INVESTIGATING THE FUNCTIONAL IMPLICATIONS OF THE *CLOCK* 3111T/C SINGLE-NUCLEOTIDE POLYMORPHISM

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Introduction: Bipolar disorder (BD) is a debilitating mental illness proven to be very difficult to treat. By studying the molecular mechanisms that underlie BD, we can learn how to improve treatments. The CLOCK protein is a key transcription factor involved in the persistence of circadian rhythms. Studies have found that manipulations of the *Clock* gene are sufficient to produce manic-like behavior in mice. The *Clock* 3111T/C single-nucleotide polymorphism (SNP) is a variation of the human *Clock* gene that is associated with increased frequency of mania in BD patients. In this study, we sought to examine the functional implications of the *Clock* 3111T/C SNP on *Clock* and *Per2* expression over 24 hours.

Materials and Methods: Plasmid construction: The human *Clock* gene was cloned and site-directed mutagenesis was performed to produce the 3111T and 3111C versions of the gene. An *Npas2* shRNA was also constructed in order to knockdown *Npas2* expression, which has been shown to be significantly upregulated in *Clock* KO MEFs. MEFs: Mouse embryonic fibroblasts (MEFs) were collected from *Clock* Knockout (KO) mice. *Clock* KO MEFs were used to avoid confounds of endogenous *Clock* expression. Transfections: MEFs were transfected with either the *Clock* T or C constructs, in addition with an *Npas2* shRNA. Quantitative PCR: RNA from transfected MEFs was isolated, and then reverse-transcribed to cDNA. Quantitative polymerase chain reaction (qPCR) was performed over a 24 hour period.

Results: Quantitative PCR revealed significantly increased levels of *Clock* and *Per2* mRNA over a 24-hour time course in MEFs expressing the 3111C SNP, as compared with 3111T.

Conclusions: The CLOCK protein controls expression of genes beyond the circadian clock. These include dopamine regulators, which are known to be involved in decreased depression-like behaviors and increased impulsivity/drug intake. From this, it is clear that altered *Clock* expression may have tremendous implications for a variety of diseases, including BD.

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1. INTRODUCTION

Bipolar Disorder (BD) is a severe and chronic psychiatric disease that afflicts approximately 1-3% of the United States population (Kupfer et al., 2011). BD incurs substantial societal burdens including a devastating global cost of illness primarily due to patients' lower levels of functioning, greater severities of disability, longer duration of illness, and ultimately greater losses in productivity when compared to other mood disorders (Budde et al., 2003; Pini et al., 2005). BD therapies currently in use include mood stabilizers such as lithium and valproate, however these are effective for only a portion of patients (for review, see McClung, 2007). The underlying cause of BD is unknown, though there is a growing body of evidence linking disruptions in circadian rhythms with the disease.

Disruption of sleep and circadian rhythms are common to many psychiatric disorders, and severe circadian rhythm disruptions in a variety of measures are prominent symptoms in patients with BD. It has been shown that in individuals with BD, mood episodes are affected by light and also follow seasonal patterns (for review, see McClung, 2007). 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 rhythm amplitude, which may be very important for their

therapeutic effects (Atkinson et al.,1975; Johnsson et al., 1983; Klemfuss, 1992; Kripke et al., 1978; Dokucu et al., 2005; Gould and Manji, 2005; Iwahana et al., 2004). Here, we investigate the role of a single-nucleotide polymorphism in the circadian gene, *Clock*, that has been associated with BD (Benedetti et al., 2003). By studying molecular mechanisms that may underlie BD symptoms, we can gain a better understanding of its causes and how to most effectively treat patients with this crippling disease.

Circadian rhythms are regulated by a molecular clock, which consists of several 'clock' genes (i.e. *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-hour period in the absence of environmental input (Takahashi et al., 2008). Within the suprachiasmatic nucleus (SCN) and other regions, circadian rhythms are controlled by the molecular clockwork, which is comprised of a series of autoregulatory transcriptional-translational feedback loops. The transcription factors CLOCK (or NPAS2) and BMAL1 heterodimerize and activate 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 (for review, see McClung, 2011). Recent human genetic studies have linked elements of the molecular clockwork to BD. Polymorphisms in *Clock* and other circadian genes have been found to be associated with various aspects of bipolar disorder (Lamont et al., 2010; Soria et al., 2010; Mansour et al., 2006; Mansour et al., 2009; Kripke et al., 2009; Sjoholm et al., 2009; Lamont et al., 2007). In addition, rhythm disruptions and sleep disturbances are common in BD and often precipitate manic or depressive episodes (Harvey et al., 2011).

