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Clock Genes may Influence Bipolar Disorder Susceptibility and Dysfunctional Circadian Rhythm

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Abstract

Several previous studies suggest that dysfunction of circadian rhythms may increase susceptibility to bipolar disorder (BP). We conducted an association study of five circadian genes (*CRY2*, *PER1-3*, and *TIMELESS*) in a family collection of 36 trios and 79 quads (Sample I), and 10 circadian genes (*ARNTL, ARNTL2, BHLHB2, BHLHB3, CLOCK*, *CRY1*, *CSNK1D*, *CSNK1E*, *DBP*, and *NR1D1*) in an extended family collection of 70 trios and 237 quads (Sample II), which includes the same 114 families but not necessarily the same individuals as Sample I. In Sample II, the Sibling-Transmission Disequilibrium Test (sib-tdt) analysis showed nominally significant association of BP with three SNPs within or near the *CLOCK* gene (rs534654, $p = 0.0097$; rs6850524, $p = 0.012$; rs4340844, $p = 0.015$). In addition, SNPs in the *ARNTL2*, *CLOCK*, *DBP*, and *TIMELESS* genes and haplotypes in the *ARNTL*, *CLOCK*, *CSNK1E*, and *TIMELESS* genes showed suggestive evidence of association with several circadian phenotypes identified in BP patients. However, none of these associations reached gene-wide or experiment-wide significance after correction for multiple-testing. A multi-locus interaction between rs6442925 in the 5′ upstream of *BHLHB2*, rs1534891 in *CSNK1E*, and rs534654 near the 3′ end of the *CLOCK* gene, however, is significantly associated with BP ($p = 0.00000172$). It remains significant after correcting for multiple testing using the False Discovery Rate method. Our results indicate an interaction between three circadian genes in susceptibility to bipolar disorder.

Keywords

single nucleotide polymorphism; linkage disequilibrium; haplotype; bipolar disorder; circadian rhythm

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INTRODUCTION

Bipolar disorder (BP), also known as manic-depressive illness, affects approximately 1% of the general population [Weissman et al., 1996], and has a strong genetic component [Hayden and Nurnberger, Jr., 2006]. Dysfunction of circadian rhythms is hypothesized to play a role in the pathophysiology of BP (see reviews [Jones, 2001; Mansour et al., 2005; McClung, 2007; Wehr et al., 1983]). First, BP patients frequently demonstrate biological rhythm-related symptoms, including diurnal variation of mood, the periodicity of exacerbations and remissions of disease, and sleep disturbance (e.g., a decreased need for sleep during mania, insomnia or hypersomnia during depression). The sleep disturbance in BP has been hypothesized to be caused by abnormal circadian function (dysfunctional timing of sleep, changed amount of sleep, and instable social rhythms or zeitgebers). Sleep disturbance possibly promotes emotion dysregulation [Harvey et al., 2006]. However, the causal relationship between sleep disturbance (or rhythm disturbance to some extent) and emotional problems may be bidirectional [Dahl, 2004]. Secondly, some treatments for mood disorders may exert their roles through modulating circadian rhythms and/or alleviating sleep disturbance [McClung, 2007]. For example, the mood stabilizer lithium, an inhibitor of glycogen synthase kinase 3 beta (GSK3B), can cause a phase delay in the circadian rhythms of BP patients [Kripke et al., 1978]. GSK3B regulates multiple molecules of the circadian clock and may represent a target of novel drugs for mood disorders [Gould et al., 2006; Iitaka et al., 2005; Yin et al., 2006]. Thirdly, several genetic association and gene expression studies have suggested that circadian genes may underlie the development of mood disorders including BP and the disturbances of rhythms seen in those patients [Mansour et al., 2005; Mitterauer, 2000]. Mansour et al. [Mansour et al., 2006] found suggestive association between individual single nucleotide polymorphisms (SNPs) in *ARNTL*, *PER3*, and *TIMELESS* genes and BPI. Nievergelt et al. [Nievergelt et al., 2006] reported haplotypic association of *ARNTL* and *PER3* genes with BP. A SNP (T3111C; rs1801260) in the 3′ flanking region of the *CLOCK* gene was reported to be associated with sleep dysregulation in BP and major depression [Serretti et al., 2003; Serretti et al., 2005], as well as with high illness recurrence of BP [Benedetti et al., 2003]. Ogden *et al* found that valproate decreased the expression of *CSNK1D* and *CRY2* in the mouse amygdala while methamphetamine down-regulated *ARNTL* expression in the mouse prefrontal cortex [Ogden et al., 2004], implicating them as candidate genes for mood disorders. Moreover, gene expression data from the Stanley Medical Research Institute's (SMRI) brain collections (https://www.stanleygenomics.org/) reveals abnormal expression of several circadian genes in BP patients in contrast to mentally healthy controls (Supplementary Table I), implying a possible dysfunction of the circadian clock in the pathophysiology of BP. Furthermore, transgenic mice carrying a mutation in the *Clock* gene display a human mania-like behavioral profile, which reverts to nearly normal levels after chronic administration with lithium. These abnormal behaviors can also be rescued by expressing a functional CLOCK protein via viral-mediated gene transfer specifically into the ventral tegmental area of mutant mice [Roybal et al., 2007]. *Gsk3b*-overexpressing transgenic mice also show hyperactivity and mania-like behavior [Prickaerts et al., 2006]. Finally, evolutional data suggests possible biological connections between evolutionary variants of circadian rhythm genes and behavior and/or mood [Fitzpatrick et al., 2007; Sandrelli et al., 2007; Sher, 2000; Tauber et al., 2007].

