Chronotype and Cellular Circadian Rhythms Predict the Clinical Response to Lithium Maintenance Treatment in Patients with Bipolar Disorder

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Abstract:

Bipolar disorder (BD) is a serious mood disorder associated with circadian rhythm abnormalities. Risk for BD is genetically encoded and may overlap with the biological systems that maintain circadian rhythms. Among mood stabilizer treatments, lithium is effective across the depressive, manic and maintenance phases, but only a minority of BD patients fully respond to monotherapy. Presently, we hypothesized that lithium-responsive BD patients (Li-R) would show characteristic differences in chronotype and cellular circadian rhythms compared to lithium non-responsive individuals (Li-NR). Selecting patients from a prospective, multi-center, clinical trial of lithium monotherapy, we examined morning vs. evening preference (chronotype) as a dimension of circadian rhythm function in 183 Li-R and Li-NR patients with BD. In a subset of 59 patients, we measured circadian rhythms in fibroblasts longitudinally over 5 days using a bioluminescent reporter (*Per2-luc*). In this manner, we estimated circadian rhythm parameters (amplitude, period, phase) and the pharmacological effects of lithium on rhythms in living cells from Li-R and Li-NR donors. Compared to Li-NRs, Li-Rs showed a difference in chronotype, with higher levels of trait morningness. Evening chronotype was associated with increased mood symptoms at baseline, especially depression and insomnia. Cells from Li-R patients were more likely to exhibit a short circadian period, a linear relationship between period and phase, and a period shortening effect of lithium. Common genetic variation in the IP_3 signaling pathway may account for some of the individual differences in the effects of lithium on cellular rhythms. We conclude that circadian rhythms may influence response to lithium in maintenance treatment of BD. *Pharmacogenetics of Bipolar Disorder* (PGBD), Clinical Trials Registry: NCT0127253

Introduction

Bipolar disorder (BD) is a disabling psychiatric disorder that affects 1-2% of the population (Baldessarini *et al*, 2003; Merikangas *et al*, 2011). BD is defined by the presence of recurrent depressive and manic/hypomanic episodes, as well as disruptions in rhythmic behaviors. For instance, depressive episodes are characterized by changes in sleep, daytime energy, motivation, and appetite; while mania is defined by decreased need for sleep and increased nocturnal activity. Even during euthymic periods, BD patients commonly show late onset of morning activity, intrusions of sleep during the day, a preference for evening activity, indicative of circadian rhythm abnormalities consistent with delayed phase and/or long period (Gonzalez *et al*, 2014; Harvey, 2008; Jones *et al*, 2005; McKenna *et al*, 2014; Pagani *et al*, 2016).

Circadian rhythms are maintained in a cell autonomous fashion by a molecular feedback loop comprised of ~20 "clock genes" (Partch *et al*, 2014). The essential elements of the loop include the CLOCK/BMAL1 protein complex that drives expression of PER1/2/3 and CRY1/2. Through negative feedback of BMAL1/CLOCK, PER and CRY inhibit their own expression over ~24 hr. cycles leading to rhythms in expression. In animals, disruption of clock genes has been used to model mania (Roybal *et al*, 2007) and depression (Landgraf *et al*, 2016b), and clock gene mutants show abnormal responses to lithium in models of mania (Schnell *et al*, 2015). In humans, variation in clock genes has been linked to morning/evening preference ("chronotype") both in the general population (Lane *et al*, 2016) and in circadian rhythm disorders (Patke *et al*, 2017; Toh *et al*, 2001). Since the circadian clock is cell autonomous, it can be studied in peripheral cultures from humans using bioluminescent reporters. Using this approach,

we have shown previously that compared to controls, circadian period is typically longer in cells from BD patients, consistent with the behavioral data observed in human subjects indicating a predisposition for evening activity in BD (McCarthy *et al*, 2013).

Mood stabilizer medications positively impact the course of BD. However, many BD patients fail to respond adequately to pharmacotherapy (Perlis *et al*, 2006). Lithium is the best studied, and arguably most effective mood stabilizer, and yet only about 30% of patients with BD respond fully to lithium (Licht *et al*, 2001). Like the risk for BD which is largely genetic (McGuffin *et al*, 2003), lithium response is heritable (Grof *et al*, 2002), and influenced by genetic variation (Song *et al*, 2015). Recent genome-wide association studies have identified loci that predict lithium response (Hou *et al*, 2016), but the biological processes governed by these genes remain obscure.

Previous preclinical studies have focused on lithium's ability to inhibit inositol monophosphates (IMP) (Berridge *et al*, 1989) and glycogen-synthase kinase 3 (GSK3) (Klein and Melton, 1996), molecules with possible links to circadian rhythms. While the role of IMP in circadian timing is relatively unexplored, our recent work indicates inositol metabolism affects rhythms (Wei *et al*, 2018). GSK3 is known to regulate the stability and turnover of clock proteins, and GSK3 inhibition has effects on circadian rhythm amplitude and period (Harada *et al*, 2005; Hirota *et al*, 2008; Iitaka *et al*, 2005; Yin *et al*, 2006). These changes in period may facilitate phase shifting, improve entrainment and have important implications for lithium's effects on mood. In the dopamine transporter (DAT) knockdown model of mania, mice have a longer circadian period, and the period was shortened by the mood stabilizer valproic acid (Landgraf *et al*, 2016a). Similar period shortening effects of valproic acid were observed in gene expression rhythms in

cells from BD patients (Landgraf *et al*, 2016a), an effect on rhythms similar to selective GSK3 inhibitors (Hirota *et al*, 2008). In humans, clinical interventions including partial sleep deprivation and phototherapy both advance circadian phase and facilitate the therapeutic effects of lithium (Wu *et al*, 2009). These data together indicate that period shortening and/or phase advancing may have beneficial effects on mood, possibly by correcting mismatches between the environmental light/dark cycle and endogenous rhythms that occur in the context of long period and/or phase delays.

Based on the observations that lithium response is genetically encoded, that circadian rhythm abnormalities are central to BD, and that lithium affects the circadian clock, we hypothesized that lithium responsive BD patients (Li-R) would have identifiable differences in circadian rhythms compared to lithium non-responders (Li-NR). Moreover, we expected that we could use identifiable circadian biomarkers to better predict therapeutic outcomes after lithium pharmacotherapy.

Methods

Lithium Clinical Trial. Clinical response to lithium was determined prospectively through the PGBD multi-center treatment trial (Oedegaard *et al*, 2016). To reduce heterogeneity of drug effects on circadian rhythms, we considered only subjects taking lithium at entry.

Determination of clinical response to lithium. Subjects were transitioned to lithium monotherapy over 12-weeks. Subjects able to stabilize on lithium monotherapy were classified as lithium responders (Li-R) and entered maintenance treatment. Subjects who were unable to stabilize were classified as lithium non-responders (Li-NR) and

discontinued from the study. Subjects who left the study for intolerable side effects or reasons unrelated to their clinical response to lithium were excluded from the analysis. Subjects in maintenance were evaluated in person every two months, until either the occurrence of a depressive/manic episode (defined by DSM-IV criteria), or the end of the 2-yr. follow-up period. The duration of stability (days) was calculated for each subject the duration of stability (days) was calculated for each subject as a dimensional measure of response.