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 (Nievergelt et al., 2006; Benedetti et al., 2008). Recent genome-wide association studies (GWAS) have also implicated circadian modulators such as *ARNTL* and *DEC1* as significantly associated with BD (McCarthy et al., 2012). These results are particularly important given data from family and twin studies that have established BD's genetic heritability as up to 85%, suggesting a strong genetic component of this disease (McGuffin et al., 2003). Interestingly, the *Clock* 3111T/C single-nucleotide polymorphism (SNP) is a genetic variant of the human *Clock* gene that has been associated with bipolar mania in BD patients. Specifically, this T→C polymorphism (rs1801260) associates with increased actimetric and sleep disturbances and incidences of manic episodes in bipolar patients (Benedetti et al., 2003; Benedetti et al., 2007; Seretti et al., 2005). The prevalence of the 3111C has been reported to be between 19-21% in American populations (Desan et al., 2000). BD patients carrying the 3111C allele experience higher rates of insomnia and sleep disturbances, and differences between the 3111C and 3111T allele carriers were attenuated with ongoing chronic lithium treatment (Seretti et al., 2005). No research group, however, has yet sought to determine the molecular mechanism through which this SNP can affect individuals with BD. The 3111T/C SNP is located within the *Clock* gene's 3' untranslated region (3'UTR). The 3'UTR is a region that has been shown to be very important for mRNA stability and modifications to this region can lead to either increased stability or degradation of gene products (Hentze et al., 1996; Zaidi et al., 1994).

Moreover, preclinical studies have shown that manipulations of the circadian gene, *Clock*, are sufficient to produce a behavioral phenotype sharing several features with bipolar mania. Mice with a dominant-negative *Clock* gene mutation exhibit a manic-like phenotype, including hyperactivity, reduced anxiety and depression-like behaviors, and increased drug and alcohol intake (McClung et al., 2005; Roybal et al., 2007; Ozburn et al., 2012; Ozburn et al., 2013). Similar to human BD patients, these mutant mice are responsive to chronic lithium, as well as valproate, treatment (Coque et al., 2011; Dzirasa et al., 2010; Logan et al., in prep).

Together, these results have led us to hypothesize that the 3111C SNP in the *Clock* gene decreases *Clock* mRNA and/or protein levels, and that this could be a possible mechanism through which this polymorphism can affect mood in individuals with BD. In this study, we investigated the effects of the human 3111T/C SNP on *Clock* mRNA and protein in a mammalian cell line by examining the differences between the 3111T and 3111C variants of *Clock*. The human *Clock* gene was cloned and transfected into *Clock* Knockout (KO) mouse embryonic fibroblasts (MEFs). Quantitative real-time polymerase chain reaction (RT-PCR) studies were completed to quantify mRNA levels, allowing us to measure differences in *Clock, Per2,* and *Npas2* mRNA expression and stability between the two variants of the 3111T/C SNP. Circadian gene expression assays were performed to understand the effects of the 3111T/C SNP on gene expression over a 24-hour time course. Additionally, we performed gel electrophoresis and quantitative Western Blotting to measure CLOCK protein levels. Surprisingly, our results revealed that 24-hour *Clock* mRNA levels were significantly increased in the 3111C SNP when compared to the 3111T variant. We also found that 24-hour *Per2* gene expression was increased in cells expressing *Clock* 3111C, suggesting that the CLOCK protein's transcriptional activity was also increased by the polymorphism.

2. MATERIALS AND METHODS

2.1 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 *E. 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 version of the *Clock* gene 3'UTR (forward primer: 5'-GAGGTGATCATAGGGGCATAGCCAGTTCTGACAGTG-3', reverse primer: 5'-CACTGTCAGAACTGGCTATGCCCCTATGATCACCTC-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).

