Functional Implications of the CLOCK 3111T/C Single-Nucleotide Polymorphism

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Circadian rhythm disruptions are prominently associated with bipolar disorder (BD). Circadian rhythms are regulated by the molecular clock, a family of proteins that function together in a transcriptional–translational feedback loop. The CLOCK protein is a key transcription factor of this feedback loop, and previous studies have found that manipulations of the Clock gene are sufficient to produce manic-like behavior in mice (1). The CLOCK 3111T/C single-nucleotide polymorphism (SNP; rs1801260) is a genetic variation of the human CLOCK gene that is significantly associated with increased frequency of manic episodes in BD patients (2). The 3111T/C SNP is located in the 3′-untranslated region of the CLOCK gene. In this study, we sought to examine the functional implications of the human CLOCK 3111T/C SNP by transfecting a mammalian cell line (mouse embryonic fibroblasts isolated from Clock−/− knockout mice) with pcDNA plasmids containing the human CLOCK gene with either the T or C SNP at position 3111. We then measured circadian gene expression over a 24-h time period. We found that the CLOCK 3111C SNP resulted in higher mRNA levels than the CLOCK 3111T SNP. Furthermore, we found that Per2, a transcriptional target of CLOCK, was also more highly expressed with CLOCK 3111C expression, indicating that the 3′-UTR SNP affects the expression, function, and stability of CLOCK mRNA.

Keywords: circadian, clock, single-nucleotide polymorphism, gene expression, bipolar disorder, cell culture

INTRODUCTION

Bipolar disorder (BD) is a severe and chronic psychiatric disease that afflicts approximately 1–3% of the United States population (3). BD incurs substantial societal burdens, including a devastating global cost of illness primarily due to patients’ lower level of functioning, greater severity of disability, longer duration of illness, and ultimately greater loss in productivity when compared patients with other mood disorders (4, 5). BD therapies currently in use include mood stabilizers, such as lithium and valproate; however, these are effective for only a portion of patients (6). The underlying cause of BD is unknown, though there is a growing body of evidence linking disruptions in circadian rhythms with the disease (7).
Disruption of sleep and circadian rhythms are common to many psychiatric disorders, including BD, which presents with severe circadian rhythm disruptions as a prominent symptom. It has been shown that in individuals with BD, mood episodes are affected by light and also follow seasonal patterns (6, 8–10). In addition to fluctuations in mood, BD patients typically exhibit irregularities in important physiological processes that are largely regulated by the body’s circadian rhythms (such as sleep, diurnal activity, body temperature, and blood pressure cycles). Mood stabilizers, such as lithium, are known to restore some of these disrupted rhythms in BD patients by producing a strong phase-delay in rhythms and increasing rhythmic amplitude, which may be very important for their observed therapeutic effects (11–17).

Circadian rhythms are regulated by a molecular clock, which consists of several core “clock” genes (e.g., *Clock, Npas2, Bmal1, Per1*, and *Per2*) that are expressed throughout the body. These elements interact with each other through a series of transcriptional and translational feedback loops that are regulated over a 24-h period in the absence of environmental input (18). Within the suprachiasmatic nucleus (SCN) and other regions, circadian rhythms are controlled by the molecular clockwork, which comprises a series of autoregulatory transcriptional–translational feedback loops. The transcription factors CLOCK or NPAS2 heterodimerize with BMAL1 and regulate the transcription of target genes, including the *Period (Per)* and *Cryptochrome (Cry)* genes, which act to inhibit the activity of the CLOCK/BMAL1 complex. Several pre-clinical studies have identified an important role for circadian genes in mood-related behaviors. Recent human genetic studies have linked elements of the molecular clock to BD. Polymorphisms in *Clock* and other circadian genes have been found to be associated with various aspects of BD (19–25). In addition, rhythm disruptions and sleep disturbances are often precipitated by manic or depressive episodes (26). By studying molecular mechanisms that may underlie such BD symptoms, we can gain a better understanding of its causes and how to most effectively treat patients with this crippling disease.

The importance of circadian genes in BD has been suggested by several human genetic studies that have identified significant associations between mutations or polymorphisms of circadian genes and BD. The *Per* genes, which act as key repressors of the circadian transcriptional–translational feedback loop in humans, have been found to associate with mood disorders and their age of onset (27, 28). Recent genome-wide association studies (GWAS) have also implicated circadian modulators, such as *ARNTL* and *DECI*, as significantly associated with BD (29). These results are particularly important given that data from family and twin studies that have established BD’s genetic heritability as up to 85%, suggesting a strong genetic component (30). Here, we investigate the role of a single-nucleotide polymorphism (SNP) in one circadian gene, *Clock*, that has been associated with BD (2).