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master pacemaker of the circadian clock and the initiating site of transcriptional-translational self-regulatory feedback loops in mammals, where multiple molecules interact to maintain the behavioral and physiological rhythms [Bell-Pedersen et al., 2005]. The cycle begins with heterodimerization of CLOCK and ARNTL1, which binds to E-box sequences of a number of circadian genes including *PER* and *CRY* and activates their transcription. PER and CRY proteins slowly accumulate in the cytosol and are phosphorylated by CSNK1D, CSNK1E, or GSK3B. Upon entering the nucleus, PER and CRY form heterodimers and inhibit their own transcription

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through binding to CLOCK-ARNTL complex, thus creating a negative feedback loop. In addition, the ARNTL and CLOCK complex can also activate the transcription of the orphan nuclear receptor gene *REVERBa* and the retinoic acid receptor-related orphan receptor gene *RORA*, which accumulate at different speeds, and can repress and activate the transcription of *ARNTL*, respectively. These molecules create an adjoining negative feedback loop. Thus, positive and negative loops of the circadian clock are linked (Supplementary Figure I) [Bell-Pedersen et al., 2005]. Other circadian proteins, including BHLHB2 and BHLHB3, may directly or indirectly interact with regulators within the feedback loops and influence the output of behavioral and physiological rhythms [Honma et al., 2002].

Our group previously proposed a neurobiological system (or pathway)-based strategy for association studies in mood disorders, including BP [Hattori et al., 2005]. It is based on the hypothesis that genetic variation in multiple genes from one or several relevant pathophysiological pathways could account for disease susceptibility [Hattori et al., 2005]. The internal trigger theory, or more specifically, the circadian rhythm disruption hypothesis of mood disorders [Grandin et al., 2006], postulates that abnormality of the circadian pacemaker (i.e. SCN) caused by genetic variation underlies the biological and social rhythm disruptions which trigger mood episodes in patients with unipolar and bipolar disorders. We tested this hypothesis by analyzing 15 circadian rhythm pathway genes in BP (Table I). We utilized annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [Kanehisa et al., 2006] and Protein ANalysis THrough Evolutionary Relationships (PANTHER) [Mi et al., 2007] databases to select the candidate genes for association analysis. Of these genes, 11 are reviewed as the main circadian genes in mammals [Cermakian and Boivin, 2003]; *BHLHB2*, *BHLHB3,* and *TIMELESS* have shown a regulatory function for mammalian biorhythm [Barnes et al., 2003; Honma et al., 2002]; *ARNTL2* may also have ARNTL-like transcription-activating function [Okano et al., 2001; Schoenhard et al., 2002].

MATERIALS AND METHODS

We initially genotyped 19 SNPs within five circadian genes in a modest-sized BP sample set (Sample I). Upon completion of genotyping, additional BP family samples became available. Therefore, we analyzed the remaining 62 SNPs within ten circadian genes in the extended BP family sample (Sample II).

Sample I

A total of 416 samples were included: 6 trios and 8 quads from the Clinical Neurogenetics (CNG) pedigrees and 30 trios and 71 quads from the National Institute of Mental Health (NIMH) Genetics Initiative pedigrees waves 1 and 2. The CNG pedigrees are described in detail elsewhere [Berrettini et al., 1991]. Background and detailed clinical assessment for the NIMH Genetics Initiative have been previously described [Nurnberger, Jr. et al., 1997]. Briefly, all subjects affected with BP were assessed with the Diagnostic Instrument for Genetic Studies (DIGS) [Nurnberger, Jr. et al., 1994] and the Family Interview for Genetic Studies (FIGS) by a clinically trained professional. Subsequently, two clinicians made separate reviews of all available information including DIGS and FIGS data and medical records, and made a final diagnosis using a best-estimate procedure. All individuals are of European descent based on self-reported ancestry and further confirmed by STRUCTURE [Pritchard et al., 2000] analysis using 254 unlinked SNPs (data not shown). Of 194 offspring, 154 meet DSM-III-R criteria for BPI, 29 for BPII, and 11 for schizoaffective disorder bipolar type (SAB); 105 are females and 89 are males.