2.2 SHRNA CONSTRUCTION

A small hairpin RNA (shRNA) was constructed against the *Npas2* gene by selecting a 24 base sequence (5'-GAACACTGGATTCTTCCTGTTAAC -3') in the 3'-UTR. For the scrambled (Scr) shRNA, a random sequence of 24 bases (5'-CGGAATTTAGTTACGGGGATCCAC-3') that had no sequence similarities with any known genes/mRNA was used (Mukherjee et al., 2010). An anti-sense sequence of the selected mRNA region followed by a miR23 loop of 10 nucleotides (CTTCCTGTCA) 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 Sap I and Xba I 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).

2.3 PREPARATION OF MEFS

Mouse embryonic fibroblasts (MEFs) were isolated and prepared from *Clock* Knockout mice at 13-14 day post-coitum as described in (Jozefczuk et al., 2012). *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 individually separated. The head and red organs were removed before the embryos were finely minced using a sterile razor blade. Minced tissue was treated with 0.05% Trypsin-EDTA (Gibco, Invitrogen) and DNase I (USB), and incubated for 15 minutes at 37°C. Cells were centrifuged at 600 x g for 5 minutes, and supernatant was carefully removed before resuspending the MEF cell pellet in a warm solution of media containing high-glucose Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine (catalog no. 11965-084, GIBCO), supplemented with 10% FBS (catalog no. 16000-044, GIBCO), 100 units/ml penicillin, 100 units/ml streptomycin, and 1mM sodium pyruvate (catalog no. 11360-070, GIBCO). MEFs were stored at -80°C.

2.4 CELL CULTURE/TRANSFECTIONS

MEF cells were cultured at 37°C with 5% CO_2 in high-glucose DMEM with L-glutamine, supplemented with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 1mM sodium pyruvate. Cells were grown to 80-100% confluence before being split using 0.25% Trypsin-EDTA with phenol red (catalog no. 25200-056, GIBCO). MEFs were transfected with plasmid construct(s) using Lipofectamine LTX (catalog no. 15338-100, Invitrogen) and Opti-MEM I Reduced Media Serum (catalog no. 31985-062, Invitrogen) according to the manufacturer's instructions. Following transfections, cells were collected for RNA isolation in qPCR or Western Blot studies.

2.5 RNA ISOLATION/CDNA SYNTHESIS

Approximately $1x10^5$ cells were transfected with 2μg of the 3111T or 3111C versions of the *Clock* SNP. 72 hours following transfection with expression constructs, cells were selected using 100μg/ml Geneticin Selective Antibiotic (catalog no. 10131-035, GIBCO) to generate stable lines. After selection, RNA from the MEFs was isolated using TRIzol reagent (catalog no. 15596-026, Ambion), according to the manufacturer's instructions. Briefly, cells were treated with TRIzol reagent and chloroform, and RNA was isolated with the addition of the carrier glycogen, followed by precipitation in isopropanol and centrifugation. 75% ethanol was used to wash the RNA pellet, which was re-suspended in nuclease-free H_2O. Total RNA (200ng) was treated with DNase I (Catalog no. 18068-015, Invitrogen), and then reverse-transcribed to cDNA using the SuperScript III First-Strand Synthesis System (catalog no. 18080-051, Invitrogen) according to manufacturer's protocols.

2.6 REAL-TIME PCR

Real-time quantitative polymerase chain reaction (RT-PCR) was completed using the 7900HT Fast Real-Time PCR System (catalog no. 4329003, Applied Biosystems). cDNA samples were prepared with the Power SYBR Green qPCR Master Mix (catalog no. 4367659, Applied Biosystems) along with oligo primers for *hClock* (forward primer: 5'-ATGGGCCAGGTGGTGACTGCAT-3', reverse primer: 5'-TGACCCAGCCACCGCAACAAT-3'), *mNpas2* (forward primer 5'-GACACTGGAGTCCAGACGCAA-3', reverse primer 5'-

AATGTATACAGGGTGCGCCAAA-3'), *G418-resistance gene* present in pcDNA3.1(-) (forward primer: 5'-CGCATGATTGAACAAGATGGATTGC-3', reverse primer: 5'GTTCATTCAGGGCACCGGACA-3'), *mPer2* (forward primer 5′-GAGTGTGTGCAGCGGCTTAG-3′, reverse primer 5′-GTAGGGTGTCATGCGGAAGG-3′) and *m18s* (forward primer 5'- ACCGCAGCTAGGAATAATGGA-3'; reverse primer 5'-GCCTCAGTTCCGAAAACCA-3'). PCR reactions 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 described in (Tsankova et al., 2004). Gel electrophoresis of the samples was then performed in order to further confirm the correct size of the amplified PCR products.

