormalities in patients with depressions (Kripke *et al.*, 2003). Some observers suspect that the powerful antidepressant and pro-mania effects of bright light are mediated through the circadian clock (Lewy *et al.*, 1998).

In the past few years, genes constituting important components of the mammalian circadian clock have been discovered. If there is a genetic variation in the circadian

0955-8829

system conferring susceptibility to bipolar illness, then it would probably occur in one of these clock genes, such as *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *ARNTL* (*Bmal1*), *Clock*, *CSNK1E* (Lowrey and Takahashi, 2000) or several others that have a less recognized role [e.g. *NPAS2* (Johansson *et al.*, 2003) or *GSK3B* (Gould and Manji, 2002)]. Moreover, a clock gene variation associated with bipolar disorder would be most probably found in a gene located in a chromosomal region that displays linkage in bipolar families. Following this logic, we focused on the human *cryptochrome 1* gene (*Cry1*). Substantial evidence has been presented for linkage of bipolar disorder to the long arm of chromosome 12 near the location of *Cry1* at 12q23-q24.1 (for example, Craddock *et al.*, 1994; Dawson *et al.*, 1995; Detera-Wadleigh, 1999; Morissette *et al.*, 1999; Degn *et al.*, 2001; Johansson *et al.*, 2001; Jones *et al.*, 2002; Curtis *et al.*, 2003; Ewald *et al.*, 2003). Although the highest LOD scores for linkage to bipolar disorder may be localized some megabases away from the exact site of *Cry1*, and there have been many failures to confirm linkage in this region, we considered that the linkage evidence was sufficiently encouraging to make *Cry1* an interesting candidate gene.

The cryptochromes (*Cry1* and *Cry2*) are blue-light-responsive proteins found in bacteria, plants, *Drosophila*, and mammals (Hsu *et al.*, 1996; Deisenhofer, 2000). In the mammalian circadian system, the cryptochromes are thought to inhibit effects of the BMAL1–CLOCK complex in promoting transcription of the *Per* genes (Griffin *et al.*, 1999; Okamura *et al.*, 1999; Shearman *et al.*, 2000; Schibler *et al.*, 2001; Yu *et al.*, 2002; Van Gelder, 2002). This essential role in a negative feedback loop of the circadian clock may be performed by heterodimers of cryptochromes and PER proteins. In knockouts of both *Cry1* and *Cry2*, the mouse circadian system becomes arrhythmic (Okamura *et al.*, 1999; van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999). The possibility that cryptochromes function as photoreceptors in mammals has been raised (for example, Van Gelder *et al.*, 2003). It is also possible that cryptochromes participate in the dual-oscillator circadian organization that mediates photoperiodism (Daan *et al.*, 2001; Hastings, 2001; Tournier *et al.*, 2003). Finally, cryptochromes appear to influence sleep patterns (Wisor *et al.*, 2002).

To examine the possible role of *Cry1* in bipolar disorder, we considered its linkage with closely adjacent polymorphic markers. To recognize any sequence variants, we resequenced parts of *Cry1* in patients from bipolar families showing positive linkage to *Cry1*, and then studied the possibility of linkage disequilibrium between polymorphisms in *Cry1* and affective phenotypes.

Methods

Subjects and assessment

All subjects provided written informed consent through local Institutional Review Board approved procedures prior to participation. Subjects were recruited as part of two multi-site collaborations to collect families for linkage studies of bipolar disorder. Set 1, the UCSD/UBC/UC family set, was collected at the University of California in San Diego, the University of British Columbia and the University of Cincinnati. Families were ascertained through a bipolar I or bipolar II proband and selected for the presence of at least two other mood-disordered family members. Ascertainment and diagnostic methods have been described previously (Kelsoe *et al.*, 2001). Briefly, all subjects were directly interviewed using the Structured Clinical Interview for DSM-III-R by interviewers who had undergone an extensive training course. Information from the interview, other family informants, and medical records were then reviewed by a panel of clinicians in order to make a best estimate diagnosis. Set 2 consisted of 106 families from the NIMH Genetics Initiative for Bipolar Disorder first wave pedigree collection. Ascertainment and diagnostic methods have been described previously (Edenberg *et al.*, 1997) and are similar to those already described except that all families were ascertained through a bipolar I proband, and the Diagnostic Interview for Genetic Studies was employed.

