Molecular Analyses of Circadian Gene Variants Reveal Sex-Dependent Links Between Depression and Clocks

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Molecular analyses of circadian gene variants reveal sex-dependent links between depression and clocks

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An extensive literature links circadian irregularities and/or sleep abnormalities to mood disorders. Despite the strong genetic component underlying many mood disorders, however, previous genetic associations between circadian clock gene variants and major depressive disorder (MDD) have been weak. We applied a combined molecular-functional and genetic association approach to circadian gene polymorphisms in sex-stratified populations of control subjects and case subjects suffering from MDD. This approach identified significant sex-dependent associations of common variants of the circadian clock genes hClock, hPer3 and hNpas2 with major depression and demonstrated functional effects of these polymorphisms on the expression or activity of the hCLOCK and hPER3 proteins, respectively. In addition, hCLOCK expression is affected by glucocorticoids, consistent with the sex-dependency of the genetic associations and the modulation of glucocorticoid-mediated stress response, providing a mechanism by which the circadian clock controls outputs that may affect psychiatric disorders. We conclude that genetic polymorphisms in circadian genes (especially hClock and hPer3, where functional assays could be tested) influence risk of developing depression in a sex- and stress-dependent manner. These studies support a genetic connection between circadian disruption and mood disorders, and confirm a key connection between circadian gene variation and major depression.

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INTRODUCTION
Circadian rhythms pervasively control the behavior, physiology and biochemistry of humans, including the timing of sleep. Circadian irregularities and/or sleep abnormalities have been linked to mood disorders such as major depressive disorder (MDD), bipolar disorder and seasonal affective disorder by studies of jet lag, sleep deprivation, chronotype, stress and comorbidity of mood disorders with advanced or delayed sleep phase syndrome.1–6 Indeed, altered sleep/wake cycles are a critical feature for diagnosis of many mood disorders in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Several effective pharmacological and non-pharmacological therapies for mood disorders, including bright light, sleep deprivation and/or phase-resetting paradigms, appear to function by modulating circadian parameters.3,4,6,7 Moreover, a recent study of postmortem human brains reported that daily rhythmic patterns of gene expression in the brain are seriously disrupted and/or desynchronized in MDD subjects,8 further emphasizing the correlation between abnormal circadian rhythms and the risk and severity of mood disorders.

There is also a strong genetic component underlying many mood disorders,9,10 and there have been a few tantalizing suggestions of links between clock gene polymorphisms and bipolar disorder.11–13 Nevertheless, most association studies to date have concluded that genetic associations between circadian clock gene variants and MDD are weak at best.4,11–15 However, in psychiatric disorders such as MDD, bipolar disorder, schizophrenia and so on, phenotyping the overt manifestations of the disorder may lead to misleading interpretations of etiology. In other words, overlapping and/or common symptoms for a psychiatric disorder may group syndromes that do not have a common mechanistic cause. Therefore, interpreting diagnoses/phenotypes can be problematic for genetic studies of psychiatric disorders, especially in meta-analyses where the standard of phenotyping may be very different among studies. Moreover, the previous genome-wide association studies (GWAS) might have missed significant associations because GWAS studies often include so many subjects and phenotypes that significant associations and biology can be lost in multiple testing corrections. In addition, several disorders associated with disruptions in circadian systems, including MDD, display significant disparities in prevalence and/or severity between the sexes.16 This could be, in part, due to sex-specific genetic effects that have not been generally explored in GWAS studies.

The process of re-examining the molecular impact of a polymorphism in the three-prime untranslated region (3'-UTR) of the human CLOCK protein led us to a re-evaluation of the published GWAS studies for clock gene polymorphisms in association with MDD. We found significant sex-dependent associations of MDD with the circadian genes hClock, hPer3 and hNpas2. In the currently accepted molecular model of the clock network and disease, there are genetic associations with circadian rhythms detected by transcription factors that regulate circadian homeostasis. These associations may be missed in a sex- and stress-dependent manner. These studies support a genetic connection between circadian disruption and mood disorders, and confirm a key connection between circadian gene variation and major depression.
Sex-dependent links between depression and clocks

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To assess statistical association between the analyzed SNPs and MDD, single locus analyses were performed in the combined data set (n = 592 cases, 776 controls). Allelic and genotypic chi-square analyses were performed, using PLINK. Where appropriate, Fisher’s exact test of association was performed. To investigate the possibility of potentially differing effects of the analyzed SNPs on MDD between the genders, sex-separated analyses were also performed (males, n = 94 cases, 253 controls; females, n = 498 cases, 523 controls) for all markers that showed a P-value < 0.2 in chi-square genotypic or allelic tests in the full data set. Correction for multiple testing of chi-square P-values was performed using false discovery rate (q = 0.1). An alternative single locus analysis, prevalence-based association testing, based on the principles of Hardy–Weinberg equilibrium has been shown in simulation studies to be more powerful than chi-square testing under some genetic models and was also performed to provide independent evidence for association. Association analysis methods are described fully in the Supplementary Materials and Methods.