Chronotype Analysis. At baseline, subjects completed the Basic Language Morningness scale (BALM), a validated, 13-item self-reported measure of chronotype (Brown, 1993; Rhee *et al*, 2012). Higher BALM values correspond to a greater level of morningness.

Analysis of Depressive and Manic Symptoms, and Suicide History. Subjects completed the 16 item Quick Inventory of Depressive Symptomatology Self-Report (QIDS-SR16) (Rush *et al*, 2003), the Clinician Administered Rating Scale for Mania (CARS-M) (Altman *et al*, 1994), and a detailed self-report of past suicidal behaviors. Scoring was conducted in accordance with the respective protocol for each scale. Some severity categories were combined to maintain adequate sample size. To minimize survival bias, only baseline mood measures were considered.

Cell Culture. 59 study participants with informative clinical outcomes donated biopsies for fibroblast cell lines. (Supplementary methods).

Measurement of Cellular Circadian Rhythms. Cellular rhythms were measured in parallel under lithium (1mM) treated and untreated (baseline) conditions (McCarthy *et*

al, 2013). Data were fit to a damped sine wave and rhythm parameters were calculated using commercial software (Lumicycle Analysis). Time of peak signal (first peak after synchronization) was used as a phase marker.

Drugs. Cells originating from the same plate were treated simultaneously in parallel with the active drug and vehicle controls. Lithium chloride was purchased from Sigma. 2-Aminoethoxydiphenylborane (APB), and [1-(4-Hydroxyphenoxy) ethylidene] bisphosphonic acid (L-690330) were purchased from Tocris Biosciences. Concentrated drug solutions were added to the growth media and diluted to the desired concentration. Drugs were dissolved in water or DMSO.

Gene Knockdown. Knockdown of *Itpr3* was performed using siRNA in mouse NIH3T3 cells (Wei *et al*, 2018). Commercially available siRNAs (Dharmacon) were used according the manufacturer's protocol. *Bmal1* (also called *Arntl*, M-040483-01-0005) was targeted as a positive control, and siRNA that does not target any known transcript was used as a negative control (D-001206-14-05).

Measurement of Cellular IP_3. NIH3T3 cells were treated with lithium 10mM or vehicle for 24-hr. IP_3 was measured using ELISA following the manufacturer's protocol (Life Span BioSciences).

Genotyping. BD subjects were genotyped at ~420K single nucleotide polymorphisms (SNP) on PsychChip by TGEN (Phoenix, AZ). Data were deposited on the Genetic Cluster Computer (http://www.geneticcluster.org) for quality control, processing and analysis, using the PGC pipeline (https://github.com/nievergeltlab). Imputation was

based on 1000 Genomes phase 3 (1KGP phase 3) using the first five ancestry informative principle components as covariates (Nievergelt *et al*, 2013).

Multifactorial model of lithium response. We developed post-hoc four criteria by which to score donors on cellular circadian factors associated with lithium response: 1) Period <25.8 hr. (i.e. shorter than median). 2) Period >25.8 hr. and corrected by lithium to < 25.8 hr. 3) Period >25.8 hr. and Tm <0.79 (top quartile phase advanced). 4) Rare allele *ITPR3* genotype. The first three criteria were considered protective factors, while the fourth was considered a risk factor for non-response. One point was assigned for each protective factor, and one point subtracted for each risk factor (range of scores -1 to 3). Mean scores were compared across the two groups using a 2-tailed t-test. Specificity and sensitivity for Li-NR were calculated based on the absence of any protective feature (score <1).

Statistical Analyses. Chronotype analysis was conducted in SPSS (version 20) using ANCOVA, corrected for age, race and sex. Cellular analyses were conducted to examine three hypotheses evaluating how circadian rhythms could relate to treatment response: baseline period, baseline phase, and pharmacological effects of the drug on period/phase. Given our modest sample size, and that the dependent variables were not independent, a stringent correction for multiple comparisons was not employed. The cellular rhythm analyses were completed using GraphPad Prism (version 5.0) using a two-tailed t-test or one-way analysis of variance, and univariate regression analyses of phase and period. Group differences in linear regressions were assessed for statistical significance using Fisher's r to z transformation. Genotypic analysis of period was conducted using linear regression under a dominant model. All analyses used $\alpha < 0.05$.

Results:

Clinical trial subjects. 183 lithium-treated subjects were considered for chronotype analysis (Table 1). The majority (N=124) advanced to the maintenance treatment, and 84 had informative clinical outcomes by the conclusion of the study. Others left the study for a variety of reasons unrelated to mood (Table 2).

Chronotype is associated with lithium response. Chronotype in people with BD is a stable trait (Supplementary Results, Figure S1). Past work indicates that low morningness is associated with depression (Lane *et al*, 2016), and worse treatment outcomes in major depression (Chan *et al*, 2014). Therefore, we examined chronotype to determine if it was a predictor of lithium response in BD. Using the categorical measure of lithium response, Li-R subjects were significantly higher in morningness compared to Li-NR (mean BALM=38.6 vs. 34.72, $p<0.02$, Figure 1A). We then analyzed separately subjects who failed to stabilize initially, relapsed during maintenance, and achieved long-term stability. There was no difference in chronotype between those who failed to stabilize and those who relapsed (mean BALM 35.31 vs. 35.29), but those who remained stable showed significantly higher morningness compared to the other two groups (Figure 1B, mean BALM 39.58 vs. 35.3, $p<0.05$).

In order to understand the particular factors driving the association between chronotype and lithium response, we analyzed specific symptoms of BD. We found that more severely depressed subjects were lower in morningness (Figure 1C). The most strongly associated depressive features were two sleep items: excessive sleepiness and delayed sleep onset (Table 3). Other functions that vary rhythmically over the day like

energy, concentration and psychomotor retardation showed similarly strong negative associations. However, additional depressive symptoms affecting processes that are not commonly considered rhythmic, including feelings of sadness, and low self-esteem were also negatively associated with morningness.

There was an overall trend for patients more severely affected with mania to show reduced morningness scores (Figure 1D), and several individual manic symptoms on the CARS-M mania scale, showed significant negative association with BALM, indicating that decreased need for sleep, disorganization and disorientation were all greater in subjects with low morningness (Table 4).

Finally, we examined suicide attempt (SA) history and chronotype. Subjects who endorsed one or more previous SA were significantly lower in morningness compared to subjects who had not attempted suicide (Mean BALM: SA=34.6 vs. no SA=38.0, $p<0.05$, Figure 1E).

Cellular rhythms. Evening chronotype could be explained by long circadian period and/or phase delay, either of which, either individually or in combination may lead to later onset of activity and poor entrainment to a 24 hr light/dark cycle. Period could also shift as a pharmacological effect of lithium. We have also shown previously that BD patients show irregular amplitude responses to lithium (McCarthy *et al*, 2013). Therefore, we examined each of these *Per2-luc* rhythm parameters (amplitude, period, phase) in fibroblasts under both lithium-treated and untreated conditions using fibroblasts from Li-R and Li-NR subjects (N=59).

