

Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia

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We hypothesize that circadian dysfunction could underlie, at least partially, the liability for bipolar 1 disorder (BD1). Our hypothesis motivated tests for the association between the polymorphisms of genes that mediate circadian function and liability for BD1. The US Caucasian patients with BD1 (DSM-IV criteria) and available parents were recruited from Pittsburgh and surrounding areas ($n = 138$ cases, 196 parents) and also selected from the NIMH Genetics Collaborative Initiative ($n = 96$ cases, 192 parents). We assayed 44 informative single-nucleotide polymorphisms (SNPs) from eight circadian genes in the BD1 samples. A population-based sample, specifically cord blood samples from local live births, served as community-based controls ($n = 180$). It was used as a contrast for genotype and haplotype distributions with those of patients. US patients with schizophrenia/schizoaffective disorder (SZ/SZA, $n = 331$) and available parents from Pittsburgh ($n = 344$) were assayed for a smaller set of SNPs based on the results from the BD1 samples. Modest associations with SNPs at *ARNTL* (*BmaL1*) and *TIMELESS* genes were observed in the BD1 samples. The associations were detected using family-based and case–control analyses, albeit with different SNPs. Associations with *TIMELESS* and *PERIOD3* were also detected in the Pittsburgh SZ/SZA group. Thus far, evidence for association between specific SNPs at the circadian gene loci and BD1 is tentative. Additional studies using larger samples are required to evaluate the associations reported here.

Keywords: *ARNTL*, association, bipolar I disorder, circadian, genetic, linkage, *PER3*, schizoaffective disorder, schizophrenia, *TIMELESS*

Received 6 October 2004, revised 16 March 2005, accepted for publication 4 April 2005

The inherent cyclical nature of bipolar 1 disorder (BD1) invites analogies with circadian variation. Patients with BD1 who have relapsed manifest marked changes in diurnal activities such as sleep, activity, appetite and the diurnal secretion of hormones (Kennedy *et al*. 1996; Kripke *et al*. 1978; Leibenluft *et al*. 1996; Mendlewicz 1982; Nurnberger *et al*. 1990; Sarai & Kayano 1968; Schreiner *et al*. 2001). Normalization of these abnormalities usually accompanies clinical stabilization, and mood-stabilizing drugs are known to modulate circadian rhythms (Abe *et al*. 2000; Basturk *et al*. 2001; Campbell *et al*. 1989; Manji *et al*. 2001; Mellerup *et al*. 1976). Hence, circadian abnormalities have long been suggested to be critically involved in the pathogenesis of BD1, especially rapid cycling BD1 (Healy 1987; Mitterauer 2000).

Our recent work supports the circadian hypothesis. We compared 'morningness–eveningness' (M/E), a stable, heritable trait reflecting circadian phase among patients with BD1, unscreened controls and patients with schizophrenia (SZ) or schizoaffective disorder (SZA). Scores on the composite scale (CS) (Smith *et al*. 1989), which was used to evaluate M/E, were significantly different among BD1 cases compared with the controls or SZ/SZA patients, when age was considered. Younger BD1 patients and those with rapid mood swings were more significantly likely to have lower CS scores. Thus, distinct patterns of M/E were noted among BD1 patients and among BD1 subgroups (Mansour *et al.* in press). The impact of medication, mood state and chronicity on CS scores is presently being evaluated.

The biology and genetics of human circadian rhythms have been clarified considerably. Circadian rhythms originate in the suprachiasmatic nucleus. They are regulated by an intrinsic circadian oscillator based on interlinked autoregulatory transcriptional/translational feedback loops (Cermakian & Boivin 2003). Self-regulatory feedback loops are thus set up and entrain circadian rhythms in numerous tissues in mammals. A large number of proteins critical for circadian function have been identified, and intensive efforts are in progress to identify other agents. These proteins mediate the feedback loops underlying circadian rhythms through intricate interactions (Holzberg & Albrecht 2003).

