UNIVERSITY OF CALIFORNIA, SAN DIEGO

Circadian Rhythm Amplitude and Neuronal Survival

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

in

Biology

by

Melissa Jayne Le Roux

Committee in charge:

Professor David K. Welsh, Chair
Professor Susan S. Golden, Co-Chair
Professor Nicholas C. Spitzer

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The Thesis of Melissa Jayne Le Roux is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego
2016

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ABSTRACT OF THE THESIS

Circadian Rhythm Amplitude and Neuronal Survival

by

Melissa Jayne Le Roux

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Professor David K. Welsh, Chair

Professor Susan S. Golden, Co-Chair

Bipolar Disorder is a serious neuropsychiatric disorder that causes impairment in mood regulation. Patients with Bipolar Disorder have structural brain atrophy, reduced hippocampal volume, and suffer from disturbances in circadian rhythms. The mood-stabilizer lithium is a mainstream treatment for the disorder, and has been shown to

increase hippocampal gray matter volume in MRI studies. However, the mechanism of this neuroprotective effect is unclear. Lithium is also known to amplify circadian rhythms. Therefore, we sought to investigate whether modulation of circadian rhythm amplitude by molecular or pharmacological means impacts neuronal survival. Using an immortalized hippocampal cell line, we performed bioluminescent reporter assays of *Per2* expression to find drugs that impacted rhythm amplitude, and ran viability assays in parallel to determine the effect of the drug on neuronal survival. We report, with lithium, verapamil, and a small sample of drugs targeting the clock genes REV-ERBα and RORα, that those that increase rhythm amplitude tend to promote neuronal survival. Conversely, we have seen that drugs that decrease rhythm amplitude tend to decrease survival. However, Bmal1 knockdown reduced amplitude but increased neuronal survival. The data suggest that there is overlap between circadian rhythm and cell survival systems, but that the relationship is not simple. Non- specific drug effects may complicate the interpretation of some results. Further studies are needed to clarify the connection.

INTRODUCTION

Introduction to Bipolar Disorder and Lithium

Bipolar Disorder (BD) is a relatively common mood disorder that affects 1-2% of the U.S. population (Goodwin, 2003). The disorder has a strong genetic component, with heritability explaining up to 87% of the variance (Cardno, 1999). Patients with BD experience major shifts in mood, suffering from depressive episodes and periods of abnormally elevated or irritable mood known as mania (DSM-5). The suicide risk in bipolar disorder is among the highest of all psychiatric disorders with an estimated 8-20% lifetime risk, 10-20 times greater than that of the general population (Goodwin, 2003). Currently the mainstream treatment for BD is lithium, a monovalent cation that functions as a mood stabilizer, preventing manic and depressive episodes, and reducing suicide risk (Gonzalez, 2006). Although lithium is considered a first line of treatment for the disorder, the drug has many non-specific effects, and consequently individuals are at risk for a variety of side effects while on the drug, such as weight gain, hyperparathyroidism, nontoxic goiter, subclinical hypothyroidism, tremor and renal failure (Reviewed by Klein, 2001). Furthermore, lithium has a narrow therapeutic index and a high risk of toxicity. It known that lithium has many molecular targets, but it is currently unclear which are responsible for lithium's therapeutic effects (Reviewed by Klein, 2001).

Behavioral Circadian Rhythm Abnormalities in Bipolar Disorder

A circadian rhythm is a rhythmic variation in physiology or behavior of a living organism that recurs with a period of ~24 hours. Circadian rhythms help an organism to anticipate predictable changes in the environment that result from the daily rotation of the earth about its axis. As such, they function to synchronize physiological states such as wakefulness and hunger to environmental events like the light/dark cycles (Ko, 2006). Circadian rhythms are endogenously generated and self-sustained, meaning that they don't require input from the environment to proceed. However, they can be entrained to the environment by "zeitgebers", external cues that synchronizes an organism's internal time keeping mechanisms to the Earth's 24-hour cycle (Toh, 2008). The main zeitgeber is light, but other cues such as temperature, food intake, exercise, and social cues can influence the timing of circadian rhythms (Toh, 2008). Patients with BD have alterations in these normal rhythmic behaviors, often having disrupted sleep and wake cycles, irregular appetite and activity regulation and abnormal rhythms in blood pressure, core body temperature and hormone secretions (Reviewed by McClung, 2008). Furthermore, activities known to disrupt the circadian clock such as shift work and traveling across time-zones tend to increase episodes in mood disorder patients (McClung, 2013).

Actigraphy studies have found a number of circadian phenotypes that are associated with BD. Patients have been reported to have a significantly lower 24-hr activity rhythm amplitude than controls, which indicates their activity is less consolidated and unstable over the course of the day (Jones, 2001/ Salvatore, 2008). Recently, a study by Pagani obtained actigraphy data for members of 26 Costa Rican and Colombian

pedigrees enriched for BD. The group defined 73 activity-related phenotypes, and found 49 of these were heritable. Twelve of these heritable phenotypes (~25%) were significantly associated with BD, with most being related to activity consolidation. The authors found genome-significant linkage for the Interdaily Stability (IS) phenotype, defined as the degree of variability of activity level on an hourly basis from day-to-day (Pagani, 2016). The largest peak was near the terminus of chromosome 12p. The authors suggest several genes in this region could contribute to this phenotype including *CACNA1C*, the alpha subunit of the voltage gated L-type CaV1.2 channel, which has been associated with BD in multiple genome-wide association studies (Cross Disorder Group, 2013/ Ferreira, 2008/ PGCBDWG, 2011).

Clinically, lithium helps correct the circadian rhythm abnormalities in BD (Reviewed by McCarthy 2012). Studies have shown that lithium lengthens the period of behavioral rhythms in humans. For example, lithium was shown to delay circadian phase of temperature and REM sleep in a patient with BD (Kripke, 1989). The same period lengthening effect has been seen in primates, and rodents (Kripke, 1980/ Welsh, 1990).

Molecular Circadian Clock

The central clock is a group of cells in the hypothalamus called the suprachiasmatic nucleus (SCN) (Welsh, 2010). The SCN acts as the "master pacemaker" in the body, and receives direct input from intrinsically photoreceptive retinal ganglion cells (ipRGC) in the retina, so its cells can be entrained to the light/ dark cycle. The cells in the SCN are coupled, meaning that their circadian rhythms are in phase, even in

constant darkness. The coupling of SCN neurons is thought to occur by intercellular signals, and is a unique feature of the SCN that contributes to its robustness as the central clock (Welsh, 2010). The SCN sends a variety of signals, via neural connections or hormones, to synchronize and set the circadian phase of peripheral tissues. This

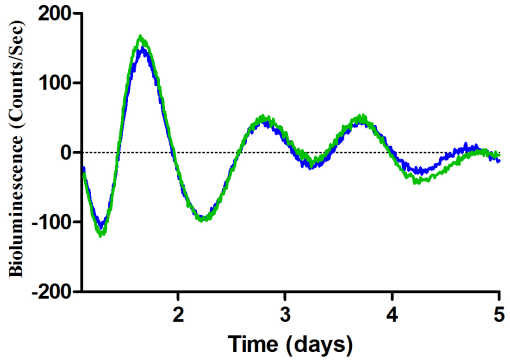

Figure 1: *Per2* rhythms recorded in vitro from two plates (green, blue) of immortalized mouse hippocampal neurons.

coordination of peripheral rhythms allows circadian rhythms to be observed on the whole-animal level, such as behavioral rhythms (Welsh, 2010). Without the input from the SCN, most cells within peripheral tissues lose synchrony.

The molecular time-keeping mechanism in cells, including those in the SCN, is comprised of "clock genes", which form a transcription-translation negative feedback loop (TTFL). These genes oscillate with a period of ~24 hours (Figure 1) and are responsible for circadian rhythms observed at the tissue and behavioral level (Reviewed by Ko, 2006). CLOCK and BMAL1 are transcriptional activators that form the main positive component of the loop (Ko, 2006). CLOCK and BMAL1 dimerize and activate transcription of their target genes, including *Per1, Per2, Cry1, and Cry 2.* The PER and

CRY protein products form the main negative component of the loop. After PER and CRY proteins accumulate in the cytoplasm to a certain level, they dimerize and are transported back into the nucleus, where they inhibit the action of the CLOCK/BMAL dimer, ultimately inhibiting their own transcription (Ko, 2006). Other accessory feedback loops exist and intertwine with the core loop. Notably, BMAL/CLOCK also drive transcription of *Rev-erbα and Rorα*, which form another regulatory loop. REV-ERBα represses, while RORα activates *Bmal1* transcription, providing a mechanism by which BMAL1 rhythm amplitude can be regulated (Mohawk, 2012). In addition to playing a role in the time keeping mechanism, these clock genes also regulate transcription of thousands of genes outside of the core loop called "clock controlled genes". These genes are not directly involved in time keeping, but oscillate rhythmically in a tissue specific manner (Bozek, 2009).