Interestingly, the *Clock* 3111T/C SNP is a genetic variant of the circadian gene (*Clock*) containing fragment was ligated into the pcDNA3.1(−) plasmid (catalog no. V795-20, Invitrogen) digested with *Apa1* and *Not1* restriction enzyme sites (5′ end and 3′ end, respectively) and treated with calf intestinal phosphatase to prevent self-ligation. The ligated plasmids were transformed into One-Shot TOP10 Chemically Competent *Escherichia coli* cells (catalog no. C4040-10, Invitrogen). We performed site-directed mutagenesis of the *Clock* construct at the site of the 3111T/C SNP using the Quikchange II XL Site-Directed Kit (catalog no. 200521, Agilent) to perform a C to T transformation, creating a 3111T/C variant of the *Clock* gene 3′-UTR (forward primer: 5′-GAGGTGATCATAGGGGCATAGCCAG TTCTGACAGTG-3′, reverse primer: 5′-CACCTGTCGAAACT GGATGATGCCCTATGATACCTC-3′). The full construct was verified through a series of restriction enzyme digests and complete sequencing of the *Clock* gene (including cloning junctions) by the University of Pittsburgh Genomics and Proteomics Core Laboratories. Isolated and sequence-verified clones were grown in *E. coli* and plasmids were isolated using QIAGEN’s QIAprep Spin Miniprep kit (catalog no. 27104) and endonuclease-free Plasmid Maxi Kit (catalog no. 12162).

MATERIALS AND METHODS

Plasmid Construction

The pBluescript II plasmid containing the full human *Clock* gene (product i.d. ORK00509, Kazusa), was digested with *Apa1* and *Not1* restriction enzymes. The DNA fragment containing the *Clock* gene was isolated using agarose gel electrophoresis and the QIAQuick gel purification kit (catalog no. 28704, QIAGEN). The 5kb *Clock* containing fragment was ligated into the pcDNA3.1(−) plasmid (catalog no. V795-20, Invitrogen) digested with *Apa1* and *Not1* restriction enzyme sites (5′ end and 3′ end, respectively) and treated with calf intestinal phosphatase to prevent self-ligation. The ligated plasmids were transformed into One-Shot TOP10 Chemically Competent *Escherichia coli* cells (catalog no. C4040-10, Invitrogen). We performed site-directed mutagenesis of the *Clock* construct at the site of the 3111T/C SNP using the Quikchange II XL Site-Directed Kit (catalog no. 200521, Agilent) to perform a C to T transformation, creating a 3111T/C variant of the *Clock* gene 3′-UTR (forward primer: 5′-GAGGTGATCATAGGGGCATAGCCAG TTCTGACAGTG-3′, reverse primer: 5′-CACCTGTCGAAACT GGATGATGCCCTATGATACCTC-3′). The full construct was verified through a series of restriction enzyme digests and complete sequencing of the *Clock* gene (including cloning junctions) by the University of Pittsburgh Genomics and Proteomics Core Laboratories. Isolated and sequence-verified clones were grown in *E. coli* and plasmids were isolated using QIAGEN’s QIAprep Spin Miniprep kit (catalog no. 27104) and endonuclease-free Plasmid Maxi Kit (catalog no. 12162).
shRNA Construction
A small hairpin RNA (shRNA) was constructed against the Npas2 gene by selecting a 24 base sequence (5′-GAAACACGTGCTCTCTGTTAAC-3′) in the 3′-UTR (41). For the scrambled (Scr) shRNA, a random sequence of 24 bases (5′-CGGAATTTAGCTGGGATCCAC-3′) that had no sequence similarities with any known genes/mRNA was used (42). An anti-sense sequence of the selected mRNA region followed by a miR23 loop of 10 nucleotides (CTTCCCTGTC) was added at the 5′ end of the above sequences. The miR23 loop facilitates the transfer of the hairpin RNA out of the nucleus. These shRNAs were designed as synthetic duplexes with overhang ends identical to those created by SapI and XbaI restriction enzyme digestion. The annealed oligonucleotides were cloned into the adeno-associated virus (AAV) plasmid expressing enhanced green fluorescent protein (GFP) (catalog no. 240075, Agilent Technologies).