Although the phase and amplitude measured from circadian rhythms undoubtedly reflect a mixture of influences both from the endogenous circadian pacemaker and from exogenous influences such as posture, behavior and environment, there are clear, significant heritable influences on circadian function. For example, twin studies have suggested a heritability of approximately 74% on growth hormone

 doi: 10.1111/j.1601-183X.2005.00147.x

secretion during the wake period and, to a certain extent, on the 24 h secretion (Mendlewicz *et al*. 1999). Heritable influences on other circadian measures are also available. Twin studies have suggested a heritability of approximately 44% for M/E, a measure based on preference for mornings or evenings (Vink *et al*. 2001). This convenient measure of circadian function is associated with interindividual differences in the phase (timing) of physiological circadian rhythms, for example, plasma cortisol and core body temperature, as well as behavioral rhythms related to eating, exercise and performance (Baehr *et al*. 2000; Horne & Ostberg 1976; Matsumoto *et al*. 1996; Monk *et al*. 1991; Nebel *et al*. 1996).

Efforts to understand the phenotypic variation associated with circadian gene polymorphisms are under way. A missense mutation in the *PERIOD2* (*PER2*) gene appears to cause familial advanced sleep-phase syndrome in some pedigrees (Toh *et al*. 2001). Variations in the *CLOCK* and *PER3* genes are associated with the delayed sleep-phase syndrome and sleep dysregulation in mood disorders (Archer *et al*. 2003; Ebisawa *et al*. 2001; Serretti *et al*. 2003). Polymorphisms at the *CLOCK* locus could also confer susceptibility to seasonal affective disorder (SAD) (Johansson *et al*. 2003). A polymorphism in the 3′ flanking polymorphism of the *CLOCK* gene has been investigated extensively. It was reported to be associated with diurnal preference (Katzenberg *et al*. 1998), but the association could not be detected in a subsequent study (Robilliard *et al*. 2002). This polymorphism does not appear to confer susceptibility to major depressive disorder (Desan *et al*. 2000), although a recent study among BD1 patients suggested an association with the recurrence of manic or depressive episodes (Benedetti *et al*. 2003). Thus, associations between circadian gene polymorphisms and mood morbidity are plausible.

We addressed the possibility that the genetic susceptibility to BD may be attributed partly to variations in genes that mediate circadian function. If established, such associations would provide additional evidence for circadian disruption in the pathogenesis of BD. To obtain credible evidence, we investigated associations using family-based and case–control samples. It has been suggested that some genetic susceptibility factors may be shared between BD and SZ, and circadian dysfunction has been reported among patients with SZ/SZA (Katz *et al*. 2001). Hence, we also investigated patients with SZ/SZA.

Materials and methods

Clinical data

Pittsburgh-based samples

When appropriate, written informed consent was obtained from participants, according to the guidelines of the University of Pittsburgh Institutional Review Board. Individuals with BD1, SZ or SZA (DSM-IV criteria) and their available parents participated. Details of ascertainment and diagnosis have been described elsewhere (Chowdari *et al*. 2002; Ranade *et al*. 2003). Briefly, consenting patients were interviewed using the Diagnostic Interview for Genetic Studies (DIGS), a structured diagnostic interview schedule (Nurnberger *et al*. 1994). Additional clinical information was obtained from available clinical records and from relatives. This information was synthesized and a consensus diagnosis was assigned. The parents provided blood samples, but detailed diagnostic interviews were not conducted. To contrast genotype distributions of the probands with those from the population, we obtained cord blood samples from live births at Magee-Women's Hospital, Pittsburgh. No information apart from ethnicity was available for these samples.

