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Contributions of circadian clock genes to cell survival in fibroblast models of lithium-responsive bipolar disorder

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Contribution statement

Conceptualization, investigation, formal analysis, and validation were performed by HKM, ADM, and MJM. Methodology was developed by HKM and HW. Data curation (including clinical data) was performed by CMN, AXM, PDS, MA, EG, MGM, JRK, and MJM. Project administration was done by HKM. Visualization of data was done by HKM, ADM and MJM. Writing of the original draft was done by MJM and ADM. All authors contributed to writing by reviewing and editing the manuscript. Resources and software were provided by MJM. Supervision was provided by HKM and MJM. Funding acquisition was done through grants to MJM and JRK.

Conflict of interest

MJM served as a consultant to Alkermes pharmaceuticals for work unrelated to the current research. None of the other authors have conflicts of interest to report.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2023.04.009.

Abstract

Bipolar disorder (BD) is characterized by mood episodes, disrupted circadian rhythms and gray matter reduction in the brain. Lithium is an effective pharmacotherapy for BD, but not all patients respond to treatment. Lithium has neuroprotective properties and beneficial effects on circadian rhythms that may distinguish lithium responders (Li-R) from non-responders (Li-NR). The circadian clock regulates molecular pathways involved in apoptosis and cell survival, but how this overlap impacts BD and/or lithium responsiveness is unknown. In primary fibroblasts from Li-R/Li-NR BD patients and controls, we found patterns of co-expression among circadian clock and cell survival genes that distinguished BD vs. control, and Li-R vs. Li-NR cells. In cellular models of apoptosis using staurosporine (STS), lithium preferentially protected fibroblasts against apoptosis in BD vs. control samples, regardless of Li-R/Li-NR status. When examining the effects of lithium treatment of cells *in vitro*, caspase activation by lithium correlated with period alteration, but the relationship differed in control, Li-R and Li-NR samples. Knockdown of *Per1* and *Per3* in mouse fibroblasts altered caspase activity, cell death and circadian rhythms in an opposite manner. In BD cells, genetic variation in *PER1* and *PER3* predicted sensitivity to apoptosis in a manner consistent with knockdown studies. We conclude that distinct patterns of coordination between circadian clock and cell survival genes in BD may help predict lithium response.

Keywords

Bipolar disorder; Circadian rhythm; Apoptosis; Lithium

1. Introduction

Bipolar disorder (BD) is a neuropsychiatric condition, characterized by recurrent episodes of depression and mania (Baldessarini et al., 2020). Risk for BD is heritable, with 70–80% of the variance explained by genetic factors (Stahl et al., 2019; McGuffin et al., 2003). Treatment with lithium can positively impact the course of BD, but only 1/3 of patients remain stable on long-term lithium monotherapy (Rybakowski et al., 2001). Recent studies have identified genetic markers of lithium response (Hou et al., 2016; Amare et al., 2018; Song et al., 2015; Amare et al., 2020), but neurobiological understanding of these predictors remains preliminary. Loss of brain volume is one key pathological feature of BD. Brain imaging studies reveal cortical and sub-cortical gray matter loss in BD (Hibar et al., 2018, 2016), while postmortem BD brains and cellular models show reduced neuron size and loss of dendritic spine density in cortical and limbic structures (Gigante et al., 2011; Tobe et al., 2017; Konopaske et al., 2014). Lithium induces dendritic sprouting in mice (Kim and Thayer, 2009), and in BD patients, gray matter loss is reduced by lithium (Hibar et al., 2018). Therefore, restoration or preservation of neurons against toxic exposures (i.e. “neuroprotection”) may be an important therapeutic action of lithium, and preserved gray matter has been proposed as a feature that may distinguish lithium-responders (Li-R) from non-responders (Li-NR) (Moore et al., 2009; Lyoo et al., 2010; Athey et al., 2021). Lithium promotes neuroprotection by inhibiting glycogen synthase kinase B (GSK3B) with subsequent anti-apoptosis effects on genes like BAX and BCL2 (Einat et al., 2003; Manji et

