UNIVERSITY OF CALIFORNIA SAN DIEGO

Gene Expression Analysis of Circadian Clock Genes in a Human Stem Cell-Derived Neuronal Model of Bipolar Disorder

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

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by

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The Thesis of Angelica Rose Ramos Luis is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Gene Expression Analysis of Circadian Clock Genes in a Human Stem Cell-Derived Neuronal Model of Bipolar Disorder

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Master of Science in Biology

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Professor Michael J. McCarthy, Chair
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Bipolar disorder (BD) is a neuropsychiatric disorder that causes depressive and manic mood episodes and greatly increases the risk of suicide. People affected by BD are known to possess circadian rhythm abnormalities, and while it has been suggested that these alterations in circadian rhythms are correlated to the symptomatic mood episodes, it is unknown how these disruptions are caused or how they specifically relate to the pathogenesis of BD. To investigate the molecular

mechanisms of circadian rhythms in BD, we analyzed the circadian gene expression patterns in induced pluripotent stem cell (iPSC)-derived neural progenitor cells (NPCs) and neurons from BD subjects compared to healthy subjects. Gene expression was measured across a 24-hour cycle by performing qRT-PCR on the core clock genes that control the transcriptional-translational negative feedback loop (TTFL). Expression of two clock genes in particular, *CRY1* and *PER2*, was found to be highly expressed in BD NPCs compared to control NPCs, demonstrating that disruption of the clock may begin early on in neuronal development. Further gene expression analyses of differentiated neurons revealed that this *CRY1* and *PER2* finding was consistent across cell types. No other clock genes were found to have expression differences in either cell types, demonstrating some degree of specificity regarding *CRY1* and *PER2*'s association with BD. Our analysis shows that looking into the mechanisms of circadian gene regulation in BD is a promising area of study in uncovering the molecular workings and developmental manifestation of BD.

INTRODUCTION

1.1 Bipolar Disorder

Bipolar disorder (BD) is a debilitating neuropsychiatric disorder that affects about 2-5% of adults (Hilty et al., 2006). Affected people are subjected to frequent hospitalizations, healthcare expenditures, and increased risk of suicide (Latalova et al., 2014). BD is defined by two distinct mood phases: a manic phase and a depressive phase (Latalova et al., 2014). The manic phase is characterized by elevated mood, persistent euphoria, excessive involvement in activities, and a reduced need for sleep, while the depressive phase is characterized by low mood, anhedonia, distractibility, and insomnia/hypersomnia (Latalova et al., 2014; Harvey et al., 2009). While the exact cause of BD is unknown, genetic factors (such as genetic epistasis, copy number variants, and epigenetic variation) and environmental stress are thought to play a role in its manifestation (Hilty et al., 2006; Maletic et al., 2014). About 80% of the risk for BD is thought to be genetic (Ament et al., 2015) and previous studies indicate that adult neurogenesis and impaired neuroplasticity are associated with psychiatric disorders such as BD (Schoenfeld et al., 2015), indicating the importance of studying BD from a developmental standpoint. While it is unknown which brain region BD is most strongly associated with, specificities in phenotype based on the phase of the illness may be attributed to altered prefrontal cortex (PFC) activity, as it has been noted that bipolar mania is associated with increased dorsolateral PFC activity and bipolar depression with decreased dorsolateral PFC activity (Maletic et al., 2014). Additionally, the heritability of BD suggests a genetic basis (Barnett et al., 2009), but past GWAS studies have failed to identify genes responsible for BD (Maletic et al., 2014). The limited understanding of the biological basis of BD often makes it difficult to diagnose patients who may show symptoms similar to another psychiatric disorder, and therefore deliver effective treatments. Understanding the basis of BD would allow for better pharmacological treatments and may be directly

translational to an improved quality of life BD patients. While the mechanism of BD is unknown, a non-adaptive circadian system is one of the biomarkers, with disruptions in the clock contributing to the symptomatic mood episodes.

1.2 The Role of the Clock in Bipolar Disorder

Circadian rhythms – internal 24-hour oscillations which regulate mental and physical conditions – are known to be associated with BD (Gold et al., 2016). However, the circadian abnormalities in BD are quite variable and may depend on the phase of the illness (depressed, manic, euthymic) in which behaviors are measured. This is most clearly demonstrated by the changes in sleep associated with depression and mania, but also differences in activity, hormonal regulation, appetite and body temperature (Maletic et al., 2014; Gold et al., 2016). Moreover, these phenotypes associated with BD have been observed to be related to different aspects of circadian rhythms including entrainment, amplitude, phase, and period. Weakly entrained rhythms in BD patients are implicated with rhythm abnormalities such as meal timing and sleep patterns (Maletic et al., 2014). Large recent GWAS studies that have looked into the relationship between the circadian clock and BD have identified low amplitude rhythm abnormalities as a marker for psychiatric disorders including BD in humans (Rock et al., 2014; Lyall et al., 2018; McCarthy et al., 2019). Other studies have found circadian phase to be advanced during mania and delayed during mixed mania and depression (Moon et al., 2016), and period to be reduced in BD patients compared to controls (Moon et al., 2016).

These disturbances in different aspects of the circadian clock indicate the possibility of disruptions in the clock as one of the causes for the genetic basis of BD, however it remains unknown how these rhythm abnormalities arise in the first place (Geddes et al., 2013; Brady et al., 2015). The association of various circadian abnormalities with BD as well as the genetic risk of BD suggest that

looking into the genes that regulate circadian rhythms may reveal a mechanistic link between BD and the clock, as well as insight into the genetic basis of BD.

1.3 Circadian Rhythms

Based on the well-established association between BD and circadian rhythms, an analysis of the genes modulating the clock may reveal important implications regarding the manifestation of BD. Circadian rhythms are controlled by core circadian clock genes – *PER1/2/3*, *CRY1/2*, *BMAL1*, *CLOCK*, *REV-ERBα/β*, and *RORA/B/C* – in the transcriptional-translational negative feedback loop (TTFL). The TTFL regulates the translation of the core circadian clock genes, controlling when they are expressed or repressed across the 24-hour circadian cycle. The positive limbs of the clock *BMAL1* and *CLOCK* heterodimerize and activate the transcription of the negative limbs of the clock, *CRY* and *PER* (Becker-Weimann et al., 2004). *CRY* and *PER* then form a complex in the cytoplasm and translocate to the nucleus to repress the activity of *BMAL1* and *CLOCK*, which in turn inhibits their own transcription (Becker-Weimann et al., 2004). *REV-ERBs* and *RORs* additionally regulate the circadian clock, feeding back to negatively and positively regulate *BMAL1* transcription (Mohawk et al., 2012).

These core clock genes in the TTFL play important roles at the transcriptional level, activating biological processes such as neuronal firing rates and neurotransmitter release (Jones et al., 2015). While it has been found that some of these core clock genes are associated with BD in candidate gene studies (Maciukiewicz et al., 2014, Benedetti et al., 2015, Oliveira et al., 2018, Charrier et al., 2017), larger GWAS studies have been more equivocal in identifying clock genes (McCarthy et al., 2019). Moreover, in mice, studies have shown that disrupting the core clock genes results in diminished circadian rhythms (van der Horst et al., 1999, Bunger et al., 2000, Bae et al., 2001), and disrupting the circadian oscillation of some of the core clock genes at the RNA level

induces depressive symptoms (Landgraf et al., 2016). This analysis in mice suggests that alterations in the core clock genes may be responsible for the manifestation of BD in humans (McCarthy et al., 2012a). Few studies on circadian rhythms have been conducted in humans (McCarthy et al. 2012a), therefore it is an immediate goal of the McCarthy Lab to analyze the interplay between BD and rhythmicity in humans at the RNA level, and to investigate the role of the TTFL in regulating BD. Additionally, one of the most widely used treatments for BD, lithium, is known to impact the clock by lengthening period and increasing the amplitude of circadian rhythms (McCarthy et al., 2013). The association between BD, circadian rhythms, and lithium's effects on amplitude and period suggests that looking into lithium's effects on BD may provide insight into the mechanism of BD.

