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L-Type Calcium Channel $Ca_V1.2$ and Circadian Rhythms in Bipolar Disorder Patient Fibroblasts

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

in

Biology

by

Victoria Shannon Nudell

Committee in charge:

Professor Michael J. McCarthy, Chair
Professor Nicholas Spitzer, Co-Chair
Professor Shelley Halpain

The Thesis of Victoria Shannon Nudell is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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University of California San Diego

2018

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ABSTRACT OF THE THESIS

L-Type Calcium Channel $Ca_V1.2$ and Circadian Rhythms in Bipolar Disorder Patient Fibroblasts

by

Victoria Shannon Nudell

Master of Science in Biology

University of California San Diego, 2018

Professor Michael J. McCarthy, Chair
Professor Nicholas Spitzer, Co-Chair

Bipolar disorder (BD) is a psychiatric disorder characterized by recurrent periods of depression and mania, accompanied by major disruptions in activity and sleep patterns. The circadian clock controls these behavioral rhythms however; genome-wide association studies have failed to identify any of the essential "clock genes" that regulate rhythms as major genetic contributors in BD, but have associated the genes encoding L-type calcium channels (LTCCs) as important risk factors. *CACNA1C* encodes $Ca_V1.2$, a LTCC essential for entrainment of the circadian clock. In post mortem

brain studies, those with BD risk-associated variants in *CACNA1C* show alterations in gene expression. However, it is not known if abnormal expression of calcium channels mediates the circadian disruptions observed in BD patients. We utilized a viral *per2::luciferase* reporter to measure circadian rhythms in vitro and evaluate the role of a BD-associated risk allele in *CACNA1C* (rs4765913) and its role in human fibroblasts during phase-shifting. We found that antagonizing ryanodine receptors lengthens period whereas, blocking LTCCs shortens of the rhythm of *per2::luc* expression in mouse fibroblasts. Interestingly, the period changing effects of calcium channel blockers were insignificant when applied in conjunction with a temperature cycle. *CACNA1C* genotype (rs4765913) predicted the ability of human fibroblasts from BD patients to entrain to temperature cycles. These findings give insight as to the role of *CACNA1C* genotype in the ability of fibroblasts to entrain to daily stimuli, and how the abnormal entrainment displayed in BD-associated risk allele carriers could contribute to the circadian abnormalities observed in BD patients.

INTRODUCTION

1.1 Bipolar Disorder

Bipolar disorder (BD) is a neuropsychiatric disorder characterized by recurrent symptoms of depression and mania, and elevated risk of suicide (DSM-V). The risk for BD is largely inherited and genetic studies in patients with BD have implicated many different genes (Xu et al., 2017, Ikeda et al., 2018, Harrison et al., 2018). The current evidence suggests that the risk for BD is polygenic, and distributed across many risk alleles, some of which may overlap with other psychiatric disorders like major depression and schizophrenia (Consortium, 2013, Dedic et al., 2018, Charney et al., 2017, Reble et al., 2017). Therefore despite considerable research interest, the mechanism of BD remains unclear, and diagnosis and pharmacological treatment of this disease is complex (Geddes and Miklowitz, 2013, Brady and Keshavan, 2015). A hallmark of the disorder is the presence of abnormalities in circadian rhythms, which can lead to sleep disturbances and reduced daytime activity (Geoffroy et al., 2015, Pagani et al., 2016, Alloy et al., 2017, Melo et al., 2017). The rhythmic abnormalities in patients and the association of some core clock genes with BD in candidate gene studies has lead to studies evaluating the interplay between the circadian clock and bipolar disorder (Maciukiewicz et al., 2014, Benedetti et al., 2015, Oliveira et al., 2018, Charrier et al., 2017), but these efforts have failed to confidently identify the connection between mood disorders and the circadian clock. However, an alternative possibility is that other BD risk genes, not commonly linked with circadian functions, have pleiotropic effects and impact rhythms through modulation of core clock functions and/or by affecting inputs into the clock.

1.2 The Circadian Clock

The mammalian circadian clock is a self-sustained, tightly controlled, complex network of transcription-translation feedback loops (TTFLs) (Panda et al., 2002, Lowrey and Takahashi, 2004).

These interlocking TTFLs serve to regulate gene expression and cellular activity such as neuronal firing rates and neurotransmitter release (Jones et al., 2015). The core loop of the mammalian TTFL as discovered in mice is composed of the genes and protein products of *Per1, Per2, Cry1, Cry2, Bmal1*, *Clock*, *Rev-erbα,* and *Rorα,* which are also known as "core clock genes". The same molecular machinery builds the components of the circadian clock in humans, although most studies encountering rhythmic variants have been completed in mice due to the ease of access of an animal model.

In mice, mutations and abnormalities in core clock genes can diminish and even eliminate rhythmicity in the clock (van der Horst et al., 1999, Bunger et al., 2000, Bae et al., 2001). Evaluation of rhythmicity of the clock must be completed in constant conditions to verify the self-sustaining cycle of the TTFL. This is necessary to ensure that observed phenotypic oscillations are a direct result of the intrinsic clock of the organism, and not a result of some intervening stimulus creating an artifact of the clock readout, a phenomena commonly referred to as "masking". Removal of a masking stimulus will result in phenotypic oscillations consistent with patterns observed before the altering event, showing that the molecular clock has not been adjusted. In circadian biology, constant conditions are commonly referred to as "freerunning conditions", where the circadian clock is free to run on its own intrinsic timing without any environmental input. In this way, the rhythmic activities of an animal may be monitored for their timing allowing analysis of the freerunning period (FRP), which is naturally encoded to be about 24 hours in mammals. Alternatively, experimental conditions are often shown in a notation similar to "12:12 LD", which is the shorthand to denote a light/dark cycle of 12 hours of light followed by 12 hours of darkness. It is no coincidence that this cycle is framed over the course of 24 hours, as it is meant to emulate the day/night cycle of the Earth.

While the intrinsic molecular clock may encode activities that are rhythmic approximately every 24 hours, a key feature of the mammalian circadian system is the ability to entrain to stimuli in the environment. Entrainment results in the adjustment of the molecular clock to adhere to the periodicity of an entraining stimulus, sharpening the precision of the rhythmic molecular feedback timing. These adjustments of the central circadian clock are measured by comparison of "phase" before and after a stimulus. The phase of the clock is defined as the starting point of the cycle relative to a fixed event (e.g. sunrise). Cycles may be moved forward so they occur earlier (phase advanced) or backward to occur later (phase delayed) in response to an environmental stimulus. The ability of the clock to adjust is essential, as the inability to adapt to the changing environment could increase the likelihood of disease (Wolff et al., 2013). Choosing one representative core clock gene and following its waveform of expression as a guide to the internal time set by the organism is a common method to monitor the timing of the clock and evaluating entrainment. In this way, transfection with a lentiviral *Per2::luciferase (Per2::luc)* reporter can be used to allow a real-time readout of bioluminescence to represent rhythmic gene expression over extended time periods and allow analysis of phase (Beaulé et al., 2011).

The "pacemaker" clock of the mammalian brain is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Kafka et al., 1985, Ralph et al., 1990, Sujino et al., 2003). Once entrained to a particular light cycle, the SCN functions as the central pacemaker to coordinate rhythms in peripheral tissues outside of the SCN, and to direct rhythms in physiology and behavior. Interference with SCN function leads to desynchronization of rhythms in gene expression and behavior (Yoo et al., 2004). Importantly, due to redundancy in the system, most modifications to the core molecular clock do not result in arrhythmicity, but instead cause changes in period and/or entrainment. A notable example of a clock variation that maintains rhythmicity but with an abnormal relationship to the environment can be seen in the advanced phase of circadian gene expression

discovered in a family with strange sleeping habits. Familial advanced sleep phase syndrome (FASPS) presents in patients with abnormally early sleep onset and wake times, and is caused by a missense mutation to casein kinase Iε (CKIε) association region of *PER2* (Toh et al., 2001). CKIε inhibits nuclear entry of PER2 via post-translational phosphorylation, leading to a conformational change in its nuclear localization sequence and ultimately degradation (Vielhaber et al., 2000). By mutating the binding region of CKIε on *PER2*, the protein product is no longer regulated by phosphorylation and will enter the nucleus to alter the transcriptional activity of circadian genes, resulting in an overall shortening of circadian period of gene expression and altered phase relationship as seen in Figure 1.1 (Xu et al., 2005). The increased rate of nuclear entry of PER2 underlies the advanced phase of sleep/wake phenotype observed in patients with FASPS.