Molecular Circadian Abnormalities in BD

Past research in BD often focused on SCN function because of its role as the

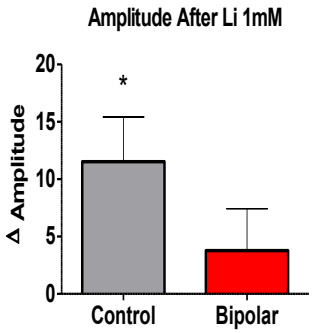

Figure 2: Li amplitude response in BD fibroblasts. Adapted from McCarthy, 2012

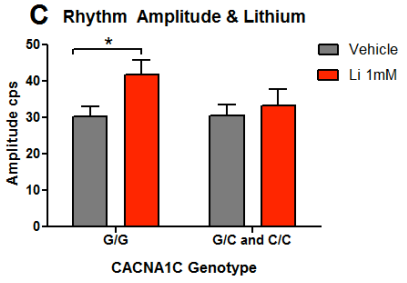

Figure 3: CACNA1C variant predicts Li amplitude response in fibroblasts. Adapted from McCarthy, 2016

"master clock" (Reviewed by McCarthy, 2012). However, while genetic defects in the clock can render tissues arrhythmic, the SCN is more resistant to perturbation due to its network properties (Welsh, 2010/McCarthy, 2012). Liu et al. found *Per1*, *Per2*, and *Cry1* knockouts abolish rhythms in peripheral cells, but the SCN can still sustain rhythms (Liu, 2007). Because of this, researchers have postulated that clocks outside of the SCN, which may be more susceptible to genetic or environmental insults, could be involved in the pathophysiology (McCarthy, 2012). These may include brain areas like the ventral tegmental area, nucleus accumbens, amygdala and hippocampus, regions that are critically involved in mood regulation. Genetic abnormalities involved in BD that impact cellular circadian rhythms could be masked in the SCN by its unique network properties, but may be evident and a cause of pathology in peripheral clocks. Moreover, many brain clocks in mood regulating areas may share features with other peripheral clocks, including those from accessible tissues like skin. Fibroblasts, easily obtainable from patients, have been studied and a number of abnormalities in circadian rhythms have been associated with BD. McCarthy et al (2013). studied the effects of lithium on *PER2* rhythm amplitude in fibroblasts. In vitro, lithium is known to increase *Per2* rhythm amplitude and lengthen *Per2* period in multiple tissues such as the SCN, lung tissues, and fibroblasts (Li, 2012). McCarthy found lithium increased *PER2* rhythm amplitude in control fibroblasts but failed to amplify rhythms in BD fibroblasts (Figure 2). Interestingly, this difference in ability to increase amplitude was linked to *CACNA1C* genotype, suggesting a role for the L-type calcium channel subunit strongly linked to the disorder (McCarthy, 2016). Carriers of the BD associated rs4765913 allele failed to

increase amplitude (Figure 3). Presently, it is not known why there is a difference in ability to increase amplitude in BD or the clinical significance to this finding.

Structural Abnormalities in Bipolar Disorder

In addition to circadian rhythm abnormalities, there are also significant structural changes in BD. A review of morphometric post-mortem brain studies by Gigante et. al. found decreased density of neurons and glia and decreased size of neurons in frontal and subcortical areas in those with BD (Gigante, 2011). A large, recent study with a sample size of over 4000 convincingly demonstrated that patients with BD have significantly reduced hippocampal and thalamic volumes (Hibar, 2016). Based on findings such as these, it is hypothesized that those with BD are more vulnerable to neuronal volume loss, but it is unclear why. Explanations include less dendritic arborization, decreased size of the soma, or perhaps even neuronal death.

One hypothesis proposed is that patients with BD are prone to oxidative stress caused by abnormal glutamate signaling (Gigante, 2011). Over activation of glutamate receptors can result in neuronal damage or death through excitotoxicity, whereby excess activation of N-methyl-D-aspartic acid (NMDA) receptors leads to toxic accumulation of calcium in cells. Calcium triggers activity of kinases such as protein kinase C, and calcium/calmodulin-dependent protein kinase II, as well as phospholipases, and proteases. Overactivity of these enzymes leads to generation of reactive, toxic species, which damage the cell membrane, cytoskeleton, and DNA, eventually causing programmed cell death (Hajek, 2014). Elevated levels of glutamate and associated

metabolites have been found in the plasma in those with BD (Altamura, 1993). Also, MRI studies have shown increased glutamate levels as measured in the whole brain, regardless of the medication status of the individuals (Gigante, 2012). The use of ketamine, an antagonist of the N-methyl-D-aspartic acid (NMDA) receptor subtype of glutamate, as an antidepressant for the treatment of BD supports this theory of abnormal glutamate signaling (Miladinovic, 2015).

Circadian Clock Genes are Linked to Cellular Defense and Death

Many environmental insults including UV ray exposure and temperature are linked to the light-dark cycle. Cellular defense mechanisms against these insults put metabolic demands on cells, and to optimize the use of limited resources, some of these mechanisms are under circadian control, with peak activity at times when they are likely to be needed. For example, in mice epidermal cells, there is a circadian rhythm in nucleotide excision repair, and when the epidermis is exposed to UV-B in the early morning when excision repair function is low, skin cancer develops at a higher rate (Gaddameedhi, 2011). Apoptotic pathways are regulated by the clock. It has been suggested that CRY may play a role in inhibiting p53-independent apoptosis pathways (Lee, 2011), and PER2 negatively regulates the anti-apoptotic protein BCL-2 (Magnone, 2015). Mutations in *Per2* have been found to increase resiliency to oxidative stress, possibly through down-regulation of p53 pro-apoptotic pathways. Per2 has also been found to be a component of the p53 pathway, suggesting a bidirectional interaction (Berns, 2004/Magnone, 2015). The clock also regulates levels of neurotropic factors, which support the survival of and growth of neurons and development of synapses.

BDNF has been found to oscillate rhythmically in the SCN and hippocampus (Liang, 1998/ Berchtold, 1999). In turn, BDNF and NGF impact the clock by regulating circadian phase in SCN (Liang, 1998/Bina, 2006).

Lithium Influences Circadian Rhythm Amplitude and has Neuroprotective Effects

Lithium influences circadian rhythms at both the behavioral and molecular level. As mentioned earlier, in vitro, *lithium increases Per2 rhythm amplitude.* It is unclear which target is responsible for this effect, but lithium inhibits several enzymes involved in clock function including glycogen synthase kinase beta (GSK3β) (Klein) and inositol monophosphotases (IMP) (Hallacher, 1980). GSK3β phosphorylates several clock proteins including BMAL1 (Sahar, 2010), CRY2 (Harada, 2005), PER2 (Iitaka, 2005) and REV- ERBα (Yin, 2006), while preliminary data from our lab suggests signaling through the IP3 pathway is involved in regulating rhythms as well.

Lithium is also known to have complex neuroprotective and neurotrophic effects (Manji, 1999). Chronic lithium treatment increases the levels of BCL-2, a major neuroprotective protein, in rat frontal cortex, hippocampus, and striatum (Manji, 1999). In cerebellar granule cells, lithium decreases levels of p53 and BAX, two pro-apoptotic proteins (Chen, 1999). Lithium has also been found to increase viability of neurons in vitro when faced with a number of toxic conditions including ischemia (Nonaka, 1998/ Wada, 2005), glutamate excitotoxicity (Hashimoto, 2002/ Nonaka, 1999/ Wada, 2005), and growth factor withdrawal (Bhat, 2000/ Wada, 2005).

Lithium's neuroprotective effects are evident in the clinic. Lithium has been shown to increase gray matter volume in patients with BD, and this increase is associated with positive clinical outcomes (Lyoo, 2010). Interestingly, an MRI study by Hajek (2014) found that BD patients who had been treated with lithium had significantly larger hippocampal volumes than controls, while untreated BD patients had the lowest hippocampal volumes.

Are Circadian Rhythm Amplitude and Neuronal Protection Linked?

We hypothesized that lithium's ability to increase circadian rhythm amplitude contributes to its ability to protect neurons. The present study aims to investigate if altering circadian rhythm amplitude through means other than lithium can also modulate neuronal survival. We used two approaches, first drugs that target the clock TTFL such as the REV-ERBα agonist GSK4112 or REV-ERBα antagonist SR8278, which have been shown to increase and decrease rhythm amplitude respectively in fibroblasts (Unpublished). Next, we used drugs that affect rhythms by affecting inputs to the clock. Certain alleles of L-type calcium channel (LTCC) subunits render cells unable to increase circadian rhythm amplitude in response to Lithium (McCarthy, 2016; Fig. 3). It is expected that inhibition of LTCC during conditions of stress such as glutamate excitotoxicity, ischemia, etc. will decrease viability of neurons facing stressors. We expect that decreasing rhythm amplitude will increase vulnerability of neurons to oxidative stress. Conversely, increasing rhythm amplitude will protect cells. We expect rendering the clock arrhythmic with siRNA will increase vulnerability to death.

MATERIALS AND METHODS

Immortalized mouse hippocampal cell line culture

To model conditions in the hippocampus, an important brain region in BD, we used an immortalized embryonic mouse hippocampal E-14 cell line (CLU198), purchased from Cedarlane Cellutions Biosystems Incorporated. This cell line was chosen because it expresses a wide array of hippocampal markers including genes and receptors suggested to play a role in neuroprotection such as BDNF, BDNF-R, AMPA-R3, AMPA-R4, and NMDA-R1 (Gingerich et al. 2010). In our hands, the hippocampal cell line was easy to culture, had efficient transfection rates, and had robust expression of the *Per2*::luc reporter.

The immortalized mouse hippocampal neurons (IMHNs) were grown from frozen cryovials to confluence in 100 mm plates in standard culture media: DMEM (Gibco 11316) with 10% fetal bovine serum (Gibco 10437), glutamine 2 mM (Gibco 25030) and antibiotics penicillin, streptomycin, and amphotericin (Gibco 15140). The cells were maintained in a Nuaire Water-Jacketed IR Autoflow Automatic CO_2 incubator at 37°C and 5% CO_2. The cells were passaged as needed using a 0.0025% Trypsin/0.001% EDTA (R001100 Thermo Scientific) PBS solution to release the cells.