Functional analyses

The P\textsubscript{Np}
\textsubscript{osc}–Fluc::3'-UTR firefly luciferase reporter for the 3'-UTR of hClock (containing the major allele T) was provided by Dr Malcolm von Schantz. To create a minor allele reporter, a T to C mutation at position 3111 (rs1801260) was introduced by site-directed mutagenesis (Stratagene, San Diego, CA, USA) and verified by sequencing. To create P\textsubscript{Np}
\textsubscript{osc}–Fluc plasmids so that the luciferase expression is under the control of an endogenous hClock promoter, a fragment extending from the hClock upstream region (~1908 from the transcription start site) to the first intron (+101 from the transcription start site) was amplified by PCR from human cell genomic DNA and was used to replace the SV40 promoter of the P\textsubscript{Np}
\textsubscript{osc}–Fluc-T/C plasmids. The hPer3 expression plasmid under the control of the CMV promoter (P\textsubscript{CMV}–hPer3) was provided by Dr Joon-Kyu Lee. A G to C mutation (rs228697) in the coding region of hPer3 was introduced to change the proline at position 856 to an alanine residue by site-directed mutagenesis (Stratagene) that was verified by sequencing. Cell culture, immunoprecipitation and functional assay methods are fully described in the Supplementary Materials and Methods.

Statistical analyses for functional assays

The statistical analyses for functional assays were performed by two-tail unpaired t-test, by one-way analysis of variance with Tukey post hoc or by two-way analysis of variance with Bonferroni post hoc test where appropriate. Welch’s correction for unequal variances was applied to t-tests. Details of statistical analyses used for particular experiments are described in the corresponding figure legends. For more information, see Supplementary Materials and Methods.

RESULTS

Molecular analyses of hClock variant shows enhanced transcriptional activity

A common polymorphism in hClock located in the 3'-UTR of the gene (rs1801260) has been associated with chronotype. The major allele at this site (position 3111 of hClock) is a T nucleotide and the minor allele is a C. A sequence analysis predicts that this variant will have significant effects on the secondary structure of the hClock transcript (analysis not shown). However, in a previous study of potential functional impact of this polymorphism, a reporter composed of firefly luciferase (Fluc) driven by the SV40 promoter and linked to the 3'-UTR of hClock with and without the rs1801260 SNP was tested in Cos-1 cells and the polymorphism was found to have no significant effect upon expression/turbover of the Fluc:3'-UTR fusion construct. We reasoned that this result might have been due to the cell type used for that test, namely Cos-1 (monkey kidney) cells. Because the expression of the proteome can vary significantly among mammalian cell lines, tissues and species, and the expression/turbover of molecular circadian clock components could be strongly influenced by the expression of other proteins, reporter constructs with human variants should be tested in a variety of cell lines with special focus upon cell lines derived from humans.
We therefore re-tested the potential effect of the 3111C SNP with a FLuc reporter (1) in a variety of cell lines derived from humans (2) where the 3'-UTR was coupled with the promoter region of hClock, including 102 bp of its 5'-UTR (as compared with the originally tested PSV40 construct** (Figure 1a). This re-examination revealed that the PsV40:FLuc::3'-UTR reporter exhibited significantly higher expression of FLuc protein when it is coupled to the 3'-UTR bearing the minor allele. Figures 1b and c depict expression results for two human cell lines, HepG2 and 293T, derived from different tissues (from liver for HepG2, from embryonic kidney for 293 T). We also re-tested the original PSV40::FLuc::3'-UTR reporter in 293 T cells (Figure 1d) and in U2OS cells (a human osteosarcoma cell line, Supplementary Figure S2), and found that FLuc was expressed at significantly higher levels when coupled to the 3111C-bearing 3'-UTR (P < 0.01). However, when the PsV40:FLuc::3'-UTR reporter was expressed in the originally tested monkey Cos-1 cell line, there was no significant enhancement of FLuc expression by the SNP (Supplementary Figure S2), which agrees with the previous report.22 Therefore, although there is no difference between the hClock versus SV40 5' regions (promoters) in their interaction with the 3'-UTR, the 3111C SNP significantly enhances the expression of the FLuc-3'UTR reporter in cell type/tissue-dependent manner. Taken together, the data indicate that in human cells the rs1801260 SNP enhances expression of the FLuc reporter and therefore strongly suggest that the rs1801260 SNP will modify expression of the hCLOCK protein, which is a key molecular component of the human circadian system.17-20 Because the clock (via hCLOCK/ARNTL-mediated transcription) regulates expression of at least 15% of mammalian mRNAs in tissues throughout the body,3,24 enhanced levels of hCLOCK are likely to modify these circadian output processes.