For the microsatellite genotyping we used the 52 families from Set 1. These pedigrees consisted of 356 subjects, with an average of 6.9 members/family (range 3–33). Mutation resequencing was performed on a subset of Set 1 with even sex ratios: on 25 bipolar probands from families with suggestive linkage to *Cry1*, one schizophrenic subject, and on one single control and one pool of 23 controls with no history of psychiatric illness. For the association study, a sample of 159 families (564 individuals), each consisting of one or two affected (bipolar I or II) children and their parents was chosen (53 triads from Set 1, 106 triads from Set 2). Probands ($n = 14$) from Set 1 had also been sequenced in the mutation screening and were used for quality control.

DNA extraction

Blood was obtained from all subjects for the immortalization of lymphoblastoid cell lines, and DNA was prepared by phenol/chloroform extraction from cultured cells.

Microsatellite genotyping

The two *Cry1* flanking microsatellite markers D12S317 (104.10 MB on the UCSC genome assembly Human July 2003, 114.28 cM on Marshfield map) and D12S353 (106.53 MB, 115.18 cM) were chosen for genotyping. Polymerase chain reaction (PCR) reactions were run in a total volume of 15 µl, containing 10 ng (for D12S317) or 100 ng (for D12S353) DNA, 0.5 µM each primer (the

forward primer was labeled), 0.25 mM dNTPs, 0.75 U AmpliTaq Gold (Perkin-Elmer), and 1 × PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3, 0.001% gelatin). Reactions were run in an MJ Research PTC-100 thermal cycler with the following cycle parameters: 1 × 95°C, 10 min; followed by a touchdown protocol [starting at an annealing temperature of 68°C (D12S317) or 65°C (D12S353) and decreasing by 1°C every two cycles, followed by 10 cycles at 60°C] with denaturing at 94°C for 1 min, annealing for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 30 min. PCR products were separated by electrophoresis and detected using an Applied Biosystems 377 automated sequencer with Genescan and Genotyper software (Applied Biosystems, Foster City, CA, USA). Markers were pooled along with a molecular weight standard (TAMRA; PE Applied Biosystems, Foster City, CA, USA) for multiplex detection. A standard sample (CEPH GM7050) was used to assure consistency between different gels.

Mutation screening

The genetic structure of *Cry1* (NCBI LocusID 1407, genomic contig NT 019546), which was determined at the time of the study by aligning the mRNA sequence with genomic sequences (GenBank accession numbers D83702, AC078929, AC007541), includes 12 exons and 102180 base pairs (bp) from the start of the 5′ untranslated region (UTR) to the end of the 3′ UTR. Screening for mutations was performed by amplifying and sequencing interesting genomic regions of the *Cry1* gene (Table 1): 16 fragments of 500–700 bp lengths were designed to cover all exons plus 150 bp flanking intronic regions on each side, the 5′ UTR and 3′ UTR, and approximately 1 kb of the promoter region; six additional fragments were designed within the 70 610 bp long intron 1. Primer sequences for the 22 fragments were picked with Primer3 software (http://www.genome.wi.mit.edu) and are presented in Table 1.

Amplification was performed in 60 µl reactions under two different conditions: (1) with 0.4 µl AmpliTaq Gold (Perkin-Elmer), 1 × AmpliTaq Gold PCR buffer, 1 mM $MgCl_2$, 100 ng DNA, 0.17 µM each primer, 0.21 mM dNTPs, and 2.4 µl dimethylsulfoxide; or (2) with the FailSafe PCR system (Epicentre, Madison, WI, USA), including 120 ng DNA, 0.6 µl enzyme mix, 0.3 µM F and R primer each, and 30 µl PreMix G. A thermocycler was used with the following parameters: 1 × 94°C for 2 min; 35 x (or 39 × for Epicentre) at 92°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min, and a final extension at 72°C for 30 min. PCR products were purified with the QIAquick 96 PCR purification kit (Qiagen, Valencia, CA, USA). They served as templates for sequencing reactions with BigDye Terminator Cycle Sequencing v2.0 reagents (PE Applied Biosystems), including 6 µl template, 4 µl reaction mix, 4 µl 5 × buffer, and 1 µM primer in a 20 µl reaction. Cycle sequencing was performed with the following parameters: 1 × 95°C, 3 min; 25 × 96°C for 45 s, 50°C for 45 s, 60°C for 4 min, and 1 × 60°C for 10 min. Fluorescent-labeled sequencing products were purified with the DyeEx 96 kit (Qiagen) and electrophoretically separated and detected using an ABI 377 automated DNA sequencer. The ABI sequencing software v.3.3 (PE Applied Biosystems) was used for lane tracking and first pass base calling.