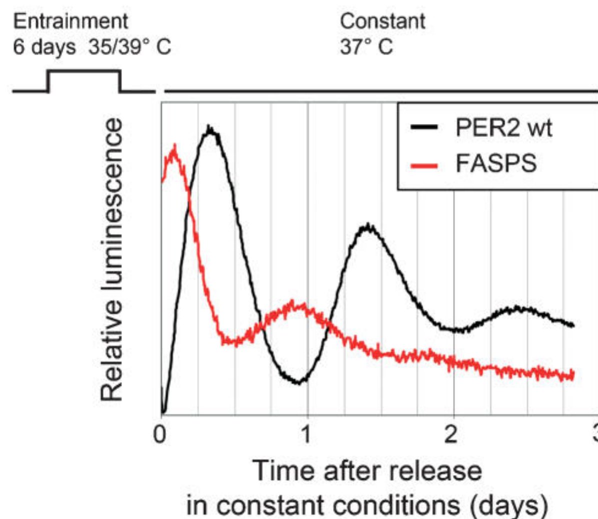

Figure 1.1: FASPS mutation leads to advanced phase and shortened period of *Per2* expression in mouse fibroblasts. NIH3T3 cell lines expressing either wild type *Per2* or FASPS mutated *Per2* were entrained to 12:12 (35C : 39C) temperature cycles for 6 days, then bioluminescence recordings were taken at a stable 37C using a *Per2::Luciferase* reporter. Taken from Vanselow et al., 2006.

1.3 Molecular Mechanisms of Entrainment

How does the circadian clock adapt to changes in environmental time cues? The mammalian circadian clock may be entrained to environmental stimuli called “zeitgebers”, a German word meaning “time-giver”. Common zeitgebers include light, as in the case of photoentrainment, and

more studies are being completed evaluating temperature changes as another input for entraining the clock. Methods being developed include the use of single temperature pulses as well as repeated temperature cycles, both of which have been shown to alter the phase of circadian gene expression in vivo and in vitro (Tamaru et al., 2011, Saini et al., 2012, Brown et al., 2002, Abraham et al., 2018, El Allali et al., 2013, Yoshikawa et al., 2013, Ohnishi et al., 2014, Ruby et al., 1999). Advancements in the area of non-photic entrainment are increasing the ability to evaluate rhythmic activity and adjustment of phase in peripheral tissues where light is not an adequate stimulus for manipulating the clock. The pathway of entrainment involving temperature has not been fully revealed, but is thought to be mediated via heat shock factor 1 (HSF1) interactions with clock machinery (Saini et al., 2012, Tamaru et al., 2011). Further discoveries in this area will strengthen the understanding of the multi-input system that contributes to entrainment of rhythms in peripheral tissues.

The neuronal machinery responsible for conveying light stimuli to the SCN has been highly characterized, and begins in the retina. Photic information encoded by intrinsically-photosensitive retinal ganglion cells projects along the retinohypothalamic tract (RHT) to the SCN to synchronize internal daily rhythms with environmental light levels (B., 2017). Signaling down the RHT to the SCN is mediated primarily by the neurotransmitter glutamate, which activates the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and *N*-methyl-D-aspartate (NMDA) receptors on the postsynaptic membrane (Colwell, 2001, Mizoro et al., 2010, Golombek and Rosenstein, 2010). Cytosolic calcium levels are increased by influx via NMDA receptors and subsequently the voltage gated calcium channel $Ca_V1.2$ (Irwin and Allen, 2007, Colwell, 2001). $Ca_V1.2$ is the primary L-type calcium channel (LTCC) in the mammalian central nervous system, and expression of the alpha-1 subunit of $Ca_V1.2$ (*CACNA1C*) is essential for the successful transmission of light information to the central clock (Pennartz et al., 2002). Association of calcium-dependent signal transduction elements such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to the

cytoplasmic domain of $Ca_V1.2$ (Abiria and Colbran, 2010, Kon et al., 2014) allows the direct activation of CaMKII and subsequently the mitogen-activated protein kinase (MAPK) signal transduction cascade (Nomura et al., 2006, Goldsmith and Bell-Pedersen, 2013). Ultimately, these pathways end with the phosphorylation of cyclic AMP response element-binding protein (CREB), activating the transcription of clock genes *Per1* and *Per2* in the nucleus to induce their expression in response to light (Tischkau et al., 2003, Colwell, 2011).

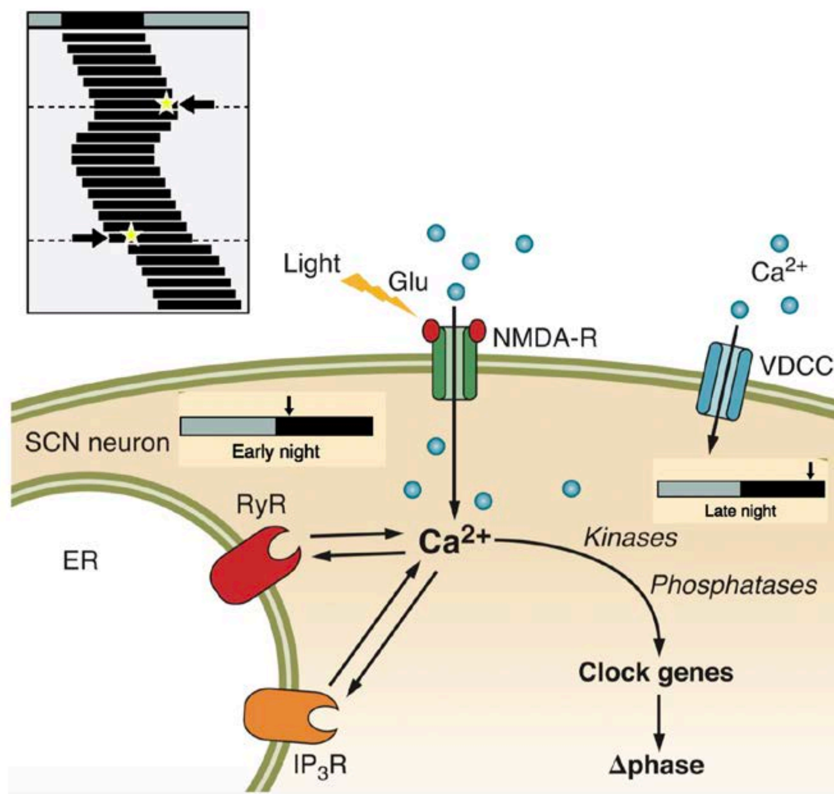

Figure 1.2: The organization of calcium sources within SCN neurons responsible for adjusting the phase of circadian gene expression. Adapted from Golombek et al., 2010.

Changes in intracellular calcium serve to mediate the coupling of light stimulation and transcriptional activation of clock genes in a phase dependent manner, where light in the *early* subjective night results in phase delays, whereas light in the *late* subjective night results in phase advances (see Figure 1.2) (Schmutz et al., 2014, Aguilar-Roblero et al., 2016). Interestingly, phase

delays are encoded by endogenous calcium release via ryanodine receptors (RyRs) (Irwin and Allen, 2007) and phase advances are mediated by exogenous calcium influx via voltage-dependent LTCCs (Kim et al., 2005). The SCN can discriminate between these two calcium sources to trigger different cellular responses to each (West et al., 2001, Lundkvist et al., 2005). Discoveries in the area of the molecular pathway of circadian entrainment are complicated in that both RyRs and LTCCs display their own pattern of circadian expression, thus making these calcium channels both inputs and outputs of the clock (Aguilar-Roblero et al., 2007, Ko et al., 2009, Pfeffer et al., 2009). This study will focus on the L-type calcium channel Ca$_V$1.2, which is associated with the phase advancing pathway for clock adjustment. Ca$_V$1.2$^{-/-}$ mice show altered abilities to respond to a light pulse in the late night, with smaller phase changes compared to controls (Figure 1.3) (Schmutz et al., 2014).

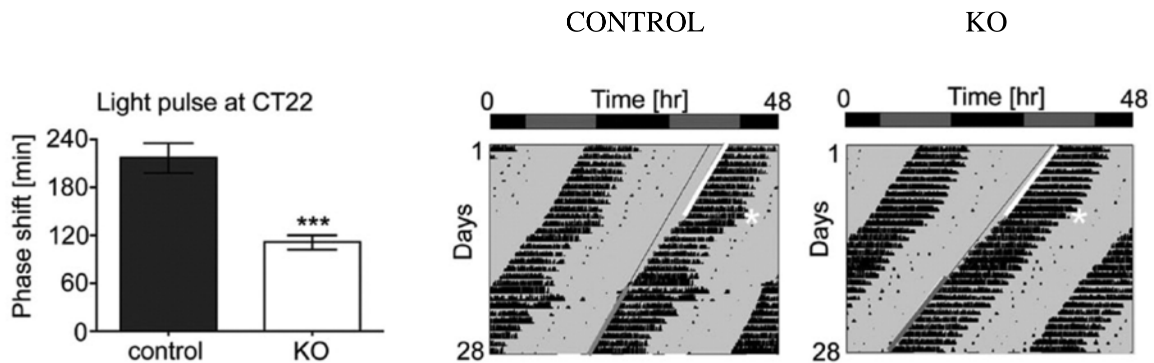

Figure 1.3: ***Ca$_v$1.2*** **knockout (KO) mice show smaller phase shifts after a light pulse at late subjective night. Shown are the quantifications of the phase shifts observed after brief light pulses presented at CT22 (left panels) and representative locomotor activity records showing the locomotor activity before and after light administration for control (middle panel) and Cav1.2 KO mice (right panel). White lines in the activity recordings represent the onset of activity before the light pulse, and grey lines represent the onset after the light pulse. White asterisks indicate the timing of the light pulse. Data are represented as mean ± SEM (*n* = 10-12), and significant differences were determined by unpaired *t* test (*** *p* < 0.001). Taken from Schmutz et al, 2013.**

The altered ability to phase advance found in mice lacking *CACNA1C* highlights the critical role of this calcium channel in the phase advancing mechanism. However, it is unclear whether single phase shifts induced by light in the late night proceed by a singular adjustment of the *phase* of gene

expression, or if this change arises by changing the *speed* of rhythmic gene expression similar to that seen in freerunning FASPS. The way the circadian clock entrains to cycles, as well as the way it responds to novel stimulus is widely debated in the field, as no mechanistic finding has allowed an explanation for how the clock adjusts its phase but many theories have been presented (Roenneberg et al., 2010, Bordyugov et al., 2015). The consistent association of *CACNA1C* single nucleotide polymorphisms (SNPs) with BD and the knowledge that this gene significantly contributes to the ability of the circadian clock to phase advance warrants an investigation of how these risk alleles may contribute to the circadian disruptions characteristic of BD.