Genetic association analyses in humans

This newly described functional impact of 3111C of hClock motivated us to re-evaluate its association with MDD. As compared with previous GWAS or meta-analyses,13-15,35,36 the benefit of a more focused hypothesis-driven candidate gene study based on prior biological knowledge is the greatly reduced multiple testing burden, especially when coordinated biological information can be collected. We, therefore, undertook a candidate gene study that focused upon a small number of genes with common polymorphisms (including rs1801260) in circadian clock genes to test an association with MDD, performing both combined and gender-stratified analyses, the latter based on the >50% higher prevalence of MDD in females. Our study sample included a subset of 1368 self-described Caucasian subjects derived from the depression (MDD) and control samples collected by the NIMH (USA) Center for Collaborative Genetic Studies.25 A diagnosis of subtype 1 MDD (melancholia) was used to define MDD case status; participants with no evidence of any form of MDD, either by clinical diagnosis or via targeted survey questions, were labeled study controls. After quality control procedures, 592 MDD cases and 776 MDD controls remained for analyses (further described in Supplementary Materials and Methods and demographic information in Supplementary Tables S1A and S1B). Samples were genotyped for a total of 32 genetic variants from eight circadian genes common in the Caucasian population27 (Supplementary Table S2). These markers were chosen based on their common frequency in the Caucasian population and/or their potential functional significance based on well-characterized roles in regulating circadian rhythms and/or sleep disorders.27,28 After quality control, 18 genetic variants remained for analysis in our data set (Supplementary Tables S3A and D). Genotypic and allelic chi-square (χ²) analyses (or where appropriate Fisher's exact tests) were performed in the full data set and in gender-stratified subsets of the data (see analysis flow chart, Supplementary Figure S3) to identify significant associations between genetic variants and susceptibility to MDD (Table 1, nonsignificant associations shown in Supplementary Table S4). Five variants had genotypic or allelic χ² P-values less than 0.05 in the full and/or sex-stratified data sets (rs228697, rs17031614, rs4851377, rs34705978 and rs1801260). The variants with P < 0.2 in the full data set were further assessed in gender-stratified subsets of the data to explore the genetic factors that may associate with MDD in males or females only. Of the three variants significant in the total data set (rs228697, rs17031614 and rs4851377), only rs228697 in hPer3 associated in a gender-specific analysis (female P = 0.041; Table 1). However, sex-stratified analyses identified two variants that were significantly associated with MDD in only one gender; the hClock SNP rs1801260 was associated with MDD in males (P = 0.028), and rs34705978 in hNPAS2 in females (P = 0.034; Table 1). Of the two variants in hPer3, rs228697 (0.007 < P < 0.011) and rs17031614 (0.017 < P < 0.026), only the association with rs228697 remained significant after correction for multiple testing (Table 1). In addition, a single variant in hNPas2,
rs4851377 (P = 0.034) was associated with MDD susceptibility in the full data set, however significance did not remain after multiple testing correction.

MDD associates with a hClock variant in males and a hPer3 variant in females.

Several studies, including a recent meta-analysis, failed to report an association between MDD and the rs1801260 SNP of hClock.4,5,13-15,35,36 However, the majority of these studies were in Asian populations and did not include gender-stratified analyses. Because MDD is more common in females, it is highly likely that MDD data sets are heavily biased towards females and if rs1801260 were interrogated in such a data set, the association signal would likely have been masked, as it was in our combined male/female analysis (Table 1). In addition, if the effect of rs1801260 on susceptibility to MDD is also population specific, the lack of association in Asian populations may not be generalizable to other continental populations.35 Our discovery of a functional impact of this SNP on luciferase activity when expressed in human cells (Figure 1) encouraged us to re-evaluate previous association reports and include this marker in our association analyses, leading to the identification of a significant association by χ² analysis between rs1801260 in hClock and MDD in male study participants (Table 1).