To analyze for single nucleotide polymorphisms (SNPs), chromatograms were then transferred to a UNIX workstation (Sun Microsystems, Santa Clara, CA, USA), base called with Phred, assembled with Phrap, scanned by PolyPhred, and the results were viewed with the Consed program as described in Nickerson *et al.* (1997).

SNP genotyping

Twelve SNPs (SNPs 1, 4, 5, 7, 8, 9, 10, 11, 13, 14, 15, 16) were chosen for genotyping according to their frequency ($\geq 4\%$) and relative position to each other. They were genotyped with the Masscode System (Qiagen Genomics), using cleavable mass spectrometry tags in multiplex reactions (Kokoris *et al.*, 2000). PCR reactions with external primers resulted in single-band products for all SNPs. Preliminary quality controls for the complete primer sets (two external and two hemi-nested primers) showed a low cluster quality for SNPs 7 and 10, and these SNPs were excluded from further genotyping.

Statistical analysis

The computer program CERVUS 2.0 (Marshall *et al.*, 1998) was used to calculate observed (H_O) and expected (H_E) heterozygosities and to test for deviation from Hardy–Weinberg equilibrium at all loci.

CRI-MAP 2.4 (Phil Green, http://compgen.rutgers.edu/multimap/crimap/index.html) was used to detect microsatellite genotyping errors (option 'chrompic') and all genotypes of candidate data errors were verified. Parametric two-point linkage analyses were performed using the LINKAGE package (http://linkage.rockefeller.edu). Allele frequencies for the markers were estimated from the families using DOWNFREQ 1.1 (J. Terwilliger). For both a narrow (bipolar I plus bipolar II plus schizoaffective) and a broad (bipolar I plus bipolar II plus schizoaffective plus recurrent major depression) diagnostic model, the disease has been modeled in three ways: (1) autosomal dominant with high penetrance (AD85), (2) autosomal dominant with medium penetrance (AD50), and (3) autosomal recessive with medium penetrance (AR50) (Kelsoe *et al.*, 2001). Penetrance and disease allele frequencies were adjusted for each genetic model to yield an approximately 5% phenocopy rate and disease prevalences of 1% for the narrow and 2% for the broad diagnostic model. An age-of-onset curve was

Table 1 Mutation screening in *Cryptochrome 1*: primers and polymerase chain reaction (PCR) conditions for 22 genomic regions