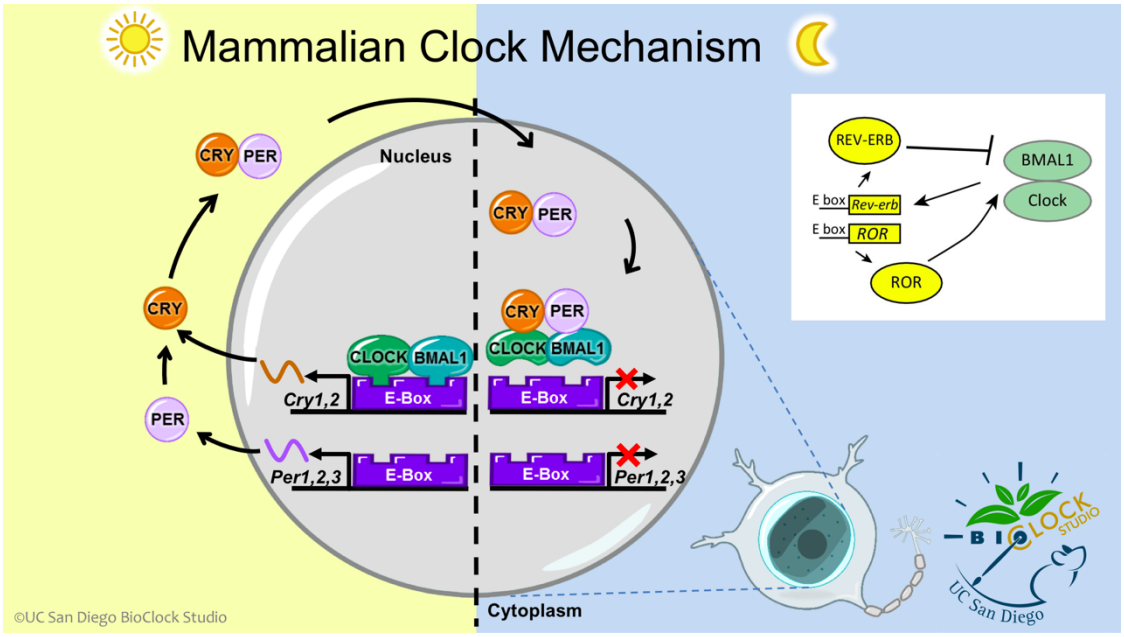


Figure 1.1: The transcriptional-translational negative feedback loop. Adapted from UC San Diego BioClock Studio and Mattis et al., 2016.

1.4 Lithium Responsiveness

Lithium is known to clinically correct the circadian rhythm abnormalities in BD, enhancing the resynchronization of the dampened rhythms by lengthening period and increasing amplitude in the circadian rhythms of BD fibroblasts (McCarthy et al., 2012b). However, lithium as BD treatment has its drawbacks; it has toxic side effects such as cognitive dulling, excessive urination, nausea, thyroid failure, and kidney failure (Gitlin, 2016), and is only effective for a subset of patients (McKnight et al., 2012). BD patients that respond to lithium are known as lithium responders (LR), and BD patients that do not respond to lithium are known as lithium non-responders (NR) (Rybakowski, 2014). Clinically, NRs often show more associated anxiety, substance use disorder, irritability, and family history of non-response (Young et al., 1993). While it is not clearly understood what causes lithium responsiveness in BD patients, it has been shown that NRs and LRs possess different neuron characteristics in vitro (Stern et al., 2017; Mertens et al., 2015), and lithium responsiveness can be predicted by observing the electrophysiological properties of IPSC-derived neurons grown from BD patients (Stern et al., 2017). Thus, analyzing the circadian gene expression differences in the neurons of LR and NR patients may provide deeper insight into the molecular gears of BD, revealing how different circadian clock alterations may lead to the two different sub-types of BD (lithium responsiveness vs lithium non-responsiveness). Additionally, uncovering what causes a BD patient to respond to lithium will allow for better administration of treatments so that patients are not given ineffective medications.

The mechanism by which lithium influences circadian rhythms is not fully known, but studies have shown that lithium inhibits glycogen kinase-3B (GSK-3β), which phosphorylates many of the rhythmic clock proteins involved in the TTFL, namely *BMAL1* (Sahar et al., 2010), *CLOCK* (Spengler et al., 2009), *CRY2* (Kurabayashi et al., 2010), *PER2* (Kaladchibachi et al., 2007), and *REV-ERBα* (Wang et al., 2006). One of the clock genes in particular, *REV-ERBα*, has been shown to degrade in

response to GSK3β inhibition, suggesting a relationship between *REV-ERBα* and GSK3β (Yin et al., 2006). Moreover, a functional genetic variant of *REV-ERBα* was found to be associated with good lithium treatment response through a candidate gene association study of Li responsiveness (McCarthy et al., 2011). These findings suggest that *REV-ERBα* may play a key regulatory role in the determination of lithium responsiveness (McCarthy et al., 2011), and that looking into *REV-ERBα* may provide insight into the molecular workings of BD.

1.5. Modeling Bipolar Disorder

Modeling BD has always been a challenge due to the wide variety of behaviors affected, and the cyclical nature of transitioning between the two mood phases: mania and depression (Gould et al, 2007). Most animal models for BD have only been able to model specific behavioral characteristics associated with either mania or depression, such as altered locomotor activity, increased or decreased goal-directed activity, enhanced sexual behavior, aggression, or appetite changes. (Roybal et al., 2007; Beyer et al., 2017; van Enkhuizen et al., 2015). One animal model in particular, the ClockΔ19 mouse, possessed a mutation in one of the core circadian clock genes *Clock* at intron 19 (Roybal et al., 2007). This mutation disrupted the circadian rhythms within the mouse, resulting in the ClockΔ19 mouse portraying the manic characteristics of BD (Roybal et al., 2007). However, as a model for BD, the ClockΔ19 mouse was unable to model the symptoms of the depressive phase that also characterizes BD.

Aside from animal models, studies on BD have also been conducted on humans in vivo, but are not viewed as practical due to the necessity of controlled lab lighting conditions, considerable subject commitment, and high expense to analyze circadian rhythms through forced desynchrony (Saini et al., 2015). Additionally, these efforts have been thought to negatively affect psychiatrically ill people (Saini et al., 2015), demonstrating the need for good in vitro models of BD. For in vitro studies

of BD, these cellular models mainly comprised of lymphoblastoid cell lines and fibroblasts (Viswanath et al., 2015). However, these cellular models are not ideal for modeling BD, as they lack expression of key neurotransmitters and do not form synaptic connections. At most, these cellular models have only been able to provide preliminary insight into BD research. In the McCarthy Lab, we utilize what we expect to be a more valid model for BD that closely represents the biological disorder in both genotype and phenotype: human induced pluripotent stem cell (hiPSC) derived neurons.

While studies have shown that bipolar fibroblasts possess altered circadian rhythms (McCarthy et al., 2013), studying BD in hiPSC-derived neuronal cells may reveal deeper implications regarding the pathology of BD because hiPSC-derived neurons have been found to form synaptic connections (Yang et al., 2011) and capture the full phenotype of BD while possessing all of the clinical characteristics and genetic factors that come from the patient (Soliman et al., 2017). Because our model is specific to each patient, the distribution of lithium responders and non-responders can be considered, allowing for specific gene expression analyses comparing lithium responders and lithium non-responders. While it is unknown which types of neurons BD specifically affects, postmortem and magnetic resonance spectroscopy (MRS) studies have shown that frontal cortical levels of glutamate were elevated in BD and MDD individuals compared to controls, indicating the possibility of region-specific abnormalities in the glutamatergic system resulting from mood disorders (Jun et al., 2014; Hashimoto et al., 2007). This association between BD and the glutamatergic system suggests that cortical glutamatergic neurons are a valid neuron type as a model for BD.

Looking into the core clock genes (*PER1/2/3*, *CRY1/2*, *BMAL1*, *CLOCK*, *REV-ERBα/β* and *RORA/B/C*) that control circadian rhythms in BD neurons may elucidate the mechanism of gene expression in the TTFL. Moreover, using a highly feasible protocol that differentiates hiPSCs into neurons (Grainger et al., 2018) (Figure 1.2), each stage of neural development can be analyzed. The suggestion that BD is associated with adult neurogenesis and impaired neuroplasticity (Schoenfeld et

al., 2014) indicates immediate relevance in studying the pathogenesis of BD, as uncovering the stage in neuronal development at which the circadian clock becomes rhythmic may provide insight into the manifestation of BD. Therefore, our study analyzes circadian gene expression patterns in NPCs as well as neurons so that we can look into this adult neurogenesis. Our study aims to analyze the differences in the expression of rhythmically expressed clock genes in cells grown from healthy patients, LRs, and NRs to uncover the mechanistic link between the regulation of circadian rhythms and BD.