There were no significant demographic differences between Li-R and Li-NR cell line donors (Table 5). As expected, the duration of stability on lithium (Mean ± SEM) was significantly different ($p<0.001$) with 568.9 ± 39 days for Li-R (N=44) vs. 97 ± 9 days for Li-NR (N=15). BALM scores among the subset of BD cell line donors reflected the same overall trends observed in the larger clinical cohort, with higher morningness scores in the Li-R group (Mean BALM Li-R 37.3 vs. Li-NR 31.9, $p=0.05$). BALM score was correlated with phase in the expected direction ($r = -0.18$), but the relationship was not statistically significant. BALM score was not significantly correlated with any other cellular rhythm parameter.

Amplitude. There was no significant difference in baseline amplitude between Li-R and Li-NR, and no effect on amplitude from lithium, indicating that the drug treatment did not increase amplitude in either group of BD cells (Figure 2A). This finding independently replicates our previous work showing that unlike cells from controls in which lithium typically increases amplitude by 30-40%, lithium does not increase amplitude in fibroblasts from BD patients (McCarthy *et al*, 2013).

Period. There was a difference in period between groups that trended towards significance (mean period: Li-R 25.5 ± 0.14 vs. Li-NR 26.0 ± 0.17 hr. $p=0.08$, Figure 2B). Further inspection revealed that the periods were not uniformly distributed between groups. Using a median split, a majority (25/29 or 86.2%) of short period (< 25.8 hr.) samples came from Li-R donors, while the majority of Li-NR cells (11/15 or 73%) had long periods (Figure 2C). These results indicate that the odds of non-response to lithium are significantly higher in cell donors with long period (OR = 3.6, $p<0.05$). However, there was overlap between Li-R and Li-NR. The results indicate that short period is

strongly predictive of response, and that while long period may be a relative risk factor, alone it is insufficient to explain non-response, and that additional mitigating factors may confer responsiveness to some subjects with long period.

Phase. There were no baseline differences in phase between Li-R and Li-NR, and lithium had no significant effect on phase. However, the relationship between period and phase differed between cells from Li-R and Li-NR. In cells from Li-R there was an inverse linear relationship between period and phase. Li-R cells with long period were typically phase advanced. In Li-NR cells this relationship was absent. Unlike Li-R cells, Li-NR cells with a long period were no more likely to be phase advanced than cells with a short period. The difference in the variance explained by these relationships was significantly different ($z=2.01$, $p<0.05$) between Li-R and Li-NR (Figure 3A, 3B).

Pharmacological effects of lithium. We hypothesized that if a short circadian period predicts clinical stability, then Li-R cells, especially those with a longer period, would exhibit greater shortening of circadian period by lithium compared to Li-NR cells. Selective inhibition of GSK3 shortens circadian period (Hirota *et al*, 2008), whereas lithium, a non-selective GSK3 inhibitor has been shown to paradoxically lengthen period in fibroblasts in a concentration dependent manner (McCarthy *et al*, 2013). Therefore, individual differences in the response of cellular period length to lithium may be relevant, possibly reflecting the varying balance of lithium's pharmacological effects on GSK3 vs. competing pathways. To determine if lithium may reverse the long period abnormality associated with Li-NR, we examined the degree to which period changed in Li-R and Li-NR cells after treatment with lithium. In Li-R cells there was a positive correlation ($r=0.27$, $p=0.06$) between period length and the degree to which lithium

shortened period (Figure 4A). This relationship was attenuated in the Li-NR cells (r=0.15, p=0.58, Figure 4B). To investigate further, we used a median split to sub-divide Li-R/Li-NR into long and short period sub groups. Compared to Li-R cells with a short period, Li-R cells with long period shortened period to a greater degree (t=1.7. p<0.05, Figure 4C, 4D). In Li-NR cells, lithium had no effect on period length in either group. Therefore, in the subset of Li-R cells with a longer circadian period, lithium treatment favors period shortening.

Period change by lithium is affected by IP_3. We have shown previously that the -50T/C variant in GSK3B (rs334558) is associated with altered sensitivity to the period lengthening effect of lithium at a high concentration (10mM) in BD cells (McCarthy *et al*, 2013). Because lithium also inhibits inositol monophosphate (IMP), we tested whether inhibition of IMP affects circadian rhythms, and whether genetic variation in the IP_3 system explains any of the observed differences in period among Li-R/Li-NR. To facilitate these mechanistic studies, we used a mouse NIH3T3 fibroblast line stably transfected with the Per2-luc reporter, a cellular model that recapitulates key features of lithium's effects on rhythms in human cells (McCarthy *et al*, 2015; McCarthy *et al*, 2016; Wei *et al*, 2018). The selective IMP1/2 inhibitor L-690330 caused a concentration dependent lengthening in circadian period in NIH3T3 cells (Figure 5A, 5B). IMP inhibition is predicted to reduce the turnover of 1,4,5-trisphosphate (IP_3), and increase its intracellular levels. Supporting this hypothesis, we found that 24-hr treatment of NIH3T3 cells with lithium 10mM (a concentration that lengthens period) increased IP_3 levels as expected (Figure 5C). Accordingly, we hypothesized that intracellular IP_3 receptors (IP3R) may explain some of the period lengthening effect of lithium. In line

with this expectation, the IP3R antagonist APB caused a concentration-dependent period shortening and attenuated the period lengthening effect of lithium (Figure 5D). Furthermore, genetic knockdown of *Itp3r*, the predominant IP_3 receptor gene in NIH3T3 fibroblasts also blocked the period lengthening effect of lithium (Figure 5E, 5F). The homologous human gene, *ITPR3* harbors a genetic variant with suggestive evidence for association with BD in GWAS (rs11758031, $p<6x10^{-4}$ in PGC-BD1). To test the role of *ITPR3* in human fibroblasts from BD patients, we examined whether this genetic variant is associated with period. We found that when treated with lithium, cells harboring one or more *ITPR3* minor A alleles showed significantly longer period (Mean ±SEM) compared to those with two common alleles (minor: 26.04±0.26 vs. common: 25.44±0.13, Figure 5G). Unlike at higher concentrations, the -50T/C variant in *GSK3B* (rs334558) was not associated with period change after lithium 1mM (Figure 5H).

Modelling lithium response using circadian factors. Based on our results, we explored the overall relationship of the identified circadian variables to lithium response. We developed a preliminary model to predict clinical lithium response (see Methods) that could assess all four variables (baseline period, period after lithium, phase, and *ITPR3* genotype) in our cohort of cell line donors. After adding each factor, the mean (± SEM) composite score among Li-R was 0.85± 0.15. Of 40 Li-R cell line donors, 33 (83%) had a score of 1.0 or more, indicating a preponderance of protective factors. Among Li-NR, the mean score was 0.07± 0.07 (Figure 6). Among 15 Li-NR cell line donors, 12 (80%) scored zero or less, indicating a preponderance of risk factors (Figure 6). Accordingly, our four-factor model had a specificity of 83% and sensitivity of 80% for

identifying Li-NR. Notably, among Li-R only 1 (3% of Li-R cell lines) had more than one protective factor, indicating minimal overlap among factors.