al., 2000; Klein and Melton, 1996; Sarkar et al., 2005), and developmental regulators like P53, CHEK2 and ATM, that regulate apoptosis and dendritic spine dynamics (Konopaske et al., 2014; Glausier and Lewis, 2013). These molecular mechanisms are insufficiently studied in clinical BD samples, but existing data support their role in lithium response. For instance, integrative brain imaging and gene expression studies of BD patients have shown that the P53 cell survival pathway may contribute to some of the effects on gray matter following lithium treatment (Anand et al., 2020). Gene expression studies in BD patient lymphocytes found associations between cell survival pathways and Li-R (Beech et al., 2014; Lowthert et al., 2012), and in iPSC-derived neurons, Li-R show differences compared to Li-NR in calcium signaling and CRMP2, a cytoskeletal protein enriched in dendrites (Tobe et al., 2017; Mertens et al., 2015).

Circadian rhythm disruption is pervasive in BD, but heterogeneous with variability that may contribute to differences in treatment response to lithium. We found previously in a prospective clinical trial of BD that evening chronotype predicted lithium non-response (McCarthy et al., 2018), and both fibroblasts and induced pluripotent stem cell (iPSC)-neurons from Li-R had shorter periods in circadian rhythms compared to Li-NR (McCarthy et al., 2018; Mishra et al., 2021). BD patient cells have lower amplitude rhythms under entrained or lithium treated conditions (Mishra et al., 2021; McCarthy et al., 2013; Nudell et al., 2019), and in BD patient neurons, Li-NR amplitude is reduced even further compared to Li-R (Mishra et al., 2021). These data indicate that like neuroprotection, circadian rhythms may contribute to lithium responsiveness. The extent to which cell survival and circadian pathways overlap in larger cohorts of BD patients remains unknown.

Numerous biological pathways vary in activity over regular 24 h cycles due to influence from the circadian clock, including genes involved in cellular responses to stress, inflammation, neurogenesis, neuronal differentiation (Bouchard-Cannon et al., 2013; Kimiwada et al., 2009) and apoptosis (Magnone et al., 2014; Sato et al., 2009; Im et al., 2010). With evidence that the circadian clock is linked to pathways regulating apoptosis, we hypothesized that circadian “clock genes” alter the susceptibility of cells to apoptosis and/or protection by lithium and may offer a novel strategy for treating BD (Porcu et al., 2019). Using fibroblasts from Li-R and Li-NR BD patients and controls, we aimed to assess the organization of genes in the circadian clock and cell survival pathways in controls, Li-R and Li-NR, and assess differences in vulnerability to apoptosis and the protective effects of lithium in these groups. Additionally, in mouse and patient cells, we sought to determine if circadian clock genes play a role in cell survival by altering expression and looking at associations with common genetic variants. Our work concludes that circadian clock genes interact distinctly with survival pathways in cells from controls, Li-R and Li-NR, and that variation in circadian clock genes, especially *PER1* and *PER3* have effects on apoptosis and cell survival that may be relevant to determining lithium response.

2. Method

2.1. Determination of lithium response

Skin biopsies to grow fibroblast cell lines were obtained from controls, or patients with BD type I who participated in the Pharmacogenomics of Bipolar Disorder (PGBD) lithium

monotherapy trial (Oedegaard et al., 2016). Lithium response was determined prospectively by longitudinal clinical evaluation to identify the occurrence of mood episodes. Non-response was defined as the inability to stabilize or the occurrence of a mood relapse within the initial 12 weeks of lithium treatment. Age and sex matched control subjects were evaluated using The Structured Clinical Interview for DSM-IVTR. Subjects with any psychiatric illnesses were excluded. The majority of participants were of European ancestry and most BD patients were on medication at the time of biopsy. Characteristics of the cell line donors are shown Table S1.

2.2. Fibroblast cultures

In order to grow cells in sufficient numbers for genetic analysis, the fibroblast model was selected. Fibroblast lines were established for each donor using standard methods (Takashima, 2001). Human fibroblasts were transduced with the *Per2*-luc reporter to assess circadian rhythms (McCarthy et al., 2013). In some studies, we used an NIH3T3 mouse fibroblasts that stably express *Per2*-luc under hygromycin selection (McCarthy et al., 2015).