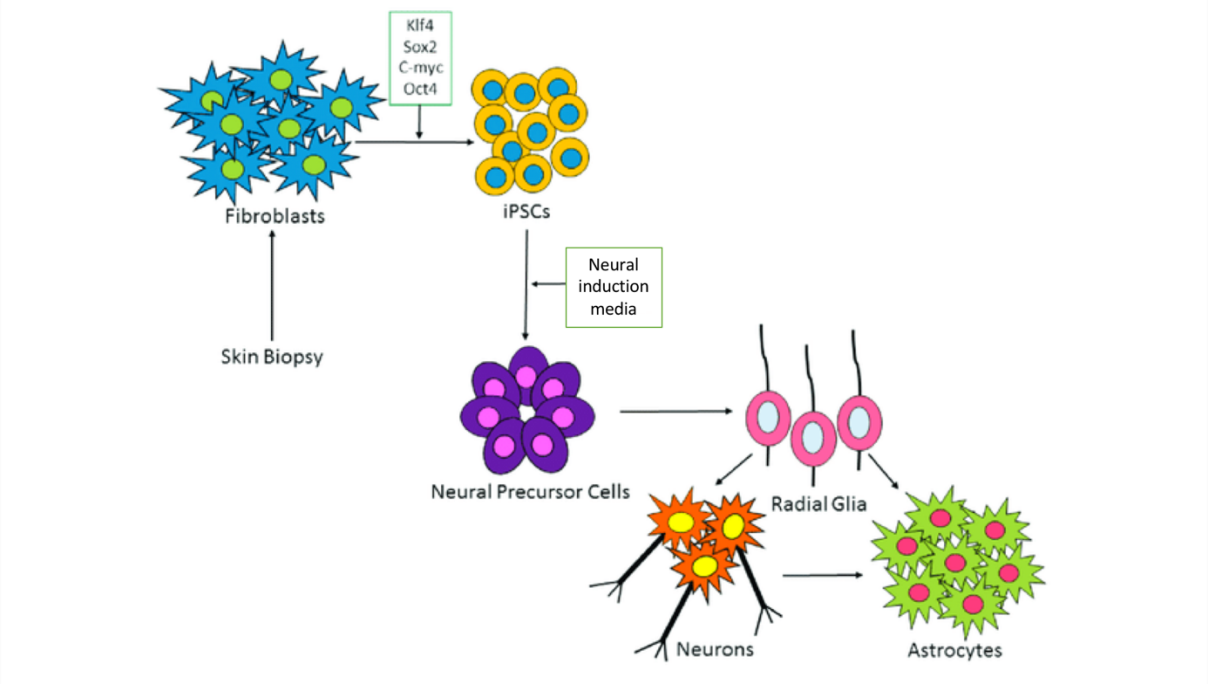


Figure 1.2: Generation of neurons from human fibroblasts using hiPSC technology. Adapted from Grainger et al., 2018.

MATERIALS AND METHODS

2.1 Human Subjects

BD subjects were patients that participated in lithium monotherapy clinical trials, and control subjects were recruited from a MRI study of BD conducted by Lisa Eyler at UCSD. Human subject protocol has been described in detail in Oedegaard et al., 2016. Subjects approved of all procedures in the form of written consent. All BD subjects were diagnosed with type I BD. Additionally, they were Caucasian, male, and ranged in age from 20-70 years old (Table 1.1). After BD subjects spent four months on lithium treatment, lithium responders (LR) and lithium non-responders (NR) were identified as those who remained clinically stable, and/or scored as stable or improved on a Clinical Global Impressions scale (Mertens et al., 2015).

Table 1.1: Clinical characteristics of subjects with BD

Subject	Response Category	Age	Gender	Race	Psychosis
1	LR	57	M	Caucasian	N
2	LR	65	M	Caucasian	N
3	NR	54	M	Caucasian	Unknown
4	NR	22	M	Caucasian	Y
5	NR	69	M	Caucasian	N

Table 1.2: Clinical characteristics of control subjects

Subject	Age	Gender	Race
1	56	M	Caucasian
2	73	M	Caucasian
3	38	M	Caucasian

2.2 hiPSC Cell Culture

Human iPSC (hiPSC) lines used in this study were obtained from the lab of Dr. Fred Gage at the Salk Institute (Mertens et al., 2015). Fibroblasts from control subjects and patients with BD (Table 2.1, 2.2) were reprogrammed using Cyto-Tune Sendai reprogramming kit (ThermoFisher Sci). hiPSC colonies were cultured on Matrigel-coated 6-well plates (Corning) using mTESR1 medium (Stem Cell Tech #85850) and Anti-Anti (Bio Core). hiPSCs were maintained in high quality by cleaning any spontaneously differentiated cells using L-shaped glass pipettes under a stereo microscope (Olympus). hiPSCs were passaged manually every 7–8 days with Collagenase IV (200 U/ml), and semi-dissociated colonies were gently lifted using cell scrappers (Corning). During maintenance, hiPSC cultures remained in a Nuaire Water-Jacketed IR Autoflow CO2 incubator with 5% CO2 at 37C. hiPSC colonies were monitored daily under the stereo microscope for quality and morphology. mTESR1 media was changed every day until hiPSCs were confluent to start neuronal differentiation. For long term storage, hiPSCs were cryopreserved using 70% Knock-out Serum Replacement (Fisher Scientific #10828010) , 20% mTesR1 (+) Media, and 10% DMSO (Sigma).

2.3 Embryoid Bodies and Neural Rosette Cell Culture

To start neuronal differentiation from hiPSCs, we used an embryoid bodies (EBs) based protocol (Mishra et al., 2016). Four to six wells of confluent hiPSC colonies were gently dissociated using collagenase IV (Stem Cell Tech #7909), and colonies were lifted using a cell scrapper. Collagenase solution was then washed twice using DMEM/F12 (Bio Core #11330032). To begin generation of EBs, hiPSCs were transferred to ultralow attachment plates (Corning) in mTeSR1 medium, and the next day mTESR1 medium was replaced with neural induction medium (NIM) containing DMEM/F12/Glutamax, with N2 and B27 (without Vitamin A) supplements and Penicillin-Streptomycin (all ThermoFisher Sci). EB colonies were maintained in suspension for two to seven

days and then plated onto polyornithine (PORN)/laminin (Invitrogen)-coated plates to begin differentiation into neural rosettes (NRs). NRs formed within one week from differentiating EBs, and during this period, NIM media changes were conducted every other day.

Visible rosettes were selected under a stereo microscope (Olympus). Selected rosettes were manually picked, dissected, and transferred onto PORN/laminin-coated 24-well plates containing N2/B27 + FGF2 media: NIM and 20 ng/ml FGF2 (ReproCELL USA #03-0002). Neural rosettes were then dissociated using accutase (Stem Cell Technologies) to form proliferative neural progenitor cell (NPC) lines.

2.4 Neural Progenitor Cell (NPC) and Neuron Cell Culture

NPCs were maintained as high density cultures in N2/B27 + FGF2 media plated on PORN/laminin-coated plates, and media was changed every other day. NPCs were split ~1:3 every five to ten days using accutase (Stem Cell Tech #7920) for expansion. For the final stage of differentiating NPCs into cortical glutamatergic neurons, NPCs were plated on matrigel (Corning #354230) coated 35mm dishes at density of 400x10^3 cells/well in N2/B27 + FGF2 media. The next day, terminal differentiation was initiated by switching the media to neuronal differentiation medium (NDM) containing N2/B27 medium, 20 ng/ml brain derived neurotrophic factor, 20 ng/ml glial cell line-derived neurotrophic factor (both Peprotech), 1 mm dibutyryl-cyclic adenosine monophosphate, and 200 nM ascorbic acid (both Sigma). Neurons were maintained in these culture conditions for four to ten weeks, and half media changes were conducted each week.