NIMH Collaborative Genetics Initiative family-based sample

Pedigrees having probands with BD1 (DSM-IV criteria) were ascertained at Indiana University, Johns Hopkins University, Washington University and the Clinical Neurogenetics Branch of the NIMH Intramural Research Program. The DIGS was the primary interview schedule. The families were ascertained if they included two or more affected first-degree relatives. Pedigrees are sequentially extended through first-degree relatives of specific individuals with specific diagnoses. Pedigrees in which both parents of the proband have SAB (SZA, bipolar type) or BD1 are excluded.

Laboratory

Genomic DNA was extracted from venous blood samples using the phenol–chloroform method.

SNP selection and genotype assays

We selected 46 single-nucleotide polymorphisms (SNPs) at eight circadian genes: aryl hydrocarbon receptor nuclear translocator-like *BmaL1* (*ARNTL*), circadian locomotor output cycles kaput protein (*CLOCK*), Period1, Period2 and Period3 (*PER1*, *PER2* and *PER3*), Cryptochrome1 and 2 (*CRY1* and *CRY2*) and TIMELESS (*TIMELESS*) (Tables 1 and 2). Additional details are available online (see *Supplementary material*).

SNP selection

We resequenced all exons, exon–intron boundaries, selected intronic fragments, 5′ upstream sequences (approximately 4 kbp) and 3′ downstream sequences (3 kbp) for all eight circadian genes (Fig. 1). In addition, all introns were resequenced at *TIMELESS*, *PER1* and *CRY1*: amplicons approximately 500 bp were generated using the polymerase chain reaction (PCR) for genomic DNA samples pooled from 93 cases and a separate pool of 93 community-based controls. The amplified fragments were sequenced using an ABI 3700 DNA sequencer (ABI Inc.; Foster City, CA, USA). We aimed for an average spacing of 1 SNP/25 kbp. SNP selection was also contingent on informativeness (minor allele frequency $> 5\%$) and on assay availability. Among 48 SNPs that were analyzed initially, four were discarded because of

Table 1: Candidate genes, SNPs and overall results of association analyses

						SZ/SZA	
Gene	Location	Genomic size (bp)*	Number of SNPs analyzed	Pittsburgh BD1 CC	BD1 TDT	CC	TDT
BmaL1 (ARNTL)	11p15	109 460	10	+	+	–	–
Clock *(CLOCK)*	4q12	114 337	10	–	–	–	–
Period1 *(PER1)*	17p13.1-17p12	15 888	6	–	–	–	–
Period2 *(PER2)*	2q37.3	44 406	2	–	–	–	–
Period3 *(PER3)*	1p36.23	60 853	6	+	–	+	+
Cryptochrome1 *(CRY1)*	12q23-q24.1	102 180	4	–	–	NA	NA
Cryptochrome2 *(CRY2)*	11p11.2	70 188	2	–	–	NA	NA
TIMELESS *(TIMELESS)*	12q12-q13	33 010	4	–	+	–	+

BD1, bipolar I disorder [note: only BD1 cases from the Pittsburgh sample were used for association analysis using the Trends test (CC), whereas the transmission disequilibrium test (TDT) included the Pittsburgh and NIMH samples]; +, significant association detected ($P < 0.05$) (corrections for multiple comparisons not applied); –, significant association not detected; NA: not analyzed; SZ/SZA, schizophrenia/schizoaffective disorder.

The following SNPs were genotyped in the SZ/SZA and the NIMH samples: *CLOCK*: rs6828570; *PER1*: rs10462024; *PER2*: rs2304670. No SNPs were genotyped at *CRY1* and *CRY2*.