Discussion

We have shown that chronotype and cellular circadian rhythms predict lithium response in BD. Higher morningness was associated with fewer depressive and manic symptoms, fewer suicide attempts and longer duration of mood stability. In cells, short circadian period either at baseline. In cells with a long period, pharmacologically induced period shortening by lithium or phase advance was also associated with favorable clinical response. Finally, we identified IMP inhibition as a novel mechanism by which lithium affects circadian period, suggesting that genetic variation in the IP_3 pathway could affect clinical response to lithium by a circadian mechanism. Taken together, our data suggest a model by which lithium response could be predicted using circadian factors.

Most previous studies of lithium response utilized retrospective clinical assessment and subjects treated with multiple psychotropic medications. A strength of our study is that our relatively large cohort of BD subjects was evaluated prospectively on a standardized lithium monotherapy protocol and that cells were available from these well characterized donors. Our study also has limitations. We did not have cell lines from every clinical trial subject and accordingly, the sample available for cellular analyses, while comparatively large for this kind of study, is incomplete and had relatively few Li-NR lines. In addition, our cellular model of the 24-hr circadian cycle has caveats. Estimates of circadian parameters calculated *in vitro* likely differ from those obtained *in vivo* due to artifacts associated with the cell culture environment and the use of peripheral cells. Moroever,

with particular respect to phase, the reference point *in vitro* is the synchronizing biochemical stimuli associated with medium change, whereas *in vivo*, phase reference point is determined by light exposure. While the chronotype findings in BD patients largely support our cell-based conclusions, underlying mechanistic differences in phase setting could limit their overall generalizability *in vivo*. Finally, without an independent cohort, we were unable to validate our predictive model in an independent data set.

We reported previously that compared to controls, cells from BD patients had longer circadian periods and were more likely to show a period lengthening effect from lithium at a therapeutically relevant concentration, especially in cells from BD patients with a history of suicide attempt (McCarthy *et al*, 2013). These previous studies were conducted in cells collected from hospitalized patients with complex medication regimens. Therefore, it was previously impossible to determine the relationship between circadian rhythms and lithium response. The present study extends this previous work, and demonstrates that period modulation by lithium may have relevance not only as a discriminator of BD from control cells, but also for predicting therapeutic outcomes to lithium monotherapy. Our previous work also found that lithium amplifies circadian rhythms in control cells, but not in BD cells (McCarthy *et al*, 2015; McCarthy *et al*, 2016; McCarthy *et al*, 2013). In this study, we found that the attenuation of this circadian amplitude phenotype in BD was present regardless of Li-R/Li-NR status. Therefore, it is a phenotypic marker of BD that may point to molecular mechanisms underlying the illness, but is not of any apparent prognostic value for predicting lithium response.

Mood implications for circadian misalignment. Circadian rhythms entrain to environmental cues, most notably light. The intrinsic human circadian oscillator runs

with a period of 24.2 hr (Czeisler *et al*, 1999). Therefore, there is typically a slight mismatch between the period of the endogenous rhythm and the 24 hr light/dark cycle. Phase advancing in response to light allows the circadian clock to overcome this mismatch and entrain to a 24-hr cycle. However, with large mismatches between the light/dark cycle and the endogenous rhythm, entrainment is incomplete and results in syndromes such as advanced sleep phase (Toh *et al*, 2001) and delayed sleep phase (Patke *et al*, 2017) disorders. With extreme mismatches, the failure to entrain is compete and the endogenous rhythm "free runs" independently of the light/dark cycle. Healthy subjects maintained in a laboratory on free running conditions on a 28 hr. light/dark cycle demonstrate signs of severe physiological stress, including changes in autonomic function, cortisol, and sleep (Scheer *et al*, 2009). Similar studies in healthy subjects reveal rhythmic changes in affect and pronounced mood impairments (Santhi *et al*, 2016). Therefore, misalignment of the circadian clock causes physiological stress and negatively impacts mood. While subjects living in typical environmental conditions are unlikely to encounter large clock/light mismatches, BD subjects do have a longer period (McCarthy *et al*, 2013), and participate in behaviors that may undermine efficient circadian entrainment, such as less daytime activity and limited exposure to light and may have mild clock/light mismatches over extended periods of time (Pagani *et al*, 2016). Therefore, we propose that the combination of longer period and compromised circadian entrainment causes some BD subjects to experience physiological distress, altered sleep and/or affect regulation. The degree to which lithium facilitates or opposes entrainment may affect the clinical course of lithium treatment.

Inositol monophosphatase and circadian rhythms. Lithium differs from selective GSK3 inhibitors with respect to its circadian effects, causing period lengthening rather than shortening. IMP inhibition by lithium and subsequent accumulation of intracellular IP_3 may underlie this effect, and variation in *ITPR3* may affect the degree to which lithium lengthens period. Therefore, the degree to which mood stability is maintained in BD may relate to genetic variation in the IP3 pathway. In particular, lithium response may be enhanced in individuals where the balance of lithium's effects is shifted toward GSK3 inhibition (resulting in short period) and away from IMP inhibition (resulting in long period). The rs11758031 A/G intronic nucleotide variant may be an example of one such genetic factor that influences the circadian effects of lithium. The SNP has been shown to be functional, affecting the expression of *ITPR3* and the *IP6K3*, an adjacent gene involved in signal transduction (https://www.gtexportal.org/home). We have previously shown that *IPMK*, a gene involved in both GSK3 and inositol signaling may influence the period lengthening effect of lithium (Wei *et al*, 2018). Accordingly, further work on the inositol pathway as it relates to lithium is warranted.

The role of the clock in mood stability. Presently, we cannot determine whether the circadian influence on lithium response is essential in promoting mood stability. By period lengthening, lithium could cause circadian misalignment, leading to a state of social, environmental and/or physiological distress, leaving only subjects who remain entrained to benefit from lithium's therapeutic benefits. In these ways, the circadian influence on lithium response may be mediated by a lack of circadian "side effects" and be permissive rather than causal. Alternatively, the clock genes may be causally involved, preferentially activating neuroprotective processes in Li-R BD patients. Clock

proteins like PER2, BMAL1 and REV-ERBα regulate neurogenesis (Bouchard-Cannon *et al*, 2013; Schnell *et al*, 2014), and neuroprotective molecules involved in lithium's therapeutic mechanism such as BDNF are rhythmically expressed (Angelucci *et al*, 2003; Begliuomini *et al*, 2008; Berchtold *et al*, 1999; Liang *et al*, 1998). Therefore, the clock may alter lithium's pharmacological actions directly through neurogenesis and/or neuroprotection, potentially suggesting a direct role for the clock genes in modifying the therapeutic response to lithium.

Conclusions. Circadian period and phase predict lithium response. Future experiments are needed to address the determine if entrainment is the primary mechanism, and whether circadian clocks are essential for lithium response.

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Demographic Features of Chronotype Sample	
Age (Mean ± SEM), years	46 ±1.16
Age Range, years	19 - 77
Sex Male, number (%)	89 (48.6)
Sex Female, number (%)	94 (51.3)
Self-Reported Ancestry	
Caucasian, number (%)	160 (87.4)
African, number (%)	16 (8.7)
Asian, number (%)	3 (1.6)
Other, number (%)	4 (2.2)

Table 1. Demographic features of BD patients assessed for chronotype. All subjects were on lithium at the start of the trial.