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Markers and Genotyping for Sample I

Nineteen SNPs at five genes were selected for genotyping (Table I and Supplementary Table II). SNPs were chosen from public databases using a series of bioinformatics tools, including the SNP Information Mining Pipeline (SIMP) and the Gene Information Mining Pipeline (GIMP), which were developed in our laboratory (http://bioinfo.bsd.uchicago.edu/index.html) and described elsewhere [Shi et al., 2007]. Briefly, SNPs with minor allele frequency (MAF) greater than 0.1 in HapMap Caucasians were examined for linkage disequilibrium (LD). We used the ldSelect algorithm to select tag SNPs (tSNPs), which are SNPs that capture most of the genetic variation in a region due to being in high LD with other SNPs [Carlson et al., 2004]. One tSNP from each LD bin was selected based on a criterion of $r^2 \geq 0.85$. We selected tSNPs and singleton SNPs (only one SNP in each LD bin) for genotyping with TaqMan 5′ exonuclease assays. Allelic discrimination analysis was accomplished on the Prism 7900HT Fast Real-Time PCR system using the software SDSv2.2.1 (Applied Biosystems Inc., Foster City, CA, USA). We used PedCheck1.1 to detect any Mendelian inconsistencies [O'Connell and Weeks, 1998], and Merlin to detect unlikely recombinants [Abecasis et al., 2002]. All genotype errors were manually resolved by checking the raw genotype data, either assigning a genotype as missing (if unable to be resolved) or correcting the genotype prior to statistical analysis. The average rate of success for each genotyped SNP was > 99.5%.

Sample II

Sample I, except for one quad, is included in Sample II (same families but not necessary the same individuals). Sample II consists of 1158 individuals, including 6 trios and 7 quads from the CNG Series [Berrettini et al., 1991], 39 trios and 185 quads from the NIMH waves 1–4, and 25 trios and 45 quads from the Chicago-Hopkins-Intramural Program (CHIP) series [Potash et al., 2007]. Ascertainment and diagnostic methods were similar to those described for Sample I, except that improved DIGS interviews and DSM-IV diagnosis criteria were used while collecting extended samples. All samples are of European descent identified by methods as used for Sample I. Of 544 affected offspring from 70 trios and 237 quads, 481 had BPI, 37 had BPII, and 26 had SAB; 326 are females and 218 are males.

Markers and Genotyping for Sample II

Tag and singleton SNPs in ten genes (Table I and Supplementary Table IV) were selected using the procedure similar to that for Sample I. These SNPs were included in a genotyping service contract using the Illumina BeadArray technology [Oliphant et al., 2002]. Tag SNPs that failed to pass the Illumina check system using a proprietary algorithm were replaced with alternative SNPs reside within the same LD bins.

For quality control of genotyping, 46 CEPH samples, 15 control samples (11 from the NIMH series, 1 from the CNG series, and 3 from the CHIP series), and 45 blank controls (using water instead of DNA) were used. Sixty-two SNPs in the circadian genes were successfully genotyped in 1279 samples (1279/1288 = 99.3%). For these 1279 samples, the average rate of success for genotyped the 62 SNPs was > 99.77%. Mendelian inconsistent or unlikely recombinant genotypes were assigned as missing prior to final statistical analysis.

Sub-phenotype Analyses

Insomnia is one of the most common symptoms of sleep disturbance in BP patients. Its main etiology is thought to be an altered endogenous oscillating circadian rhythm or a misalignment between the intrinsic sleep-wake propensity and the 24-hour social and physical environment [El-Ad, 2007; Lu and Zee, 2006]. Perturbations of endogenous circadian rhythm may also contribute to diurnal variation of mood and rapid cycling of mood status [Feldman-Naim et

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al., 1997; Papadimitriou et al., 2005]. Therefore, these sub-phenotypes in BP patients can be regarded as circadian phenotypes. We assume that circadian rhythm disturbances as recorded for severe episodes are a stable characteristic that may be related to susceptibility genes. Six circadian sub-phenotypes, including early insomnia, middle insomnia, late insomnia, insomnia in mania, diurnal variation of mood, and rapid cycling were analyzed. Sub-phenotype information was ascertained through the DIGS or the Schedule for Affective Disorders and Schizophrenia-lifetime version (SADS) interview questions answered by each BPI, BPII, and SAB patient. However, because different versions of the DIGS interview were used over the patient collection period, not every patient had information on every circadian phenotype. We used those families with at least one child suffering from both BP and circadian disturbance for sub-phenotype analysis. Rapid cycling was determined using two different methods: answering “yes” to the question “Have you ever switched back and forth between feeling high to feeling normal or depressed” (RC1), and by identifying individuals who had four or more mood disturbances (depressive, manic, and/or hypomanic episodes) per year (RC2). Patients from CNG, CHIP, and NIMH waves 1–4 had sub-phenotype information for early insomnia (317 affected offspring), insomnia in mania (536 affected offspring), and rapid cycling (222 affected offspring for RC1, 47 affected offspring for RC2). Patients from CNG and NIMH waves 1–4 had sub-phenotype information for middle insomnia (226 affected offspring), late insomnia (177 affected offspring), and diurnal variation (173 offspring affected by worse mood in the morning, 122 offspring affected by worse mood in the afternoon/evening).