1.4 L-Type Calcium Channels and the Brain

Both human and animal studies have informed our understanding of the widespread importance of $Ca_V1.2$ activity in the mammalian brain, and its role in regulating mood and behavior. Genome-wide association studies (Starnawska et al., 2016, Ikeda et al., 2018) and gene ontology studies (Ryan et al., 2006, Askland et al., 2009, Holmans et al., 2009) have repeatedly implicated the alpha-1 subunit of $Ca_V1.2$ (*CACNA1C*), and to a lesser extent, $Ca_V1.3$ (*CACNA1D*), as risk associated genes in BD. BD's most implicated SNP (rs1006737) is found in intron 3 of the *CACNA1C* gene (Eckart et al., 2016), whereby channel function remains unaffected but regulation of gene expression is changed. Another SNP (rs4765913) is in linkage disequilibrium with rs1006737 and has also been strongly implicated in BD (Franke et al., 2010). In neurons, these two isoforms play an essential role in regulating intracellular calcium levels, gene expression, membrane polarization and neurotransmitter release (Flavell and Greenberg, 2008). The SCN is only one region where the excitation-transcription coupling completed by $Ca_V1.2$ has been researched, and this LTCC has been shown to have functions in other brain areas. Mice with region specific knockouts of *CACNA1C* in the pre-frontal cortex, hippocampus, and cerebral cortex show heightened anxiety behaviors (Moosmang et al., 2005, Lee et al., 2012, Kabir et al., 2016). Neuroimaging research has allowed the

evaluation of how *CACNA1C* genotype influences the processing of emotionally salient stimuli in humans, and revealed that carriers of a BD-associated risk allele (rs1006737) present altered structure and function in regions associated with the regulation of depression and anxiety (Kabir et al., 2016). Studies conducted on post mortem brain samples show that carriers of the *CACNA1C* risk allele associated with BD have altered levels of calcium influx in the cerebellum, which may be explained by a change in regulation of *CACNA1C* expression (Gershon et al., 2014).

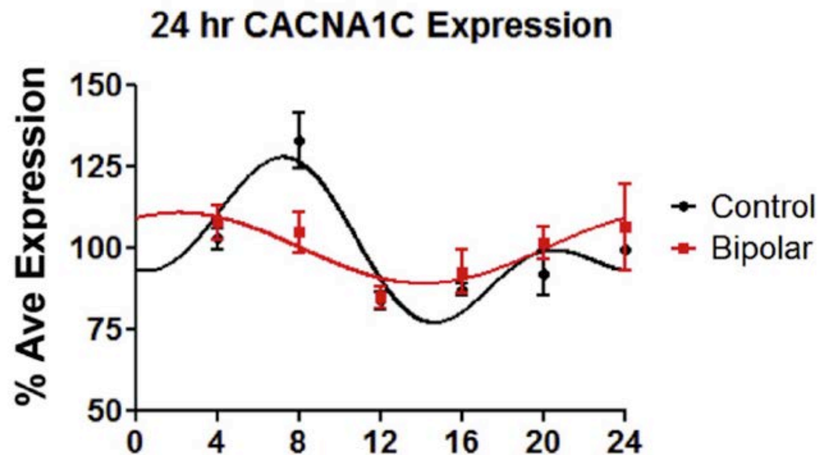

Figure 1.4: Expression of CACNA1C is rhythmic in both control and bipolar patient fibroblasts, although BD patient cells display lower amplitude and altered temporal expression. Taken from McCarthy et al., 2016.

Further investigation using primary human fibroblasts revealed that *CACNA1C* exhibits a circadian pattern of expression; with an abnormal phase relationship and amplitude of expression observed in carriers of this BD associated risk allele compared to controls (Figure 1.4) (McCarthy et al., 2016). These studies indicate a clear connection between *CACNA1C* and BD. Interestingly, the deficits observed in lithium induced amplitude modulation were linked to ERK signaling, a feature that along with calcium channels overlaps with circadian entrainment. Based on this observation, this

study hopes to gain further mechanistic insight into the molecular basis underlying circadian differences between BD and controls.

1.5 Modeling Bipolar Disorder

With heterogeneous clinical presentations, subjective mood states and polygenic genotypes, mood disorders face various challenges when it comes to creating valid and dependable experimental models. Moreover, BD presents the unique need for a dynamic disease model that can display both manic and depressive behavioral phenotypes interspersed with healthy intervals. Typically, animal model research in BD is completed independently, with studies completed separately for the depressive phenotype and another model for the manic phenotype (Beyer and Freund, 2017). One of the foremost animal models of bipolar mania is the *ClockΔ19* mouse strain, in which the core molecular circadian gene *Clock* is mutated at intron 19, eliminating the rhythmic DNA binding and transcriptional activating properties of its protein product. Interestingly, the CLOCKΔ19 mutant displays manic characteristics in common with BD and is responsive to lithium therapy, the most common pharmacological treatment prescribed for BD (Logan and McClung, 2016, Kristensen et al., 2018), for the correction of these behaviors. The *ClockΔ19* mouse model is limited to the study on manic phenotypes, as this strain does not exhibit the cycling of depressive behavior that would also be expected to accompany mania in BD.

Alternatively, there is an argument for the use of primary human fibroblasts from BD patients as a model for mechanistic discoveries; as such cells would retain the polygenic characteristics of the patient. In vitro modeling of psychiatric conditions is limited due to tissue availability, and the use of fibroblasts for this research circumvents this issue where a non-invasive biopsy may be collected from a large group of controls and patients, lending additional statistical power to results. Cellular models can provide preliminary insights and directions for research as we develop animal and cell

models that more closely represent BD in genotype and phenotype. While primary human fibroblasts may not participate in electrochemical communication in the same manner as neurons, they provide an opportunity to evaluate hypotheses in an accessible tissue that carries a polygenic BD background. Fibroblasts have been established as autonomous, self-sustained oscillators that are dependent on membrane potential and calcium dynamics (Nagoshi et al., 2004, Noguchi et al., 2012, Tsuchiya and Nishida, 2003), characteristics that lend themselves to validate psychiatric disorder discoveries in this cell type. Additionally, human fibroblasts have also been shown to express $Ca_V1.2$ L-type calcium channels, making this accessible cell type an option for evaluating BD and circadian rhythms (Figure 1.4) (McCarthy et al., 2016).

The studies discussed here prompt more lines of questioning: are characteristics of BD correlated with *CACNA1C* risk allele genotype? What role does *CACNA1C* play in the circadian rhythms of BD patients? If $Ca_V1.2$ is integral to adjusting phase advancing, do BD associated risk alleles influence the ability of the clock to adjust in BD patients? The aim of this study is to better understand how the *CACNA1C* risk alleles contribute to the adjustment of the circadian clock in BD patient fibroblasts, and to validate an in vitro model for evaluation of phase advancing in peripheral tissues. To do this, collaborators shared primary patient fibroblasts collected from BD patients and controls which were then genotyped for the *CACNA1C* SNP rs1006737 (Oedegaard et al., 2016, Milivojevic et al., 2011). Our hypothesis was that carriers of the CACNA1C risk allele (rs1006737) display lower levels of entrainment to temperature cycles, as the abnormal expression of $Ca_V1.2$ in these cells could alter their ability to entrain to environmental stimuli.

MATERIALS AND METHODS

Evaluation of rhythms was completed via transfection with a viral *per2::luciferase* reporter, and recording of subsequent bioluminescence. Pharmacological agents to manipulate calcium levels were used to evaluate the role of different calcium sources on fibroblast rhythms; the RyR antagonist dantrolene, and the LTCC antagonist verapamil.