Location and name	Start position (ATG = 1)	Fragment length (base pairs)	PCR primers[a]	PCR conditions (see text)	Sequencing primers
Promotor P-3	−1892	542	F, tgctaggaatgggcactttt R, tcctggacttcctggaatttt	1	F; R
Promotor P-2	−1489	548	F, tgcagttcactgtcgctgga R, ggatgagcacggggatgc	2	F; R
Promotor P-1	−1081	508	F, aaatcccacgggaggaatcg R, ctgcccgggtgactctgc	2	F; R
5′ UTR	−711	636	F, ccctccgagccagtgtagta R1, tccaagagaattgcctcacc R2, gaggcgcagtggaaagat	2	R1; R2 only
Exon 1	−213	575	F, cgactaggcaacctccattc R, acgacctgaaacactcggtc	1	F; R
Intron 1, I1	20 468	698	F, caatttgggtcaggggttt R, gcaatacatgtgagaatggaagac	1	F; R
Intron 1, I2	27 425	666	F, gcaacctcaacctccagtgt R, tcatgctaatggagcaacaga	2	F; R; F2, caggctggcctcaaactc
Intron 1, I3	36 238	702	F, ggaacttcctgaggcctttt R, gaattcccaaaccaaaagctaa	1	F; R
Intron 1, I4	47 888	686	F, taatgtgccgtgaggaatga R, tgaggtgcccacagttattg	1	F; R
Intron 1, I5	50 957	703	F, ccaatcctatttgtcttgagcac R, gatgtccccaatgttaaagca	1	F; R
Intron 1, I6	69 261	709	F, aggtgaaatggtgggatgtc R, tctgttgcgaagaaatggaa	1	F; R
Exon 2	70 622	517	F, tggtttttggaaaaagtatatatcaa R, ctggtagtagctgttgcttctg	1	F; R
Exon 3	87 484	593	F, ttgctttgctagattgttgcctagcat R, acctgtcattgggtcaata	1	F; R; R2, aaaaggactcacttgtctaggtcat
Exon 4	90 799	521	F, ttgatacccagaggatgtctagaag R, ccctgccctttctttctctc	1	F; R
Exon 5	91 262	535	F, ccccttttaattttgccatgt R, ttctgttaatcctccccttttca	1	F; R
Exon 6	92 577	564	F, cgagaccctgtctcaggaaa R, gttgcccataaagggaaagg	1	F; F2, aaaaatctccattctgagtgttctttt
Exon7	92 957	604	F, tccgatttggttgtttgtca R, gaaagtgtccccgtatttgg	1	F; R
Exon 8	94 660	620	F, gcagaggttggaaaagtaaattct R, caaataacgcctttgggaga	1	F; R
Exons 9 + 10	95 179	604	F, tggtcttgatttgattttggt R, ttaagctaatttcaaagacagattttt	1	F; R
Exons 11 + 12	99 748	599	F, ttaagttttcctggtttgagca R, cagatgcatgtctcttgacca	1	F; R
3′ UTR, 3′-1	100 902	486	F, cattgaattatttgcaatcaagc R, tgcaaagttagatcaaacaacatc	1	F; R
3′ UTR, 3′-2	101 284	437	F, aaagttttgaagtcagtgggaa R, tggtagctgttccttcaaaatg	1	F; R

UTR, untranslated region.
[a]F, forward (5′–3′); R, reverse (3′–5′).

included with minimal risk below age 15 years, and maximizing at the defined penetrance at age 40 years. Using the same affection stati (i.e. narrow and broad), two-point non-parametric linkage analyses were performed by MERLIN (Abecasis *et al.*, 2002).

Association of the genotyped sequence variants to bipolar disorder was tested with a Transmission Disequilibrium Test (TDT) (ETDT package; Sham and Curtis, 1995). Power calculations for the TDT with nine SNPs was calculated using Purcell *et al.*'s (2003) program for discrete traits (http://statgen.iop.kcl.ac.uk/gpc/) assuming an additive model with a disease prevalence of 1% and perfect linkage disequilibrium (LD) between marker and disease. LD between markers was analyzed with GOLD (Graphical Overview of Linkage Disequilibrium) v.1.0 (http://www.well.ox.ac.uk/asthma/GOLD; Abecasis and Cookson, 2000). Simwalk2 v.2.80 (ftp://watson.hgen.pitt.edu/pub/simwalk2; Sobel and Lange, 1996) was used to estimate haplotypes. Using founder haplotype estimates as input, LD is described here using the pair-wise standardized disequilibrium coefficient D' for its independence of allele frequency (Lewontin, 1988). TRANSMIT 2.5.2. (http://www-gene.cimr.cam.ac.uk/clayton/software/; Clayton, 1999), was used for transmission disequilibrium testing of haplotypes comprised of multiple SNPs, because this program allows for testing with marker haplotypes based on several closely linked markers and when parental genotype and/or haplotype phase may be missing. The robust variance estimator was used as it allows for multiple affected sibs within a family, even in the presence of linkage.

Results

Linkage analysis

Markers D12S317 and D12S353 have been genotyped in 343 and 345 subjects from 52 bipolar families. Both markers were highly polymorphic (D12S317, 0.793; D12S353, 0.786). Observed heterozygosities did not deviate from the Hardy–Weinberg equilibrium ($P > 0.05$). No positive LOD scores were obtained under any of the six models for the overall marker versus disease analysis (Table 2). Non-parametric linkage analyses for D12S317 resulted in a LOD = 0.17 ($Z = 0.60$, $P = 0.2$) for the narrow and LOD = 0.37 ($Z = 0.93$, $P = 0.10$) for the broad affection status, and for D12S353 in a LOD = 0.05 ($Z = 0.33$, $P = 0.3$) and LOD = 0.27 ($Z = 0.75$, $P = 0.13$), respectively.