Statistical Analysis

We considered individuals with BPI, BPII and SAB to be affected in our association analyses.

Pairwise LD, using the standard LD coefficient D′, was calculated between every pair of markers in each gene using Haploview (version 4.0) [Barrett et al., 2005]. Haplotype blocks were identified using the solid spine of LD method in Haploview [Barrett et al., 2005]. This method searches for strong LD (a solid spine of $D' > 0.80$ was set in our analysis), such that the first and last markers in a block are in strong LD with all intermediate markers, but the intermediate markers are not necessarily in LD with each other.

Allelic association of individual SNPs was tested using the sib_tdt program in the program package ASPEX 2.5 (http://aspex.sourceforge.net/). The sib_tdt tests for association independent of linkage, by following the transmission of alleles to affected offspring within families consisting of two parents and one or more affected offspring. It also determines the *p*-value for the test statistic at each SNP by calculating the empirical probability for the chi-square statistic through permutation of the parental alleles while keeping the alleles shared by siblings fixed within the family.

Haplotypic association tests were carried out using PDTPHASE, in the program package UNPHASED [Dudbridge, 2003]. PDTPHASE is an implementation of the pedigree disequilibrium test [Martin et al., 2000], with an extension to deal with missing parental genotyping data. It also includes an expectation-maximization algorithm that calculates maximum-likelihood gametic frequencies under the null hypothesis, allowing the inclusion of phase-uncertain haplotypes. PDTPHASE analyzes whole pedigrees for association, correcting for the effects of linkage. It gives results for the individual haplotypes and a global haplotype value for a particular combination of SNPs.

To evaluate interactions between genes without significant main effects, we used the “focused interaction testing framework” (FITF) method [Millstein et al., 2006]. This approach tests for association in a combination of 1, 2, or 3 loci. For this analysis, we selected one affected offspring per family as the case and created an unaffected control with the alleles that were not transmitted to the case. For tests of interactions of 2 or 3 loci, combinations are pre-screened

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by using a chi-square goodness-of-fit test in the entire sample using both cases and controls. The FITF prescreening approach tests for deviations from the expected genotype distribution and selects those that exceed a particular threshold. Interactions that exceeded this threshold are tested for association using likelihood ratio tests. We searched for gene-gene interactions in the CNG and NIMH waves 1–2 samples, as all SNPs have genotypes in these samples. We also tested, separately, gene-gene interaction among 10 genes in Sample II as this is a larger sample set. Multiple testing was corrected by the false discovery rate (FDR), which is designed to reduce the proportion of all positive results that are false and is less conservative (has a less negative effect on power) than the Bonferroni correction [Benjamini et al., 2001]. Significant results were then tested using a beta-version of MDR-PDT (http://chgr.mc.vanderbilt.edu/ritchielab/method.php?method=mdrpdt) [Martin et al., 2006], which can analyze trio (and discordant sib pair) data for gene-gene interactions using a combination of the genotype-Pedigree-Disequilibrium-Test [Martin et al., 2006] and Multifactor Dimensionality Reduction gene-interaction test [Hahn et al., 2003]. In MDR-PDT, for each trio, an unaffected child is "created" using the genotypes that have not been transmitted from the parents to the affected child. Interaction analysis is performed by comparing the genotypes of the affected child with the unaffected child, using the MDR method [Hahn et al., 2003]. The significance of the result is assessed through permutation of the child's affection status within each family.

RESULTS

Neither allelic nor haplotypic association was found in Sample I for the standard disease phenotype (Supplementary Tables II and III). However, two SNPs in *TIMELESS*, rs2291738 and rs10876890, were found to have possible association with insomnia during a manic episode in Sample I (data not shown). We then tested these and two additional SNPs (rs774026 and rs2279665) in *TIMELESS* in Sample II, which had suggestive evidence of association with BP [Mansour et al., 2006]. But the sub-phenotype association shown in Sample I disappeared in Sample II (data not shown). However, another SNP (rs2279665) showed nominal association for this same sub-phenotype (see below).