2.3. Drugs

Staurosporine (STS) was purchased from Tocris. Lithium chloride was purchased from Sigma. Preliminary studies revealed lithium 10 mM was optimal for inducing cell survival. STS concentration (62.5 nM) was based on our own preliminary and previously published studies (Batalla et al., 2015). Drugs were dissolved in assay buffers.

2.4. Gene expression

Cells were collected 24 h after a media change to synchronize circadian rhythms. RNA was prepared by RNeasy kit (Qiagen). cDNA (~750 ng) was prepared using reverse transcription (Applied Biosystems). Gene expression was estimated by quantitative real-time PCR using a CFX384 thermocycler (Bio-Rad). Taqman primers (Thermo Fisher Scientific) were used to measure expression of *ARNTL* (*BMAL1*), *ATM, BAX, BCL2, BRCA1, CHEK2, TP53(P53), PER1, PER2, PER3, NR1D1* (*REV-ERBα*), *RORA* and normalized to *GAPDH*, a non-rhythmic reference gene suitable for use in circadian rhythm studies (Kosir et al., 2010). Target gene expression was estimated by calculating 2ΔCt between GAPDH and the target (Schmittgen and Livak, 2008). All experiments were run in technical triplicates. This subset of cell survival and circadian clock genes was selected based on a review of the literature to identify genes previously linked with circadian mechanisms of apoptosis and/or lithium-induced neuroprotection, with prioritization of genes for which we found previous evidence of association with bipolar disorder and/or cellular circadian rhythms in fibroblasts (McCarthy et al., 2013; Magnone et al., 2014; Sato et al., 2009; Im et al., 2010).

2.5. Caspase and viability assays

The apoptosis assay was based on a STS-fibroblast model described previously that demonstrated relationship to psychiatric disorders (Gasso et al., 2014). Fibroblast were plated on 96 well-plates using a cell counter to standardize conditions (3000 cells/well) and treated with STS 62.5 nM. Using enzymatic bioluminescence assays, Caspase-Glo (Promega

G8091) or Celltiter-Glo Luminescent Viability (Promega, G7570), photoemissions were measured with a plate reader (Biotek Cytation 3). Modifications were made for viability assays employing siRNA. Gene knockdown was conducted in 24 well-plates (20,000 cells/well) for 48 h After STS, 500 μl of viability assay reagent was added to the plate. The plate was gently shaken for 2 min then incubated for 10 min at 25 °C. Aliquots were transferred to a 96 well plate for bioluminescence assays. To facilitate direct comparison of caspase, viability and circadian rhythms in human samples, cells used for apoptosis assays were grown from parallel cultures of the cells used in the *Per2*-luc rhythm assays. Complete rhythm and apoptosis data sets were available from Li-R ($n = 14$), Li-NR ($n = 9$), and control ($n = 6$) cell lines.

2.6. Circadian rhythm assays

Circadian rhythm experiments were performed using human fibroblast from control ($n = 15$), Li-R ($n = 44$) and Li-NR ($n = 15$) or NIH3T3 mouse fibroblasts stably transfected with the *Per2*-luc reporter in a luminometer, sampling photoemissions every 10 min (McCarthy et al., 2013). Circadian parameters were estimated by fitting data to a damped sine wave using commercial software (Lumicycle Analysis).

2.7. Genotyping

BD subjects who donated fibroblast cell lines were genotyped at ~420 K single nucleotide polymorphisms (SNP) on PsychChip by TGEN (Phoenix, AZ) as described previously (McCarthy et al., 2018).

2.8. Gene knockdown

Knockdown experiments were performed using commercial siRNA pools that bind multiple sites (Dharmacon). Cells were plated at 20,000/well and grown for 48 h Transfection Reagent (Dharmacon, T-2002–02) and siRNA were mixed with medium and incubated for 20 min at 25 °C before transferring cells to an incubator for 48 h at 37 °C. Results were compared to negative control siRNAs.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9 to conduct T-tests or analyses of variance (ANOVA) as described. Networks were constructed using a correlation matrix to identify all associations among genes. Afterwards, r values were transformed to Z-scores. Node-node relationships were matched across groups and compared using a one-way ANOVA with repeated measures. Post-hoc comparisons of pathway components across groups were made using Fisher's r to Z transformation. A Benjamini-Hochberg correction was applied to the results to limit type I error.