Reason For Termination	n=	%
Completed Without Relapse	48	26.2
Relapse After Stabilization	24	13.1
Failure To Remit	21	11.5
Lost To Follow Up	31	16.9
Side Effects/ Medical/Other	29	15.8
Non-Compliant	19	10.4
Voluntary Withdrawal	11	6.0

Table 2. Reasons for subject discontinuation from the trial.

Depression	Correlation (r=)	p-value
Total QIDS-SR	-0.20	0.01*
Sleep Onset	-0.28	0.00*
Sleep Interruption	0.07	0.35
Early Waking	0.04	0.58
Excessive Sleep	-0.31	0.00*
Feeling Sad	-0.17	0.03*
Decreased Appetite	-0.07	0.34
Increased Appetite	0.01	0.85
Decreased Weight	-0.01	0.87
Increased Weight	-0.06	0.45
Concentration	-0.17	0.03*
View of Self	-0.21	0.01*
Suicidal Ideation	-0.12	0.12
Reduced Interest	-0.14	0.08
Low Energy	-0.21	0.00*
Feeling Slow	-0.17	0.03*
Restlessness	-0.04	0.64

Table 3: Associations between depressive symptoms (QIDS-SR score) and chronotype (higher QIDS-SR indicates more depression; higher BALM indicates more morningness)

Mania	Correlation (r=)	p-value
Total CARS-M Score	-0.13	0.09
Euphoria	0.00	0.99
Irritability	-0.15	0.06
Activity	-0.06	0.46
Speech	-0.03	0.71
Thoughts	-0.03	0.66
Distractibility	-0.12	0.11
Grandiosity	-0.12	0.11
Decreased Sleep	-0.16	0.03*
Energy	-0.10	0.20
Judgement	-0.08	0.32
Disorganization	-0.19	0.01*
Delusions	-0.01	0.92
Hallucinations	-0.03	0.68
Orientation	-0.18	0.02*
Insight	-0.05	0.49

Table 4: Associations between manic symptoms (CARS-M score) and chronotype (higher CARS-M indicates more mania; higher BALM indicates more morningness)

	Male Sex(%)	Age (mean ± SEM)	BALM (mean ± SEM)	Duration of Stability(days)
Responder	29/44(55%)	47.5 ± 2.2	37.3 ±1.4	568.9
Non-Responder	10/15(66%)	50.4 ± 3.7	31.9 ±2.0	97.1
p-value	0.42	0.50	0.05*	0.00*

Table 5: clinical and demographic features of Li-R/Li-NR cell line donors

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Figure 1. Chronotype is associated with clinical response to maintenance treatment with lithium. A) Li-R subjects (entered the maintenance phase, N=121) were significantly higher in trait morningness at baseline compared to Li-NR (those who did not enter maintenance phase, N=62). B) Those who failed to complete the study due to failure to stabilize mood (N=12) and those who suffered mood relapse (N= 24) were similar to each other in morningness, and lower in morningness than those who completed the entire 2-yr study (N=48). C) Morningness (BALM score) increased with age (N=463). D) Morningness is negatively correlated with E) baseline depression (for absent/mild/moderate/severe/extreme N=83/44/21/11/3) F) baseline symptoms of mania (for absent/mild/moderate+severe N=155/8/4). G) Morningness is negatively correlated with past suicide attempts (N=126 no suicide / N=41 suicide). Error bars indicate SEM. * indicates $p<0.05$

Figure 2. Circadian rhythm parameters in cells from Li-R and Li-NR donors. A) There was no difference in amplitude between Li-R (N=44) and Li-NR (N=15) samples, either at baseline (untreated) or after treatment with lithium 1mM. B) Period length was more likely to be short in Li-R samples compared to Li-NR. Mean period Li-R 25.5 ± 0.14 vs. Li-NR 26.0 ± 0.17 hr., two-tailed T test indicates $p=0.08$ C) The proportion of Li-R and Li-NR differs significantly in donors with short and long circadian period [$X^2=4.06(1)$, OR= 3.6, * indicates $p<0.05$]. Short/Long corresponds to below/above the median period of 25.8 hr. D) Representative rhythm traces of cells from Li-R (blue) and Li-NR (red) subjects. Error bars represent SEM.

Figure 3. The phase-period relationship differs in cells from Li-NR compared to Li-R. A) In Li-R cells (N=44), samples with the longest period are phase advanced relative to those with shorter periods. B) In Li-NR cells (N=15), there exists no relationship between period and phase. C) Representative rhythm traces showing the relative phase advance of Li-R cells vs Li-NR cells with similarly long periods. D) Representative rhythm traces showing the relative phase advance of Li-R cells with long period vs Li-R cells with a short period. Arrows indicate phase marker (Tmax).

Figure 4. Lithium shortens circadian period preferentially in cells from Li-R donors. A) In Li-R subjects the period shortening effect of lithium inversely correlates with baseline period length. Li-R subjects with long (>25.8 hr.) periods show the greatest shortening effect of lithium. B) In Li-NR subjects the relationship between baseline period length and the effect of lithium on period is attenuated and no longer significant. C) Li-R cells with long period (N=18) show significant period shortening after lithium compared to Li-R cells with short period (N=25). Lithium had no effect on period length in Li-NR with long (N=11) or short (N=4) period. * indicates $p<0.05$. D) Representative rhythm traces of a Li-R cell with long period after treatment with vehicle or lithium 1mM.

Figure 5. Inhibition of IMP causes period lengthening. Despite its action as an inhibitor of GSK3, lithium lengthens circadian period at high concentrations, perhaps indicating circadian effects of IMP inhibition. A) Selective inhibition of IMP with L-690330 causes period lengthening (N=6-9/group). B) Representative trace of the period lengthening effect of L-690330. C) Treatment of mouse fibroblast cell line with lithium for 24 hr. causes an increase in IP_3 (N=7-8/group). D) Antagonism of the IP_3 receptor shortens period and reverses the period lengthening effects of lithium (N=3/group). E) Knockdown of the IP_3 receptor gene Itpr3 in NIH3T3 cells (N=8-14/group) blocks the period lengthening effect of lithium (10mM). F) Representative traces of *Itpr3* knockdown experiment. *Itpr3* knockdown attenuates the period lengthening effect of lithium. The loss of rhythm from *Bmal1* knockdown confirms efficient siRNA transfection. G) In cells from BD patients, *ITPR3* genotype at the variant rs11758031 predicts circadian period after treatment of the cells with lithium (N= 48 GG and 14GA/AA, $p<0.01$), whereas F) GSK3B genotype at rs334558 (-50T/C) does not (N=23 TT, 38 CT/CC, $p=0.65$). * indicates $p<0.05$ vs control. **indicates $p<0.05$ vs lithium treated.

Figure 6. Four cellular circadian factors predict lithium response. A) The mean sums of the four circadian factors in each of 55 individual fibroblast lines was significantly lower ($p<0.0001$) in cells from Li-NR compared to Li-R. Each circadian factor was equally weighted. Very few subjects had more than one protective factor suggesting multiple independent pathways to a favorable response. See methods for description of the model. B) The model correctly identified 12/15 Li-NR subjects and 32/40 Li-R subjects for a sensitivity and specificity each of 80%.