In Sample II, which is larger, three SNPs (rs534654, rs6850524, and rs4340844) at the *CLOCK* gene region showed suggestive evidence for transmission disequilibrium (Table II and Supplementary Table IV). Several haplotypes consisting of six SNPs at the *CLOCK* gene region also showed nominal association with disease (Table II and Supplementary Table V). None of these associations survived Bonferroni correction for multiple testing at gene- or experiment-wide levels (data not shown). No nominal disease association with the other nine circadian genes was found in this set of samples (Supplementary Tables IV and V).

Analysis of the six circadian phenotypes in combined samples resulted in four SNPs showing a nominally significant allelic association in several of the sub-phenotypes. Three SNPs within the *CLOCK* gene region had allelic associations to early insomnia (rs534654, $p = 0.021$), middle insomnia (rs534654, $p = 0.021$; rs4340844, $p = 0.0044$; rs6850524, $p = 0.023$), late insomnia (rs534654, $p = 0.0077$; rs4340844, $p = 0.04$), and rapid cycling (RC1) (rs534654, $p = 0.026$). One SNP at the *TIMELESS* gene also showed a nominally significant allelic association with insomnia in mania (rs2279665, $p = 0.024$). One SNP in *ARNTL2* (rs922270, $p = 0.02$) and one SNP in *DBP* (rs3848543, $p = 0.024$) showed nominally significant allelic association with the diurnal phenotype of having a worse mood in the afternoon/evening. Two SNPs in *ARNTL2* showed nominally significant allelic association with rapid cycling (RC2) (rs4963954, $p = 0.023$; rs1048155, $p = 0.048$). After correcting for multiple testing, none of the allelic associations were significant.

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Four haplotype blocks had nominally significant association, but did not survive correction for multiple testing. A haplotype block of 6 SNPs within the *CLOCK* gene region was nominally significant for late insomnia (rs534654, rs2412648, rs4340844, rs11735267, rs6850524, rs7660668, $p = 0.036$). Three haplotype blocks were nominally significant for rapid cycling: a 2-SNP haplotype in *CSNK1E* (RC1: rs6001093-rs135757, $p = 0.038$), a 3-SNP haplotype in *TIMELESS* (RC1: rs774026-rs2279665-rs10876890, $p = 0.025$), and a 3-SNP haplotype in *ARNTL* (RC2: rs6486121-rs12421530-rs3816360, $p = 0.039$).

There was no gene-gene interaction between 15 circadian genes (data not shown). However, a significant multi-locus interaction between rs6442925 in *BHLHB2*, rs1534891 in *CSNK1E*, and rs534654 near 3′ of *CLOCK* gene was identified in Sample II ($p = 0.00000172$), which remained significant after correction for multiple testing using a false discovery rate method (FDR cutoff p-value of 0.00000177) (Table III). This was also significant in the MDR-PDT analysis with a $p < 0.00012$ (uncorrected for multiple testing).

DISCUSSION

The present study did not identify significant allelic or haplotypic association with BP using 81 SNPs located in 15 circadian genes. However, the *CLOCK* gene showed suggestive nominal evidence of association in both allelic and haplotypic analyses (Table II). The interaction of rs534654 in the *CLOCK* gene region with rs6442925 in the *BHLHB2* gene and rs1534891 in the *CSNK1E* gene was significantly associated with BP in Sample II, after correction for multiple testing (Table III). This interaction was also significant in an MDR-PDT analysis.

CLOCK, a transcription factor containing a basic helix-loop-helix (bHLH)-Period-Arnt-Single-minded (PAS) domain, is an essential positive regulator of the mammalian circadian feedback loop in the SCN. The T3111C polymorphism (rs1801260), at the 3′ untranslated region of the *CLOCK* gene, has been associated with a high recurrence rate of BP [Benedetti et al., 2003; Serretti et al., 2003], sleep disturbances in patients with major depression or BP [Benedetti et al., 2007a; Serretti et al., 2003], improved insomnia in BP patients during antidepressant treatment [Serretti et al., 2005], as well as moral valence decision in depressed patients [Benedetti et al., 2007b]. However, other studies have not detected an association with unipolar or bipolar disorders [Bailer et al., 2005; Desan et al., 2000; Johansson et al., 2003; Mansour et al., 2006; Nievergelt et al., 2006; Serretti et al., 2003]. SNP rs7660668, which is in complete LD with T3111C based on HapMap Phase II Caucasian data ($r^2 = 1$, LOD = 30.65), is not associated with BP (Table II) or circadian sub-phenotypes in BP in our sample (data not shown), as suggested by Benedetti et al. [Benedetti et al., 2007a; Benedetti et al., 2007b], given the limited power of our study. We note another limitation that our study was based on subjective measures of circadian rhythm disturbance while Benedetti et al. [Benedetti et al., 2007a; Benedetti et al., 2007b] used objective measures including actigraphy and functional magnetic resonance imaging.