Table 2 Linkage analysis for bipolar disease and two microsatellite markers

Marker	theta = 0	theta = 0.05	theta = 0.1	Genetic model
Narrow diagnostic model[a]				
D12S317	−10.42	−4.46	−2.56	AD85
D12S353	−13.15	−4.8	−2.58	
D12S317	−7.05	−3.24	−1.77	AD50
D12S353	−8.06	−3.07	−1.47	
D12S317	−9.26	−5.39	−3.27	AR50
D12S353	−8.94	−4.71	−2.6	
Broad diagnostic model[b]				
D12S317	−14.94	−6.88	−3.79	AD85
D12S353	−21.39	−10.11	−6.03	
D12S317	−8.94	−4.53	−2.44	AD50
D12S353	−12.29	−6.28	−3.74	
D12S317	−9.83	−6.34	−4.16	AR50
D12S353	−10.71	−6.62	−4.22	

LOD scores at different recombination fractions (thetas) are given for six different transmission models.
[a]Affected = bipolar I plus bipolar II plus schizoaffective.
[b]Affected = bipolar I plus bipolar II plus schizoaffective plus recurrent major depression.

Mutation screening in *Cry1*

Although the linkage analysis showed negative overall LOD scores in our sample set for both microsatellites (D12S317 and D12S353) flanking *Cry1*, this gene was chosen for further analysis because of its close correspondence to a strong linkage hotspot for bipolar disorder in other studies. At the single family level, slightly positive LOD scores in the range of 0.01–0.27 were obtained for 23 of the 52 genotyped bipolar pedigrees under model 2. One affected proband from each of these families was selected for subsequent mutation screening.

Sequencing of 22 fragments of genomic DNA resulted in 9679 bp of high-quality double-stranded sequence, corresponding to 9.5% of the *Cry1* gene. These sequences covered all 12 exons, the 5′ and 3′ UTRs, a 1.31 kb sequence of the promoter region (i.e. upstream of the 5′ UTR), and some intronic regions. This mutation screen in 27 subjects with the power to identify variances with a frequency of approximately 2% led to the detection of a total of 16 base substitutions and one 3 bp insertion (Table 3). All mutations have been reported to dbSNP and were assigned the following accession numbers ss12586661–ss12586677. The only coding sequence variant identified (SNP 14), a C→T substitution in exon 5, was found to be synonymous. Most SNPs occurred at a relatively high frequency, and the high density of SNPs with most distances < 20 kb made this selection suitable for LD studies.

SNP genotyping

Ten sequence variants of the *Cry1* gene were genotyped in 159 parent proband triads. Genotyping quality, assessed by the percentage of calls (> 90%), Hardy–Weinberg equilibrium, and cluster score values were in the acceptable range for nine SNPs. SNP 11 was not in Hardy–Weinberg equilibrium ($P < 0.0005$) and

Table 3 Location and characteristics of 16 single nucleotide polymorphisms (SNPs) and one insertion detected among 27 subjects in the 9.7 kb sequence of *Cryptochrome 1*

SNP	DbSNP	Location	Position (base pairs)	Frequency of rare allele	Variant	Distance to next SNP (base pairs)
1	ss12586661	867 bp 5′ of 5′ UTR	−1449	0.37	C/T	28
2	ss12586662	839 bp 5′ of 5′ UTR	−1421	0.02	A/G	1089
3	rs 3809235	250 bp within 5′ UTR	−332	0.37	A/G	22
4	rs 3809236	272 bp within 5′ UTR	−310	0.04	C/T	135
5	rs 3809237	407 bp within 5′ UTR	−175	0.33	A/G	49
6	rs 3809238	456 bp within 5′ UTR	−126	0.02	C/T	27 767
7	rs 1921135	27 484 bp 3′ of exon 1	27 642	0.37	G/A	20 462
8	ss12586668	22 665 bp 5′ of exon 2	48 104	0.33	G/A	3229
9	rs 1017168	19 436 bp 5′ of exon 2	51 333	0.30	T/G	18 223
10	rs 1420399	1213bp 5′ of exon 2	69 556	0.11	C/T	254
11	rs 1861591	959 bp 5′ of exon 2	69 810	0.37	A/G	21 589
12	ss12586672	194 bp 5′ of exon 5	91 399	0.02	C/G	49
13	ss12586673	145 bp 5′ of exon 5	91 448	0.37	A/G	184
14	ss12586674	39 bp within exon 5	91 632	0.33	C/T	9496
15	rs 1056560	168 bp within 3′ UTR	101 128	0.37	G/T	156
16	ss12586676	324 bp within 3′ UTR	101 284	0.11	A/C	323
17	ss12586677	13 bp 3′ of 3′ UTR	101 607	0.37	GTG insertion	–