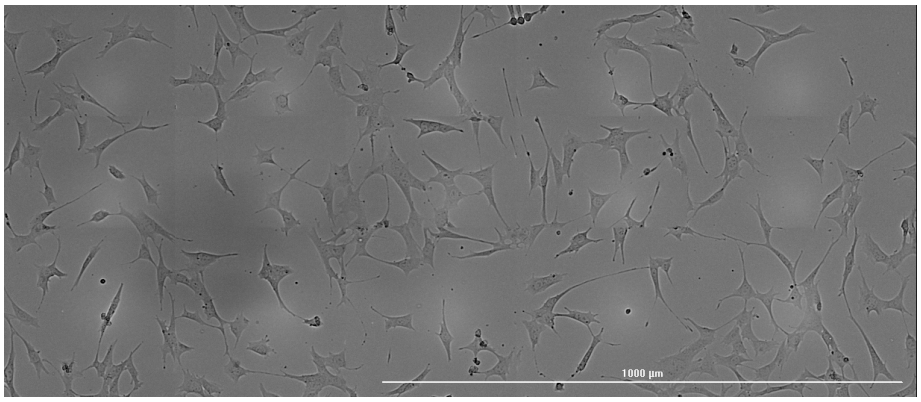

Figure 4: Images of IMHN at 60% confluency taken with a BioTek Cytation 3 imaging reader under a bright-field

Production of an IMHN line stably expressing the Per2::luc circadian reporter gene

In order to produce an IMHN line that stably expressed the bioluminescent *Per2* circadian reporter gene, cells were infected with lentiviral *Per2::luc* and lines were selected for use based on the quality of their rhythms recorded during luminometry screening experiments. The *Per2::luc* reporter construct contains the gene encoding luciferase under the promoter for the *Per2* gene. If integrated successfully, the amount of luciferase expression should correlate with *Per2* expession: luciferase expression will be high when *Per2* expression is high, and vice versa.

Transfection with Per2::luc reporter gene

The cells were transferred to two 6 well plates (35mm wells holding 2ml each) and grown to 50-60% confluence. Viral media was prepared. 20ul of an HIV *Per2::luc* lentivirus (1000x) and 20ul polybrene (1000x) were added to 20mL standard culture media. 2mL of viral medium was added to each well. The cells were allowed to grow to 100% confluence for 2 days.

The *Per2::luc* virus construct contained an antibiotic resistance gene to blasticidin, so blasticidin HCl at 0.01ng/ml (prepared from 10ng/ml stock, Thermo Fisher R21001) was used to select for cells that had integrated the viral DNA. The cells were allowed to grow for another 2 days in the selection medium. Cell survival was assessed visually with a microscope, and cell death was noted in all wells indicating the blasticidin was killing off cells that had not integrated the viral DNA. After 2 days, the medium and dead cells were aspirated and fresh selection medium was added. The cells were allowed to grow to confluence in the 35mm wells and little cell death was noted indicating all of the cells left had integrated the viral DNA. After confluency was achieved, all of the cells in each well were transferred to a 100mm plate (1 plate per well, 12 100mm plates total containing 1 line each). Each line was grown to confluence in the 100mm plate.

Recording of Per2 Expression Rhythms and Selection of Per2::luc Lines for Further Use

When confluent, cells were transferred to 35mm individual dishes for *Per2* rhythm recordings in a luminometer. The cells were grown to 80-90% confluence in the

35mm dish before the experiment. Rhythms were recorded from 35 mm plates over 5 days with a luminometer (Actimetrics). Rhythms were measured using a 24 well plate format luminometer (Actimetrics) over 5 to 7 days. Luminometers were housed in a dry incubator and maintained in room air at 35°C. Immediately before rhythm recording, medium was replaced with "recording medium": 1M HEPES-buffered (Gibco 15630), serum and phenol-red free recording medium supplemented with B-27 (50x Gibco 17504-044) containing 1 mM luciferin (Biosynth International). This medium was needed because the plates were sealed before being placed in the luminometer. The buffering capacity of HEPES is independent of the CO_2 concentration and is added to keep the pH in physiological range.

Photoemissions (counts per second) from each sample were recorded every 10 min over the duration of the experiment and logged automatically for subsequent analyses. To reduce variability, the first 24 h of each experiment were excluded. Background was subtracted using a 24h running-average and data were fit to a damped sine curve by the least squares method using commercial software (Lumicycle Analysis). Rhythm parameters (period, amplitude, goodness-of-fit) were estimated for the rhythm trace obtained from each well.

Two lines (lines #7, #10) out of the 12 that were screened displayed nice *Per2* rhythms; the rhythms were consistent across replicates, period was around ~24 hours, and goodness of fit was >75%. The lines were split, frozen down in multiple cryovials, and used for subsequent luminometry and cell viability experiments

Glutamate stock preparation for cell viability experiments

L-Glutamic acid in powder form (C5H9NO4, MW=147.13) was ordered from Tocris Bioscience (Cat. No. 0218). Stock drug solutions were prepared in 100 mM NaOH.

Preparation of glutamate stock solutions:

150 mg of L-Glutamic acid was weighed into a 15 ml conical tube on an analytical balance, and dissolved in ~4 ml of 150mM NaOH. The solution needed to be heated at 37°C and vortexed vigorously in order for the L-glutamic acid to dissolve. The solution was then transferred to a small 10 mL beaker. The pH of the initial solution measured with a pH meter was ~12. In order to correct the pH, ~1.7ml of 1M HCl was added to solution until the final pH was 7.4. Volume was then adjusted such that the molarity of the final solution was 180mM. The pH-corrected glutamate solution was then filtered at 0.4 microns. 100 ul aliquots were prepared and stored at -20° C for up to one month.

Drug preparations for luminometry and cell viability experiments

All drugs were ordered from Tocris Bioscience, and filtered at 0.4 microns before use. The drugs did not require special preparation as they went into solution easily and did not influence the pH. Vehicle controls were prepared for drugs dissolved in DMSO.

Lithium chloride- purchased from Sigma (CAS Number 7447-41-8). A 1M stock was prepared in dIH_2O.

GSK4112 (Cat. No. 3663) – 25mM stock was prepared in DMSO

SR8278 (Cat. No. 4463)- 25mM stock was prepared in DMSO.

SR1078 (Cat. No. 4874)- 23mM stock was prepared in DMSO.

SR1001 (Cat. No. 4868)- 20mM stock was prepared in DMSO.

Verapamil hydrochloride (Cat. No. 0654)- 10mM stock prepared in dIH_2O.

SiRNA preparations/transfection for luminometry and cell viability experiments

SiRNA, transfection reagent (DharmaFECT1, Catalog #T200102), and OptiMEM (Gibco 31985070) were ordered from GE Healthcare. siRNA pools (SMARTpool), that bind to four distinct sites within a transcript, were used to maximize knockdown. The siRNA pools used were *Arntl* (*Bmal1*) siRNA (M-040483-01-0005) and a non-targeting negative control siRNA (D-001206-14-05). siRNA pool stocks were prepared at 20uM with RNA H_2O according to the manufacturers protocol.

On transfection day, OptiMEM was warmed to 37C, siRNA was diluted to 5uM with RNA H_2O. Then, transfection solutions of 11ul siRNA, 4.4 ul reagent, and 440ul

OptiMEM were prepared. The solutions were mixed and incubated at room temperature for 20 minutes. Then, 1760ul of DMEM was added to each tranfection tube. The final concentration of siRNA was 0.025uM, and the reagent was at 0.2%. 200ul of the respective tranfection solution was added to each well, and cells were allowed to grow in the transfection solution for two days.

Luminometer Experiments

Experiments were performed to assess the effect of each drug on *Per2::luciferase* expression amplitude, using a luminometer to record luminescence. The luminescence was caused by activity of the enzyme on its substrate luciferin.

Luminometry procedure for Lithium experiments

IMHNs were plated at $1.5x10^4$/ml into a 24 well plate (500ul per well). The next day, the neurons were treated with 1 or 10mM lithium. The neurons were allowed to incubate in the media for two days. On the day of the experiment, cells were synchronized with 0.5uM dexamethasone in standard culture medium for 1 hour. Immediately afterwards and preceding rhythm recording, the medium was changed to recording medium with 1 or 10mM lithium. The plates were sealed and rhythms were recorded as described in the "Recording of *Per2* Expression Rhythms" section.

Luminometry data were analyzed for amplitude using a non-linear least-square minimization. Although amplitude is the main parameter measured and presented in this thesis, period and goodness-of-fit were estimated as well.

Luminometry procedure for GSK4112, SR8278, SR1078, SR1001, and verapamil experiments

IMHNs were plated at $5.0x10^4$/ml into a 24 well plate (500ul per well). On the day of the experiment, cells were synchronized with 0.5uM dexamethasone in standard culture media for 1 hour. Immediately afterwards and preceding rhythm recording, neurons were treated with drugs for respective experiment at various concentrations made in recording media (see results section), plates were sealed, and put directly into the luminometer for rhythm recordings. The rhythms were recorded as described in the "*Recording of Per2 Expression Rhythms*" section. The rhythms were analyzed as described in the "*Luminometry procedure for Lithium experiment"* section.

DMSO solvent controls were prepared for all drugs except verapamil (dissolved in dH_2O).