The SNP rs534654, in the *CLOCK* gene region, has the strongest individual association signal in our study (allele A was under-transmitted in Sample II [Table II]). It is located in an intron of the TPA regulated locus gene *TPARL* (also known as transmembrane 165, *TM165*). A recent genome-wide association study (WTCCC) [Wellcome Trust Case Control Consortium, 2007] also showed nominally significant allelic association between rs534654 and BP. However, allele A was overrepresented in patients (OR, 1.12; 95% CI, 1.01–1.24; $p = 0.026$), thus a possible flip-flop association was found [Lin et al., 2007]. This SNP is 8 kb away from the 3′ end of the *CLOCK* gene and is not in strong LD with other common SNPs within the *CLOCK* gene region (Supplementary Figure II). Furthermore, this SNP was found to interact with rs6442925 in *BHLHB2* and rs1534891 in *CSNK1E* in Sample II (Table III). Therefore, it is possible that another causative variant, which is in LD with rs534654, influences genetic

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risk for BP through regulating the expression or function of *CLOCK*, and/or interacting with other genes [Lin et al., 2007].

Based on allelic and haplotypic association results in our own data and in other reports [Mansour et al., 2006; Nievergelt et al., 2006; Wellcome Trust Case Control Consortium, 2007], common variants in the *CLOCK*, *BHLHB2* and *CSNK1E* genes do not have major effects on BP susceptibility. However, the 3-locus interaction suggests that each gene has a weak effect, which additively or synergistically contributes to increased risk for BP. In addition, several lines of evidence suggest possible biological interactions between these three proteins in the circadian pathway (see summary of protein function in Table I and Supplementary Figure I). First, these three genes are simultaneously expressed in multiple human brain regions including the hypothalamus wherein the SCN resides (http://www.ncbi.nlm.nih.gov/sites/entrez?db=geo). Second, it has been shown that BHLHB2 represses CLOCK/ARNTL-induced transactivation of the *Per1* promoter through direct protein-protein interactions with ARNTL and/or competition for E-box elements in mice [Honma et al., 2002]; CSNK1E can phosphorylate mammalian PERIOD proteins and the phosphorylated PER/CRY complex can inhibit CLOCK-controlled gene expression including that of *BHLHB2* [Ko et al., 2002; Ueda et al., 2005]. Third, decreased expression of the *CLOCK* and *BHLHB2* genes have been found in postmortem brains of patients with BP (Supplementary Table I). Therefore it is possible that a disrupted circadian rhythm may be caused by the accumulation of minor abnormalities from each gene in the system. However, the statistical interactions shown in our study should be interpreted with caution, until there are replications in independent samples, and more evidence on its biological significance.

Recently, two groups reported that *ARNTL* and *PER3* show suggestive evidence for association with BP [Mansour et al., 2006; Nievergelt et al., 2006]. Some BP families examined in both studies come from the CNG and/or NIMH wave 1 collections, which overlap with our selected samples. We have not detected similar association signals, although those associated SNPs are all tagged by SNPs we tested (which are in complete or very strong LD with SNPs examined in our study, based on LD structure from HapMap data with MAF > 0.1 in Caucasians, Supplementary Figure II).

Long stretches of genotype homozygosity and Mendelian inconsistencies of genotypes in families are indicators of large deletions or copy number changes. We found loss of heterozygosity (LOH) in two gene regions: *ARNTL* and *ARNTL2*. *ARNTL2* region also has Mendelian inconsistencies which further support the deletion. These findings need further confirmation using other methods and to be tested in extended families.

Several key regulators in circadian feedback loops have been associated with abnormal circadian phenotypes such as sleep disturbances [Archer et al., 2003; Ebisawa et al., 2001; Pereira et al., 2005]. As circadian dysrhythmias are frequently seen in BP patients [Mansour et al., 2005; Wirz-Justice, 2006], and are thought to be an endophenotype of BP [Hasler et al., 2006], it would be interesting to interrogate the relationship between variation in circadian genes and circadian dysrhythmias comorbid to BP [Benedetti et al., 2007a; Serretti et al., 2003; Serretti et al., 2005]. We explored six circadian sub-phenotypes in individuals with BP, and tested whether any sub-phenotype was associated with any of the allelic or haplotypic variation in 15 circadian genes. We detected suggestive associations between circadian sub-phenotypes in BP and variants in several circadian genes (*ARNTL*, *ARNTL2*, *CLOCK*, *CSNK1E*, *DBP*, and *TIMELESS*). However, no significance remained after correction for multiple testing. This may be ascribed to limited samples in sub-phenotype analysis, weak effects of these genes, and/or phenotype heterogeneity. For example, in the present study, the sleep disturbance phenotypes were obtained based on interview data from patients using DIGS or SADS, not diagnosed using objective measures such as polysomnography and actigraphy