3. Results

3.1. Co-expression of circadian clock and cell survival genes in human fibroblasts

We first examined the relationship between cell survival and circadian clock genes networks in cells from BD patients and controls at a single time point using 12 genes selected from

both systems. Significant correlation in expression was identified between many of the cell survival and circadian clock genes, suggesting substantial co-regulation of the networks (Fig. 1). When comparing BD and control cells, overall organization of gene networks was found to differ significantly (one-way ANOVA, $p<0.05$ Fig. 1A, B). Mean edge strength was generally stronger in the control versus BD network (Fig. 1C). Additional analyses were conducted to assess networks in BD sub-groups, comparing cells from Li-R and Li-NR (Fig. 1D). Mean edge strength was decreased in Li-R compared the Li-NR, leading to overall significant differences in the networks (one-way ANOVA, $p < 0.05$). Pairwise comparisons of node-edge relationships were conducted post-hoc to identify specific relationships that differed between controls and BD cells, and between Li-R and Li-NR (Fig. 1D). More than 30 edges showed nominally significant or trend-level group differences. After correction for false-discovery, 14 remained statistically significant. Ten significant differences were observed between BD and control samples, and four differences were observed between Li-R and Li-NR. In both cases, these included connections within networks (e.g. *PER1-PER2* within the circadian network and *P53-BAX* within the survival network) and between networks (*PER3-CHEK2, BMAL-CHEK2*). None of the individual genes showed significant differences in expression level between Li-R and Li-NR groups (Fig. S1).

3.2. Apoptosis in bipolar disorder and control fibroblasts

Based on the differences in network organization, we hypothesized that cells from BD and controls would differ in vulnerability to apoptosis and/or the protective effects of lithium. To test this, we examined both the initiation and endpoint of apoptosis (caspase activation and viability, respectively) in fibroblasts from controls ($n = 11$) and BD patients ($n = 13$ Li-R, $n = 9$ Li-NR). After 18 h treatment with staurosporine (STS), caspase activity was strongly induced to a similar extent in control and BD cells (Fig. 2A). Interestingly, co-treatment with STS and lithium (10 mM) altered caspase activity distinctly in the two groups. In BD cells, co-treatment with lithium significantly decreased caspase activity. In controls, co-treatment with lithium increased caspase activity. Results of a two-way ANOVA revealed significant effects of drug ($p < 0.0001$), BD ($p < 0.05$) and drug x BD interaction ($p < 0.001$). However, sub-group analyses of the BD samples revealed no differences between Li-R and Li-NR ($p = 0.70$, Fig. 2B). Lithium in the absence of STS had no significant effect on caspase activity (one way ANOVA $p = 0.72$. Mean% Vehicle: Control 99.9, Li-R 96.8, Li-NR 96.8, Fig. S2A).

Viability was assessed 48 h after STS or STS + lithium treatment. After STS alone, 54% of control and 56% of BD cells remained viable (Fig. 2C). Co-treatment of fibroblasts with STS and lithium increased viability compared to STS alone, and these effects were significantly greater in BD compared to control cells (Fig. 2D). Lithium increased viability by 7% in controls whereas viability in BD cells improved by nearly twice as much, increasing survival by 12% (Two-way ANOVA, $p < 0.05$ overall, $p < 0.03$ in post-hoc tests BD vs control). However, sub-group analyses of Li-R and Li-NR revealed no significant differences in viability ($p = 0.64$). Lithium alone increased the number of viable cells by 15–20% but there were no significant group differences (one way ANOVA $p = 0.84$, Fig. S2B).