Furthermore, an independent analysis testing for association, prevalence-based association testing,47 provided additional evidence for the significant association with four variants previously identified using χ² analyses (rs228697 and rs17031614 in hPer3, and rs485133 and rs34705978 in hNPAS2; Supplementary Table S5). Prevalence-based association testing analysis in the male subset revealed a novel association between MDD and a single variant in Arntl2, rs7137588 (P = 0.008; Supplementary Table S5). Although none of the SNPs reached significance at the P < 0.05 level in the male case-only data set in the prevalence-based association testing analysis, rs1801260 of hClock was the only SNP in the male case-only subset that was marginally significant (P = 0.081, Supplementary Table S5). Finally, univariate logistic regression analyses using an additive model were performed to determine the strength and direction of effects for variants that were determined to be significantly associated with MDD in our data set (Table 2). In hPer3, rs228697 and rs17031614 were significantly associated with an increased risk for MDD in the full data set, while rs228697 was also significantly associated in the female-only subset. Moreover, rs1801260 in hClock was again significantly associated with a decreased risk for MDD in the male-only subset.

hClock variant shows differential transcriptional response to glucocorticoids.

On the basis of our results indicating a functional difference between the two alleles at 3111 in the hCLOCK 3′-UTR and the gender-specific association results, we tested whether hormones that might recapitulate the in vivo gender-specific environments affect expression in vitro. Neither testosterone nor estrogen influenced the expression of FLuc in the assay (not shown). However, the synthetic steroid glucocorticoid dexamethasone (Dex) had an unexpected effect on the expression assay (Figure 1e). In HepG2 cells that were transfected with the P₃₉₄₋₄₇₋₄₉_deptrexLuc:3′-UTR reporter, Dex from 50 to 500 nM significantly increased the expression level of FLuc relative to 0 nM Dex in the presence of the major allele (T), whereas constructs with the minor allele (C) had the opposite effect, namely Dex decreased the level of expression (P < 0.001, Figure 1e). This result is interesting for three reasons. First, analysis of transcriptional factor binding motifs of the 3′ region of hClock revealed a glucocorticoid response element (GRE) in the SNP region (Supplementary Figure S4). Previous analyses of GRE-containing promoter

### Table 1. Genetic loci significantly associated with MDD in combined data set and/or sex-separated subsets

| Chr | SNP   | Gene  | Test* | Combined P-value | Female P-value | Male P-value |
|-----|-------|-------|-------|-----------------|----------------|--------------|
| 1   | rs228697 | PER3  | Geno  | 0.011           | 0.055          | 0.440        |
| 1   | rs228697 | PER3  | Allelic | 0.007          | 0.041          | 0.255        |
| 1   | rs17031614 | PER3 | Geno  | 0.026          | 0.119          | 0.108        |
| 1   | rs17031614 | PER3 | Allelic | 0.017          | 0.076          | 0.135        |
| 2   | rs4851377 | NAPSA2 | Geno  | 0.034          | 0.081          | 0.748        |
| 2   | rs4851377 | NAPSA2 | Allelic | 0.070          | 0.125          | 0.839        |
| 2   | rs34705978 | NAPSA2 | Geno  | 0.056          | 0.034          | 0.974        |
| 2   | rs34705978 | NAPSA2 | Allelic | 0.184          | 0.121          | 0.679        |
| 4   | rs1801260 | CLOCK | Allelic | 0.402          | 0.076          | 0.007        |
| 4   | rs1801260 | CLOCK | Geno  | 0.402          | 0.076          | 0.007        |
| 11  | rs70965440 | ARNTL | Geno  | 0.152          | 0.076          | 0.462        |
| 11  | rs70965440 | ARNTL | Allelic | 0.237          | 0.111          | 0.611        |

Abbreviations: MDD, major depressive disorder; SNP, single-nucleotide polymorphism. *Describes the type of chi-square test performed; genotypic (Geno) or allelic. P-values are from chi-square test performed in the combined data set unless otherwise noted. P-values are from chi-square tests performed in the female-only subset unless otherwise noted. P-values are from chi-square tests performed in the male-only subset unless otherwise noted. Fisher’s exact P-value or one-sided Fisher’s exact P-value is shown. SNPs remained significant after multiple testing correction with false discovery rate q = 0.2. Significant P-values (P < 0.05) are shown in bold. Gender-separated studies performed only for select SNPs.

### Table 2. Logistic regression analysis of significant loci from chi-square or PRAT analyses

| Chr | SNP       | Gene  | Combined odds ratio | Combined P-value | Female odds ratio | Female P-value | Male P-value | Male odds ratio | Male P-value |
|-----|-----------|-------|---------------------|------------------|------------------|----------------|--------------|----------------|--------------|
| 1   | rs228697  | PER3  | 1.39                | 0.007            | 1.38             | 0.041          | 1.38         | 0.261          |
| 1   | rs17031614 | PER3 | 1.68                | 0.022            | 1.52             | 0.091          | 2.04         | 0.217          |
| 2   | rs4851377 | NAPSA2 | 0.87               | 0.073            | 0.88             | 0.136          | 0.97         | 0.843          |
| 2   | rs34705978 | NAPSA2 | 1.14               | 0.186            | 1.19             | 0.116          | 0.91         | 0.684          |
| 4   | rs1801260 | CLOCK | 0.89               | 0.187            | 0.96             | 0.647          | 0.66         | 0.036          |
| 12  | rs10548381 | ARNTL2 | 1.04               | 0.759            | 0.96             | 0.762          | 1.37         | 0.245          |