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{Buysse, 2006 BUYSSE2006/id;Harvey, 2006 HARVEY2006/id}, In addition, it is unknown whether insomnia in BP patients is caused by BP itself (e.g., required coinciding onset and temporal disease course by research diagnostic criteria for insomnia [Edinger et al., 2004]) or other causes such as psychiatric medication (see review [McClung, 2007]) substance abuse, or other comorbid disorders [Chokroverty, 2000]. It also should be noted that we did not identify the illness phase where sleep disturbance appeared in each BP patient, which may make the case sample heterogeneous. Therefore, comprehensive analysis of clinical data and accurate diagnosis of insomnia in BP patients are needed for rigorous genotype-insomnia association studies.

As with other biological pathways, there are no clear boundaries to define which gene should be included. Besides components of the core circadian self-regulatory feedback loops tested in the present study, other circadian genes could be investigated. For example, neuronal PAS domain protein 2 (NPAS2), can form a heterodimer with ARNTL and activate expression of *PER1*, *PER2*, and *CRY1* genes [Reick et al., 2001]. *NPAS2* has shown decreased expression in postmortem brains of patients with BP (Supplementary Table I), and has been associated with seasonal affective disorder [Johansson et al., 2003], but not with BP [Nievergelt et al., 2006]. GSK3B is interesting because of its ability to lengthen the circadian period and as a target for the action of the mood stabilizers lithium and valproic acid [Yin et al., 2006]. *GSK3B* has been associated with age at onset and response to total sleep deprivation in BP [Benedetti et al., 2004a; Benedetti et al., 2004b], increased susceptibility to BPII in females [Lee et al., 2006; Szczepankiewicz et al., 2006b], and response of patients with BP or depression to lithium treatment [Adli et al., 2007; Benedetti et al., 2005]. However other studies did not detect association with response to lithium treatment [Lee et al., 2006; Michelon et al., 2006; Szczepankiewicz et al., 2006a] or the disease phenotype of BP [Lee et al., 2006]. Therefore this study of 15 candidate genes may not be considered as a complete coverage of the whole circadian rhythm system. More genes should be investigated in the future to help us to achieve better understanding of relationships between circadian rhythm system and mood disorders.

Our sample has modest power to detect allelic association with small effect sizes. For example, using PBAT (www.biostat.harvard.edu/~clange/default.htm), under a multiplicative model, at $p < 0.05/n$ (where n is the number of SNPs tested), we had 80% power to detect odds ratios (ORs) of 2.2 and 2.0 for minor allele frequencies of 0.1 and 0.5 in Sample I (36 trios and 79 quads), and ORs of 1.7 and 1.5 in Sample II (70 trios and 237), respectively. Since Sample I has obviously lower power, we checked all the nineteen SNPs tested only in Sample I, in the WTCCC data [Wellcome Trust Case Control Consortium, 2007]. They all had $p > 0.05$ when allelic frequencies are compared between cases and controls using chi-squared test (data not shown).

Finally, our association study interrogated disease and sub-phenotype association at individual SNPs, which is a test of the common variants-common disease model (e.g., MAF > 0.1) [Chakravarti, 1999; Reich and Lander, 2001]. This model is consistent with meta-analyses of genetic association data in multiple common diseases, as reviewed elsewhere [Bertram et al., 2007; Lohmueller et al., 2003]. However, there is an alternative and not mutually exclusive multiple rare variants-common disease (MRV/CD) model, which hypothesizes that under conditions of higher mutation rates and moderate “purifying” selection, considerable allelic and locus heterogeneity could be generated, and multiple rare variants might underlie susceptibility to common diseases [Pritchard, 2001; Pritchard and Cox, 2002]. This hypothesis has received increasing experimental support [Cohen et al., 2005; Cohen et al., 2004; Cohen et al., 2006; Fearnhead et al., 2004; Johnson et al., 2007; Meyer et al., 2005; Romeo et al., 2007; Zhu et al., 2005]. Thus, extensive resequencing may be necessary to further clarify the

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roles of circadian genes in influencing genetic susceptibility to BP and dysfunctional rhythms in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table I