*Genomic sequence, including exons and introns (http://www.ncbi.nlm.nih.gov, build 33).

inconsistent genotyping assays (rs2304675 A/G at *PER2*, rs2292912 G/C at *CRY2* and *rs*707465 A/G and rs1773134 A/T at *PER3* gene). Thus, 44 SNPs were used. They included 31 SNPs listed in public databases (http://www.ncbi.nlm.nih.gov/genome/guide/human/) and 13 novel SNPs (details online at http://www.pitt.edu/~nimga, see *Supplementary*

Table 2: Analysis of single-nucleotide polymorphisms (SNPs) at *ARNTL*, *PERIOD3* and *TIMELESS*

					Pittsburgh SZ/SZA	
Gene	SNP ID	Allele*	Pittsburgh Trends test	BD1 TDT	Trends test	TDT
ARNTL	rs2279287	A/G	0.056	0.322	0.173	0.348
	rs1481892	C/G	0.018	0.452	NA	NA
	rs7107287	G/T	0.033	0.047	NA	NA
	rs4757142	G/A	0.0008	0.597	NA	NA
	rs895682	A/C	0.230	0.008	0.846	0.697
	rs1982350	T/C	0.005	0.162	0.255	0.561
	rs2896635	A/T	0.162	0.235	0.772	0.933
	rs2278749	C/T	0.161	0.162	NA	NA
	rs969486	C/T	0.501	0.994	NA	NA
	rs2290035	T/A	0.090	0.181	0.689	0.935
PER3	rs228729	C/T	0.080	0.836	0.028	0.914
	rs10462018	C/T	0.780	0.303	0.878	0.197
	rs10462020	T/G	0.070	0.784	0.080	0.317
	rs2859387	A/G	0.039	0.803	0.168	0.020
	rs10462021	C/T	0.281	0.680	0.913	0.485
	rs4908699	A/G	0.470	0.398	0.161	0.352
TIMELESS	rs2279665	G/C	0.717	0.047	NA	NA
	rs774026	C/T	0.835	0.314	0.352	0.039
	rs2291738	A/G	0.328	0.029	0.256	0.126
	rs2291739	A/G	0.812	0.089	NA	NA

BD1: bipolar I disorder [note: only BD1 cases from the Pittsburgh sample were used for association analysis using the Trends test (CC), whereas the transmission disequilibrium test (TDT) included the Pittsburgh and NIMH samples]; NA, not analyzed; SZ/SZA: schizophrenia/schizoaffective disorder.

*The alleles at each SNP are listed. Elsewhere, the alleles are numbered '1' or '2' in the order listed here. Corrections for multiple comparisons were not applied.

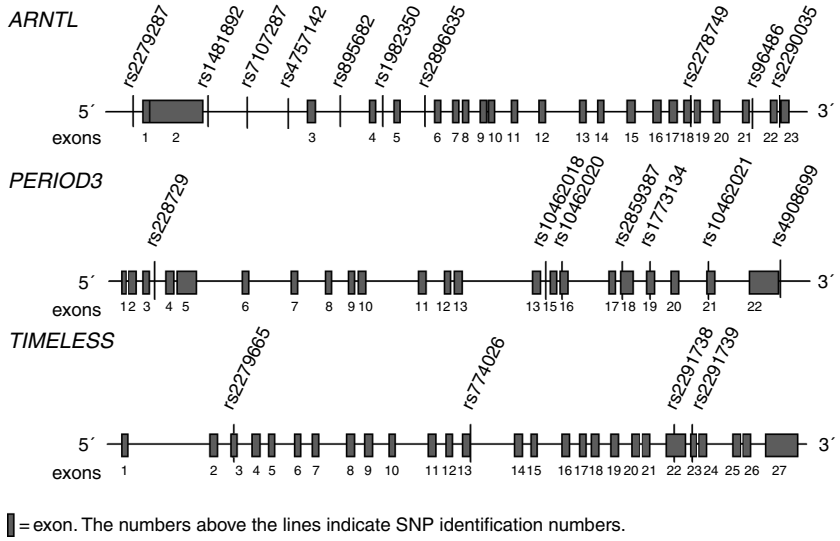

Figure 1: Structure of *ARNTL, PERIOD3* and *TIMELESS* genes.

material). Genomic DNA from a panel of six individuals was resequenced for each SNP. These samples served as positive controls for subsequent genotype assays.