Abbreviations: PRAT, prevalence-based association testing; SNP, single-nucleotide polymorphism. *Indicates that exact logistic regression analysis was used. aExact logistic regression was attempted but was not computationally feasible, therefore standard logistic regression analysis using a dominant genetic model was applied. An odds ratio > 1 indicates that possessing the minor allele at this variant increases the odds of MDD; an odds ratio < 1 indicates that possessing the minor allele at this variant decreases the odds of MDD. Significant P-values (P < 0.05) are shown in bold.
sequences predict the T to C polymorphism at 3111 in hClock converting the putative GRE from a 'repressed GRE motif' toward an 'activated GRE motif' (Supplementary Figure S4). On the basis of the glucocorticoid responses of many genes, this conversion will reverse the glucocorticoid response. We also observed a reversal in the response to glucocorticoids for the rs1801260 variant in the 3′-UTR of the hClock gene (Figure 2e). Second, cortisol levels differ between females and males, and this difference correlates with the sex-dependent association of MDD with rs1801260 (Table 1). Finally, recent studies indicate that modulation of glucocorticoid-mediated stress response may constitute a common mechanism by which the circadian clock affects psychiatric disorders. Overall, the data suggest that sex differences in glucocorticoid levels may influence expression of hClock and thereby lead to sex-dependent effects on clock-influenced gene expression pathways, thereby altering susceptibility to MDD in a gender-specific manner.

hPer3 variant is a stronger transcriptional repressor

Coordination of functional assays was also examined for variants in hPer3, hNpas2 and Arntl2 that showed significant association results (Tables 1 and 2, Supplementary Table S5). Appropriate functional tests are difficult to design for the variants rs17031614 (synonymous codon in hPer3), rs4851377 and rs34705978 (intron variants in hNpas2), and rs10548381 (upstream variant in Arntl2). However the rs228697 SNP is a missense mutation in the coding region of hPer3 that changes a proline at position 856 to an alanine residue. Proline residues often have profound effects upon protein structure, so we tested the impact of this polymorphism in a transcriptional assay designed to test the regulation of E-box-containing promoters by CLOCK and BMAL1. PER3 is known to repress CLOCK/BMAL1 transactivation; therefore we tested the repressive capability of hPer3 with and without the rs228697 SNP. As shown in Figures 2a and b, the variant-containing hPER3 is a stronger repressor of CLOCK/BMAL-stimulated transcription on two different E-box cis-reporters (P_{P2,8} and P_{N2,8}). As compared with the protein encoded by the common allele, the rs228697 variant stabilizes hPER3 (Supplementary Figure S5) and recruits more PER2 into a transcription repression complex (Supplementary Figure S6). Because PER3, PER2 and PER1 interact to synergistically promote nuclear translocation, the rs228697 variant augmentation of hPER3 stability and PER complex formation predict enhanced repressive activity, as depicted in Figures 2a and b. Moreover, when hPER3 is transiently expressed in mammalian fibroblasts, the rs228697 variant causes a significant dose-dependent lengthening of the circadian period as compared with the native version of hPer3 (Figures 2c–e). Although this period effect is statistically significant, the small magnitude of the period effect indicates that the primary impact of the rs228697 hPer3 variant is likely to be upon clock-regulated transcription pathways (as in Figures 2a and b) rather than upon the central clock mechanism itself.

DISCUSSION

Molecular assays of functional activity affected by hClock and hPer3 polymorphisms

Functional and association analyses indicate that common polymorphisms in core circadian genes affect the activity of the encoded proteins and provide a connection between clock function and MDD. Critical is the sex-dependent nature of these associations with MDD, which is a sexually dimorphic disorder. The rs1801260 allele is protective in males, so males with this polymorphism are less likely to present with MDD. The rs1801260 polymorphism in the 3′-untranslated region (3′-UTR) of the hClock gene stabilizes a reporter construct when expressed in the human cell lines (Figure 1), and higher abundance of the hCLOCK protein is known to alter circadian period and phase. Glucocorticoid levels exhibit age-dependent differences between males and females. Moreover, 3′ noncoding regions of other transcripts contain glucocorticoid-responsive elements that regulate activity. As glucocorticoids reciprocally modulated the expression of the 3′-UTR reporter in vitro in an allele-specific manner (Figure 1e), our results revealed a potential glucocorticoid regulatory region in the 3′-UTR of the hClock transcript, which resonates with recent proposals suggesting that glucocorticoid-mediated stress response constitutes a common mechanism by which the circadian clock affects psychiatric disorders including MDD. Finally, the 3′-UTR of the mouse homolog of Clock is significantly different from that of hClock, and, in particular, mClock does not include a homologous region to that of the rs1801260 region of hClock. Consequently, although mClock-mutant mice have been proposed as models of mania, they will not allow an adequate test of a human stress/MDD connection mediated through the rs1801260 region of the hClock 3′-UTR.