Preparation of Mouse Embryonic Fibroblasts
Mouse embryonic fibroblasts (MEFs) were isolated and prepared from homozygous Clock knockout (KO) mice at 13–14 day post-coitum (Jackson Labs, Bar Harbor, ME, USA, stock#010925) (43). All experiments were approved by the Institutional Animal Care and Use Committee and adhered to NIH Guidelines. Clock KO MEFs were used to avoid confounds of endogenous Clock gene expression when measuring expression levels in MEFs transfected with the 3111T/C constructs. Briefly, uterine horns were dissected, and embryos were subjected to agarose gel electrophoresis to determine specificity of the amplified product. Gene expression was quantified using the ΔΔCT (CT = cycle threshold) method as previously described (44). Gel electrophoresis of the samples was then performed in order to further confirm the correct size of the amplified PCR products. Efficiencies of qPCR primers used in this study were calculated by means of construction of a standard curve as previously described (45). Samples of experimental MEF cDNA were serially diluted and prepared with the Power SYBR Green qPCR Master Mix (catalog no. 4367659, Applied Biosystems). Dilution series from 1:1 to 1:64 were used for transfected genes (hCLOCK, G418-resistance gene), while dilution series from 1:1 to 1:8 were used for endogenous genes (mPer2, Npas2). qPCRs were run in duplicate, followed by a dissociation reaction, and were subjected to agarose gel electrophoresis to determine specificity of the amplified product. Gene expression was quantified using the ΔΔCT (CT = cycle threshold) method as previously described (44). Gel electrophoresis of the samples was then performed in order to further confirm the correct size of the amplified PCR products. Efficiencies of qPCR primers used in this study were calculated by means of construction of a standard curve as previously described (45). Samples of experimental MEF cDNA were serially diluted and prepared with the Power SYBR Green qPCR Master Mix (catalog no. 4367659, Applied Biosystems). Dilution series from 1:1 to 1:64 were used for transfected genes (hCLOCK, G418-resistance gene), while dilution series from 1:1 to 1:8 were used for endogenous genes (mPer2, Npas2). qPCRs were run in duplicate, followed by a dissociation reaction. Gene expression was quantified using the ΔΔCT method and plotted against the logarithm of the dilution factor. The calculated efficiencies for the primer sets used are as follows: hCLOCK 88%, G418 93%, mPer2 110%, and Npas2 78%. The observed relatively low expression levels of Npas2 indicate that CLOCK KO MEFs do not express high levels of Npas2 to compensate for the loss of CLOCK. The hCLOCK and G418 primer sets used for determination and comparison of CLOCK3111T and CLOCK3111C levels have similar efficiencies; therefore, we conclude that these findings do not affect interpretation of the data.
Circadian Gene Expression Assay
Approximately $1 \times 10^4$ cells were plated in 12-well plates, and were doubly transfected with 1 μg each of pcDNA3.1(−) (containing the 3111T, 3111C, or no insert), and a plasmid containing Npas2 shRNA or a Scr shRNA. A caveat of using Clock KO MEFs was that previous research has shown that KO of the Clock gene can result in increased Npas2 expression, possibly as a compensatory mechanism (46). Because, as a CLOCK homolog, NPAS2 also regulates Per2 gene expression, an Npas2 shRNA was co-transfected along with either versions of the 3111T/C SNP when measuring Per2 circadian gene expression in qPCR studies. Seventy-two hours following transfection, MEFs were subjected to serum shock to synchronize individual molecular rhythms as previously described (47). Briefly, cells were serum shocked using DMEM supplemented with 50% horse serum for 30 min to induce expression of circadian genes and synchronize the molecular clock (48). After the serum shock treatment, MEFs were switched to recovery media containing high-glucose DMEM with 1-glutamine supplemented with 2% B-27 (catalog no. 17504-044, GIBCO), 350 mg/l sodium bicarbonate, 0.25% penicillin/streptomycin, and 10 mM HEPES (catalog no. 15630-106, GIBCO). Cells were collected every 3 h for the next 27 h (e.g., circadian time CT3, CT6, CT9, CT12, etc.) and processed for RNA isolation/cDNA synthesis. Samples were then used to measure circadian gene expression (as described in Section “Quantitative Real-Time PCR”).