Genotype assays

Assays were based on multiplex PCR followed by single-base extension analysis (SnaPshot assays, ABI Inc.; see *Supplementary material*). We denoted alleles at each polymorphism as '1' or '2' for convenience. All allele calls were rechecked independently, blind to diagnostic status. Genotypes were then evaluated for Mendelian consistency using PEDCHECK (O'Connell & Weeks 1998). In case of any discrepancy, samples were retyped. All clinical and genetic data were double checked to guard against data entry errors.

Statistical analysis

Transmission distortion at individual SNPs was tested initially using the transmission disequilibrium test (TDT) as implemented in the GENEHUNTER software package (Kruglyak 1999). Using the count of transmitted alleles from heterozygous parents to their affected offspring, the TDT assesses distortion from the Mendelian expectation of equal transmission rates for all alleles at a locus. The TDT detects association in the presence of linkage, and its statistic cannot be confounded by population substructure (Ewens & Spielman 1995; Spielman *et al*. 1993). The possibility of transmission bias could be evaluated in three family-based samples: families containing individuals diagnosed with BD1 from Pittsburgh; families containing individuals diagnosed with BD1 from NIMH and families containing individuals diagnosed with SZ from Pittsburgh. From a purely phenomenological point of view, there would be no reason to analyze the bipolar samples separately; however, there would also be little reason to combine the SZ and bipolar samples. For this reason, we analyzed transmission bias of the bipolar samples jointly, taking into account the origin of the samples, and we analyzed transmission bias for SZ separately. For the joint analysis of each locus, we recorded transmission of the same allele from parents to their affected offspring for each sample. We then fit a logit model in which congruence with Mendelian transmission was tested, as well as whether the transmission rate was the same for samples from both sites.

When significant transmission distortion was detected, we explored the bounds of the associated genomic region by evaluating the transmission of haplotypes composed of two to four contiguous SNPs from the gene (i.e. a 'sliding window' to two to four SNPs; Nair *et al*. 2000). When significant distortion of haplotype transmission was detected, we also analyzed the data using TRANSMIT for a global test of association for haplotypes (Clayton 1999); its test statistic, for h haplotypes, is distributed as chi-square with $(h - 1)$ degrees of freedom under the null hypothesis of no transmission distortion. TRANSMIT has the advantage of using all data to estimate haplotype frequencies and account for haplotype uncertainty by averaging over all possible configurations of parental haplotypes.

To evaluate whether the genotype distribution differed significantly among individuals diagnosed with BD1 vs. the population in general, we contrasted the observed genotyped distribution for cases with the sample from neonates by using the Cochran–Armitage trend test (Devlin *et al*. 2001). This test evaluates whether the counts of the minor allele at a locus (0, 1 or 2 minor alleles) can predict status, case or population sample; its test statistic is chi-square distributed with df $= 1$. Again, when a significant difference was detected, we explored the bounds of the associated

genomic region by evaluating differences in haplotype distributions when they were composed of two to four contiguous SNPs from the gene. These sliding window analyses were conducted using SNPEM (Fallin *et al.* 2001), which uses the expectation-maximization algorithm to estimate haplotype frequencies for each sample. An empirical omnibus statistic is produced based on the permutation of status with a user-specified number of permutations – in this case 10 000.

The reader will recognize the exploratory nature of the analyses. For this reason, we have not corrected for multiple comparisons.

Results

All genotype distributions were in Hardy–Weinberg equilibrium for case-, parental- and population-based samples, with the exception of rs1481892 at *ARNTL*. A heterozygote deficit was noted for this SNP in the neonatal sample ($P = 0.030$) and in parents of NIMH patients ($P = 0.042$). For details of all analyses reported herein, including linkage disequilibrium analyses, please see Results online (http://www.pitt.edu/~nimga).