UTR, untranslated region.

comparison with known genotypes of 14 sequenced samples revealed genotyping errors. This SNP was excluded from further analysis. Allele frequencies of the remaining nine SNPs (Table 4) corresponded relatively well with the allele frequencies determined by sequencing of 27 subjects.

LD analyses

Association of the nine *Cry1* SNPs to bipolar disorder was analyzed with a linkage disequilibrium test in 159 affected families. The results of the ETDT analysis are presented in Table 4. No evidence for association between bipolar disease and any of these sequence variants was found ($P > 0.05$ in all cases).

The power of this analysis with nine SNPs was calculated for 150 triads at a disease prevalence of 1% in the general population under an additive model and assuming the candidate gene to be the disease locus (Fig. 1). The power was $> 80\%$ to detect alleles at low frequencies of 0.1 with high genotype relative risks (GRR > 2), but not much power was estimated for alleles with more modest relative risks.

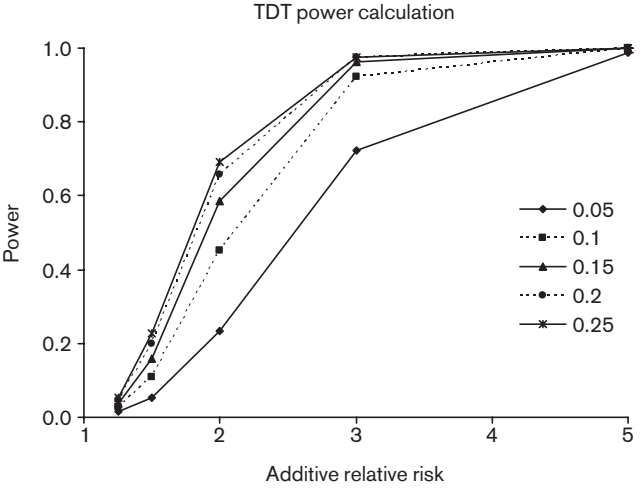
Multi-allelic haplotypes comprised of several SNPs are more informative than individual SNPs. In order to determine most informative haplotypes, however, LD relationships between the SNPs have to be taken into consideration. Following these initial analyses, LD between the nine markers was therefore analyzed using SimWalk2 to construct founder haplotype estimates, and GOLD to calculate pair-wise disequilibrium measures. All markers were found to be in strong or even perfect LD with each other. With the exception of the SNP4/SNP16 pair ($P = 0.438$), association was significant in all cases ($P < 0.05$).

Because there was no apparent substructure with recombination hotspots apparent in *Cry1*, all nine SNPs were analyzed together using TRANSMIT. One hundred and fifty-five families with transmission to affected offspring ($n = 232$) were analyzed. Of the 512 possible marker haplotypes only 17 were observed, and 13 of them were rare (< 1%). There was no significant association of any of the single haplotypes or the global distribution to bipolar disorder ($P > 0.1$ in all cases).

Table 4 **Transmission disequilibrium test analyses using ETDT: successful trials, the number of alleles passed from parent to affected offspring, and empirical *P* values (Monte Carlo approach with 1000 replicates) are given for each of the nine single nucleotide polymorphisms (SNPs)**

SNP	Frequency of rare allele in 159 triads	Trials	Allele 1 passed	Allele 2 passed	*P* empirical
1	0.460	219	111	108	0.890
4	0.046	43	23	20	0.755
5	0.394	181	90	91	1.000
8	0.397	205	101	104	0.888
9	0.366	203	98	105	0.683
13	0.459	222	110	112	0.946
14	0.401	206	103	103	1.000
15	0.460	190	84	106	0.116
16	0.019	14	8	6	0.774

Fig. 1



Power calculation for Transmission Disequilibrium Tests (TDTs) using nine single nucleotide polymorphisms in *Cryptochrome 1*. The power for 150 triads and a disease prevalence of 1% in the general population is shown for five different high-risk allele frequencies for increasing additive relative risks.