Statistics
All data are presented as the mean ± SEM. Two-way analysis of variance (ANOVA) and Student’s t-tests were performed to determine significant differences between experimental groups and time points. Statistical outliers were identified as ±2× SD of the mean and removed prior to further data analysis.

RESULTS
Npas2 Gene Expression in MEF Cells and Knock-Down
NPAS2, a homolog of CLOCK, can heterodimerize with BMAL1 and positively regulate the transcription of Per and Cry genes. Since it is possible that Npas2 may be upregulated in Clock KO MEFs, Npas2 expression in WT and Clock KO MEFs was measured using qPCR (Figure 1A). After normalizing CT values to GAPDH, we performed a two-tailed unpaired Student’s t-test on our preliminary data, which revealed a statistically significant difference between mean ΔCT values $[n = 2, \text{Figure 1A}]$. These data show a 4.59-fold increase in Npas2 expression in Clock KO MEFs compared to WT MEFs (Student’s t-test, $P < 0.05$). We used an shRNA to knockdown expression of Npas2. Npas2 shRNA resulted in a decrease in Npas2 expression as compared with Scr shRNA, as qPCR studies revealed that Npas2 gene expression was undetected after 40 cycles of PCR in cells expressing the Npas2 shRNA. Representative data from ZT21 is shown (Figure 1B).

CLOCK 3111T/C SNP Gene Expression (Single Time Point)
To better understand the effects of the CLOCK 3111T/C SNP on mRNA expression and stability, we transfected Clock KO MEFs with either version of the SNP and sought to measure differences in CLOCK mRNA levels between cells expressing the 3111C and 3111T alleles (at a single time point). Using primers for the human CLOCK gene, our studies revealed that cells expressing the 3111C allele showed a 4.17-fold increase in CLOCK mRNA levels (Student’s t-test, $P < 0.05, n = 8$, Figure 2). Transfection efficiency was not directly evaluated; we used antibiotic selection to ensure proliferation of only transfected cells. Furthermore, we used the housekeeping gene, G418 (the gene in the pcDNA plasmid that is responsible for conferring antibiotic resistance to Geneticin) for calculating the dCT.

Twenty-Four-Hour Gene Expression Levels of CLOCK 3111T/C and Per2
To test the functional implications of the CLOCK 3111T/C SNP on CLOCK mRNA expression and stability over a 24-h time period, we transfected CLOCK KO MEFs with either version of the SNP and sought to determine if there were differences in CLOCK mRNA levels between cells expressing the 3111C and 3111T alleles. Transfection efficiency was not directly evaluated; we used the housekeeping gene, G418 (the gene in the pcDNA plasmid that is responsible for conferring antibiotic resistance to geneticin) for calculating the dCT. Our findings show that MEFs transfected with the 3111C construct showed significantly increased levels of CLOCK mRNA at every time point measured, excluding CT18 ($n = 4/group/timepoint$, Figure 3). Using a two-way ANOVA, we found a highly significant SNP × time interaction; SNP × time [$F = (21, 93) = 5.34, P < 0.0001$]. The main effect of the SNP was highly significant; $[F = (3, 93) = 156.45, P < 0.0001]$, as was the main effect of time; $[F = (7, 93) = 16.68, P < 0.0001]$. Bonferroni post hoc test results reveal significant differences in CLOCK expression in MEFs co-transfected with 3111T or C SNP constructs (along with Npas2 shRNA; Figure 3). We were primarily interested in these group comparisons (due to the upregulation of Npas2 in Clock KO MEFs). There were no significant differences in CLOCK expression seen in cells transfected with or without the Npas2 shRNA.

To test the CLOCK 3111T/C SNP’s effects on CLOCK protein function, 24-h Per2 expression was measured in Clock KO MEFs co-transfected with the 3111T or 3111C constructs and the Npas2 shRNA ($n = 4/group/timepoint$, Figure 4). As a downstream transcriptional target of the CLOCK protein, Per2 is expressed as part of a transcriptional–translational feedback loop in the molecular clock. Therefore, alterations to Per2 expression indirectly reveal differences in CLOCK’s transcriptional activity between cells expressing the T or C variant of the SNP. Npas2 shRNA plasmid was co-transfected along with the human CLOCK constructs in order to eliminate the possible confound of Per2 expression driven by NPAS2. Using a two-way ANOVA, we found a significant interaction between SNP and time: SNP × time [$F = (21, 95) = 8.52, P < 0.0001$]. We also found that the main effect of the SNP was highly significant; $[F = (3, 95) = 523.66, P < 0.0001]$. 