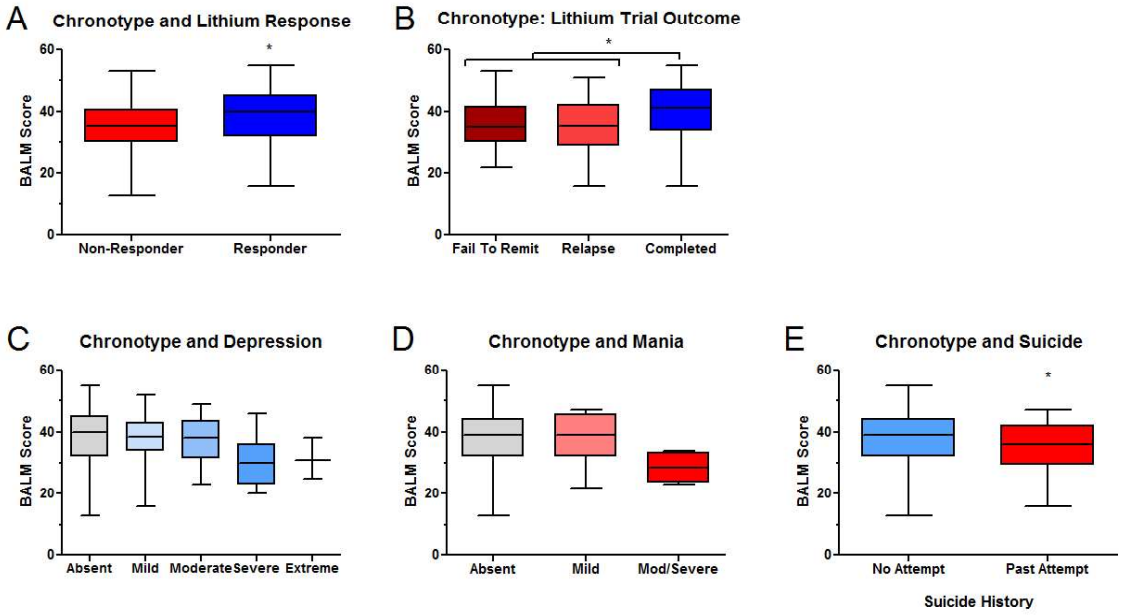
A
Chronotype and Lithium Response
BALM Score
Non-Responder
Responder
B
Chronotype: Lithium Trial Outcome
BALM Score
Fail To Remit
Relapse
Completed
C
Chronotype and Depression
BALM Score
Absent
Mild
Moderate
Severe
Extreme
D
Chronotype and Mania
BALM Score
Absent
Mild
Mod/Severe
E
Chronotype and Suicide
BALM Score
No Attempt
Past Attempt
Suicide History

Figure 1

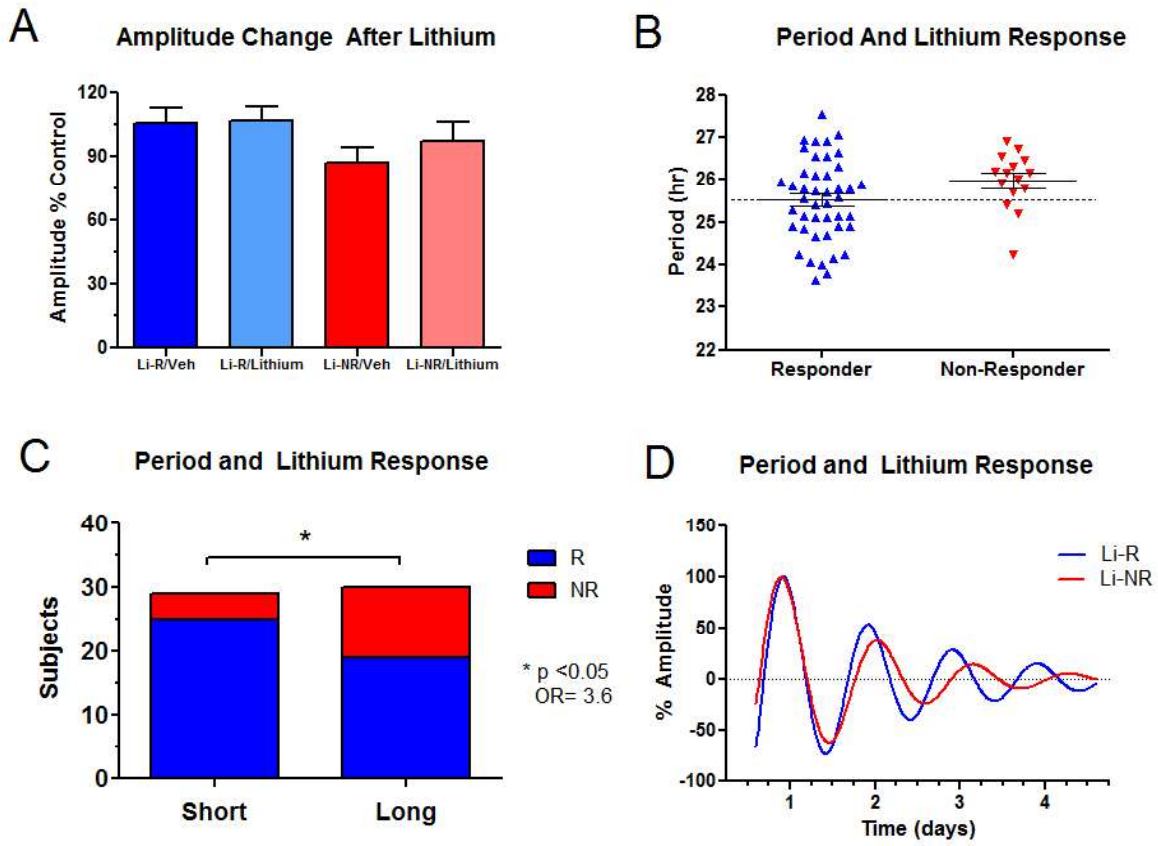
A
Amplitude Change After Lithium
Amplitude % Control
120
90
60
30
0
Li-R/Veh
Li-R/Lithium
Li-NR/Veh
Li-NR/Lithium
B
Period And Lithium Response
Period (hr)
28
27
26
25
24
23
22
Responder
Non-Responder
C
Period and Lithium Response
Subjects
40
30
20
10
0
*
Short
Long
R
NR
* p <0.05
OR= 3.6
D
Period and Lithium Response
% Amplitude
150
100
50
0
-50
-100
1
2
3
4
Time (days)
Li-R
Li-NR