2.5 Immunostaining

Immunostaining was conducted on iPSCs, NPCs, and neurons to determine the stage specific markers for each cell type. iPSCs were grown on Matrigel-coated coverslips, and NPCs and neurons

on PORN/laminin-coated coverslips. On day one of immunostaining, cells were aspirated of their media, washed in DPBS (ThermoFisher Sci), and fixed with 4% PFA. Following a 30 minute incubation and two DPBS washes, cells were permeabilized using 0.2% TritonX-100 (Sigma #X100) diluted in DPBS. The coverslips were moved to a wet chamber where non-specific proteins were blocked using 5% donkey serum (Jackson ImmunoResearch #017-000-121). Following a one hour incubation, primary antibodies (Table 2.5) diluted in 2% donkey serum were added to each coverslip, and coverslips were left to incubate overnight at 4C. On the next day, three washes using 0.2% TritonX-100 diluted in DPBS were conducted on each coverslip. Immediately after, secondary antibodies (1:500) diluted in 2% donkey serum were added to each coverslip and left to incubate for two hours at room temperature. Coverslips were then washed with DPBS, incubated in DAPI (ThermoFisher Sci #D1306) for five minutes, washed in dH2O, and mounted using aqueous mounting medium (Polysciences, Inc #18606-20). Immunostained neurons were visualized using fluorescence microscopy (Leica Microsystems #C2343-33722164) the following day.

Table 1.3: Details on the antibodies used during immunostaining

Target	**Species**	**Company**	**Cat #**	**Dilution**
Tuj-1	Mouse	Biolegend	801201	1:300
GFAP	Goat	Abcam	ab53554	1:600
Nestin	Mouse	Millipore	MAB5326	1:200
Sox2	Rabbit	Cell Signalling Tech	3579S	1:200
Tra 1-60	Mouse	Millipore	MAB4360	1:300
Nanog	Goat	R&D Systems	AF1997-SP	1:400

2.6 Experimental Plating, RNA Isolation and cDNA Synthesis

To prepare the materials for gene expression analysis, hiPSCs, NPCs, and neurons were experimentally plated and subjected to RNA isolation and cDNA synthesis. To begin experimental plating, confluent wells for each cell line were obtained to be plated. For hiPSCs and NPCs, cells were split using collagenase (hiPSCs) or accutase (NPCs), and were counted and plated on four duplicate six-well plates for 400x10^3 cells/well on the same day for time course studies. Replicates of two were plated for each line and time point. Following plating, cells were analyzed via microscope for two to five days to ensure proper plating, cell type, and growth. To synchronize the cells to the same circadian cycle, the neuronal media was changed with neuronal media that included forskolin (Tocris), and cells were kept in new neuronal media for 16 hours undisturbed. Following the 16 hour incubation, each plate was frozen at one of four time points – 6 hours, 12 hours, 18 hours, or 24 hours – by extirpating the neuronal media, washing with cold DPBS, extirpating all media completely, and placing cells in -80C. For neurons, cells were plated on 35 mm dishes with replicates of 2 for each line and time point, and were frozen at six to eight weeks into differentiation in the same 35mm dishes they were initially plated on.

To perform RNA extraction, RNeasy Mini Kit (Qiagen #74104) was utilized. First, frozen cells were lysed in their wells using lysis buffer mixed with B-Mercaptoethanol, and gently scraped using a cell lifter. Cellular materials from the same line and time point were pooled together and collected in eppendorf tubes on ice. Eppendorf tubes were centrifuged to remove the pellet, and one volume of 70% ethanol was added to the supernatant. The contents of the tube were then transferred to RNeasy Mini spin columns. Following centrifugation, columns were washed once with Buffer RW1 and twice with Buffer RPE. To extract the RNA from the columns, the columns were centrifuged with 50uL RNase free water. The flow through containing the isolated RNA was then added back into the columns and centrifuged again to increase the RNA concentration. Total RNA was quantified using

Nanodrop 2000 (ThermoFisher Sci), and cDNA was synthesized via reverse transcription of 500 ng of total cellular RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems #4368814) and T100 Thermal Cycler (Bio-Rad #1861096).

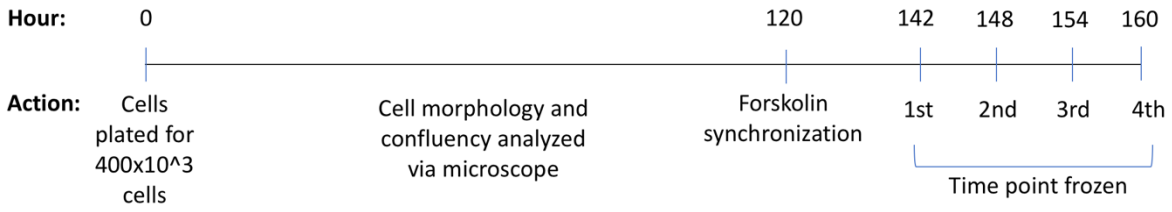


Figure 2.1: Timeline of NPC time-course experimental design

2.7 Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

In order to measure the gene expression patterns of our cells, we used a C1000 Touch Thermal Cycler with a CFX384 Real-Time System (Bio-Rad). Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using the pre-made Taqman primers for *PER1/2*, *BMAL1*, *CRY1/2*, *CLOCK*, *RORA*, *REV-ERBα* and *GAPDH* (ThermoFisher Sci) to measure the amount of cellular cDNA in each cell line at various time points across the 24 hour circadian cycle. In order to control for batch effects, one control and two BD samples were run at the same time in each experiment to directly compare gene expression differences between BD cases/controls, and all four time points for each cell line were run at the same time to compare gene expression between time points. Replicates of three were run for each reaction, and outlier Ct values (>1 Ct) were removed before analysis. The transcript level of each gene was normalized to a non-rhythmic control housekeeping gene, *GAPDH*, following the comparative CT method (Livak et al., 2001; Kosir et al., 2010). Data was normalized to the control gene expression for each probe in each qRT-PCR experiment.

2.8 Data Analysis and Statistical Methods

Statistical analyses were performed in GraphPad Prism, version five. Two-way analysis of variance (ANOVA) tests were utilized to identify significant differences by diagnosis (control versus BD), time, and the interaction between them (diagnosis x time). One-way ANOVA tests were utilized to identify significant differences between groups (iPSCs, NPCs, and neurons). Significance was denoted by a p-value ≤ 0.05, and data shown are displayed as mean ± standard error of mean (SEM).

RESULTS

3.1 Immunohistochemistry Image Analysis Confirms Successful Generation of BD Model

To validate our cellular differentiation and neuronal BD model, we performed immunostaining on three of the key stages of neuronal differentiation to verify cell identity: hIPSC stage, NPC stage, and neuron stage (Figure 3.1A-C). Immunohistochemistry analysis confirmed that our differentiation protocols were successful, with hiPSCs expressing pluripotency markers Nanog and Tra 1-60, NPCs expressing the neuro-ectodermal marker Nestin and progenitor marker Sox2, and neurons expressing neuron-specific Class III β-tubulin. These results indicate the cells we grew were successfully reprogrammed into iPSCs, and effectively differentiated along the neuronal developmental pathway to NPCs and neurons, making them suitable for qRT-PCR time course experiments.