The re-analyses of association of circadian gene polymorphisms with MDD also revealed potential associations within hPer3 and hNpas2 (Table 1, Supplementary Table S5). As in the case with rs1801260, these associations were sex-dependent, but unlike hClock's rs1801260, SNPs within hPer3 (rs228697) and hNpas2 (rs34705978) strongly associated with MDD in females (Table 1). The NPAS2/ARNTL1 complex is a key regulator of circadian transcriptional activation; the rs4851377 and rs34705978 SNPs of hNpas2 are both intronic and might influence expression of the NPAS2. The PER3 protein is part of the negative feedback process that controls circadian transcriptional networks and its effects are highly tissue dependent; knockout of Per3 in mice does not cause significant effects on the central clock mechanism in the brain, but it has large effects on clock-regulated gene expression in other tissues such as liver, colon, esophagus and adipose tissue. Although the hPer3 variant that we studied here has a significant influence on circadian period (Figures 2c–e), this effect is smaller than its action on PER-modulated gene expression (Figures 2a/b), suggesting that rs228697 hPer3 variant acts more strongly on clock-regulated output pathways than upon the central clockwork. Moreover, expression of Per3 may be sex dependent, as interactions exist between the estrogen receptor, menstrual/estrous cycles and expression of Per genes in humans and rodents. The exonic SNP rs228697 is predicted to change a proline to an alanine within the hPER3 amino acid sequence, and our functional data support the conclusion that the rs228697 variant slows hPER3 turnover, thereby enhancing the effective repressive activity of hPER3 on CLOCK/BMAL1-mediated transcriptional activity (Figures 2a and b, Supplementary Figures S5 and S6). Interestingly, rs228697 of hPer3 is significantly associated with altered phasing of daily activity/sleep timing, which together with our observations (Tables 1 and 2, Supplementary Table S5 and Figure 2) resurrects a hypothesis that MDD is at least partially due to suboptimal phasing of activity/sleep with the environment. If true, circadian phase resetting may be an effective therapy. Intriguingly, our results with rs1801260 of hClock are also consistent with a phasing interpretation because a stabilization of hClock that leads to increased protein levels should alter circadian period and phase. Indeed, the first report of rs1801260 of hClock suggested its impact upon human phase, and therefore rs1801260 of hClock and rs228697 of hPer3 are similar in sharing a delayed phase phenotype (specifically, a delayed morningness–eveningness preference). Sex-dependent association of clock gene polymorphisms with MDD

Studies of the affective disorders MDD, bipolar disorder and seasonal affective disorder have long suggested that disrupted circadian (daily) timing mechanisms may contribute to the
development or severity of depression,1–4,6,7 but genetic links between clocks and mood disorders have been tenuous.4,11,12,14,15,35,36 However, the earlier case/control attempts to associate rs1801260 in hClock used sample sizes that were relatively small and did not stratify for sex.15,16 Given that MDD is much more common in females, failure to stratify may have led to an association of rs1801260 with MDD being overlooked in males, especially as the effect of this SNP in females was so close to unity (odds ratio = 0.95). Moreover, meta-analyses have suffered from inconsistent phenotype criteria and the enhanced multiple testing burden that is incumbent in tests of polymorphisms that we report herein reveal core circadian genes to be targeted for functional and clinical studies to understand the connections between the circadian system and MDD.8

We propose that the impact of the hClock and hPer3 SNPs on transcription and/or expression (Figures 1 and 2) may not be on the core circadian oscillator, but on global output transcriptional pathways17,23,24 that are mediated sex-dependently by the circadian system. This interpretation is consistent with (and potentially explanatory for) observations of disrupted transcriptional patterns in the brains of humans suffering from MDD.8 These output pathways can include targets of CLOCK-mediated E-box transcription such as the neuropeptide cholecystokinin that is involved in mood disorders.50 Moreover, this clock system controls neurotransmitters and their receptors (for example, serotonin) that are known to be modulators of mood.4 As such, the polymorphisms that we report herein reveal core circadian genes to be targeted for functional and clinical studies to understand the connections between the circadian system and MDD, which may ultimately lead to noninvasive therapies that ameliorate the symptoms of MDD.51