2.7 CIRCADIAN GENE EXPRESSION ASSAY

Approximately 1x104 cells were plated in 12-well plates, and were doubly-transfected with 1μg each of pcDNA3.1(-) (containing the 3111T, 3111C or no insert), and a plasmid containing an *Npas2* shRNA with a GFP reporter or a scramble plasmid with a GFP reporter. A caveat of using *Clock* KO MEFs was that previous research has shown that KO of the *Clock* gene results in increased *Npas2* expression, possibly as a compensatory mechanism (Ozburn and Falcon, submitted). 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. 72 hours following transfection, MEFs were subjected to serum shock to synchronize individual molecular rhythms as described

in (Izumo et al., 2003). Briefly, cells were serum shocked using DMEM supplemented with 50% horse serum for 30 minutes. After the serum shock treatment, MEFs were switched to recovery media containing high-glucose DMEM with L-glutamine supplemented with 2% B-27 (catalog no. 17504-044, GIBCO), 350mg/l sodium bicarbonate, 0.25% penicillin/streptomycin, and 10mM HEPES (catalog no. 15630-106, GIBCO). Cells were collected every 3 hours for the next 27 hours (and processed as described in RNA isolation/cDNA synthesis section). Samples were then used to measure circadian gene expression (as described in Real-Time PCR section).

2.8 WESTERN BLOT

Western Blot assays were run similar to previously published protocols (Falcon et al., 2013). However, we were unable to confirm the presence of a CLOCK band using denaturing sample lysis and SDS-PAGE protocols. Our lab has had success using the CLOCK H-276 antibody (Santa Cruz Biotechnology) for chromatin immunoprecipitation (ChIP) assays (Ozburn and Falcon et al., submitted). Since ChIP assays are carried out in non-denaturing conditions, we are in the process of carrying out this experiment again using native sample and PAGE conditions. 72 hours after transfection, cells were trypsinized and resuspended in RIPA buffer with protease inhibitors (Roche, San Francisco, CA; Sigma, St. Louis, MO). Samples were sonicated and centrifuged at 15,000xg for 15 min, supernatant was subsequently collected and protein concentrations were determined using a Bradford assay (Bio-Rad, Hercules, CA). Samples will be run on a 10% native gel (acrylamide/bisacrylamide), transferred to a 0.22μm PVDF membrane, blocked in LiCor Odyssey blocking buffer, incubated with primary antibodies (CLOCK H-276) and then incubated with fluorescent secondary antibodies (LiCor Bioscience).

Blots will be visualized and quantified using a LiCor Odyssey fluoresence imaging system. All samples will be normalized to Anti-Neomycin Phosphotransferase (Millipore).

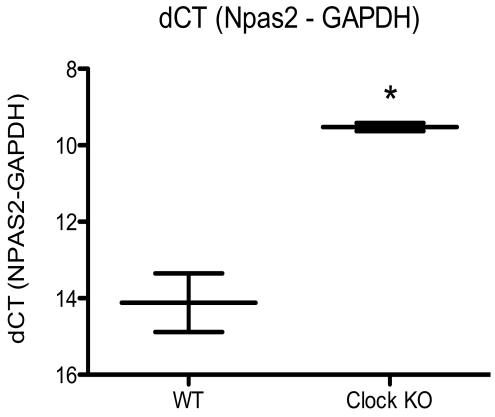
2.9 STATISTICS

All data is presented as the mean +/- standard error of the mean (s.e.m.). 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 +/- 2x standard deviation of the mean and removed prior to further data analysis.