Luminometry procedure for Bmal1 siRNA experiments

IMHN were plated at $1.0x10^4$/ml into a 24 well plate (500ul per well). The next day, at ~40-50% confluency cells were treated with respective siRNA as described under

siRNA preparations/transfection. The cells were allowed to incubate for two days in the transfection media. On the day of the experiment, cells were synchronized with 0.5uM dexamethasone in standard culture medium for 1 hour. Afterwards and immediately preceding rhythm recording, the media was changed to recording media and the plates were sealed. The rhythms were recorded as described in the luminescence section. The rhythms were analyzed as described in the "*Luminometry procedure for Lithium experiment"* section.

Cell Viability Assays

Experiments were performed to assess the effect of each drug on IMHN viability during excitotoxicity. To measure cell viability, we used an enzymatic assay that determines the number of viable cells in culture based on the quantification of ATP indicating the presence of metabolically active cells. ATP is used to drive a luciferase reaction producing luminescence.

Viability assay reagent preparation

The commercially available CellTiter-GLO Luminescent Cell Viability Assay Kit (Cat.# G7570) was ordered from Promega. The reagent was prepared according to the manufacturer's instructions, and stored at -20°C for up to one month before use.

Cell viability procedure for lithium chloride experiment

IMHNs were plated at $1.0x10^4$/ml into 96 well plates (200 ul per well). The next day, IMHN were treated with 1 or 10 mM lithium dissolved in cell culture media. Cells were then grown in presence of lithium or vehicle for two days. On day four, solutions containing 5mM glutamate with either vehicle or lithium (1 or 10mM) were prepared. The solutions were added to the appropriate wells, and allowed to incubate for 18 hours.

After 18 hours, the medium was removed, and 50ul cell culture medium (free of drug/glutamate) was added to each well. The cells were allowed to equilibrate at room temperature for 20 minutes. Then, 50ul of the Cell Titer Glo reagent was added.

The plates were read in a BioTek Cytation 3 imaging reader equipped with Gen 5 image 2.07 software. The contents of the plate were mixed for 2 minutes on an orbital shaker to induce cell lysis. The plate was then allowed to incubate at room temperature for 10 minutes to stabilize the luminescent signal. The luminescence from each well was then recorded by the reader. A high reading indicated more metabolically active cells, and higher viability in a well.

Cell viability procedure for GSK4112, SR8278, SR1078, SR1001, and verapamil experiments

IMHNs were plated at 2.0x104/ml into 96 well plates (200ul per well). The next day, neurons were treated with drugs for respective experiment at various concentrations (see results), with or without glutamate.

DMSO solvent controls were prepared for all drugs except verapamil (dissolved in dH_2O).

The cells were allowed to incubate in the media for 18 hours. Then, the procedure was repeated as described above with lithium.

Cell viability procedure for Bmal1 siRNA experiment

IMHNs were plated at $1.0x10^4$/ml into 96 well plates (200ul per well). The next day, neurons were treated with siRNA as described earlier. The neurons were allowed to incubate in the siRNA media for 2 days. On day four, the neurons were treated with 3mM glutamate. The cells were incubated in this solution for 24 hours. Then, the procedure was repeated as described above with lithium.

RESULTS

Lithium Increases Rhythm Amplitude in Immortalized Mouse Hippocampal Neurons (IMHNs)

To validate that lithium increases rhythm amplitude in the IMHN line, neurons were treated with lithium two days prior to and during *Per2::luc* rhythm recordings. 10 mM lithium increased mean amplitude by 81% compared to the control. A one-way ANOVA revealed a significant effect of lithium ($p < 0.05$). The amplitude increase is consistent with studies performed in human (McCarthy, 2013) and mouse (McCarthy, 2016) fibroblasts, although human fibroblasts seem to require a lower concentration (1 mM) of lithium than IMHNs or mouse fibroblasts to achieve a significant amplitude increase.

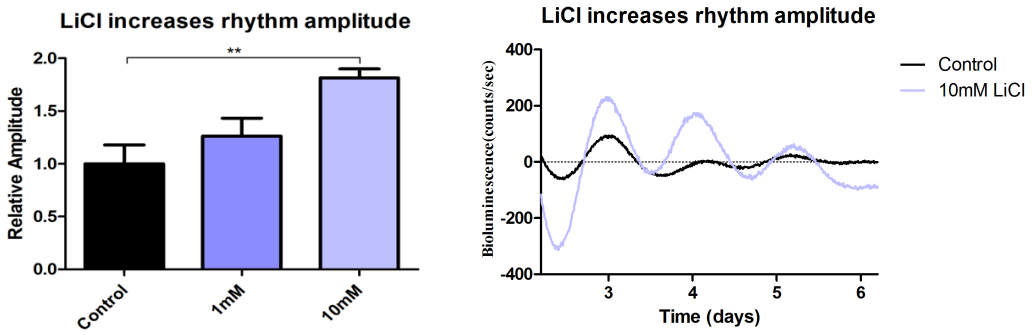

Figure 5: Lithium chloride increases rhythm amplitude in IMHNs. A Normalized mean amplitude was significantly higher than the control at the 10 mM concentration. (post hoc t-test $p<0.05$). N=6 wells for each condition. B Representative rhythm traces of a control (black) and 10 mM lithium treated well (purple).

Lithium increases viability of IMHNs during excitotoxicity

To determine if lithium has a neuroprotective effect in IMHNs, the neurons were treated with lithium two days prior to and during an 18-hour glutamate shock that is toxic to the cells. Viability was measured by an ATP sensitive enzymatic assay. The neurons were treated with lithium during an 18-hour glutamate shock. Glutamate (5 mM) decreased mean viability by 73% compared to the control condition. Mean viability increased by 1.4x when glutamate and lithium (1 mM) were added concomitantly. A two-way ANOVA reveals main effects of glutamate and lithium on viability, with a significant interaction effect, indicating that lithium improved the viability of glutamate-treated cells (p=0.05). The neuroprotective effect of lithium seen in IMHNs is consistent with that seen in rat cerebral cortical neurons (Hashimoto, 2002) and cerebellar granule cells (Nonaka, 1998).

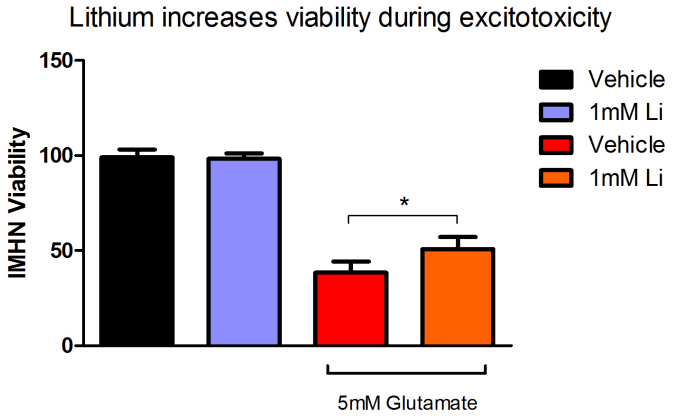

Figure 6: Lithium increases viability of IMHNs during excitotoxicity. Normalized mean viability was decreased significantly by 73% with 5 mM glutamate (post hoc t-test p<0.05) .When lithium (1 mM) was added concomitantly, mean viability increased by 11% (post hoc t-test p=.05). Analyzed by two-way ANOVA. N=10 wells of each condition.

REV-ERBα agonist GSK4112 increases rhythm amplitude in IMHNs

Lithium has many non-specific effects that may complicate the interpretation of its effects on rhythms. To minimize these non-specific effects on amplitude, we aimed to increase rhythm amplitude by targeting the clock more directly. GSK4112, an agonist of the clock protein REV-ERBα, was screened for amplitude effects. The drug has previously been shown to increase *Per2::luc* rhythm amplitude in mouse 3T3 fibroblasts (unpublished). GSK4112 increased mean amplitude by 35% at the 10 uM concentration in the IMHNs. IMHNs were treated with GSK4112 during *Per2::luc* rhythm recordings. A one-way ANOVA revealed a significant concentration dependent effect of GSK4112 ($p<0.05$) on amplitude.

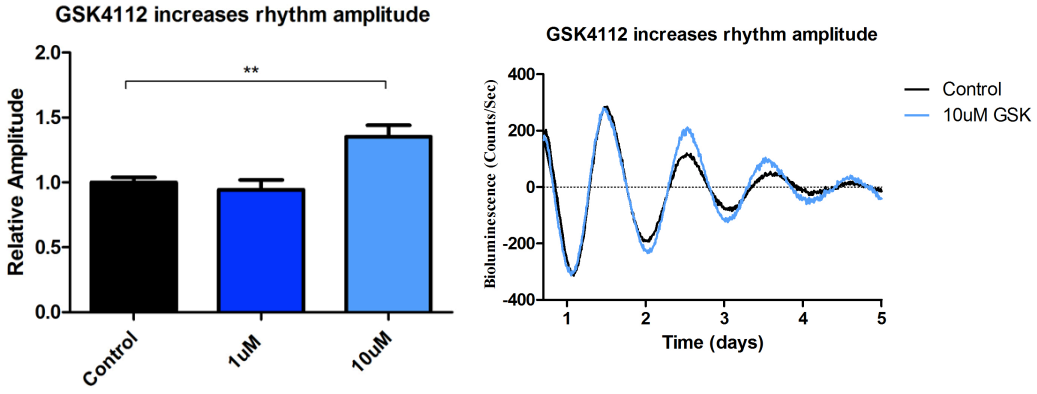

Figure 7: GSK4112 increases rhythm amplitude in IMHNs. A Normalized mean amplitude was increased significantly at the 10 uM concentration compared to the control (post-hoc t-test, $p<0.05$). N=8 wells of each condition. B Representative rhythm traces of a control (black) and 10 uM GSK4112 well (blue).