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES
1 Giedke H, Schwärzler F. Therapeutic use of sleep deprivation in depression. Sleep Med Rev 2002; 6: 361–377.
2 Levendosky RA, Dantas G, Fernandes LC, Caumo W, Torres I, Roenneberg T et al. Depression scores associate with chronicity and social jetlag in a rural population. Chronobiol Int 2013; 28: 771–778.
3 Lewy AJ. Circadian misalignment in mood disturbances. Curr Psychiatry Rep 2009; 11: 459–465.
4 McClung CA. Circadian genes, rhythms and the biology of mood disorders. Pharmacol Ther 2007; 114: 222–232.
5 Wehr TA, Goodwin FK. Circadian Rhythms in Psychology. Boxwood Press: Pacific Grove, CA, USA, 1983.
6 Wehr TA, Witz-Justice K, Goodwin FK, Duncan W, Gillin JC. Phase advance of the circadian sleep-wake cycle as an antidepressant. Science 1979; 206: 710–713.
7 Lewy AJ, Sak RL, Miller LS, Hoban TM. Antidepressant and circadian phase-shifting effects of light. Science 1987; 235: 352–354.
8 Li JZ, Bunney BG, Meng F, Hagenauser MH, Walsh DM, Vawter MP et al. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. Proc Natl Acad Sci USA 2013, 110: 9950–9955.
9 Kelsoe JR, Arguments for the genetic basis of the bipolar spectrum. J Affect Disorder 2003; 73: 183–197.
10 Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. Am J Psychiatry 2000; 157: 1552–1562.
11 Benedetti F, Serretti A, Colombo C, Barbini B, Lorenzi C, Campori E et al. Influence of CLOCK gene polymorphism on circadian mood fluctuation and illness recurrence in bipolar depression. J Affect Disord 2007; 109: 833–842.
12 Kennaway DJ. Clock genes at the heart of depression. Neuropsychopharmacology 2003; 28: 825–833.
13 McCarthy MJ, Nievergelt CM, Kelsoe JR, Welsh DK. A survey of genomic studies (R21 MH082258 from NIMH, P20 GM103534 from NIGMS and UL1 RR024975-01 from NCATS), and a Discovery Grant from Vanderbilt University. S-QS was partially supported by NARSAD Young Investigator Award #17623. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.
14 21 DeBruyne JP, Zubenko GS, Crowe RR, DePaulo RJ, Scheftner WS, Weissman MM et al. Genetics of recurrent early-onset depression (GenRED): design and preliminary clinical characteristics of a repository sample for genetic linkage studies. Am J Med Genet B Neuropsychiatr Genet 2003; 119: 118–130.
15 22 Kessler RC, Andrews G, Mroczek D, Ustun TB, Wittchen HU. The World Health Organization Composite International Diagnostic Interview Short Form (CIDI-SF). Int J Methods Psychiatr Res 1998; 7: 171–185.
16 23 Ciarleglio CM, Ryckman K, Servick SV, Hida A, Robbins S, Wells N et al. Genetic differences in human circadian clock genes among worldwide populations. J Biol Rhythms 2008; 23: 330–340.
17 24 Gamble KL, Motsinger-Reif AA, Hida A, Borsetti HM, Servick CV, Ciarleglio CM et al. Shift work in nurses: contribution of phenotypes and genotypes to adaptation. PLoS One 2011; 6: e18395.
18 25 Levinson DF, Zubenko GS, Crowe RR, DePaulo RJ, Scheftner WS, Weissman MM et al. Genetics of recurrent early-onset depression (GenRED): design and preliminary clinical characteristics of a repository sample for genetic linkage studies. Am J Med Genet B Neuropsychiatr Genet 2003; 119: 118–130.
19 26 Kessler RC, Andrews G, Mroczek D, Ustun TB, Wittchen HU. The World Health Organization Composite International Diagnostic Interview Short Form (CIDI-SF). Int J Methods Psychiatr Res 1998; 7: 171–185.
20 27 Ciarleglio CM, Ryckman K, Servick SV, Hida A, Robbins S, Wells N et al. Genetic differences in human circadian clock genes among worldwide populations. J Biol Rhythms 2008; 23: 330–340.
21 28 Gamble KL, Motsinger-Reif AA, Hida A, Borsetti HM, Servick CV, Ciarleglio CM et al. Shift work in nurses: contribution of phenotypes and genotypes to adaptation. PLoS One 2011; 6: e18395.
22 29 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007; 81: 559–575.
23 30 Dupont WD, Plummer WD. PS power and sample size program available for free on the Internet. Control Clin Trials 1997; 18: 274.