2.1 Human Subjects

Human fibroblast cell lines were obtained by punch biopsy of the deltoid and shared from previous studies. Control lines were collected by Jonathan Covault at University of Connecticut (Milivojevic et al., 2011); donors were self-identified non-Hispanic Caucasians screened with the Structured Clinical Interview for DSM-III-R or DSM-IV or the Semi-Structured Assessment for Drug Dependence and Alcoholism to exclude individuals with a substance use disorder or other major Axis I psychiatric disorder. Lines from patients with clinically diagnosed Bipolar Disorder were shared from The Pharmacogenomics of Bipolar Disorder Study (Oedegaard et al., 2016). The Diagnostic Interview for Genetic Studies was used for DSM-IV diagnoses and to collect detailed patient history.

2.2 Cell Culture

Cells were thawed from cryopreservation and cultured in 100mm dishes to confluence for each cell line before passaging. Standard cell culture media contained DMEM (Gibco 11316), 10% fetal bovine serum (Gibco 10437), 2 mM L-glutamine (Gibco 25030), and an Antibiotic-Antimycotic [Penicillin + Streptomycin + Amphotericin B] (Gibco 15240062). Passaging of cells was completed with HBSS (Gibco 14175-095) to wash, and 0.05% Trypsin-EDTA with phenol red (Gibco 25300054) for cell dissociation. Cultures were maintained in a Nuaire Water-Jacketed IR Autoflow CO_2 incubator with 5% CO_2 at 35C. Preliminary assay development was completed using commercially available immortalized mouse fibroblasts before moving into human fibroblast

experiments. NIH 3T3 immortalized embryonic mouse fibroblasts (CRL 1658) were purchased from Cedarlane Cellutions Biosystems Incorporated, and cultured in standard growth media and procedure outlined above. In order to measure rhythmic gene expression in these cells, a stable expressing line of NIH3T3 *Per2::luc* was generated using lentiviral transfection and antibiotic selection (see more below). The transfected NIH3T3 cells were cultured in standard growth media containing 200 ug/ml hygromycin B (ThermoFisher 10687010), to select for only the cells which were successful in expressing the viral construct.

2.3 Reporter Gene Transfection

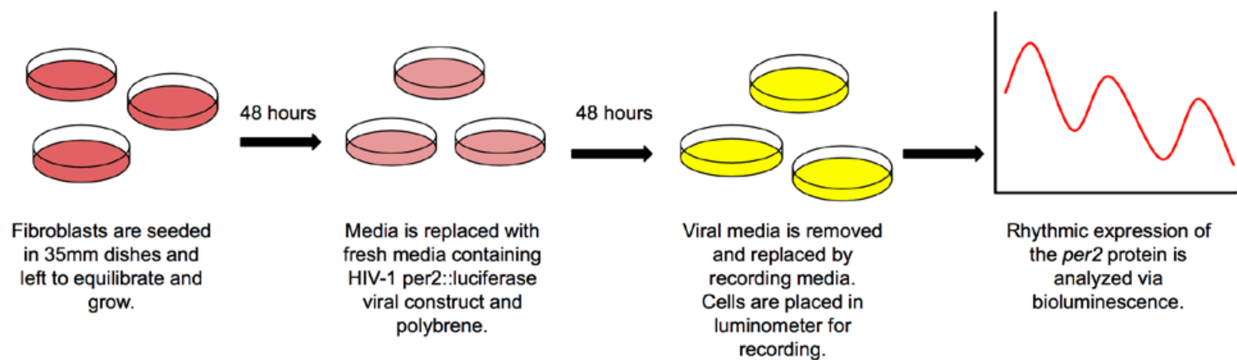

Figure 2.1: Outline of the transfection process for preparing fibroblasts for luminometry recording.

The *Per2::luc* lentiviral vector has been described in detail previously (Liu et al. 2007). This vector contains the *Per2* promoter upstream of the firefly luciferase gene, the protein product of which catalyzes a reaction with its substrate luciferin to produce light. The replication inactivated HIV-1 vector inserts the construct within the host genome, where luciferase is then expressed under the regulation of the endogenous circadian clock.

Luminometry assays required transfection with a *Per2::luc* virus to monitor the reporter gene as a measurement of circadian rhythms. Human fibroblast cells were grown to confluence in a

100mm dish before being dissociated with 0.05% trypsin, and then seeded into 35mm dishes at a 10% dilution. Dishes were placed in 35C incubator for 48 hours to allow stabilization. Following this period, transfection was completed using ~1x10^7 infectious units of *Per2::luc* HIV-1 virus per plate, with 0.1% polybrene in standard growth media. Cells incubated at 35C with 1ml of media containing virus and polybrene for 1 hour, then an additional 1ml of plain growth medium was applied to cells and incubated for an additional 48 hours at 35C to allow growth to confluence (~1.2x10^6 cells/dish).

NIH3T3 cells were treated with a *Per2::luc* HIV-1 virus with an additional gene conferring resistance to the antibiotic hygromycin (Meng et al., 2008). The addition of this resistance gene allowed the transfection and selection of a stable expressing line of NIH3T3 *Per2::luc* cells, which would not require transient transfection before each experiment. Attempts to produce stable transfected human fibroblasts lines with a *Per2::luc* virus conferring resistance to blasticidin were unsuccessful, as the lines grew poorly, and rhythms in these lines were inconsistent, with very low amplitude oscillations. Therefore, luminometry experiments with human fibroblasts were completed using transient transfection protocols that would place minimal stress on the cells, as this method does not require antibiotic selection to ensure incorporation of the viral construct.

2.4 Luminometry Assays

Immediately before placement into the luminometer, growth media was aspirated and replaced with recording media containing 1X DMEM without phenol red (Cellgro 90-013-PB), 10 mM HEPES (Gibco 15630), 14 mM $NaHCO_3$ (Gibco 25080), 50 mM glutamine (Gibco 25030), 1X B-27 (Gibco 17504-044), penicillin/streptomycin (Gibco 15140), and 1mM luciferin (BioSynth International L-8220). This media change allows the synchronization of *Per2::luc* rhythms in these cells without a need for serum shock. Drug treatments were added to the recording media, and were present for the duration of luminometer recording. All drug solvents were run in parallel as vehicle

controls. Once placed in recording media, the cells were sealed into the dishes using grease and a glass coverslip. The recording media contains a combination of HEPES and $NaHCO_3$ buffers to stabilize the pH for the duration of the recording in sealed dishes, in a dry incubator that is incompatible with CO_2 supplementation. The sealed plates were secured into a Lumi-Cycle 32-well luminometer (Actimetrics, Wilmette, IL) and bioluminescence (counts/sec) was measured for 70 seconds, every 10 minutes for a period of 5 days for analysis. The luminometer was placed inside a dry incubator, with a stable temperature of 35C for the duration of data collection.

2.5 Temperature Pulse and Temperature Cycling Experiments

Rhythm data was collected in freerunning conditions, as well as entrainment conditions. This in vitro model employed fibroblasts, which do have photosensitive machinery to allow them to entrain to light. Therefore, entrainment and phase manipulations were completed using temperature cycles and temperature pulses, which have been shown to be effective stimulus for entrainment of the circadian clock in vitro (Saini et al., 2012, Ruby et al., 1999, Ohnishi et al., 2014, Tamaru et al., 2011). We sought to measure circadian entrainment in fibroblasts by examining the response of rhythms to temperature pulses of different durations and intensities in a programmable incubator (Panasonic MIR-554-PA). All pulse experiments were completed by allowing samples to stabilize at 35C in the luminometer for 24 hours after beginning recording, then increasing the temperature for a specified intensity and duration. Once the chosen duration elapsed, the temperature was dropped back to 35C for the remainder of the experimental recording. Temperature cycling experiments were completed in a two-step square wave scheme: 12 hours at 35C followed by 12 hours at 37.5C where all recordings began with samples at 35C for 12 hours. This repeating cyclical protocol is meant to provide an entrainment stimulus similar to that of the rhythmic fluctuation of body temperature in mammals. All samples were completed in duplicate.

Data obtained from these cycling experiments in human fibroblasts allows the analysis of entrainment according to BD-associated genotype, and therefore was an integral portion of the experimental design for this study. Entrainment may be evaluated by assessing the difference between period of circadian gene expression and the period of the given entrainment stimulus, with total entrainment indicated by these two values being equal.

The expression of immediate early clock gene *PER1* was evaluated in response to temperature change as a validation of this model for entrainment of the circadian clock. Synchronizing stimulus has been shown to result in the acute induction of *PER1* (Kaeffer and Pardini, 2005), allowing the expression levels of *PER1* mRNA to serve as a control when evaluating the response of *CACNA1C* expression in a temperature cycle. This experimental design is meant to provide insight to the mechanism by which the temperature cycling paradigm is interacting with the molecular oscillators in human fibroblasts, and how *CACNA1C* expression responds to temperature changes. For time course temperature pulse gene expression experiments, human fibroblasts were cultured in parallel in 6-well plates, and then left to equilibrate for 48 hours. The temperature cycling incubator was optimized for use with a luminometer, therefore all samples required the same sealing and buffer treatment as with luminometry studies. To begin, the standard growth medium was aspirated and replaced with recording media, and plates covered with Microseal 'B' Seals (Bio-Rad MCB1001) before being placed in their separate incubators (t=0 hr). It has been shown that media changes can down-regulate expression of *per1* in rat fibroblasts, with expression levels stabilizing around 2 hours post media change *(Hirota et al., 2002)*. Thus, our first collection and RNA isolation was performed at t=2 hours. The second time point was selected as t=14 hours to allow the temperature change in the 12:12 temperature cycle to take place (t=12), and any following influences on gene expression to take full effect over the following two hours. Two six-well plates were placed at constant temperature for the duration of the experiment (35C), and two more were placed in a temperature cycling incubator (12 hours at 35C, 12 hours at 37.5C). At each time point, one plate

from each condition was promptly washed with cold PBS, sealed and placed at -80C for preservation before isolation of RNA.