REV-ERBα agonist GSK4112 increases viability during excitotoxicity

Because GSK4112 increased rhythm amplitude in the IMHNs, we wanted to determine if the drug would increase viability similar to lithium. The neurons were treated with GSK4112 during an 18-hour glutamate shock. Glutamate decreased mean viability by 87% compared to the control condition. Mean viability increased by 2.4x when glutamate and GSK4112 (10 uM) were added concomitantly. A two-way ANOVA reveals main effects of glutamate (5 mM) and GSK4112 on viability, with a significant interaction effect, indicating that GSK4112 improved the viability of glutamate-treated cells ($p<0.05$).

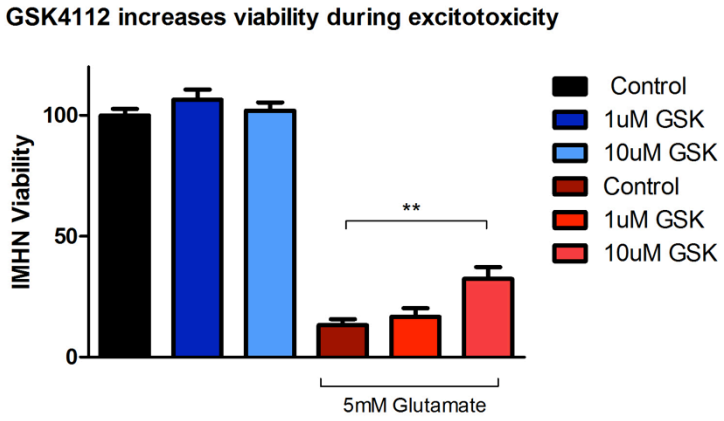

Figure 8: REV-ERBα agonist GSK4112 increases viability of IMHNs during excitotoxicity. Normalized mean viability was decreased significantly by 87% with 5 mM glutamate (post-hoc t-test, $p<0.05$). When GSK4112 (10 uM) was added concomitantly, mean viability increased significantly by 19% (post-hoc t-test, $p<0.05$). N=12 wells of each condition.

REV-ERBα antagonist SR8278 decreases rhythm amplitude

Because increased rhythm amplitude seemed to make IMHNs more resistant to excitotoxicty, we expected that decreasing rhythm amplitude would show the opposite effect, and make them more vulnerable. The REV-ERBα antagonist SR8278 was screened for effects on rhythm amplitude, and was expected to decrease amplitude as the drug has an opposite effect of GSK4112 (the REV-ERBα agonist). SR8278 has previously been found to decrease rhythm amplitude in NIH3T3 fibroblasts (unpublished). IMHNs were treated with SR8278 during *Per2*::luc rhythm recordings. There was no significant effect of the drug on mean rhythm amplitude at the 1 uM concentration. However, SR8278 decreased mean amplitude significantly by 14% at the 10 uM concentration (t-test, $p<0.05$).

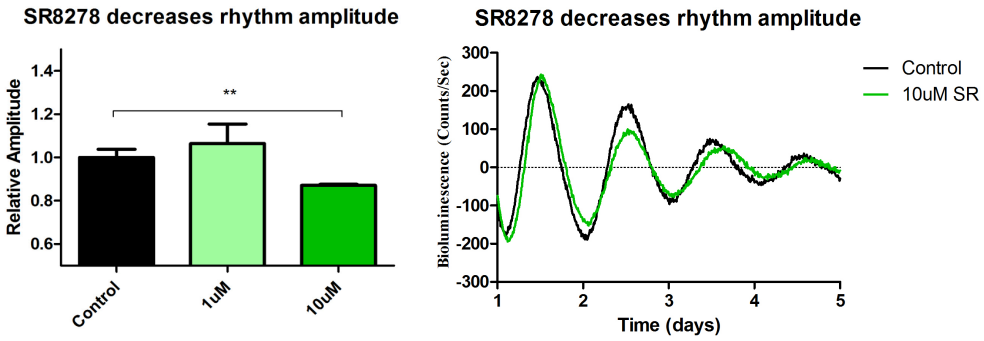

Figure 9: SR8278 decreases rhythm amplitude in IMHNs. A Normalized mean amplitude was increased significantly at the 10 uM concentration compared to the control (t-test, p<0.05). N=4 wells of each condition. B Representative rhythm traces of a control (black) and 10 uM SR8278 well (green).

REV-ERBα antagonist increases vulnerability during excitotoxicity

To determine if decreased rhythm amplitude would increase vulnerability of IMHN to excitotoxicity, the neurons were treated with SR8278 during an 18-hour glutamate shock. Glutamate (1 mM) decreased mean viability significantly by 23%. The viability was decreased by 2.3x when glutamate and 10 uM SR8278 were applied concomitantly. A two-way ANOVA revealed main effects on viability of glutamate and SR8278. Again there was a significant interaction, with the effects of SR8278 on viability primarily affecting the glutamate-treated cells ($p<0.05$).

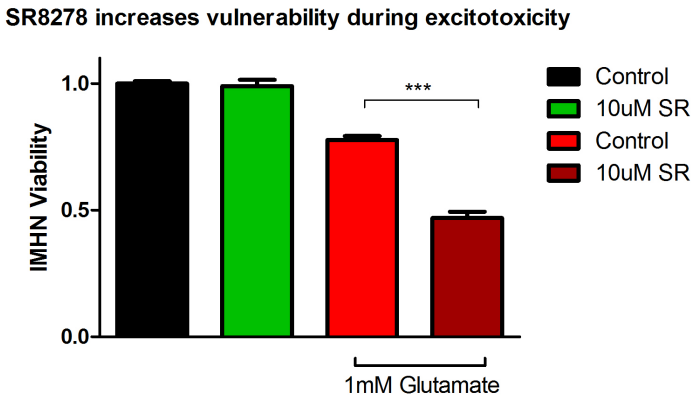

Figure 10: REV-ERBα antagonist SR8278 decreases viability of IMHNs during excitotoxicity. Normalized mean viability was decreased by 23% with 1 mM glutamate (post hoc t-test, $p<0.05$). When SR8278 (10 uM) was added concomitantly, mean viability decreased significantly by an additional 30%(post hoc t-test, $p<0.05$). N=3 wells of each condition.

RORα /γ inverse agonist SR1001 does not significantly affect rhythm amplitude

We aimed to modulate rhythm amplitude with drugs chemically distinct from the REV-ERBα agonist/antagonists. SR1001, an inverse agonist of the clock protein RORα, was screened for amplitude effects. IMHNs were treated with SR1001 during *Per2* rhythm recordings. Mean rhythm amplitude was not changed by the addition of 2 or 10 uM SR1001. A one-way ANOVA revealed there was no significant change in rhythm amplitude (p>>0.05).

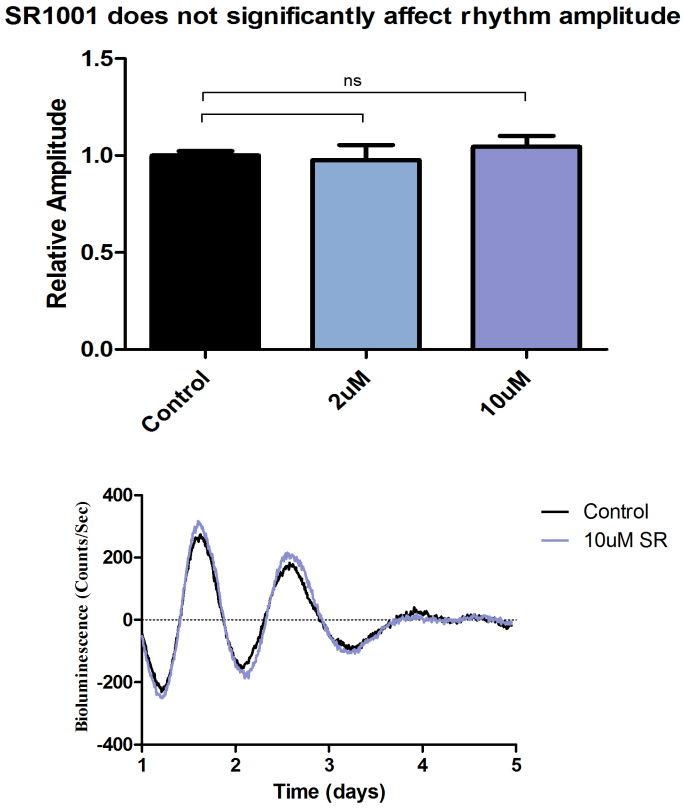

Figure 11: SR1001 does not significantly affect rhythm amplitude. A Normalized mean amplitude was not significantly affected at 2 or 10 uM (One-way ANOVA, p>>0.05). N=7 wells of each condition. B Representative traces of a control (black) and 10 uM SR1001 well (purple).

RORα /γ inverse agonist SR1001 does not significantly affect IMHN viability

Although SR1001 did not affect rhythm amplitude significantly, we wanted to screen the drug for effects on viability. IMHN were treated with SR1001 during an 18-hour glutamate treatment. Glutamate (3 mM) decreased IMHN viability by 90%. SR1001 did not significantly affect mean viability at any concentration when added concomitantly with glutamate. A two-way ANOVA revealed a main effect of glutamate only ($p<.05$). There was no significant glutamate x SR1001 interaction ($p>>.05$).