Summary of 15 Circadian Genes Tested for Association with Bipolar Disorder

Gene	Protein function	Location	Length (kb)[a]	SNPs typed
Sample I				
CRY2: cryptochrome 2	Inhibition of CLOCK-BMAL1	11p11.2	55.8	4
PER1: period1	Inhibition of CLOCK-BMAL1	17p13.1-17p12	31.9	3
PER2: period2	Inhibition of CLOCK-BMAL1	2q37.3	64.4	3
PER3: period3	Association with CRY?	1p36.23	80.5	5
TIMELESS: timeless homolog (Drosophila)	Interaction with PER1 and inhibition of CLOCK-BMAL1-induced transactivation	12q12-q13	52.2	4[b]
Sample II				
ARNTL: aryl hydrocarbon receptor nuclear translocator-like (BMAL1; MOP3)	Activation of CLOCK and CLOCK-controlled genes (with CLOCK)	11p15	129.5	14
ARNTL2: aryl hydrocarbon receptor nuclear translocator-like 2 (BMAL2)	Activation of CLOCK and CLOCK-controlled genes?	12p12.2-p11.2	107.5	13
BHLHB2: basic helix-loop-helix domain containing, class B, 2	Inhibition of CLOCK-BMAL1-induced transactivation of PER1	3p26	25.7	4
BHLHB3: basic helix-loop-helix domain containing, class B, 3	Inhibition of CLOCK-BMAL1-induced transactivation of PER1	12P11-12	24.9	3
CLOCK: circadian locomoter output cycles protein kaput	Activation of CLOCK and CLOCK-controlled genes with BMAL1)	4q12	134.3	6
CRY1: cryptochrome 1	Inhibition of CLOCK-BMAL1	12q23-q24.1	122.2	4
CSNK1D: casein kinase 1, delta	Phosphorylation of PERs, CRYs and BMAL1	17q25	49.3	3
CSNK1E: casein kinase 1, epsilon	Phosphorylation of PERs, CRYs and BMAL1	22q13.1	47.4	9
DBP: D site of albumin promoter binding protein	Output, activation of PER1?	19q13.3	26.6	2
NR1D1: nuclear receptor subfamily 1, group D, member 1 (REVERBA)	Inhibition of BMAL1	17q11.2	27.9	4

[a] Genic sequence (NCBI B35) and 10 kb upstream and downstream.

[b] Two SNPs (rs2291738 and rs10876890) were found to be associated with insomnia during mania episode in bipolar disorder patients in CNG and waves 1–2 samples, and then were genotyped in CHIP and waves 3–4 samples; other two SNPs (rs774026 and rs2279665), which showed suggestive association evidence with bipolar disorder by Mansour HA *et al*, were genotyped in CHIP, CNG, and NIMH waves 1–4 samples. These four SNPs were typed using TaqMan assays.

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Table II

Allelic and Haplotypic Association of the *CLOCK* Gene with Bipolar Disorder in Sample II

SNP	Chromosome position[a]	Alleles (1/2)	Proportion allele 1 is transmitted	T	NT	TDT *p*-value[b]
rs534654	56131148	A/G	0.40	82	122	**0.0097**
rs2412648	56161995	A/C	0.46	113	133	0.12
rs4340844	56169784	A/C	0.44	119	154	**0.015**
rs11735267	56188215	A/G	0.55	159	127	0.059
rs6850524	56222925	C/G	0.43	126	168	**0.012**
rs7660668	56242625	C/G	0.51	116	113	0.59
haplotype[c]			frequency[d]			*p*-value[b]
GAAACG			0.04			**0.025**
GACAGC			0.07			**0.028**
AAAGCC			0.19			**0.034**

[a]Based on human genome sequence (NCBI Build 35, May 2004, hg17).

[b]Nominally significant results are shown in bold type.

[c]Haplotypes are constructed by 6 SNPs at the *CLOCK* gene region.

[d]Haplotype frequencies were calculated based on the parents. Haplotype analysis gave a global p-value of 0.048. T: transmitted; NT: non-transmitted.

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Table III

Most Significant Gene-gene Interactions in Sample II

Locus 1/SNP	Locus 2/SNP	Locus 3/SNP	FDR-cutoff *p*-value	FITF *p*-value
BHLHB2/rs6442925	TPARL/rs534654	CSNK1E/rs1534891	1.77E-06	**1.72E-06**
BHLHB2/rs6442925	CRY1/rs714359	NR1D1/rs16965644	1.77E-06	8.88E-06
BHLHB2/rs6442925	TPARL/rs534654	CSNK1E/rs135757	1.77E-06	2.62E-05
BHLHB2/rs2137947	CLOCK/rs6850524	CSNK1E/rs1534891	1.77E-06	7.59E-05

FDR-cutoff *p*-value is the threshold for significance for 3-loci interaction analysis. A result is significant if the *p*-value is less than the FDR cutoff. SNP rs534654 in *TPARL* is 8 kb away from 3′ downstream of *CLOCK* gene. The program for the "focused interaction testing framework" method (FITF) was used to evaluate interactions between genes without significant main effects. A significant signal is shown in bold type.