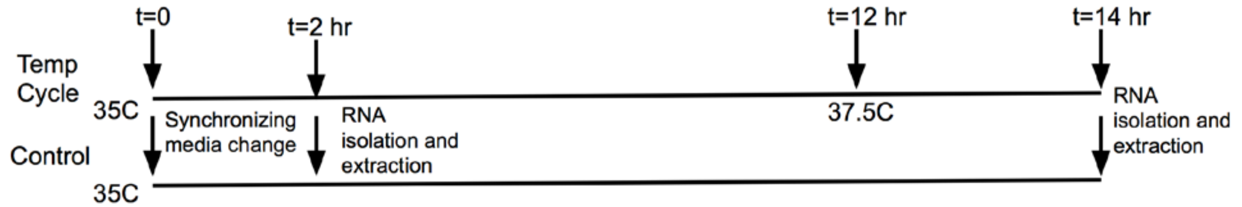

Figure 2.2: Timeline of temperature cycle time course experimental design.

2.6 Preparation of Drug Treatments

The following list contains details of all drugs used in this study, along with the corresponding solvent:

- Verapamil hydrochloride (Tocris 0654) - water
- Dantrolene, sodium salt (Tocris 0507/100) - DMSO

Drugs were dissolved in sterile water or DMSO to create stock solutions. All subsequent dilutions were completed by adding stock to explant media and then distributing this dilution across all plates of the same condition. Vehicle controls were completed for all drugs with stocks prepared in DMSO.

2.7 Human Fibroblast SNP Genotyping

Purification of DNA from human fibroblast lines was completed with the DNeasy Blood and Tissue Kit (Qiagen 69504) according to the manufacturer's protocol. SNP Genotyping was completed via quantitative real-time polymerase chain reaction (PCR) of isolated DNA, with pre-designed Taqman assays (Applied Biosystems) using a C1000 Touch Thermal Cycler with a CFX384 Real-Time System (Bio-Rad). All lines were evaluated for their genotype for *CACNA1C* SNPs (4765913 and rs1006737).

Cell lines were sorted into the following categories according to *CACNA1C* SNP genotype (rs4765913 and rs1006737): homozygous carriers of the non-risk associated allele at rs1006737 (G; common allele), and heterozygous and homozygous carriers of the risk allele at rs1006737 (A; risk allele) are combined as one experimental group. The risk allele carriers include both heterozygotes and homozygotes for the rare allele, as the minor allele frequency makes it challenging to gather data with significant statistical power if these groups are separated.

2.8 RNA Isolation and qPCR

Cells were retrieved from -80C and lysed within their wells, then immediately extracted for total RNA using RNeasy Mini Kit (Qiagen 74104) following manufacturer protocol. Total RNA yield was quantified using Nanodrop 2000 (ThermoFisher). cDNA synthesis was completed with 500 ng of total RNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814) and T100 Thermal Cycler (Bio-Rad 1861096). Quantitative RT-PCR was performed using a C1000 Touch Thermal Cycler with a CFX384 Real-Time System (Bio-Rad) using pre-made primers (Applied Biosystems) for *CACNA1C*, *PER1*, and *GAPDH*. Transcript levels for each gene were normalized to the non-rhythmic housekeeping gene GAPDH using the comparative C_T method (Schmittgen and Livak, 2008, Kosir et al., 2010). Average relative expression ratios for each gene were calculated from two PCR replicates and expressed as a percentage of the maximum ratio at peak expression.

2.9 Data Analysis and Statistical Methods

For each cell line, luminometry data were fitted to a damped sine curve by the least squares method using Lumicycle Analysis (Actimetrics). Rhythm parameters (period, amplitude, phase) were then averaged. To limit variability, the first day of recording was excluded from analysis for all

bioluminescence data. Data were filtered using moving average background subtraction. Post hoc analysis of all data was completed via T-Test, with $p<0.05$ indicating significance.

RESULTS

3.1 Calcium Channel Antagonists Modify the Circadian Period of Mouse Fibroblasts

To begin the study of calcium dynamics in the circadian rhythms of fibroblasts, we investigated the effect of LTCC antagonist verapamil on mouse fibroblasts. Bioluminescence recordings of cells in recording media containing verapamil showed significantly shortened periods of *per2* expression in mouse fibroblasts in constant conditions (35C). This result was highly reproducible and post hoc T-test indicated p=0.0004 for the 10 µM concentration of verapamil versus vehicle controls.

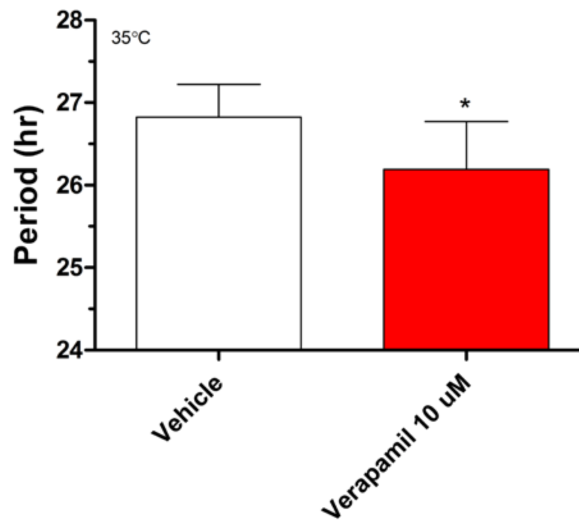

Figure 3.1: Verapamil significantly shortens the freerunning period of mouse fibroblasts.
Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days in recording media containing either water (vehicle) or 10 µM verapamil. Counts/sec were analyzed using a fitted damped sine curve, then evaluated for circadian period and averaged (n=24, n=13 respectively). Post hoc t-test indicated p<0.05 (* indicates statistical significance compared to vehicle).

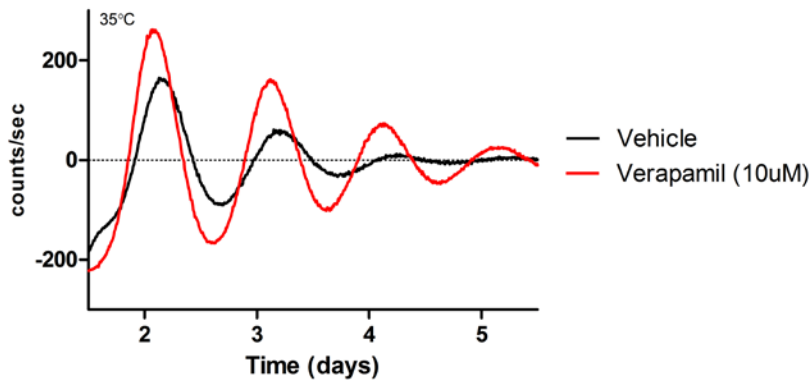

Figure 3.2: Verapamil shortens the freerunning period of mouse fibroblasts.
Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days. A single representative rhythm trace was selected from sample data of controls (black) and 10 μM verapamil treated (red).

In addition, the effect of RyR antagonist dantrolene on *per2* expression in mouse fibroblasts in constant conditions (35C) was a lengthening of the period of *per2* expression. The circadian period of vehicle controls run in parallel was on average 26.6 hrs, versus 26.9 hrs for cells with 10 μM dantrolene (Figure 3.1). The effects on period lengthening effect of 10 μM dantrolene was variable, as the standard deviation between samples is larger than the margin of difference between the control and this experimental condition (p=0.08).

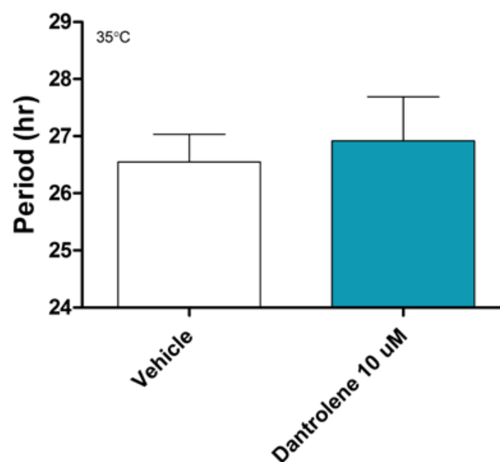

Figure 3.3: Dantrolene insignificantly lengthens the freerunning period of mouse fibroblasts.
Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days in recording media containing either DMSO (vehicle) or 10 μM dantrolene. Counts/sec were analyzed using a fitted damped sine curve, then evaluated for circadian period and averaged (n=21, n=17 respectively). Post hoc t-test indicated p>0.05.