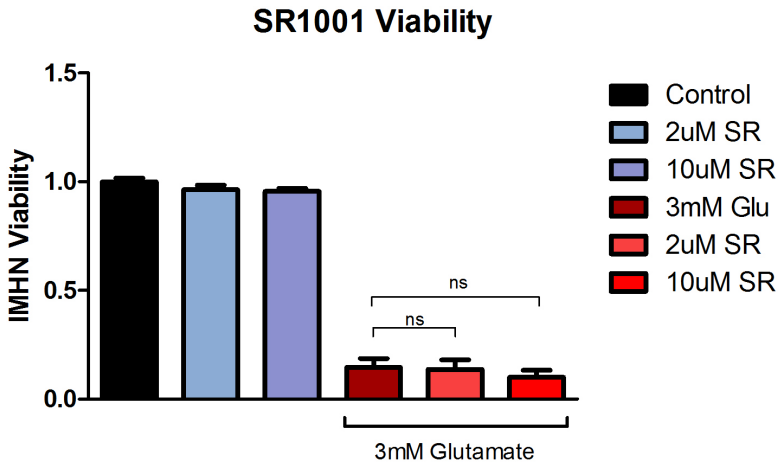

Figure 12: SR1001 does not significantly affect IMHN viability. Glutamate reduced viability significantly. SR1001 did not significantly affect mean viability when added concomitantly with glutamate (analyzed by two-way ANOVA, p>>.05). N=9 wells of each condition

RORα /γ agonist SR1078 decreases IMHN rhythm amplitude

The RORα/γ agonist SR1078 was screened for amplitude effects in IMHNs. IMHNs were treated with SR1078 during *Per2::luc* rhythm recordings. Mean rhythm amplitude was decreased at both the 2 and 10 uM concentration by 20 and 80% respectively. A one-way ANOVA revealed a significant effect of SR1078 ($p<.05$) on amplitude. However, there was suspected toxicity at the 10 uM concentration because of the very low rhythm counts. This was confirmed in the viability assay (see SR1078 viability section), so the decrease in rhythm amplitude at this concentration was likely due to reduced live neurons in the plate.

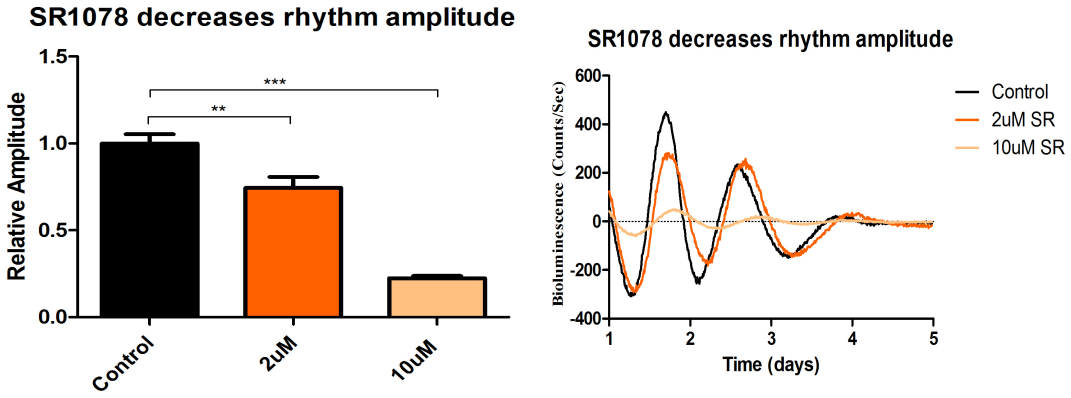

Figure 13: RORα /γ agonist SR1078 decreases IMHN rhythm amplitude. A Mean rhythm amplitude was decreased significantly at the 2 and 10 uM concentration (post hoc t-test, p<.05). N= 8 wells of each condition. B Representative rhythm traces of control (black), 2uM (orange), and 10uM (tan) wells.

RORα /γ agonist SR1078 does not have a significant effect on IMHN viability

Because SR1078 acts in a mechanism distinct from REV-ERB activity, and decreased rhythm amplitude, we were interested to know if the drug would affect IMHN survival during excitotoxicity. The neurons were treated with SR1078 during an 18-hour glutamate shock. Glutamate (1 mM) reduced mean viability significantly by 18% compared to untreated cells. When glutamate and 2 uM SR1078 were added concomitantly, mean viability was reduced slightly by an additional 5%. However, a two-way ANOVA revealed a main effect of glutamate only ($p<.05$), and no glutamate x SR1078 interaction ($p>>.05$). We confirmed that the 10 uM SR1078 was toxic to the neurons on its own (see SR1078 rhythms section), reducing viability by 15%, so the data was excluded from analysis.

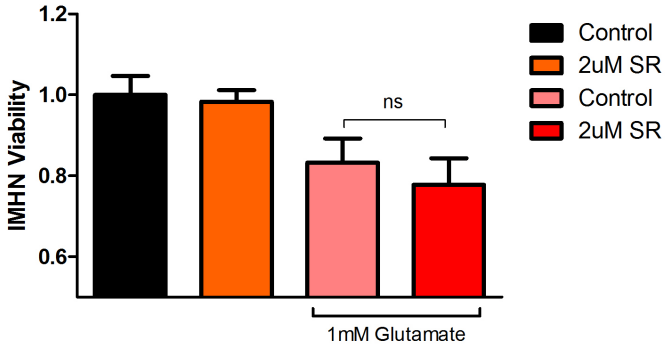

Figure 14: SR1078 does not have a significant effect on IMHN viability. Viability was decreased significantly by 18% with glutamate ($p<.05$). Mean viability decreased by an additional 5% when glutamate and SR1078 were added concomitantly to culture, but the interaction was not significant (analyzed by two-way AVOVA, $p>>.05$). N= 7 wells of each condition

LTCC Antagonist Verapamil decreases rhythm amplitude

Variants in LTCC subunits have been shown to be associated with BD and contribute to the molecular circadian abnormalities seen in BD fibroblasts, and in particular may regulate amplitude (McCarthy, 2016). Because of the association, verapamil, an LTCC blocker, was screened for amplitude effects in IMHNs. IMHNs were treated with verapamil during *Per2::luc* rhythm recordings. Verapamil decreased mean amplitude by 33% at a concentration of 1 uM, and by 39% at 10 uM. A one-way ANOVA revealed a significant effect of verapamil ($p<.05$). This amplitude finding is in agreement with that seen in other cells lines including NIH3T3 and human fibroblasts with verapamil, and the related LTCC blocker, diltiazem (McCarthy, 2016).

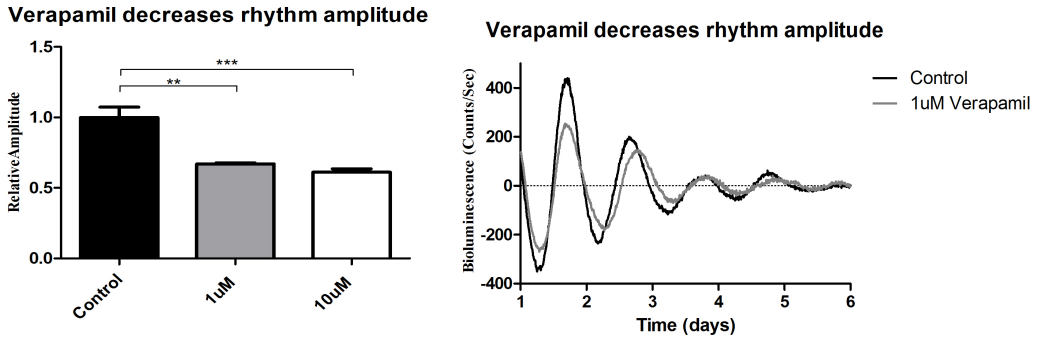

Figure 15: LTCC antagonist Verapamil decreases rhythm amplitude in IMHNs. A Normalized mean amplitude was decreased significantly at both the 1 and 10 uM concentration compared to the control (post hoc t-tests, p<.05). N=4 wells of each condition. B Representative rhythm traces of a control (black) and 1uM Verapamil well (gray).

LTCC Antagonist Verapamil increases vulnerability during excitotoxicity

Verapamil presented an interesting test of our hypothesis regarding amplitude and neuronal survival. On the one hand, verapamil decreases rhythm amplitude and so it may be expected to make cells vulnerable. On the other, it could also be expected to increase viability because excess calcium influx is thought to mediate excitotoxicity. Glutamate (3 mM) decreased mean viability significantly by 73% compared to the control neurons . Verapamil (1 uM) alone had no significant effect on mean viability. However, viability was decreased significantly by 1.14x when verapamil was added concomitantly with glutamate (post hoc t-test, $p<.05$). Statistical analyses showed that there were significant main effects on viability of verapamil and glutamate (Two-way ANOVA, $p<.05$. There was a statistical trend towards an interaction although the effect was not quite significant ($p=.08$). Therefore, in support of the amplitude hypothesis of cell survival, verapamil decreased viability during excitotoxicity.

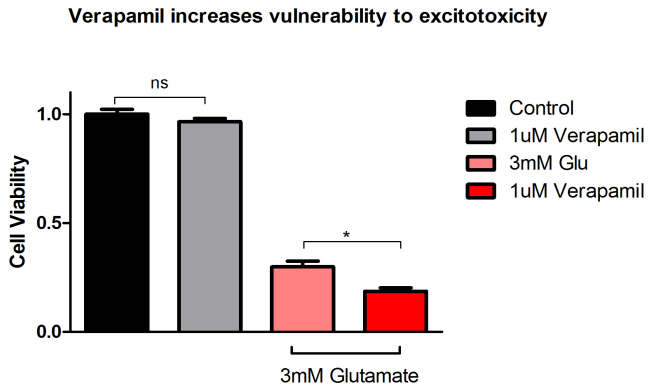

Figure 16: LTCC blocker verapamil decreases viability of IMHNs during excitotoxicity. Normalized mean viability was decreased by 73% with 3 mM glutamate (post hoc t test, p<.05). When verapamil (1 uM) was added concomitantly, mean viability decreased significantly by 10%(post hoc t-test, p<.05). N=6 wells of each condition.