3. RESULTS

3.1 *NPAS2* GENE EXPRESSION IN MEFS 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 previously measured using qPCR by Edgardo Falcón, a graduate student at UT Southwestern. 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, Figure 1 (left)). This data shows a 4.59-cycle increase in *Npas2* expression in *Clock* KO MEFs compared to WT MEFs (student's t-test, $p < 0.05$). We then designed an shRNA to knockdown expression of *Npas2*. *Npas2* shRNA resulted in a decrease in *Npas2* expression as compared with scramble 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 1 (right), representative data from ZT21).



Experimental Group	Mean CT
Clock 3111C + *Npas2* KD	**Undetermined**
Clock 3111T + *Npas2* KD	**Undetermined**
Clock 3111C + scramble	**34.12**
Clock 3111T + scramble	**37.24**

Figure 1: *Npas2* expression and Knockdown in *Clock* KO and wild-type (WT) MEFs. (Left panel) *Npas2* expression is significantly increased in *Clock* knockout MEFS. * = P < 0.05. (Right panel) *Npas2* shRNA results in significantly decreased *Npas2* expression as compared with Scramble shRNA.

3.2 *CLOCK* 3111T/C GENE EXPRESSION

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. 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).

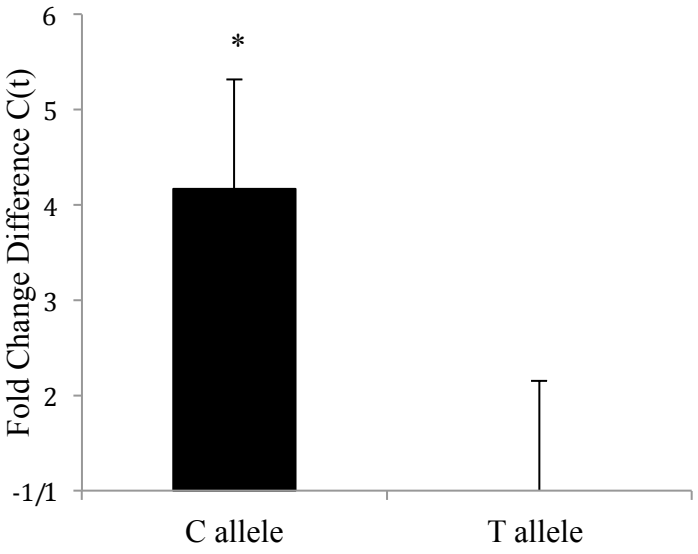


Figure 2: Fold change difference in *Clock* levels in *Clock* 3111T/C-expressing MEFs. *Clock* 3111C gene expression is significantly higher than 3111T expression. * = $P < 0.05$.

3.3 24-HOUR *CLOCK* 3111T/C AND *PER2* GENE EXPRESSION

To test the functional implications of the *Clock* 3111T/C SNP on *Clock* mRNA expression and stability over a 24-hour time period, 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. Our findings show that MEFs transfected with the 3111C construct showed significantly increased levels of *Clock* mRNA at every time point measured, excluding ZT18 (n = 12, Figure 3). Using a 2-way ANOVA, we found a highly significant SNP x time interaction; SNP x 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 (presented in Figure 3) reveal significant differences in *Clock* expression in MEFs co-transfected with 3111T or C SNP constructs (along with *Npas2* shRNA). 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.