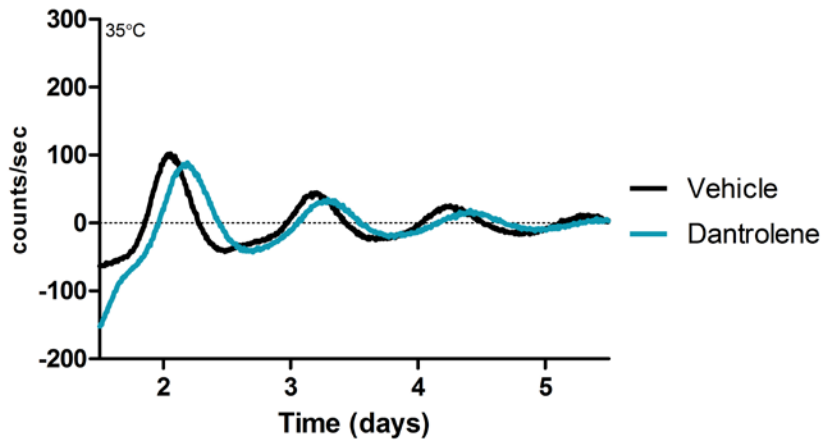

Figure 3.4: Dantrolene lengthens the freerunning period of mouse fibroblasts.
Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days. A single representative rhythm trace was selected from sample data of vehicle controls (black) and 10 µM dantrolene treated (teal).

To summarize, we found that in mouse fibroblasts long-term blockage of extracellular calcium influx via LTCCs resulted in shorter circadian period consistent with its role in facilitating phase advances in response to environmental cues, whereas blocking intracellular calcium release via RyRs resulted in longer circadian period consistent with its role in facilitating phase delays. Curiously, these results were obtained via *antagonism* of these calcium channels in vitro, and in vivo these mechanisms are mediated by *activation* of the channels.

3.2 Temperature Pulses and Cycles Modify the Circadian Period of Mouse Fibroblasts

To evaluate the characteristics of phase adaptation and entrainment in fibroblasts, we needed to investigate a method of relaying information to the circadian clock in these cells. We found many publications detailing the use of temperature as an entraining stimulus in vitro (Izumo et al., 2003, Tamaru et al., 2011, Abraham et al., 2018), some employing temperature cycles and others using single temperature pulses. To better understand how the duration and intensity of temperature pulses mediates changes to the circadian clock, we tested six different combinations of pulse length and

temperature change in mouse fibroblasts. It appears that the degree of alteration to circadian period coincides with the intensity and duration of the pulse given, as the strongest pulse of 9 hours with 37.5C delivered a significant change to circadian period (Figure 3.5).

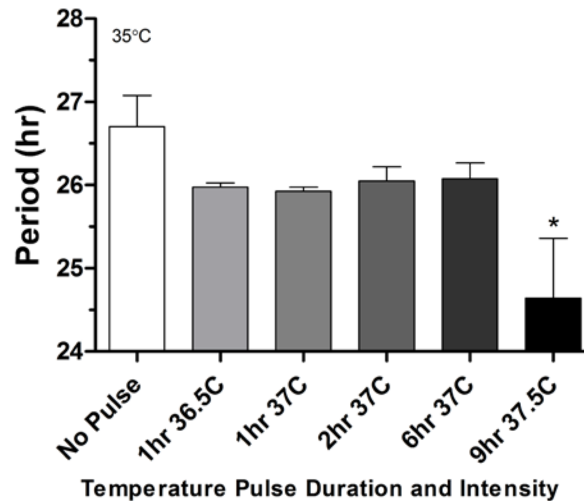

Figure 3.5: Temperature pulses can modify the period length of mouse fibroblasts.
Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days in recording media. Samples were run with varying duration and intensity of temperature change (increasing from 35C) to evaluate the effect of these parameters on circadian period. Counts/sec were analyzed using a fitted damped sine curve, then evaluated for circadian period and averaged (n=28, n=4, n=4, n=4,n=4, n=7 respectively). Post hoc t-test indicated $p<0.05$ for the 9 hr 37.5C condition (* indicates statistical significance compared to no pulse).

The strongest pulse intensity, an increase of 2.5C elicited the strongest change to circadian period. Therefore, we chose to evaluate circadian entrainment of mouse fibroblasts using temperature cycles with a 2.5C change in temperature between steps. We saw that mouse fibroblasts in a 12 hour 35C, 12 hour 37.5C cycle displayed a stronger level of entrainment than cells in a 14 hour 35C, 10 hour 37.5C temperature cycle (Figure 3.6). The degree of entrainment is determined by the reduction of the FRP to match *per2::luc* expression to the 24 hour cycle provided via temperature. Therefore, the 12:12 condition which reduced circadian period to an average 24.5 hours, down from freerunning controls at an average 26.8 hours, displays stronger entrainment than the 14:10 condition with an average period of 25.2 hours. Although both of these cycles are within a 24 hour frame, the 12:12 cycle is more effective at entraining mouse fibroblasts.

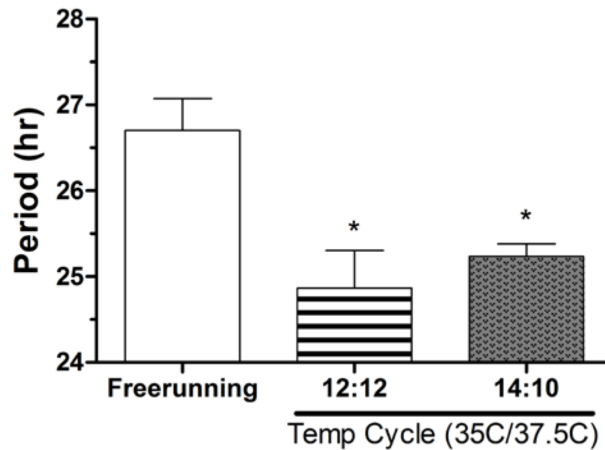

Figure 3.6: Mouse fibroblasts entrain to temperature cycles.
Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days in recording media. Samples were run in constant temperature (freerunning; 35C), 12:12 (35C : 37.5C) or 14:10 (35C: 37.5C) repeated temperature cycles. Counts/sec were analyzed using a fitted damped sine curve, then evaluated for circadian period and averaged (n=24, n=11, n=14 respectively). Post hoc t-test indicated $p<0.05$ for both the 12:12 and 14:10 temperature cycle conditions (* indicates statistical significance compared to freerunning).

3.3 Calcium Channel Antagonists Do Not Alter Circadian Period of Entrained Mouse Fibroblasts

Next, we wanted to evaluate the effect of our calcium channel blockers in conjunction with an entraining stimulus. To do this, samples were run in parallel either in freerunning conditions (35C), or the 12:12 (35C: 37.5C) temperature cycle. Application of verapamil with the temperature cycle did not significantly change the period of *per2* expression in mouse fibroblasts (Figure 3.7). In freerunning conditions the effect of verapamil is significant, but this effect is not observed when cells are placed in a temperature cycle. Once again showing that the effect of a period altering drug is overpowered by entrainment to another stimulus.

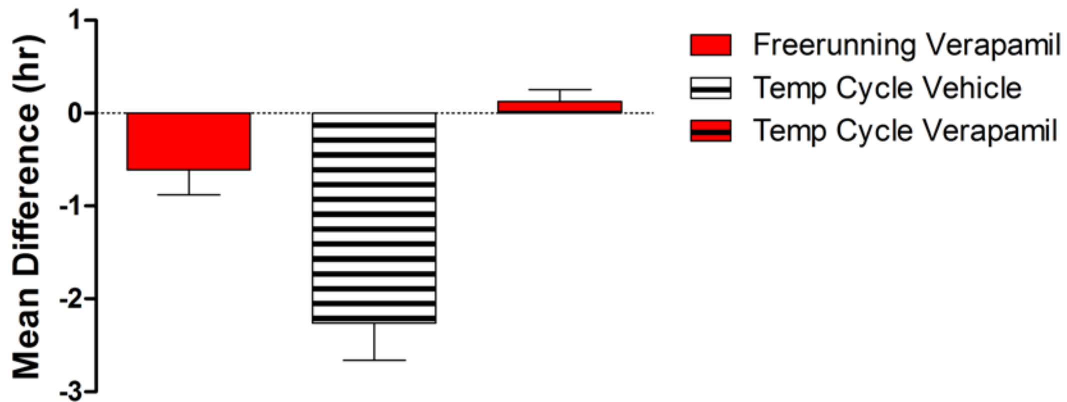

Figure 3.7: Verapamil does not significantly change circadian period of entrained mouse fibroblasts. Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days in recording media. Samples were run in constant temperature (freerunning; 35C) or 12:12 (temp cycle; 35C : 37.5C) repeated temperature cycles. DMSO (vehicle), or 10 µM verapamil were added to recording media at experiment initiation. Counts/sec were analyzed using a fitted damped sine curve, then evaluated for circadian period and averaged (n=13, n=11, n=11, respectively). Post hoc t-test indicated $p<0.05$ for only the freerunning verapamil and 12:12 vehicle temperature cycle conditions (* indicates statistical significance compared to freerunning).