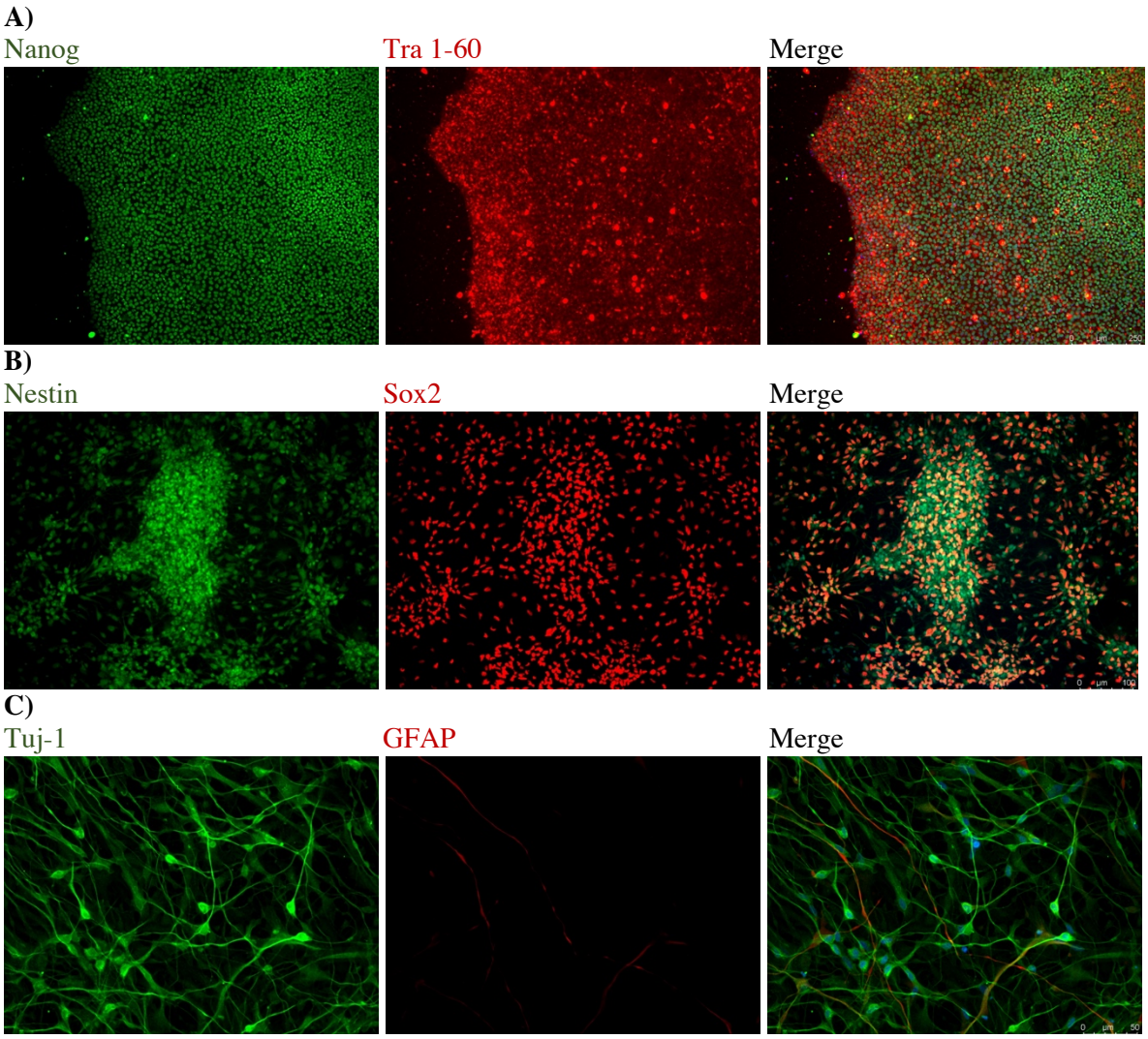


Figure 3.1: Immunohistochemistry analysis of hiPSCs, NPCs, and neurons
(A) Representative image of iPSC colony from a control line stained with pluripotency markers Nanog, and Tra 1-60, and nuclear marker DAPI. Most cells uniformly express pluripotency markers Nanog and Tra 1-60, demonstrating successful hiPSC generation.
(B) Representative image of NPCs from a control line stained with neuro-ectodermal marker Nestin, progenitor marker Sox2, and nuclear marker DAPI. NPCs are shown to grow as single cells and express the neuroectodermal marker Nestin as well as progenitor marker Sox2.
(C) Representative image of neurons from a BD line. Neurons are shown to mainly express neuron-specific class III β-tubulin (Tuj-1), present in newly generated immature postmitotic neurons and differentiated neurons. Lack of GFAP staining demonstrates divergence from the NPC stage and glial lineage.

3.2 Expression of PER2 Increases Throughout Differentiation in BD Cells

Based on the positive results from immunohistochemistry image analysis, we looked into verifying clock gene expression in iPSCs and iPSC-derived cell lines, and whether total gene expression changes over the course of neuronal development. Our preliminary findings demonstrate that all three stages of development (iPSC, NPC and neuron) show detectable levels of *PER2* expression. Moreover, *PER2* is developmentally regulated, demonstrating a trend of increasing gene expression throughout neuronal differentiation with *PER2* being expressed about 10x higher in NPCs compared to iPSC and about 30-50x higher in neurons compared to iPSC (Figure 3.2). Only the neuron group shows a trend towards rhythmic *PER2* expression (Figure 3.2), suggesting that the time keeping function of the circadian clock may become more developed throughout differentiation. Further studies are required to confirm whether this trend is present in all BD lines, and whether control lines would follow the same trend. Because of this developmental regulation of clock genes, we determined that we should study BD at both the NPC and neuron stage.

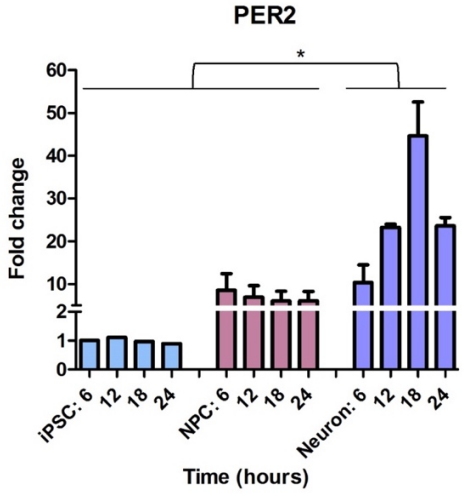


Figure 3.2: Total *PER2* gene expression increases throughout neuronal differentiation. Data was normalized to the average iPSC gene expression level of the four time points. Bars indicate standard error of mean (SEM). * Indicates one-way ANOVA revealed a significant difference between the means of two groups ($p<0.05$).
iPSC: n = 1 experiment
NPC: n = 3 experiments
Neuron: n= 2 experiments

3.3 hiPSC-derived Neurons Demonstrate Robust Circadian Rhythms

To confirm the presence of circadian rhythms in neurons, live cell bioluminescence experiments were conducted in the laboratory by my colleagues Himanshu Mishra and Noelle Ying. Using the *Per2*-luc reporter (Liu et al., 2007) to measure circadian expression of core clock gene *PER2* in neurons, we found that hiPSC-derived neurons display robust circadian rhythms indicating the presence of an intact and functional circadian clock in these cells (Figure 3.3). Further live cell bioluminescence experiments are currently being conducted in the McCarthy Lab to determine whether there are any differences in the circadian rhythms between control and BD neurons.