BD1 sample

The Pittsburgh BD1 sample was composed of 138 Caucasian cases. Both parents were available for 61 probands and one parent was available for each of 74 cases. From the NIMH sample, 96 case–parent trio families were selected. When we analyzed transmission distortion of the bipolar samples jointly, we observed significant transmission bias at four loci: *ARNTL* rs7107287 ($\chi^2 = 3.92$, df $= 1$, $P = 0.0477$); *ARNTL* rs895682 ($\chi^2 = 7.00$, df $= 1$, $P = 0.0082$); *TIMELESS* rs2279665 ($\chi^2 = 3.94$, df $= 1$, $P = 0.0472$) and *TIMELESS* rs2291738 ($\chi^2 = 4.73$, df $= 1$, $P = 0.0296$). Members of these pairs of SNPs are in linkage disequilibrium ($D' > 0.9$) (see *Supplementary data* online http://www.pitt.edu/~nimga). Another locus in *TIMELESS* showed substantial but not quite significant transmission bias rs2291739 ($\chi^2 = 2.89$, df $= 1$, $P = 0.0890$). Neither these loci nor any other locus tested showed significant differences between sites in the transmission rate.

For the contrasts of genotype distributions for individuals from the Pittsburgh sample diagnosed with BD1 vs. the population in general, significant differences were detected at four loci: *ARNTL* SNPs rs1481892 ($P = 0.018$); rs7107287 ($P = 0.033$); rs4757142 ($P = 0.00086$) and rs1982350 ($P = 0.005$) and *PER3* SNP rs2859387 ($P = 0.039$).

To explore the bounds of the associated genomic region for each of three associated genes, *ARNTL*, *TIMELESS* and *PER3*, we evaluated both transmission and distribution of haplotypes composed of two to four contiguous SNPs from each gene. The set of contiguous SNPs was formed by a sliding window of SNPs across the gene.

For *ARNTL*, these analyses revealed over-transmission of haplotypes encompassing five SNPs rs1982350, rs2896635, rs2278749, rs969486 and rs2290035. However, global tests of haplotypes involving these SNPs revealed no significant association ($\chi^2 = 9.18$, df $= 11$, $P = 0.61$). For *TIMELESS*, these analyses revealed over-transmission of haplotypes encompassing four SNPs rs2279665, rs774026, rs2291738 and rs2291739. Global tests of haplotypes involving these SNPs revealed a significant association ($\chi^2 = 9.62$, df $= 3$, $P = 0.02$). Finally, consistent with the single-locus results, our exploratory analyses revealed no transmission bias for *PER3*.

For *ARNTL*, exploratory analyses based on haplotype distributions in individuals diagnosed with BD1 vs. the population sample revealed significant differences involving four SNPs, rs2896635, rs2278749, rs969486 and rs2290035 and also another set of four SNPs in the 5′ region of the gene, namely rs2279287, rs1481892, rs7107287 and rs4757142. Neither *TIMELESS* nor *PER3* showed significant differences among the samples for haplotype distributions.

SZ/SZA sample

The sample was composed of 331 cases (327 Caucasians, 4 African-Americans). The African-American probands were used in the family-based analyses only. There were 197 patients with SZ and 134 patients with SZA. Both parents were available for 149 cases. All Caucasian cases were used for comparisons against the neonatal sample ($n = 327$). Analyses were restricted to the SNPs analyzed in the NIMH BD1 samples (Table 2). Because similar patterns were observed in both the SZ and SZA families, they are presented together.

When we analyzed transmission bias in the SZ/SZA samples, we observed significant transmission distortion at loci in two genes, *PER3* rs2859387 ($\chi^2 = 5.36$, df $= 1$, $P = 0.020$) and *TIMELESS* rs774026 ($\chi^2 = 4.25$, df $= 1$, $P = 0.039$). Contrasting genotype distributions in cases with the population-based sample did not reveal any significant differences by the trend test, although the genotype distribution for *ARNTL* rs1982350 was noted to be significantly different ($\chi^2 = 7.137$, df $= 2$, $P = 0.028$) (See *Supplementary material*).