Discussion

Although linkage of bipolar disorder to a region near the location of *Cry1* at 12q23-q24.1 has been reported by several groups (Dawson *et al.*, 1995; Detera-Wadleigh, 1999; Morissette *et al.*, 1999; Degn *et al.*, 2001; Curtis *et al.*, 2003), we found no evidence of linkage to this locus in our set of 52 bipolar families. Furthermore, mutational screening of our candidate gene enabled us to find SNPs at a density and frequency high enough to be suitable for LD studies, but no likely functional mutations were discovered. LD analyses using nine single SNPs as well as haplotypes did not show evidence for association to bipolar disease.

As with any analysis reporting a negative result, it is important to determine whether our sample provided sufficient power to detect the hypothesized effect. The power of these 52 pedigrees for linkage has been reported previously (Shaw *et al.*, 2003) and was found to be relatively low for genes of modest effect; for example, under a dominant and recessive model, respectively, the sample had expected LOD scores of 3.98 and 3.39, with 74% and 61% power to detect a LOD > 3 assuming 25% heterogeneity. Here we further used association studies

that are known to be more powerful (Risch and Merikangas, 1996). Our power calculations for the TDT analysis showed that we only have sufficient power to detect alleles with a high genotype relative risk. However, because about 50% of our parent–proband sets included two affected siblings and many of them were drawn from larger families with many affected individuals, the actual power might be higher than calculated here.

So far, *Cry1*, which is necessary for the maintenance of circadian rhythmicity (van der Horst *et al.*, 1999), has not been reported to be involved in any human disorder. However, other clock genes have been studied in mood and sleep phase disorders. A non-coding mutation in *Clock* was reported to be associated with diurnal preference (Katzenberg *et al.*, 1998) and insomnia in mood disorders (Serretti *et al.*, 2003), but not major depressive disorder (Desan *et al.*, 2000), bipolar disorder (Shiino *et al.*, 2003), or seasonality and seasonal affective disorder (SAD) (Johansson *et al.*, 2003). Small samples failed to replicate the association with diurnal preference (D'Almeida *et al.*, 2002; Robilliard *et al.*, 2003). Mutations in *Per3* are related to delayed sleep phase syndrome (Ebisawa *et al.*, 2001; Archer *et al.*, 2003) and diurnal preference (Johansson *et al.*, 2003), but not SAD and seasonality (Johansson *et al.*, 2003). Furthermore, a missense mutation in *Per2* causes familial advanced sleep phase syndrome (Toh *et al.*, 2001), but is not associated with SAD, seasonality or diurnal preference (Johansson *et al.*, 2003), and a non-synonymous mutation in *NPAS2* may be related to SAD, but not seasonality and diurnal preference (Johansson *et al.*, 2003). To date, relatively few studies have examined the role of circadian clock genes in mood disorders. It is not surprising that clock gene variants have been shown to be associated with disorders of circadian rhythmicity. More studies are required in order to determine the role of clock genes in mood disorders. Furthermore, the number of identified clock genes has been rapidly expanding. Time will be required in order to test these newly identified clock genes or clock genes yet to be identified.

We have identified a number of novel SNPs that will be useful for association analyses of this gene in a variety of phenotypes. In combination with other known SNPs, *Cry1* now has an average SNP density of 1.6 kb. We also provided data on allele frequency and LD structure that will facilitate future studies.

Although we did not find any evidence for a major role of *Cry1* in bipolar disorder, additional studies with greater power and on other populations are clearly necessary. It is possible that *Cry1* does contribute susceptibility to bipolar disorder in other populations, or in such a small portion of families that it could not be detected here. Furthermore, other circadian clock genes and their interactions with each other need to be tested before an association of circadian abnormalities with bipolar disease can be excluded.

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