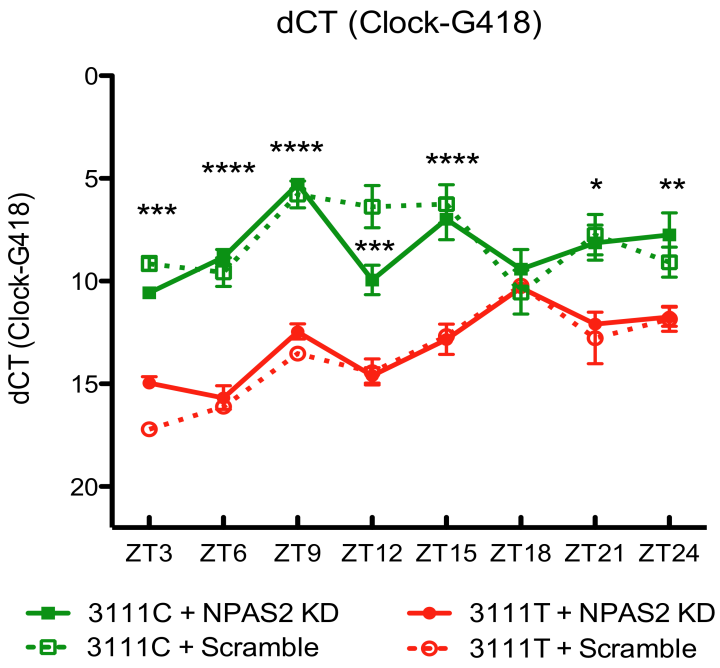


Figure 3: 24-Hour expression of Clock in 3111T/C SNP-expressing MEFs. *Clock* 3111C gene expression over 24 hours is significantly higher than *Clock* 3111T expression. **** = P, 0.0001, *** = P < 0.001, ** = P < 0.01, * = P < 0.05.

To test the *Clock* 3111T/C SNP's effects on CLOCK protein function, 24-hour *Per2* expression was measured in *Clock* KO MEFs co-transfected with the 3111T or 3111C constructs and the *Npas2* shRNA. As a downstream transcriptional target of the CLOCK protein, *Per2* is rhythmically 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 is a CLOCK homolog that also serves as a transcriptional activator for *Per2*, and the *Npas2* shRNA plasmid was co-transfected along with the human *Clock* constructs in order to eliminate confounds of *Per2* expression driven by NPAS2. Using a 2-way ANOVA, we found a significant

interaction between SNP and time: SNP x 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]. 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.

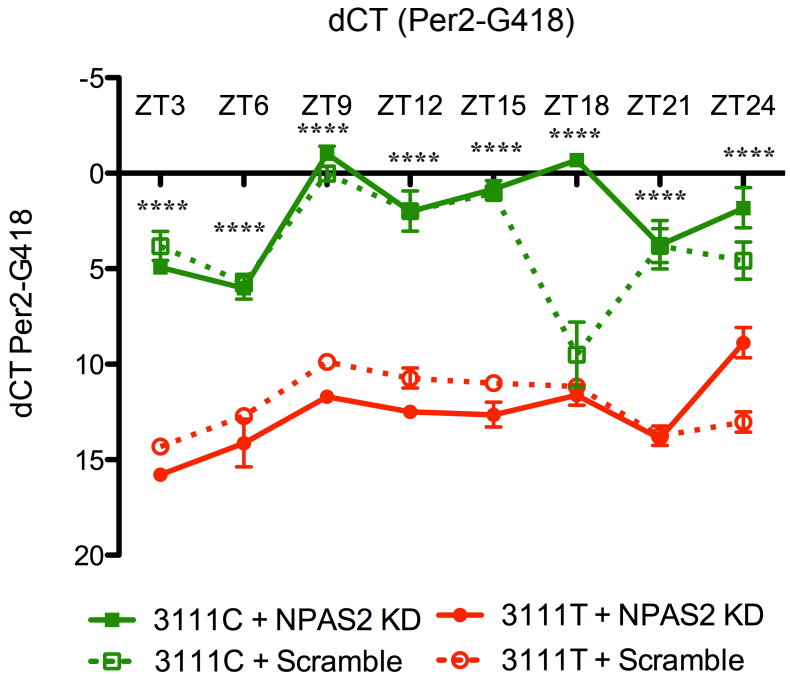


Figure 4: Fold change difference of 24-hour *Per2* expression in 3111T/C-expressing MEFs. Fold change in gene expression (for *Per2*-G418) over 24 hours reveals a highly significant interaction between the *Clock* SNP and time of day. **** = P < 0.0001.

4. 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 (Nievergelt et al., 2006; Benedetti et al., 2008). 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 (Benedetti et al., 2003; Benedetti et al., 2007; Seretti et al., 2005). 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 (for review, see Mignone et al., 2002). In addition, previous animal research has found that decreased CLOCK activity produces increased locomotor and drug- and alcohol-seeking behavior, and decreased depression- and anxiety-like behavior – hallmarks of bipolar mania (McClung et al., 2005; Roybal et al., 2007; Ozburn et al., 2012; Ozburn et al., 2013). 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 hours.