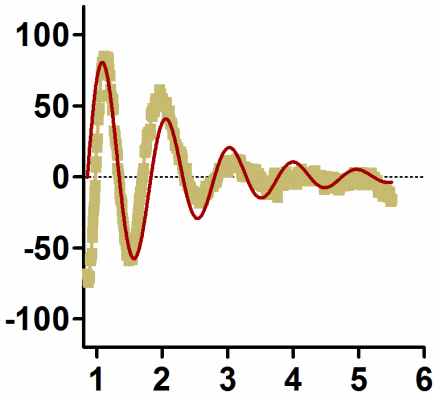


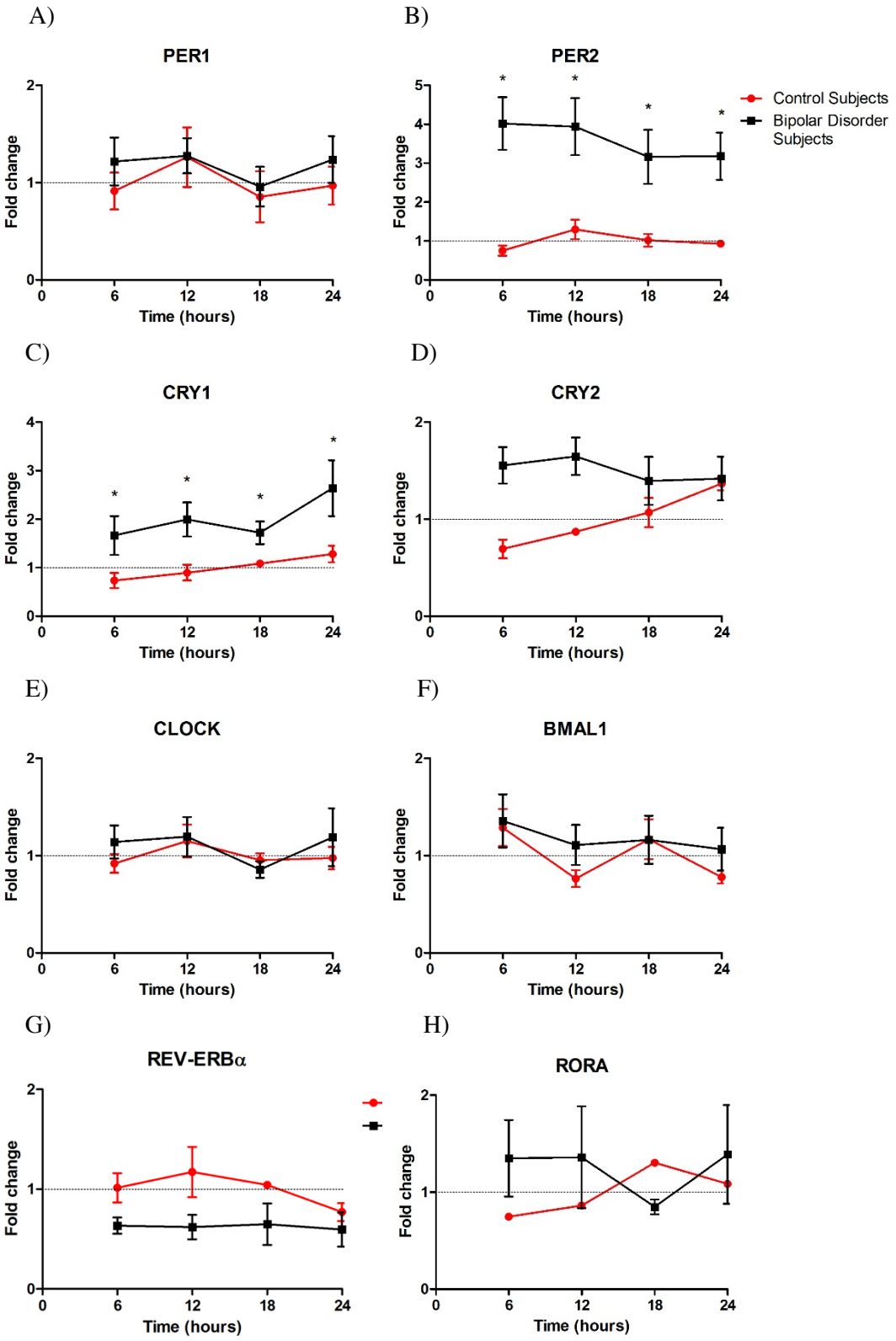
Figure 3.3: hiPSC-derived neurons display robust circadian rhythms. Control patient-derived neuron transfected with a lentiviral *Per2*-Luc construct that functions as a bioluminescent reporter of cellular circadian rhythms. Rhythms were measured over several days in cell populations using a luminometer.

3.4 Expression of CRY1 and PER2 is Significantly Higher in BD vs. Control NPCs

BD is a neurodevelopmental disorder. Therefore, it is possible that the circadian alterations in BD are present early in neuronal development, and that NPCs show rhythm abnormalities. To study the differences in gene expression levels and circadian rhythms in an early stage of neuronal development, we performed 24 hour time-course qRT-PCR analyses on NPCs from healthy control individuals and BD patients using eight core clock genes: *PER1*, *PER2*, *CRY1*, *CRY2*, *BMAL1*, *CLOCK*, *RORA*, and *REV-ERBα* (Figure 3.4A-H).

We found that gene expression levels of *CRY1* and *PER2* were expressed significantly higher in BD NPCs compared to control NPCs(Figure 3.4B-C). In contrast, the expression levels of *PER1*, *CRY2*, *BMAL1*, *CLOCK*, *RORA*, and *REV-ERBα* were similar between the two groups (Figure 3.4A, 3.4D-H). Moreover, no differences in gene expression across time were detected in either groups for any of the probes (Figure 3.2A-H). These results suggest that differences in clock gene expression may be correlated with the altered circadian rhythms in BD, as the clock genes form the TTFL that regulates circadian rhythms, and that these rhythm disturbances may be present early on in neuronal development.

Figure 3.4: CRY1 and PER2 are more highly expressed in BD vs. control NPCs. (A-H) Results of qRT-PCR time course experiments on control and BD NPCs against various circadian clock probes. Line graphs show respective gene expression data. BD NPCs are comprised of both lithium non-responders and lithium responders. Time point 0 denotes 16 hours post forskolin synchronization. * Indicates two-way ANOVA revealed a main effect of diagnosis ($p<0.05$). There was no significant effect of time, or no time x diagnosis interaction for any of the genes examined. Bars indicate standard error of mean (SEM). Data was normalized to control NPC gene expression for each probe, denoted by a horizontal dashed line.
(D, G-H) controls: n = 1-2 replicates from 1-2 donors. BD: n = 2-4 replicates from 2-3 donors.
(A-C, E-F) controls: n = 3-5 replicates from 2 donors. BD: n = 7-10 replicates from 3 donors.


A)
PER1
Fold change
Time (hours)
B)
PER2
Control Subjects
Bipolar Disorder Subjects
C)
CRY1
D)
CRY2
E)
CLOCK
F)
BMAL1
G)
REV-ERBα
H)
RORA

3.5 Expression of CRY1 and PER2 is Significantly Higher in BD vs. Control Neurons

Since differentiation of NPCs into neurons could alter their gene expression profile (Figure 3.2), we decided to investigate whether the results from the NPC qRT-PCR experiments were consistent in neurons. Based on the findings from the NPC qRT-PCR gene expression data (Figure 3.4B, 3.4C), we decided to follow up with gene expression analysis of six of the clock genes in neurons: *PER2*, *BMAL1*, *CRY1*, *CLOCK*, *RORA*, and *REV-ERBα*. Similar to the experiments performed on NPCs, 24 hour time-course qRT-PCR analyses of gene expression were conducted on neurons from healthy control individuals and BD patients. We found *CRY1* and *PER2* to be expressed significantly higher in BD neurons compared to control neurons, similar to the results found in NPCs (Figure 3.5A-B). As with NPCs, *BMAL1*, *CLOCK*, *RORA*, and *REV-ERBα* expression was similar between the two groups (Figure 3.5C-F), and no differences in gene expression across time were detected in any of the probes for each group (Figure 3.5A-F). The consistency in our results among NPCs and neurons suggests that a disruption in the negative transcriptional regulators (*CRY1* and *PER2*) may be responsible for the altered circadian rhythms in BD, and that these changes are present early in neuronal development.