To explore the bounds of the associated genomic region for each of three associated genes, we evaluated the transmission or distribution of haplotypes using the sliding window approach described previously. For *ARNTL*, no transmission bias was observed, although significant differences were observed overall for haplotypes bearing SNPs rs895682, rs1982350, rs2986635 and rs2290035 (data not shown). For *PER3*, transmission of haplotypes encompassing SNPs rs2859387, rs10462021 and rs4908699 showed significant bias (data not shown), but the contrast of haplotype distributions was not significant. Finally, for *TIMELESS*, transmission distortion was also observed for a haplotype based on SNPs rs774026 and rs2291738 (data not shown), the same haplotypes over-transmitted in the BD1 sample.

Discussion

Our study was designed to evaluate circadian gene variation in relation to BD1 liability. We evaluated several informative SNPs at each gene and analyzed two independent BD1 samples as well as a comparison SZ/SZA group. Family-based and case–control analyses were conducted at each SNP. We reasoned that such joint analysis would yield more credible results than either set of comparisons. Both the family-based analyses and the contrasts between the cases and the population-based sample suggested associations with *ARNTL*, *PER3* and *TIMELESS* in the BD1 samples, albeit with different SNPs. Some of the SNPs are in significant linkage disequilibrium; hence, such associations are not independent. The differences between the family-based and case–control analyses may reflect variations in power (Bacanu *et al*. 2000). Overall, the magnitude of the associations is modest, and corrections for multiple comparisons were not applied. Replicate studies using larger samples are required.

As in the BD1 samples, associations were detected at *PER3* and *TIMELESS* in the Pittsburgh SZ/SZA sample, using case–control comparisons and the TDT. However, the associated SNPs were different. The differences may be attributed to sample size variations or they may reflect differences in the architecture of liability conferred by the circadian genes. All these associations are modest and must be considered tentative pending replication. If replicated, our observations would lend credence to the possibility that certain genetic susceptibility factors are shared across the psychosis spectrum (Berrettini 2000).

We used an unscreened neonatal sample for contrast against our cases. As the lifetime prevalence of BD1 is approximately 1% (Kessler *et al*. 1994), the minimal loss of power incurred by including 1% false negatives is negligible (Bacanu *et al*. 2000). An unscreened sample from the same population is not expected to increase the rate of false positives.

In conclusion, the analysis of more than 1450 individuals suggested associations between three candidate circadian genes and liability for BD1. Other distinct associations were noted in SZ/SZA sample. None of these associations can be considered definitive, particularly because corrections for multiple comparisons were not applied. Nevertheless, the suggestive results warrant further investigations of the links between circadian variation and BD1 liability. Further studies using larger samples are in progress.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1: LD patterns at *ARNTL* among parents of BDI patients.

Figure S2: LD patterns at *PER3* among parents of BDI patients.

Figure S3: LD patterns at *TIMELESS* among parents of BDI patients.

Figure S4: Trends test analyses at *ARNTL* and *PER3*.

Table S1: SNPs analyzed.

Table S2: Genomic sequences flanking the SNPs, and PCR primers

Table S3: Details of SNP Genotyping Assays.

Table S4: Allele Frequencies of ANPs analyzed.

Table S5: Case-control analysis at *ARNTL, TIMELESS* and *PERIOD3*.

Acknowledgments

We thank the participants in our study. Supported in part by grants from the National Institutes of Health (MH63420, MH56242 and MH66263 to VLN; MH57881 to BD and AG13396 and AG020677 to THM), the Mental Health Intervention Research Center (MH 30915 to DJK) and NASA (NAG9-1234 to THM). We thank NIMH for providing DNA and data from the National Institute of Mental Health Collaborative Genetics Initiative (NIMH CGI) sample.

Additional details are available at our web site (http://www.pitt.edu/∼nimga)