24 31 Benjamini N, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 1995; 57: 289–300.
25 32 Ryckman KK, Jiang L, Li C, Bartlett J, Haines JL, Williams SM. A prevalence-based association test for case-control studies. Genet Epidemiol 2008; 32: 600–605.
26 33 Robillard DL, Archer SN, Arendt J, Lockley SW, Hack LM, English J et al. The 3111 Clock gene polymorphism is not associated with sleep and circadian rhythmicity in phenotypically characterized human subjects. J Sleep Res 2002; 11: 305–312.
27 34 Katzenberg D, Young T, Finn L, Lin L, King DP, Takahashi JS et al. A CLOCK polymorphism associated with human diurnal preference. Sleep 1998; 21: 569–576.
28 35 Kishi T, Yoshimura R, Fukuo Y, Kitajima T, Okochi T, Matsuanga S et al. The CLOCK gene and mood disorders: a case-control study and meta-analysis. Chronobiol Int 2011; 28: 825–833.
29 36 Desan PH, Oren DA, Malison R, Price LH, Rosenbaum J, Smoller J et al. Genetic polymorphism at the CLOCK gene locus and major depression. Am J Med Genet 2000; 96: 418–421.
30 37 Kuo T, Lewy MJ, Mayba O, Harris CA, Speed TP, Wang JC. Genome-wide analysis of glucocorticoid receptor-binding sites in myotubes identifies gene networks modulating insulin signaling. Proc Natl Acad Sci USA 2012; 109: 11160–11165.
31 38 Halbrich U, Asnis GM, Zumboff B, Smith U, Schindleder R. Effect of age and sex on cortisol secretion in depressives and normals. Psychiatry Res 1984; 13: 221–229.
32 39 Halbrich U, Lumley LA. The multiple interactional biological processes that might lead to depression and gender differences in its appearance. J Affect Disorder 1993; 29: 159–173.
33 40 Halbrich U, Ray O. Hormones, brain, and neuropsychopharmacology. Neuropsychopharmacology 2000; 23: VIII.
34 41 Landgraf D, McCarthy MJ, Welsh DK. Circadian clock and stress interactions in the molecular biology of psychiatric disorders. Curr Psychiatry Rep 2014; 16: 483.
35 42 Petersen DD, Koch SR, Ganner DK. 3’ noncoding region of phosphoepinephrine carboxykinase mRNA contains a glucocorticoid-responsive mRNA-stabilizing element. Proc Natl Acad Sci USA 1989; 86: 7800–7804.
36 43 Lilavski A, Dumbell R, Ott V, Oster H. Adrenal clocks and the role of adrenal hormones in the regulation of circadian physiology. J Biol Rhythms 2015; 30: 20–34.
37 44 Roybal K, Theobold D, Graham A, DiNieri JA, Russo SJ, Krishnan V et al. Mania-like behavior induced by disruption of CLOCK. Proc Natl Acad Sci USA 2007; 104: 6406–6411.
38 45 Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 2001; 30: 525–536.
39 46 Pendergast JS, Niswender KD, Yamazaki S. Tissue-specific function of Period3 in circadian rhythmicity. PLoS One 2012; 7: e32091.
40 47 Nakamura T, Miya N, Tsuji K, Shimazoe T, Watanabe S, Ebihara S et al. Estrogen differentially regulates expression of Per1 and Per2 genes between central and peripheral clocks and between reproductive and nonreproductive tissues in female rats. J Neurosci Res 2005; 82: 622–630.
41 48 Hida A, Kitamura S, Katayose Y, Kato M, Ono H, Kadoshima T et al. Screening of clock gene polymorphisms demonstrates association of a PER3 polymorphism with morningness-eveningness preference and circadian rhythm sleep disorder. Sci Rep 2014; 4: 6309.
42 49 Soria V, Martinez-Amorós E, Escaramís G, Valero J, Pérez-Egea R, García C et al. Differential association of circadian genes with mood disorders: CR1 and NPS2 are associated with unipolar major depression and CLOCK and VIP with bipolar disorder. Neuropsychopharmacology 2010; 35: 1279–1289.

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50 Arey RN, Enwright JF 3rd, Spencer SM, Falcon E, Ozburn AR, Ghose S et al. An important role for cholecystokinin, a CLOCK target gene, in the development and treatment of manic-like behaviors. *Mol Psychiatry* 2014; 19: 342–350.

51 Wirz-Justice A, Benedetti F, Terman M. *Chronotherapeutics for Affective Disorders: A Clinician’s Manual for Light and Wake Therapy*. Karger Press: Basel, Switzerland, 2013.

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