Knockdown of *Bmal1* reduces rhythm amplitude

Even though pharmacological modulation of REV-ERB/ROR may be more precise than lithium, off target effects of these drugs could contribute to their effects. Therefore, to reduce amplitude by more precise, non-pharmacological means, we knocked down the expression of BMAL1, a clock gene essential for sustaining rhythms (Bunger, 2000), using siRNA. *Bmal1* siRNA decreased mean rhythm amplitude significantly by 63% compared to the negative control (NC) siRNA (t-test, $p<.05$).

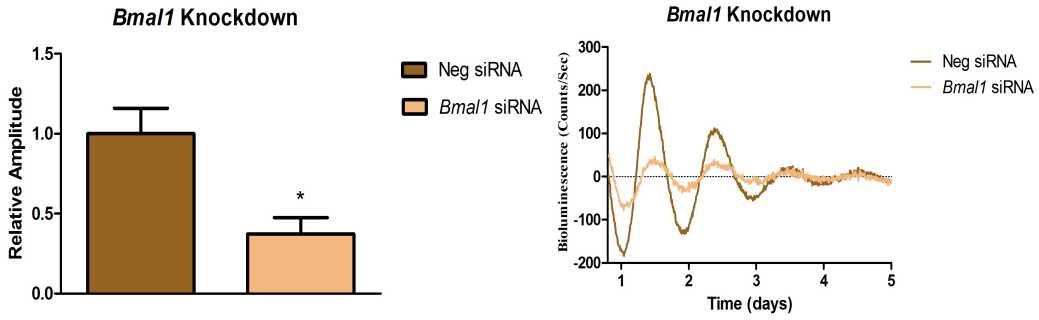

Figure 17: *Bmal1* siRNA decreases rhythm amplitude in IMHNs. A Normalized mean amplitude was decreased significantly compared to the negative siRNA control (students t-test, p<.05). N=3 wells of each condition. B Representative rhythm traces of a negative siRNA control (brown) and *Bmal1* siRNA (peach) well.

Knockdown of *Bmal1* increases viability during excitotoxicity

Based on the amplitude results of the drug studies, we expected treatment with *Bmal1* siRNA to decrease viability during excitotoxicity. Addition of glutamate (5mM) decreased viability significantly by 25% compared to cells treated with NC siRNA. In contrast to our expectations, mean viability was significantly improved by 1.14x when *Bmal1* siRNA was added concomitantly with glutamate. A two-way ANOVA revealed a main effect of glutamate only: *Bmal1* siRNA did not have a significant effect on mean viability on its own compared to the NC siRNA. A *Bmal1* by glutamate interaction was almost significant at p=.05.

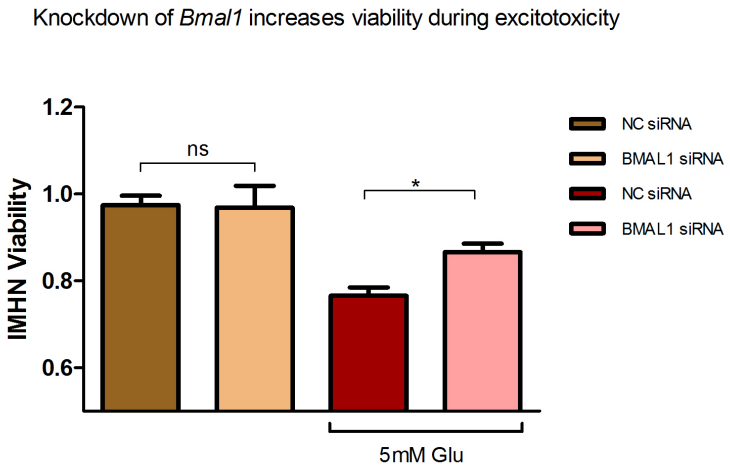

Figure 18: *Bmal1* siRNA increases viability of IMHNs during excitotoxicity. Normalized mean viability was decreased significantly by 5mM glutamate (post hoc t test, p<.05) When *Bmal1* siRNA was added concomitantly, mean viability was rescued significantly by 10% (post hoc t-test, p<.05). N=6 wells of each condition.

DISCUSSION

Major findings of study

Our aim was to investigate whether rhythm amplitude was correlated with neuronal survival in the context of glutamate excitotoxicity. We found lithium increased *Per2* rhythm amplitude in IMHNs, which is consistent with findings in other models such as mouse and human fibroblasts (McCarthy, 2013), and mouse lung fibroblasts (Li, 2012). Lithium also promoted neuroprotection against excitotoxicity in these IMHNs, consistent with other in vitro studies utilizing neuroblasts (Chuang, 2004), cerebral corticol neurons (Chuang, 2002), and striatum (Senatorov, 2004). The protective effect of lithium seems to generalize to other stressors; Lithium increases resistance of neurons to hypoxia-ischemia (Li, 2010), protects mitochondria from stressors like methamphetamine (Bachmann, 2009), and inhibits secondary injurious calcium overload in response to glycolytic inhibition (Bosche, 2013). Despite previous rhythm and viability studies with lithium, our study was the first that measured them in the same cell type under similar conditions allowing increased rhythm and viability to be correlated.

Lithium is a very non-specific drug and has many molecular targets (Klein, 2001), which could be influencing viability. We wanted to isolate amplitude as a variable, so we used drugs with pharmacological profiles distinct from lithium to modulate amplitude, and then used these same drugs in viability assays. Our initial hypothesis was that drugs

which increased rhythm amplitude would promote neuroprotection, while drugs that decreased rhythm amplitude would render the neurons more vulnerable to excitotoxicity. In support of the hypothesis, there was a correlation of *Per2* rhythm amplitude and protection against excitotoxicity with the drugs that targeted the REV-ERBα receptor; the agonist GSK4112 increased rhythm amplitude and cell viability, while the antagonist SR8278 decreased rhythm amplitude and decreased cell viability. Similarly, verapamil, the LTCC antagonist, decreased rhythm amplitude and decreased cell survival. Drugs that targeted the RORα receptor produced more ambiguous results, and did not consistently show as strong a correlation. The antagonist SR1001 did not have a significant effect on rhythm amplitude or viability, while the agonist SR1078 decreased rhythm amplitude, but had only a weak, if any effect on viability. Finally, we used *Bmal1* siRNA to weaken rhythm amplitude. Based on the reduction in amplitude caused by this treatment, we expected the siRNA to increase the vulnerability of the neurons to the stressor. However, *Bmal1* knockdown caused a significant increase in viability. These results are summarized in figure 19.

In considering the data together, support for the hypothesis is mixed. The pharmacological studies do lend support to the hypothesis that increased rhythm amplitude is neuroprotective, and that decreased rhythm amplitude renders neurons vulnerable to cell death. However, the results from siRNA studies were in disagreement with this conclusion as knockdown of rhythm amplitude with *Bmal1* siRNA increased viability. These discordant findings suggest that the relationship between rhythm

amplitude and cellular survival is more complex than our hypotheses presupposed. At the onset of the project, we assumed *Per2*::luc expression amplitude would reflect the circadian network amplitude, but this may not be the case. Nonetheless, we suggest *Bmal1* has a role in reduction of viability, and calcium influx through LTCC may play a neuroprotective role.

Drug	Rhythm Amplitude	Viability	Agreement with Hypothesis
Lithium	↑	↑	YES
GSK4112	↑	↑	YES
SR8278	↓	↓	YES
Verapamil	↓	↓	YES
SR1001	No effect	No effect	YES
SR1078	↓	No effect	?
Bmal1 siRNA	↓	↑	NO

Figure 19: Summary of amplitude and viability findings in IMHNs

Limitations of study

Despite our attempts to select drugs that target the clock directly, the drugs could have off-target effects that impact viability unrelated to their ability to modulate rhythm

amplitude. ROR and REV-ERB binding sites are not just found at the *Bmal1* promoter, but in many different genes, some of which are "clock controlled", but do not directly influence the clock itself (Duez, 2009). For example, RORα and REV-ERBα are involved in controlling many genes that influence cellular metabolism (Duez, 2009). The drug could be affecting these clock-controlled genes, which may impact viability, and at the same time influence expression of genes that modulate circadian amplitude like *Bmal1*. In other words, the drugs could modulate rhythm amplitude and cell survival through independent pathways. We found a correlation between amplitude and viability with a relatively small set of drugs (four) drugs, so the generalizability of the findings remains uncertain. The RORα/γ agonist SR1078 did decrease rhythm amplitude but did not impact neuronal survival leaving this possibility open. Repetition of our procedure with a greater number drugs would be helpful in improving the validity of our conclusions.

Rora (encoding RORα) and *Rorc* (encoding RORγ) are not highly expressed in the mouse dentate gyrus (Allen Brain Atlas), which is the orgin of the IMHN cell line. This may be reason for the low response to SR1001 and SR1078, which target RORα/γ receptors but not the RORβ receptor (which is expressed in the dentate gyrus). It would have been helpful to use drugs targeting RORβ in this line. No synthetic RORβ ligands have been developed, but it is known that all-*trans* retinoic acid and stearic acid both bind to the receptor specifically, and could potentially be used to target the receptor (Kojetin, 2011).