Concurrent application of dantrolene in conjunction with a temperature cycle did not result in a significant change in circadian period, showing that the temperature cycle driven entrainment is stronger than the effect of dantrolene (Figure 3.8).

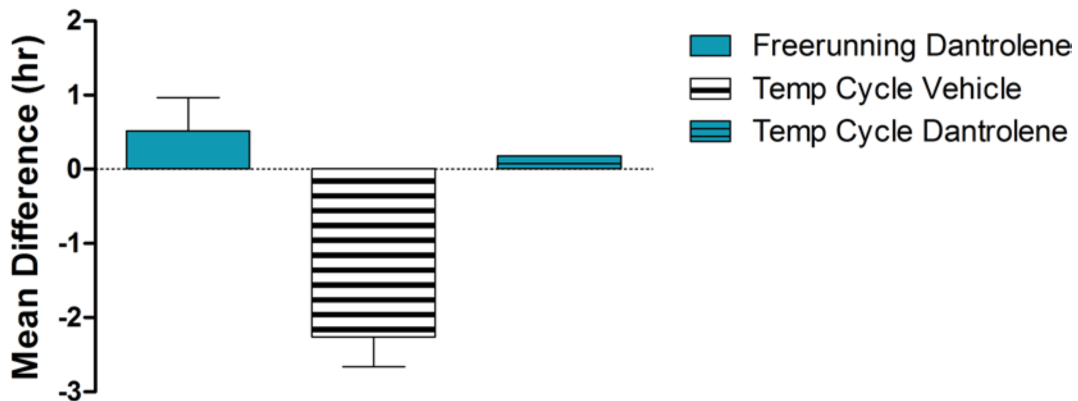

Figure 3.8: Dantrolene does not significantly change the period of entrained mouse fibroblasts. Stable transfected NIH3T3 cells expressing a *per2::luciferase* reporter were recorded for bioluminescence for 5 days in recording media. Samples were run in constant temperature (freerunning; 35C) or 12:12 (35C : 37.5C) repeated temperature cycles. DMSO (vehicle), or 10 µM dantrolene were added to recording media at experiment initiation. Counts/sec were analyzed using a fitted damped sine curve, then evaluated for circadian period and averaged (n=17, n=6, n=6, respectively). Post hoc t-test indicated $p<0.05$ for only the 12:12 vehicle temperature cycle condition (* indicates statistical significance compared to freerunning).

3.4 Human Fibroblasts Are Rhythmic and Entrain to Temperature Cycles

Now that we characterized the entrainment and effect of calcium channel drugs on mouse fibroblasts, we desired to complete entrainment assays with human fibroblasts from bipolar patients and controls. We conducted luminometry experiments with control and bipolar patient fibroblast lines in 12:12 temperature cycles then genotyped each line for *CACNA1C* genotype. We found that bipolar and control fibroblast lines displayed rhythmic, circadian expression of the *PER2::luc* reporter and this expression entrained to 12:12 temperature cycles (Figure 3.9). The entrainment of these cells is easily visualized, as the peaks and troughs of *PER2* expression match with the transition points between steps of the temperature cycle. Control patient lines exhibited an average period of 24.9 hours, with no significant different from BD patient lines with an average of 25.3 hours.

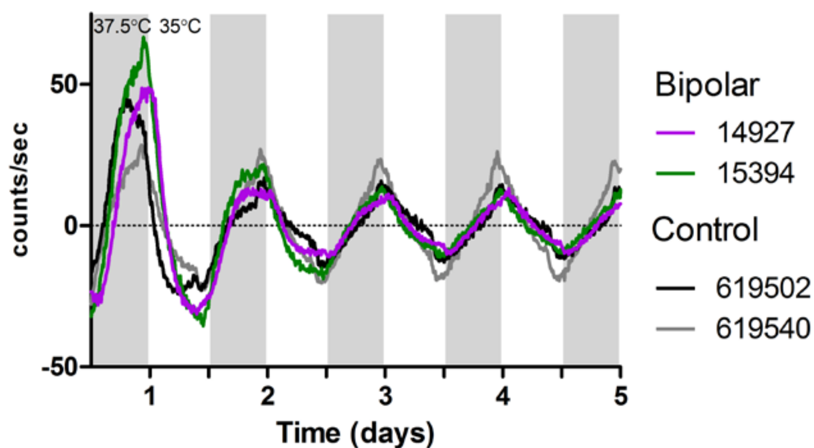

Figure 3.9: Human fibroblasts are rhythmic and entrain to temperature cycles.
Transient transfected human fibroblasts cells expressing a *PER2::luciferase* reporter were recorded for bioluminescence for 5 days. Samples were run in 12:12 (35C : 37.5C) repeated temperature cycles. Representative traces of BD patient lines (n=2) and control patient lines (n=2) are shown.

3.5 CACNA1C Genotype Predicts Entrainment of Human Fibroblasts to Temperature Cycles

Further analysis revealed a genotype difference of entrainment in bipolar patient fibroblasts, with cells homozygous for the common allele rs1006737 showing a smaller phase angle, with periods

closer to the 24 hour cycle compared to the rare allele carriers (Figure 3.10). Indeed, *CACNA1C* SNP rs1006737 genotype predicted the entrainment of bipolar patient fibroblasts. Due to the same number of cells harboring the rare variant, both heterozygotes and homozygous carriers of this risk allele are grouped together for analysis. This genotype-associated difference in entrainment was not observed in a small number of control fibroblasts. However, only two control fibroblasts were carriers of the BD-associated SNP.

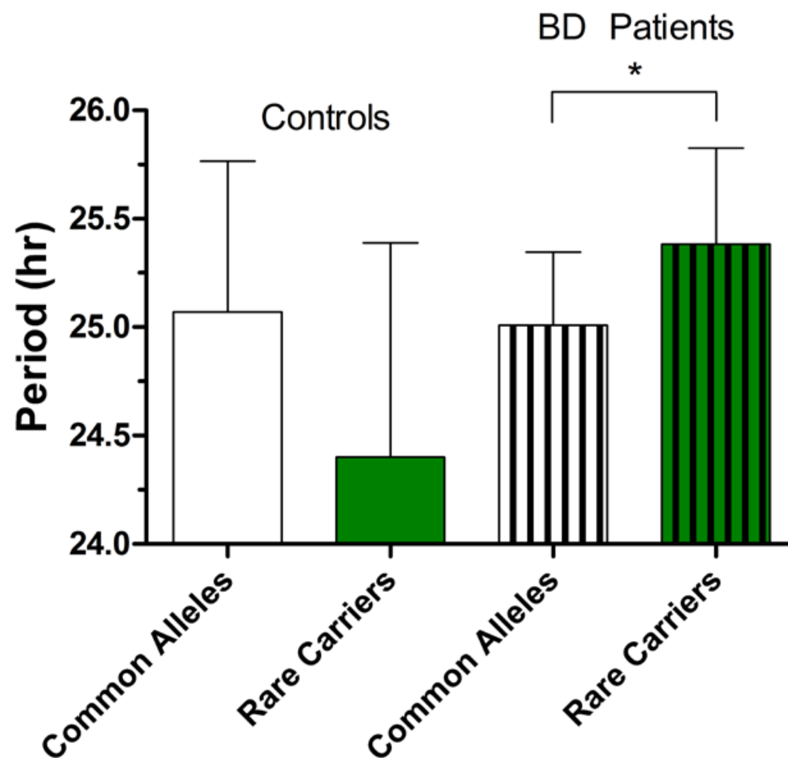

Figure 3.10: CACNA1C genotype predicts entrainment of human fibroblasts to temperature cycles. Transient transfected bipolar patient human fibroblasts cells expressing a *PER2::luciferase* reporter were recorded for bioluminescence for 5 days. Samples were run in12:12 (35C : 37.5C) repeated temperature cycles. Counts/sec were analyzed using a fitted damped sine curve, then evaluated for circadian period and averaged (n=7, n= 2, n=15, n=6 respectively). CACNA1C SNP rs1006737 genotype was found for each human fibroblast line via qPCR SNP genotyping assays. Post hoc t-test indicated $p<0.05$ for the carriers of the rare, bipolar disorder-associated rs1006737 allele compared to BD patients without the rare allele (* indicates statistical significance compared to BD patients with only common alleles).