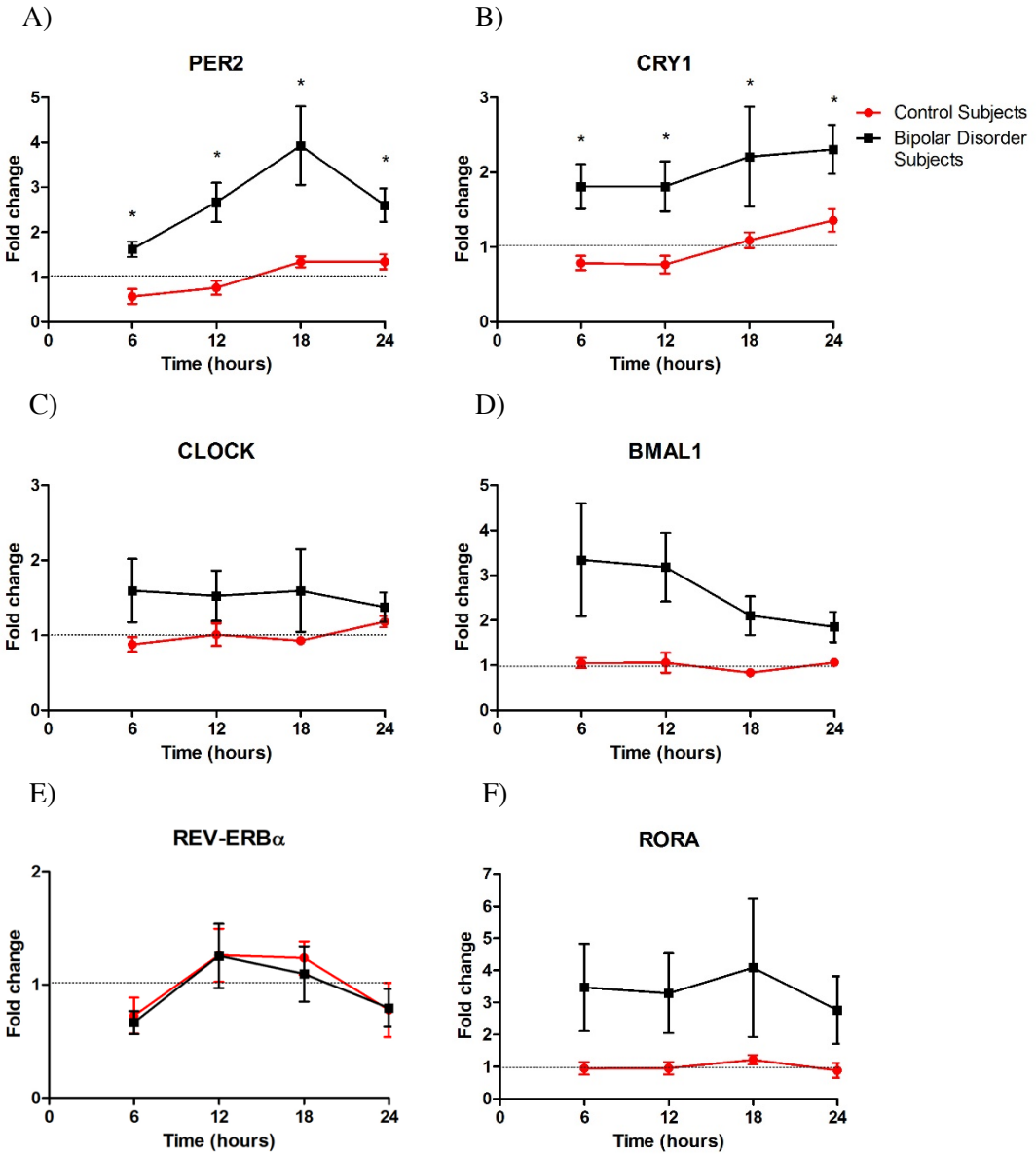


Figure 3.5: CRY1 and PER2 are more highly expressed in BD vs. control neurons. (A-F) Results of qRT-PCR time course experiments on control and BD neurons against various circadian clock probes. Line graphs show respective gene expression data. BD neurons are comprised of both lithium non-responders and lithium responders. Time point 0 denotes 16 hours post forskolin synchronization. * Indicates two-way ANOVA revealed a main effect of diagnosis ($p<0.05$). There was no significant effect of time, or no time x diagnosis interaction for any of the genes examined. Bars indicate standard error of mean (SEM). Data was normalized to control neuron gene expression for each probe, denoted by a horizontal dashed line.
(A-F) controls: n = 4-5 replicates from 2 donors. BD: n = 7-10 replicates from 5 donors.

3.6 REV-ERBα is Expressed More in LR vs. NR Neurons

It has been determined that LR and NR patients possess different neuron characteristics (Stern et al., 2017), and BD neurons as our model allows for a specific analysis of the two subtypes of patients. Therefore, we decided to look in more detail into our BD group to analyze the differences in clock gene expression between LR and NR neurons. Our preliminary findings show *REV-ERBα* to be highly expressed in LR neurons compared with NR neurons (Figure 3.6F), supporting previous studies that *REV-ERBα* is associated with lithium responsiveness in BD patients (McCarthy et al., 2011). None of the other core circadian clock genes were found to be differentially expressed between LR and NR neurons (Figure 3.6A-E). These results suggest that the expression of *REV-ERBα* may play a role in determining lithium response, supporting the relevance of continued studies on *REV-ERBα* to uncover the molecular workings of BD.

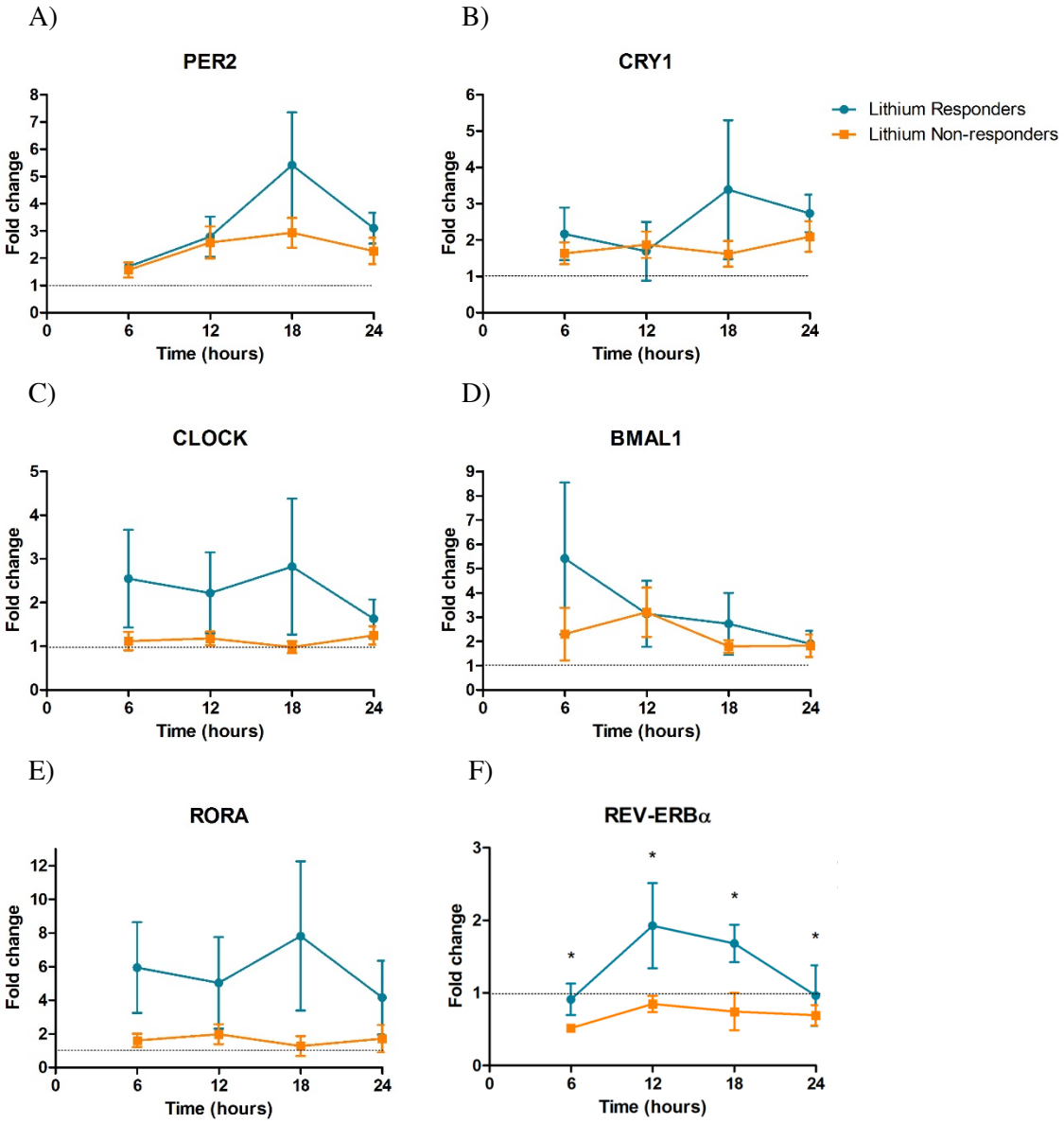


Figure 3.6: REV-ERBα is highly expressed in LR neurons. (A-F) Results of qRT-PCR time course experiments on LR and NR neurons against various circadian clock probes. Line graphs show respective gene expression data. Time point 0 denotes 16 hours post forskolin synchronization. * Indicates two-way ANOVA revealed a main effect of diagnosis ($p<0.05$). No significant difference was observed across time points for any probes. Bars indicate standard error of mean (SEM). Data was normalized to control neuron gene expression for each probe, denoted by a horizontal dashed line. **(A-F)** controls: n = 3 replicates from 2 donors. BD: n = 4-6 replicates from 3 donors.