Figure 2

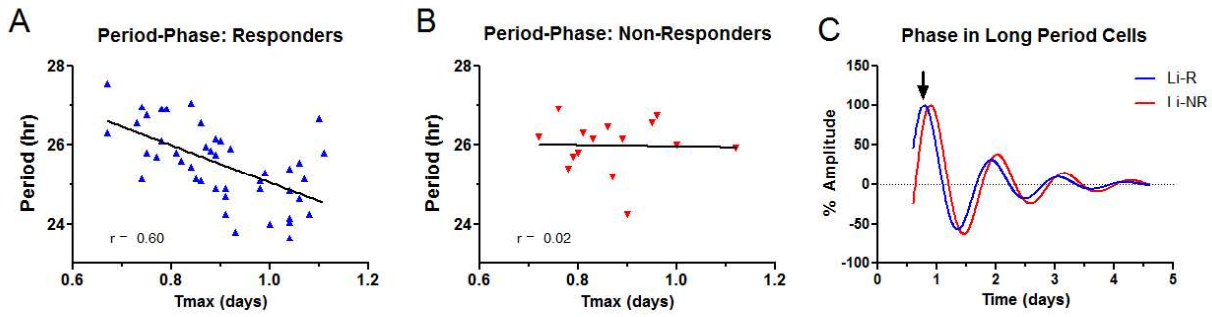
A
Period-Phase: Responders
Period (hr)
28
26
24
r = 0.60
0.6
0.8
1.0
1.2
Tmax (days)
B
Period-Phase: Non-Responders
Period (hr)
28
26
24
r = 0.02
0.6
0.8
1.0
1.2
Tmax (days)
C
Phase in Long Period Cells
% Amplitude
150
100
50
0
-50
-100
0
1
2
3
4
5
Time (days)
Li-R
Li-NR

Figure 3

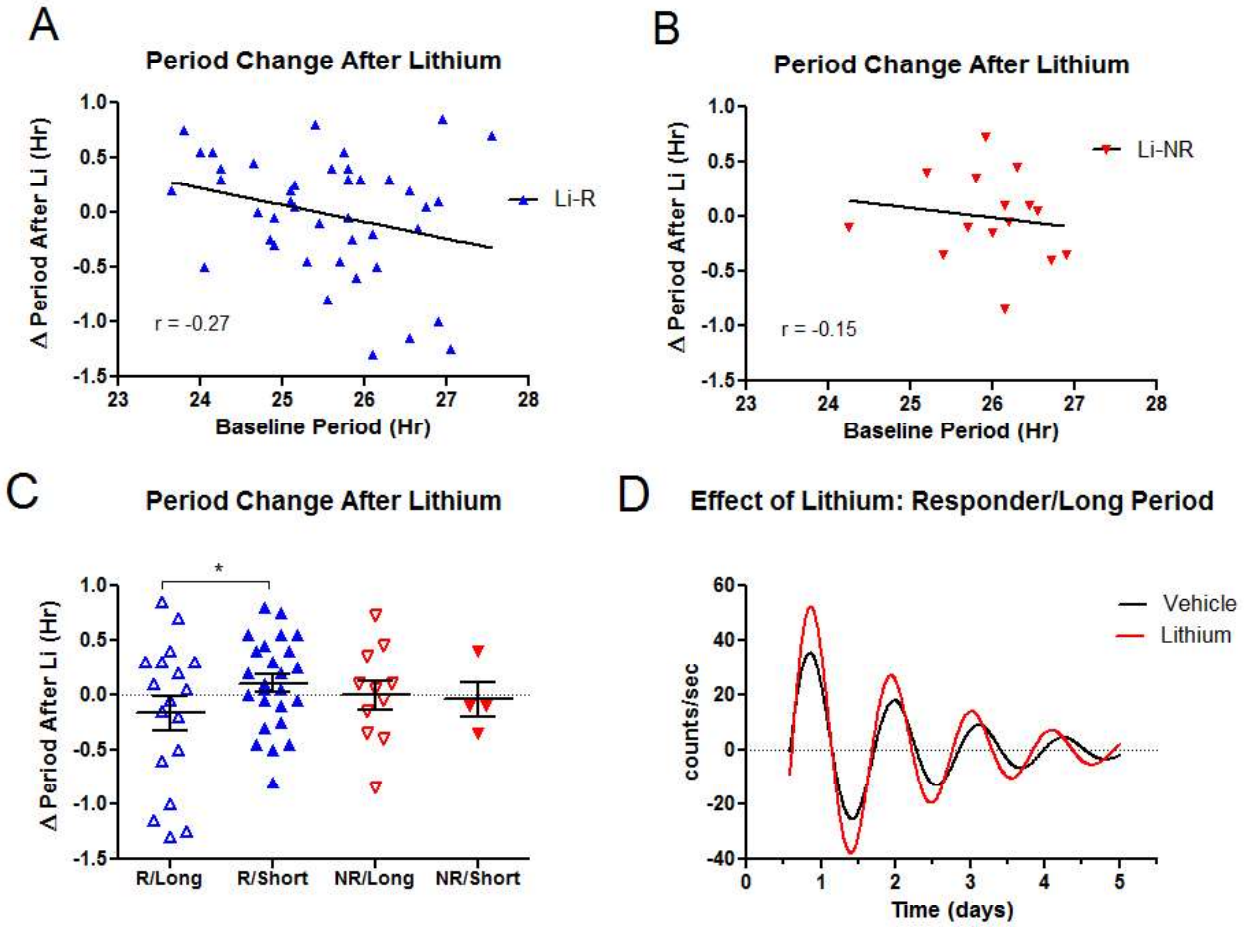
A
Period Change After Lithium
Δ Period After Li (Hr)
Baseline Period (Hr)
Li-R
r = -0.27
B
Period Change After Lithium
Li-NR
r = -0.15
C
Period Change After Lithium
*
R/Long
R/Short
NR/Long
NR/Short
D
Effect of Lithium: Responder/Long Period
counts/sec
Time (days)
Vehicle
Lithium