These findings are very exciting as there is a clear dearth in the literature of molecular research of this SNP. The possibility that a clinically-relevant SNP results in increased *Clock* expression is important to many interesting genes, including related circadian genes as well as those outside of the molecular clock. For example, in this study, we report that *Per2*, a gene positively regulated by CLOCK, exhibits significantly elevated expression over 24 hours in cells expressing the *Clock* 3111C SNP. This suggests that CLOCK protein levels are also increased.

In this study, we attempted to control for compensatory overexpression of *Npas2* in our *Clock* KO cell culture system using an shRNA to knockdown *Npas2* levels. *Npas2* knockdown reduces possible confounds on our measures of *Per2* expression. Interestingly, the dramatic change in *Per2* expression as a result of *Npas2* knockdown in 3111C-expressing cells suggests that important interactions between *Clock* and *Npas2* are critical for normal *Per2* expression. We did not expect to see a significant difference in *Per2* expression in 3111T-expressing cells co-transfected with scramble or *Npas2* shRNA. As we predicted, these cells were similar to the WT group in Figure 1 in that normal *Clock* expression was restored, reducing *Npas2* upregulation.

Changes in expression of these related circadian genes may have important implications for rhythms of critical physiological processes. Sleep/wake, hormonal, body temperature, and blood pressure rhythms, for instance, are all influenced by molecular clockwork. These changes may also be important in the understanding and development of effective treatments for mood disorders, as current therapies have been shown to strongly phase-shift circadian rhythms. For example, selective serotonin reuptake inhibitors (SSRI's) and morning-light therapy produce circadian phase shift advances in the SCN, while lithium and valproate induce phase shift delays and period-lengthening (Lewy et al., 2006; Lewy et al., 2007; Terman and Jiuan Su, 2010; Dudley et al., 1999; Sprouse et al., 2006).

Outside of the molecular clock, changes in *Clock* expression may have tremendous implications for a wide variety of genes that clearly play an important role in regulating mood and behavior. Tyrosine hydroxylase (TH), dopamine receptor D3 (DrD3), cholecystokinin (CCK), and many other regulators of dopaminergic transmission are under transcriptional control by the CLOCK protein (Arey et al., 2013; Ozburn and Falcon et al., submitted; Sidor and Spencer et al., submitted). Additionally, mice with an induced mutation of the *Clock* gene, which results in a truncated CLOCK protein with dominant-negative function, express a behavioral profile strongly resembling BD patients in the manic state that can be reversed using chronic lithium treatment. (Roybal et al., 2007). These mice show increased dopamine synthesis and dopamine firing in the ventral tegmental area (VTA), a region featuring important dopaminergic projections that have been implicated in psychiatric disorders (Coque et al., 2011). This includes an increase in TH expression in the VTA (McClung et al., 2005). Most of the manic-like behavioral phenotypes of these mutant mice are rescued with the induced expression of a functional CLOCK protein in the VTA, while knockdown of *Clock* expression in the VTA in wild-type mice produces a "mixed state" of reduced anxiety- but increased depression-like behavior (Dzirasa et al., 2010; Roybal et al., 2007; Mukherjee et al., 2010). Taking this into consideration, it becomes clear 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, including binding sites for microRNAs (miRNAs). MiRNAs are short, untranslated sequences of RNA that have been shown to have

dramatic consequences for mRNA transcript stability (for review, see Keursten and Goodwin, 2003). For instance, the 3'UTR of the pro-apoptotic gene *hid* in *Drosophila* features several miRNA binding sites for the *bantam* miRNA that induce repression of *hid* mRNA activity (Brennecke et al., 2003). 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 hours. We are continuing to investigate whether the *Clock* 3111T/C SNP results in altered CLOCK protein levels, stability, or function using native gels for Western Blotting and binding assays. These changes may be due to a number of different mechanisms, including miRNA-182 binding sites in the 3'UTR. We plan to investigate whether miRNA-182 binding is altered in the 3111T/C SNP using luciferase assays and RNA footprinting. 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. Although the 3111T/C SNP is only one polymorphism of many identified for the *Clock* gene that associates with BD, these results will help to further characterize the functional implications of this clinically-relevant SNP.

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