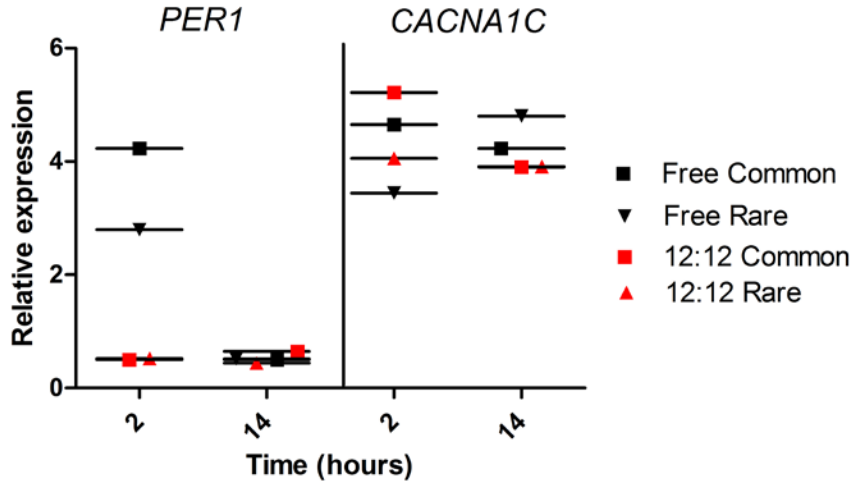

Figure 3.11: Temperature Change Does Not Induce CACNA1C Expression in Human Fibroblasts. Bipolar patient fibroblasts were run in parallel; either in freerunning conditions ("Free"), or in a 12:12 (35C : 37.5C) cycle. Cell collection and RNA isolation was performed at 2 hours and 14 hours post media change. cDNA was run with probes for GAPDH and PER1, and data analyzed using the comparative C_T method, with GAPDH as a non-rhythmic housekeeping control. *CACNA1C* SNP rs1006737 genotype was found for each human fibroblast line via qPCR SNP genotyping assays. Due to low allele frequency of the BD-associated allele, both heterozygotes and homozygous carriers of this allele are grouped together. Carriers of the rare, bipolar disorder-associated rs1006737 allele ("Rare") are compared to BD patients without the rare allele ("Common").

3.6 Temperature Change Does Not Induce CACNA1C Expression in Human Fibroblasts

To follow our discoveries of the difference in circadian entrainment between *CACNA1C* genotype in BD patient fibroblasts, the expression of immediate early clock gene *PER1* was evaluated in response to temperature change as a validation of this model for entrainment of the circadian clock. Synchronizing stimulus has been shown to result in the acute induction of *PER1* (Kaeffer and Pardini, 2005), allowing the expression levels of *PER1* mRNA to serve as a control when evaluating the response of *CACNA1C* expression in a temperature cycle. This experimental design is meant to provide insight to the mechanism by which the temperature cycling paradigm is interacting with the molecular oscillators in human fibroblasts, and how *CACNA1C* expression responds to temperature changes. All samples showed a reduction in *PER1* expression from t=2 to t=14 (Figure 3.11), and no significant difference between genotypes was detected. *CACNA1C* expression did not display any significant changes between t=2 and t=14, indicating that dynamic, short-term regulation of

CACNA1C expression is not the mechanism by which circadian entrainment to temperature cycles occurs in fibroblasts.

DISCUSSION

The work in mouse fibroblasts found that LTCC antagonism shortens circadian period, whereas RyR antagonism lengthens period. Temperature cycles were found to be an effective method of entrainment in both human and mouse fibroblasts, and work in these mouse lines showed that calcium channel antagonists do not significantly alter entrainment to temperature. Our hypothesis predicted carriers of the *CACNA1C* risk allele (rs1006737) would display lower levels of entrainment to temperature cycles, as the abnormal expression of $Ca_V1.2$ in these cells could alter their ability to entrain to environmental stimuli. Results indicate that this is true (Figure 3.10), but only in BD patient fibroblasts, not in controls. The insignificant difference in entrainment between genotypes of controls shows that *CACNA1C* genotype alone is not sufficient to explain the observed differences. Some other unknown factor must also be in play, and may attributed to BD genotype. In support of this finding, we saw that *CACNA1C* genotype does not influence *PER1* or *CACNA1C* expression after a temperature change in BD patient fibroblasts, thus the mechanism of phase alteration following a temperature change does not induce expression of either of these genes. The observed *CACNA1C* genotype difference in entrainment paints a picture where a central input component of the circadian clock is functioning abnormally in BD patients who carry the rs1006737 risk allele (Figure 3.10). The BD-associated SNP rs1006737 is known to be in a regulatory region of the *CACNA1C* gene (Kabir et al., 2017), providing a plausible explanation for the misregulation of rhythmic *CACNA1C* expression seen in BD patient fibroblasts (McCarthy et al., 2016). It cannot be confidently reported that control patients do not show the same *CACNA1C* genotype difference in entrainment as BD patients until larger sample sizes are tested.

The human circadian rhythm disorder FASPS is caused by a mutation in *PER2*, leading to period shortening in animal and cellular modes (Toh et al., 2001, Xu et al., 2005). Therefore, when

put on a recurrent light/dark (or temperature) cycle with daily resetting, short circadian period can manifest as a phase advance. The shortening of period observed in mouse fibroblasts in response to verapamil gives clues to the possible mechanism of circadian phase advancing. Blocking LTCCs and subsequent calcium influx via verapamil for the duration of recording results in a shortening of the rhythmic circadian expression of *per2*. In vitro application of glutamate in the late night to SCN neurons activates $Ca_V1.2$, allowing a calcium influx and resulting in phase advances to the clock. This advance is the result of a single shortened circadian period immediately following the stimulus, where the activity of the clock in the next 24 hours is modified. Thus, the mechanism of phase advancing involves regulation of circadian genes to speed up their rhythm. By the continuous application of verapamil to mouse fibroblasts, the clock in these cells is consistently "phase advancing", resulting in a shortened circadian period for all days of recording compared to controls. In this way, it was as if the verapamil was operating as an entraining stimulus for the clock, informing the circadian period length to consistently be shortened from the FRP. Similar effects have been observed by lowering the extracellular concentration of calcium, thereby reducing the influx mediated by LTCCs (Noguchi et al., 2012).

Previous studies have documented the effect of RyR antagonism on the circadian rhythms of fibroblasts using high concentrations of ryanodine. Noguchi et al. showed that blocking intracellular release of calcium from the ER in vitro resulted in delayed phase of *per2* expression, similar to the results presented in this study (Noguchi et al., 2012). Application of glutamate to SCN slices in the early subjective night results in phase delays of the clock, which may be attenuated by the concurrent application of RyR blocker dantrolene(Ding et al., 1998). Likewise, the long-term application of dantrolene to mouse fibroblasts results in a lengthened period of *per2* expression. In this way, the continuous lowering of intracellular calcium levels by blocking RyR calcium release creates a stable period length longer than the FRP, perhaps due to the consistent delaying signal being relayed to the

molecular clock. It has been established that ryanodine receptor expression is modulated as an output of the circadian clock and is rhythmically expressed in mouse fibroblasts (Aguilar-Roblero et al., 2007, Pfeffer et al., 2009), which highlights that RyRs relay light information to the clock and contribute to the circadian control of their own expression. This autoregulatory mechanism could explain the way light pulses in vivo cause phase adjustments to only the cycle immediately following the stimulus, altering only the phase of expression without changing the circadian period thereafter.

The use of a peripheral cell type for the evaluation of neural mechanisms will always pose the question: Do the results of studies of non-neural tissues accurately represent the molecular happenings of the brain? As previously mentioned, there are many studies that make use of fibroblasts for mechanistic circadian discoveries and the insights gained allow a pointed approach when translating these experiments to more accurate models. A specific advantage of evaluating peripheral oscillators comes into play with the unique challenge of modeling psychiatric disorders. The complex polygenic nature of these neural disorders complicates research progress at the molecular level, but unique solutions such as the use of BD patient fibroblasts overcomes these obstacles. The discoveries presented in this study serve as an example of the capacity of peripheral oscillating models to allow experimentation that is highly reproducible and capable of statistical significance. Further directions for this work could include a more thorough characterization of the response of circadian clock genes to temperature cycles. By better understanding how the clock responds to mediate entrainment in these conditions, it will lead the way for discovery of the mechanism of temperature entrainment. Additionally, studies addressing the role of *CACNA1C* genotype in fibroblast-derived human induced neurons from BD patients will lead the way in furthering the world's understanding of this heterogeneous disorder (Mattis and Svendsen, 2011, Brennand et al., 2012, Viswanath et al., 2015). Induced neurons allow research in the cell type where psychiatric disorders arise, with the genetic information to create an accurate disease state if the cells

are derived from BD patients. Given the common ectodermal origin of both neurons and fibroblasts, there is strength to the developmental accuracy of using fibroblast-derived iPSCs in modeling psychiatric disorders(Rieske et al., 2005). Recent advances in this technique include the ability to differentiate these pluripotent cells into specific neuronal cell types, which is particularly advantageous for the evaluation of mechanisms relevant to BD (Temme et al., 2016). Future potential models include the culture of neural organoids, which could allow the testing of a more physiologically accurate neuronal framework for aspects of psychiatric disorders (Adegbola et al., 2017). Alternatively, there is still an argument for the use of primary human fibroblasts from BD patients as a model for mechanistic discoveries; as such cells would retain the polygenic characteristics of the patient without requiring the level of labor needed to create a sufficient number of iPSCs (Nagoshi et al., 2004).

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