Lithium is known to lengthen the period of molecular and behavioral circadian rhythms, and this period-lengthening effect could play a role in lithium's neuroprotective properties. Therefore, other circadian rhythm parameters may also affect cell survival during excitotoxic stress. For these studies, we chose drugs that selectively modulate amplitude, but some of the drugs that we were using also affected the period and phase of circadian rhythms. These two properties were not analyzed in detail, and further work will need to be done to determine if they contribute to neuronal survival.

Reassessing the Assumptions Underlying Circadian Rhythm Amplitude

At the outset of this project, we assumed that because the core clock genes are interconnected in a coordinated network, the expression of a particular clock gene, in this case *Per2* would generalize to the amplitude of other clock genes like *Bmal1;* and that an increase in *Per2* amplitude would also signify an increase *Bmal1* amplitude, and the overall rhythm amplitude of the cell. However a survey of recent circadian literature regarding the drugs we used revealed this assumption may be too simplistic and that circadian rhythm amplitude is more than the robustness in expression oscillation of a single gene. There is evidence that lithium differentially regulates *Bmal1* and *Per2* expression. Osland et. al (2011) found in mouse fibroblasts that lithium increased rhythmic expression of *Per2* and *Cry1*, and decreased expression of *Bmal1*, *Per3*, and *Cry2*. Similarly, in studies examining the drugs used in our studies, effects differ depending on the reporter employed. We found in IMHNS and NIH3T3 cells (not shown)

that the REV-ERBα agonist GSK4112 increased *Per2::luc* expression amplitude. However, the same drug has been found to decrease *Bmal1::Luc* amplitude in Rat-1 fibroblasts (Meng et al 2008), and in in HEK293 cells (Kojetin 2011). Likewise, we found the REV-ERBα antagonist SR8278 decreased *Per2::luc* expression in IMHNS and NIH3T3 cells (not shown), but it has been found to increase *Bmal1::luc* expression in HEK293 cells (Kojetin 2011). These *Bmal1::luc* findings are consistent with past evidence suggesting that REV-ERBα inhibits *Bmal1* expression (Guillaumond 2005).

Taken together, the opposing *Bmal1*::luc and *Per2*::luc findings with each of the drugs illustrate the complexity of regulatory factors affecting the circadian clock, and suggest we cannot generalize the relative amplitude of one clock gene such as *Per2* to that of the other clock genes. Drugs that increase the amplitude of *Per2* expression could cause a reduction in *Bmal1* expression amplitude because of the negative regulatory role of PER2. Furthermore, we cannot generalize the amplitude of one clock gene to the entire clock network. For example, REV-ERBα has been suggested to be a repressor of the entire clock network and damps rhythmic processes like liver metabolism at the whole-animal level (Zhao, 2016), but our *Per2*::luc results would suggest otherwise as the agonist increased amplitude, while the antagonist decreased amplitude. It may be more logical to assess circadian network amplitude by measuring a clock output that reflects the activity of a number of clock genes. Many clock genes have roles in processes like viability, and the sum of the effects of each likely produces the phenotype. Measurement of a clock output would likely be better representation of the amplitude of the entire clock network.

Role of Clock Gene Bmal1 in Neuronal Death

Because *Bmal1* is essential for sustaining circadian rhythms in most cells (Bunger 2000) and Bmal1 knockdown caused a flattening of the *Per2* rhythms, we expected based on our original hypothesis that the siRNA would cause the IMHNs to be more vulnerable to glutamate toxicity. However, we saw the opposite, with an increase in viability of the neurons. This finding was in contrast to the pharmacological studies, and initially was puzzling. However, our *Per2*::luc reporter findings are opposite to what has been found in *Bmal1::luc* reporter studies as described in the above section. If the drugs do the opposite to *Bmal1::luc* expression as they do to *Per2*::luc expression, the results would be consistent with our *Bmal1* siRNA findings. It would be interesting to use the REV-ERBα drugs and lithium in circadian rhythm experiments with IMHNS with the *Bmal::luc* reporter to see if we could replicate these opposing amplitude effects. If Lithium and GSK4112 actually decrease *Bmal1* expression in opposition to our assumption, then the increased viability seen with these drugs fits with the *Bmal1* knockdown findings. Similarly, if SR8278 and verapamil increase *Bmal1* amplitude, the decreased viability seen would also be consistent. The findings would then suggest a role for BMAL1 in neuronal death.

BMAL1 has already been suggested to play a role in hippocampal cell death. Rakai et. al (2014) examined differences in neurogenesis and survival in the hippocampal subgranular zone in arrhythmic *Bmal1* knockout mice and their rhythmic counterparts.

While they found no difference in neurogenesis between the two groups, there was increased survival of newly generated dentate gyrus hippocampal neurons in the *Bmal1* knockout mice, and deceased pyknosis, indicating lower levels of apoptosis. This finding is consistent with our knockdown data, and is particularly interesting considering the origin of the IMHN line we used was dentate gyrus neurons. Moreover, overexpression of *Bmal1* has been found to induce p53-dependent apoptotic pathways (Jiang 2015). Our viability findings may relate to the particular drug's ability to modulate BMAL1 levels, but it is unclear as we did not look at *Bmal1* expression.

Role of LTCC in excitotoxicity

In line with our hypothesis verapamil decreased *Per2* rhythm amplitude viability during excitotoxicity. However, at the outset of the experiment, despite the fact that the drug decreases rhythm amplitude, we also considered the possibility that verapamil would cause an increase in viability as excess calcium influx is thought to mediate the toxic effects of glutamate. Glutamate stimulates sodium current through AMPA receptors and also allows calcium current through NMDA receptor activation. Influx of sodium and calcium from these channels leads to depolarization of the membrane, which also activates voltage-dependent calcium channels (LTCC), allowing further influx of calcium. Excess calcium overactivates a number of enzymes, with damage to the cell membrane, cytoskeleton, and DNA, initiating apoptosis or leading to necrosis (Mark, 2001). We expected blocking a source of calcium would decrease this process and

increase survival, but we saw an increase in cellular death. This result indicates that the source of calcium is important, and that calcium influx through LTCC may actually play a role in neuroprotection. LTCC inhibition has been found to suppress activity of BDNF, a major neuroprotective protein (Zucca, 2010). Our finding is consistent with Li et. al. (2013) who found inhibition of L-type but not N-type nor P/Q-type Ca^{2+}-channels significantly inhibited the survival of cultured hippocampal neurons subjected to ischemia. The same group found that the LTCC agonist Bay K8644 promoted protection of hippocampal neurons during ischemia (Hu, 2013).

This finding is particularly interesting considering the possible role of LTCC in BD. Variants in LTCC subunits have been found in genome wide association studies (GWAS) to be strongly linked with BD (PCG, 2011). Variants in CACNA1C, the pore forming alpha subunit, were the most strongly associated, but those in CACNA1D, and CACNB3 have been identified as conferring risk for the disorder as well (PCG, 2011). LTCC blockers have been explored as potential therapeutic agents for BD (Dubovsky 1986, Garza-Trevino 1992, Giannini 1987), and these recent GWAS findings have re-sparked an interest in their clinical use (Cipriani 2016). However, excess glutamate signaling (Gigante, 2012) and a reduction in hippocampal volume (Hibar, 2016) are also associated with BD. Therefore, our results suggest that the use of LTCC antagonists may not be helpful for treating the disorder, and may even aggravate these problems if they increase the risk of cell death from excitotoxicity. Our findings perhaps explain the findings from a recent review of studies regarding the use of LTCC antagonists for treatment of BD that found no evidence for their effectiveness (Cipriani 2016).

Do the Deficits in Amplitude Regulation Associated with LTCC variants in BD contribute to Neuronal Loss?

We have now shown that LTCC antagonists decrease rhythm amplitude in both fibroblasts (McCarthy, 2016) and IMHNs. Our previous work has suggested that variants in the CACNA1C subunits contribute to the deficits in amplitude regulation seen in fibroblasts from patients with bipolar disorder (McCarthy, 2013). In mouse fibroblasts, LTCC antagonism or knockdown of LTCC subunits inhibits lithium's ability to increase rhythm amplitude. Both of these observations suggest LTCC play a modulator role in amplitude regulation. We speculate that deficits in amplitude regulation associated with LTCC variants could play a role in the increased susceptibility to neuronal loss in BD. However, genetic variants in the LTCC subunits like CACNA1C have not been functionally characterized, and the hippocampal volume loss in BD has not been attributed to neuronal death.

Concluding Thoughts and Future Directions

We conclude that the regulation of circadian rhythm amplitude may be important for neuronal survival and death, but we cannot generalize the relative amplitude of one clock gene such as *Per2* to the entire clock network. Many clock genes have roles in

neuronal protection, and the sum of the effects of each likely produces the phenotype observed in viability studies. We suggest measuring a clock output that reflects a number of summed clock genes activities would be a better representation of circadian network amplitude. However, based on the results of the pharmacological studies, high expression of PER2 may be associated with neuroprotection, possibly by reducing BMAL1 through its feedback inhibition activity. The *Bmal1* siRNA studies suggest low levels of BMAL1 are associated with neuroprotection, likely through inhibition of apoptotic pathways.

It is interesting that we found other molecules distinct from lithium that influence both the circadian clock and viability. Our findings suggest lithium's ability to regulate circadian rhythms is important for its neuroprotective properties, even if the mechanisms are unclear. It would be interesting to study the REV-ERB agonist GSK4112 further, as an adjunct to lithum. The rhythm and viability studies suggest overlap of the molecular mechanisms of both drugs. Clinically lithum has a narrow therapeutic index, with toxic effects sometimes seen before the therapeutic effects (Klein, 2001), so a drug that boosts its actions would be beneficial to the treatment of BD.

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