DISCUSSION

4.1 Summary of Results

Our gene expression analyses of hiPSC-derived neuronal cells found that the negative transcriptional regulators of the TTFL, *CRY1* and *PER2*, are more highly expressed in BD compared with control NPCs (Figure 3.4). Previous studies in animal models found that disruptions in the core circadian clock genes induced depression-like behaviors, modelling some of the symptoms of BD (Landgraf et al., 2016), and our results support the role of the clock in BD in a human neuronal model. Additional gene expression analyses at a later developmental stage revealed that *PER2* and *CRY2* expression remains elevated in neurons from BD donors (Figure 3.5). Moreover, *REV-ERBα* expression is higher in LR neurons compared with NR neurons (Figure 3.6). Our results demonstrate the first description of circadian clock genes in human BD neurons, indicating that differences in genes may underlie differences in the clock leading to the manifestation of BD.

4.2 Strengths

The successful generation of our iPSC-derived neuronal BD model was confirmed by immunohistochemistry image analysis that verified cell identity (Figure 3.1), changes in clock gene expression over the neuronal cell lineage (Figure 3.2), and verification of a functional clock in iPSC derived neurons using long term measurement of the *Per2*-luc reporter gene (Figure 3.3). The successful development of this model may provide researchers with a cellular model of BD that better models key aspects involved in its pathophysiology compared to previous models that relied on peripheral cell types like fibroblasts. We expect neurons will more faithfully model the molecular aspects of brain function that are disturbed in BD. Previous cellular models such as fibroblasts do not express key neurotransmitter systems, do not show electrical activity, and do not form synaptic

connections, and therefore begged the question as to whether these models were accurately capturing the essential features of BD. With comparatively easier methods of expanding and maintaining the cells, these older cellular models could be studied in relatively large numbers, with good statistical power. However, the results from these models were perhaps less valid due to limitations of the cell type. It is possible that some combination of cells will be necessary to generate valid models with adequate statistical power.

We chose cortical glutamatergic neurons as our model to study BD due to the involvement of the frontal cortex in mood regulation, anatomical differences in BD, and association of glutamate levels with mood disorders (Maletic et al, 2014), but it is possible that different neurons are also involved in BD, and/or that a different neuron type may have been a better model. For instance, postmortem studies have found defects in GABAergic neurotransmission to play a role in BD (Benes et al., 2001), indicating the possibility of cortical GABAergic neurons as the more precise cell type affected by BD. An even more convincing model of circadian clock dysfunction could be neurons of the suprachiasmatic nucleus (SCN), as the SCN is the "master clock" known to oversee circadian rhythms (Gold et al., 2016). However, the SCN is a heterogeneous neuronal network made up of multiple components (Schaap et al., 2003), and there is currently a lack of established SCN neuron differentiation protocols, making the neurons of the SCN impractical to utilize as a BD model. Moreover, it has been found that an SCN independent pathway is central to light's effects on mood regulation (Fernandez et al., 2018), indicating that the SCN itself might not be the most important structure for mood regulation. Still, neurons of the SCN should be studied in the future, as its direct role in regulating circadian rhythms may reveal deeper implications regarding the clock's effects on BD. Our lab aims to eventually differentiate hiPSCs from BD patients into other types of neurons such as GABAergic neurons to analyze whether gene expression patterns differ across the types of neurons, and to investigate which neuron type BD mainly affects. Our lab also aims to differentiate hiPSCs

from BD patients into oligodendrocytes and astrocytes to see how BD affects non-neuronal cells. Despite the need for investigating other cellular models, utilizing hiPSC-derived neurons as a model for BD allows for the study of BD in a more accurate cell type, allowing our results to already support past studies that were performed on other cellular models. For instance, our significant finding of core circadian clock gene *REV-ERBα* being highly expressed in LR neurons supports the finding that circadian rhythms influence the response to lithium in BD fibroblasts (McCarthy et al., 2019).

4.3 Limitations

Our qRT-PCR gene expression analyses focused on analyzing the gene expression patterns of the core clock genes between groups throughout neural development, and our results demonstrated consistent findings on *CRY1* and *PER2* between NPCs and neurons that supported previous findings. One of the strengths of the qRT-PCR model is the ability to study many different genes at the same time, which allowed us to look into most of the core clock genes. Another aim of our lab was to study whether these clock genes were rhythmic in our control and BD cells, which is why time-course experiments were conducted for our gene expression analyses to look at rhythmicity. However, our gene expression analyses failed to detect any circadian rhythms in any of the experiments, denoted by the insignificant p-values for interaction generated using two-way ANOVA. This is likely due to the limited number of time points sampled for our time course experiments. While our 6-hour interval 24-hour time course experiments were unable to show any rhythmicity in the cells, it is likely that shorter intervals over a more extended time course would have shown rhythmicity. However, it was unfeasible to perform such time course experiments because it is difficult to grow the number of cells needed to conduct experiments on that scale, and we found it more relevant to dedicate our limited cell numbers to maximizing group analyses.

To circumvent the issue of detecting rhythms using qRT-PCR, live cell bioluminescence experiments are currently being conducted in our lab to validate the presence of circadian rhythms in our BD model. Using live cell bioluminescence experiments is more suitable for time course studies than using qRT-PCR because live cell bioluminescence uses a lentiviral *Per2*-Luc construct that functions as a bioluminescent reporter of cellular circadian rhythms, allowing rhythms to be measured over several days in cell populations (Liu et al., 2007). *PER2* is a known core clock gene, and its transcription levels are assumed to be representative of cellular circadian activity. The preliminary results from our live cell bioluminescence experiments have been positive, with neurons from one of our control lines displaying robust circadian rhythms (Figure 3.3). By studying gene expression utilizing qRT-PCR and rhythmicity using live cell bioluminescence experiments, our lab aims to obtain the best picture of the molecular framework of BD.

Aside from the lack of detecting rhythms, another shortcoming of our gene expression analyses was our limited statistical power. Our sample size is small, with our lab only possessing three control lines and five BD lines. A larger sample would provide more statistical power and higher confidence to my findings, and also may have shown significance in rhythmicity between time points. This is a general problem faced by all researchers using iPSC-models of psychiatric disorders who struggle to scale the model to achieve robust statistical effects due to the technical limitation of expanding lines with limited manpower and increased expenditures required to maintain cell lines (Hoffman et al., 2019). While the McCarthy Lab plans to obtain more control and BD lines in the near future, our preliminary findings on the core circadian clock genes using our small sample size have already been positive. This study aimed to uncover the mechanism that results in altered circadian rhythms leading to the manifestation of BD, and our results suggest that looking into the genes that control the TTFL is a good starting point.

4.4 Future Directions

The consistency in our significant findings on *CRY1* and *PER2* in both NPCs and neurons suggests the hypothesis that a disruption in the negative transcriptional regulators of the TTFL is responsible for the altered circadian rhythms in BD, and that this disruption occurs early in neuronal development, and persists in neurons. Moreover, our significant finding on *REV-ERBα* reveals the relevance of continued studies on the relationship between *REV-ERBα* and lithium response. Still, our study leaves many questions open: What exactly is resulting in the disruption of *CRY1* and *PER2*? Does the increased expression of *REV-ERBα* result in an increased response to lithium? While the McCarthy Lab aims to answer these mechanistic questions and many more, our results demonstrate the first description of circadian clock genes in human BD neurons. Additionally, our efforts in uncovering the molecular workings of BD may have implications for improving the quality of life for BD patients in the future. Our gene expression analysis shows that *CRY1* and *PER2* are differentially expressed in BD patients, indicating the possibility for using these genes as disease markers in diagnostic testing for screening BD risk. Utilizing hiPSCs to investigate BD may allow for a patient specific analysis on the effects of various treatments, allowing researchers to test new drugs without affecting the actual patient. Additionally, an understanding of the association between BD and lithium response would allow psychiatrists to identify LR and NR prior to initial lithium treatment, supporting the administration of personalized medicine.

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