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The main effect of time was also significant; \( F = (7, 95) = 23.67, P < 0.0001 \). Bonferroni post hoc testing revealed that in MEFs expressing the \( CLOCK \) 3111C variant and \( Npas2 \) shRNA, expression of \( Per2 \) mRNA is significantly increased at every time point measured.

**DISCUSSION**

Severe circadian rhythm disruptions are prominent symptoms in mood disorders, including BD and major depression. There is mounting pre-clinical and clinical data, suggesting that circadian genes play a role in a number of disease parameters, such as age of onset (27, 28). Here, we sought to study the implications of the \( CLOCK \) 3111T/C SNP, a polymorphism located in the 3′-UTR that has been shown to associate with increased frequency and severity of manic episodes and actimetric disturbances in BD patients (2, 31, 32). Alterations to the 3′-UTR have been shown to engage in transcriptional and translational regulation through various pathways, including those involving mRNA stability and degradation (49). In addition, previous animal research has found that reductions in \( CLOCK \) expression or function produces increased locomotor and drug- and alcohol-seeking behavior, and decreased depression- and anxiety-like behavior – hallmarks of bipolar mania (1, 36–38). Therefore, we sought to test the hypothesis that the 3111C variant of the 3111T/C SNP would result in reduced \( CLOCK \) expression and alter \( Per2 \) expression (as a measure of altered \( CLOCK \) transcriptional activity) when compared to the 3111T allele. Using cell culture and qPCR, we found that the 3111C variant results in significantly increased \( CLOCK \) and \( Per2 \) mRNA expression over 24 h.
CLOCK, and many other regulators of dopaminergic transmission hydroxylase (TH), dopamine receptor D3 (Drd3), cholecystokinin play an important role in regulating mood and behavior. Tyrosine have tremendous implications for a wide variety of genes that clearly chronic lithium treatment (1). These mice show increased dopamine resembling BD patients in the manic state that can be reversed using dominant-negative function, express a behavioral profile strongly which results in overexpression of a truncated CLOCK protein with reduced anxiety- but increased depression-like behavior (1, 40, 42). Taking this into consideration, it becomes easy to see how a SNP affecting Clock expression can have major functional consequences for a number of diseases.

It is still unclear as to how CLOCK mRNA levels are increased in cells expressing the 3111C variant. Changes in mRNA levels for 3111C could be due to increased stability of the mRNA. One possible mechanism to explain the increased mRNA stability is that the 3′-UTR hosts several regulatory elements controlling stability, one of which is binding sites for microRNAs (miRNAs). miRNAs are short, untranslated sequences of RNA that bind specific RNA sequences and, once bound, decrease mRNA transcript stability (57). The 3′-UTR of the CLOCK gene also features a binding site for miRNA-182 less than 40 bases from the 3111T/C SNP that may affect stability of the mRNA by preventing or activating degradation mechanisms. It is possible that the 3111C variant may interfere with this site, reducing miRNA-182 binding and thereby increasing stability and elevating levels of CLOCK mRNA.

Our studies show that the CLOCK 3111T/C SNP has important functional consequences by increasing CLOCK and Per2 mRNA expression over 24 h. These changes may be due to a number of different mechanisms, including miRNA-182 binding sites in the 3′-UTR. In the future, we plan to investigate whether miRNA-182 binding is altered in the 3111T/C SNP. Additional future studies may focus on behavioral studies involving transgenic mice that express either version of the 3111T/C SNP. Given that our studies report altered Per2 expression, the clinical relevance of the SNP may also be further studied by examining whether carriers of the 3111C allele respond differently to mood stabilizers that are known to induce changes in circadian rhythm phase and period. These results directly help to further characterize the functional implications of this clinically relevant SNP and because the 3111T/C SNP is only one polymorphism of many identified for the CLOCK gene that associates with BD, they provide a stepping off point for future SNP studies in this model.

**AUTHOR CONTRIBUTIONS**

AO, SM, and CM designed the experiments. AO, KP, PP, GK, EF, SM, and HC performed research; KP, AO, GK, EF, and CM analyzed data. AO, KP, GK, and CM wrote the manuscript.

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