Figure 4

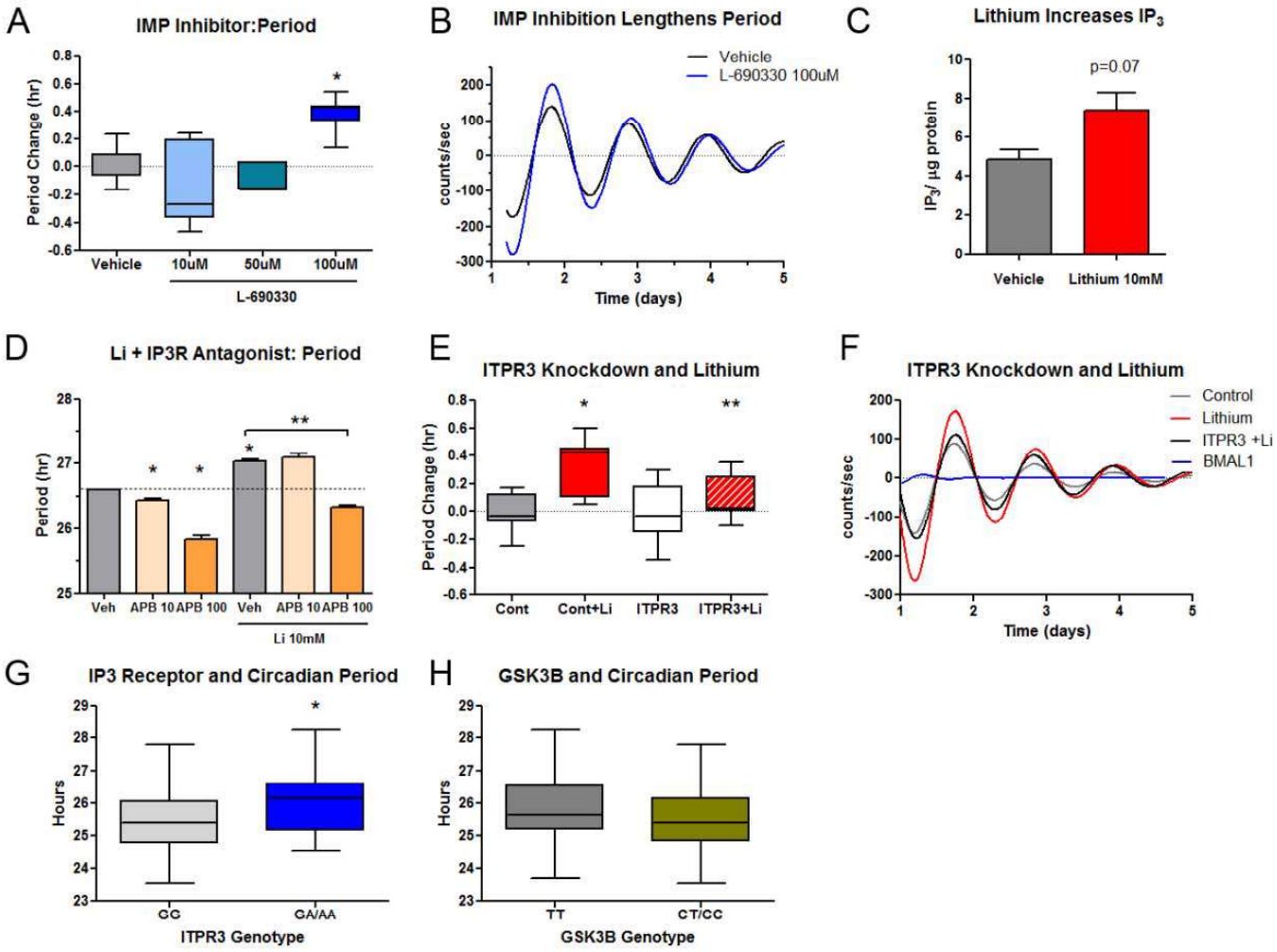
A
IMP Inhibitor:Period
Period Change (hr)
Vehicle
10uM
50uM
100uM
L-690330
B
IMP Inhibition Lengthens Period
Vehicle
L-690330 100uM
counts/sec
Time (days)
C
Lithium Increases IP3
p=0.07
IP3/ µg protein
Vehicle
Lithium 10mM
D
Li + IP3R Antagonist: Period
Period (hr)
Veh
APB 10
APB 100
Veh
APB 10
APB 100
Li 10mM
E
ITPR3 Knockdown and Lithium
Period Change (hr)
Cont
Cont+Li
ITPR3
ITPR3+Li
F
ITPR3 Knockdown and Lithium
Control
Lithium
ITPR3 +Li
BMAL1
counts/sec
Time (days)
G
IP3 Receptor and Circadian Period
Hours
GG
GA/AA
ITPR3 Genotype
H
GSK3B and Circadian Period
Hours
TT
CT/CC
GSK3B Genotype

Figure 5

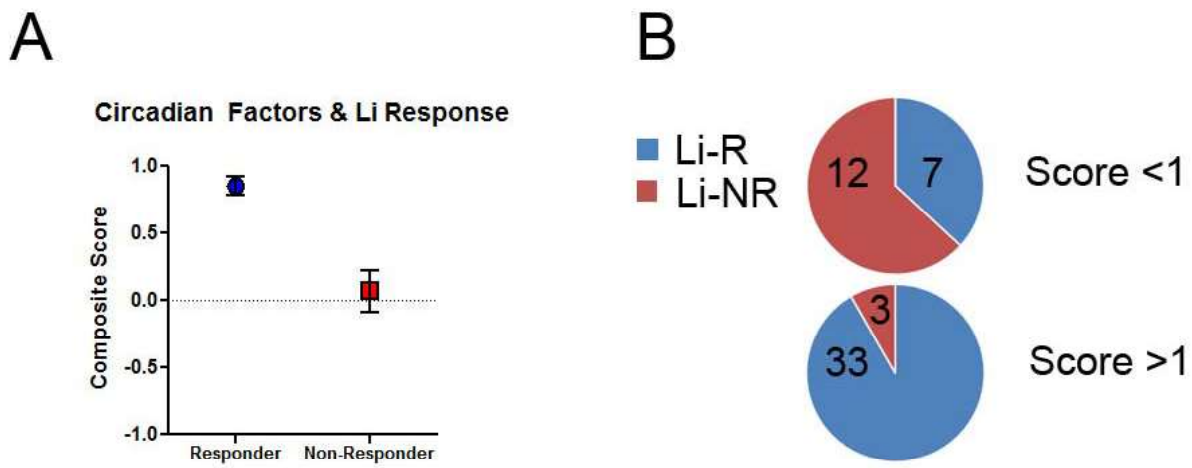
A
Circadian Factors & Li Response
Composite Score
1.0
0.5
0.0
-0.5
-1.0
Responder
Non-Responder
B
Li-R
Li-NR
12
7
Score <1
3
33
Score >1

Figure 6

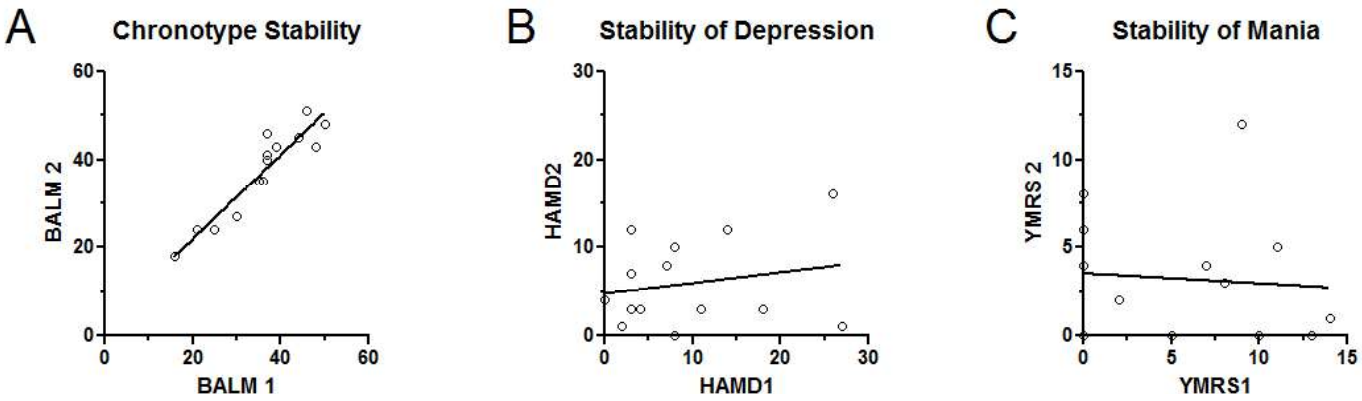

Figure S1. A) Chronotype is stable over repeated testing across several months suggesting it is a stable trait characteristic. Mood symptoms such as B) Depression (scored with the Hamilton Depression Inventory – HAMD), and C) Mania (scored with the Young Mania Scale – YMRS) show considerably more change after repeated testing. While BALM score on the first measure predicts the second measure, there is no significant correlation in mood symptoms across pre-post testing. For each scale, graphs indicate repeated measurements of the same patient on the same days. N=14 paired observations of lithium-treated BD patients conduced an average of 10 ± 1 months apart (range 5-23 months).