3.3. Circadian rhythms, caspase activity and cell viability in Li-R and Li-Nr

We next conducted additional analyses of caspase activation and viability to identify associations between cell survival and circadian rhythm parameters. Parallel cultures of fibroblasts were separately tested for circadian rhythms or caspase/viability and the data compared across assays. There was no significant association between any circadian rhythm parameter measured under vehicle-treated conditions and caspase activation or viability following *in vitro* treatment with STS or STS + lithium (Fig. S3). When the cells were treated with lithium, the change in period showed a significant negative correlation with caspase activity, whereby greater period shortening effects of lithium predicted lower levels of caspase activation ($r = -0.4$, $p < 0.05$). Further analyses revealed that this relationship differed by group. In controls, association between period shortening and caspase was strongest ($r = -0.85$, $p < 0.05$). In Li-R, this relationship was weaker with a nominal relationship in the same direction ($r = -0.46$, $p = 0.11$) whereas the correlation was entirely absent in Li-NR cells ($r = -0.03$, $p = 0.93$). These data indicate that in the absence of a trigger for apoptosis like STS, period shortening by lithium is associated with reduced caspase activity in controls and to some extent Li-R, but not in Li-NR (Fig. 3).

3.4. Effects of clock gene knockdown on apoptosis

Associations between clock genes and caspase activity prompted us to perform additional experiments to evaluate potential mechanisms between clock gene regulation of cell survival. Based on our network analysis and potential importance of circadian period, we selected *Per1* and *Per3* for further evaluation as both genes are involved in period length (Liu et al., 2007), and showed distinctive connections in controls, Li-R and Li-NR. We conducted these experiments in immortalized mouse fibroblasts stably transfected *Per2*-luc so that we could knockdown expression of *Per1* and *Per3*, induce apoptosis and monitor rhythms simultaneously in the same cellular model, a combination of procedures that is difficult to do in primary human cells. We have reported previously that circadian rhythms in this mouse model respond to lithium in a manner similar to human fibroblasts (McCarthy et al., 2015, 2016; Wei et al., 2018). Treatment with *Per1* siRNA reduced target gene expression by 80% (Fig. 4A) and had significant effects on circadian rhythms, shortening period by 1.6 h and decreasing amplitude to 63% compared to control (both $p<0.0001$, Fig. 4B–D). After 18 h of STS treatment, apoptosis in mouse fibroblasts was similar to that observed human cells, with a 224% mean increase in caspase activation resulting in an average of 75% viability in control siRNA-treated cells (Fig. 4E,F). *Per1* knocdown alone caused a modest but significant decrease in caspase activity (94% of control, Fig. 4E) and a significant increase in the number of viable cells compared to control siRNA (113% of control, Fig. 4F). Following treatment with STS, *Per1* knockdown further attenuated caspase activation (Per1 siRNA −16% vs control siRNA after STS, Fig. 4E) and significantly increased viability compared to control siRNA (viability after STS: 70% control vs 90% *Per1* siRNA, Fig. 4F). Together, these results suggest that *Per1* typically facilitates apoptosis and reducing *Per1* activity protects cells. Knockdown reduced *Per3* expression by >50% (Fig. 4G). In circadian rhythm assays, *Per3* knockdown increased amplitude by 25% and modestly but significantly shortened period by 0.1 h showing a distinct phenotype from *Per1* with an attenuated effect on period and opposite effect on amplitude (Fig. 4H–J). *Per3* knockdown alone had significant effects on caspase activation and viability that were distinct from *Per1*

knockdown. In contrast to *Per1, Per3* knockdown at baseline caused a significant increase in caspase activation of 18% causing a 12% decrease in cell viability compared to control siRNA treated cells (Fig. 4K–L). In the apoptosis model, *Per3* knockdown was additive with STS, increasing caspase activation by an additional 27% and reducing viability further by 8% compared to control siRNA (Fig. 4K–L). The results indicate that both *Per1* and *Per3* regulate apoptosis and that under baseline and apoptosis conditions *Per3* provides a proliferative/cellular protective effect opposite to that of *Per1*.

3.5. Genetic variation, apoptosis and circadian rhythms

Finally, we set out to determine if common genetic variation in circadian clock genes predicted viability in human cells from BD patients. Previous work suggests that *PER1/3* regulate apoptosis, possibly through interaction with *GSK3B* (Magnone et al., 2014; Sato et al., 2009; Im et al., 2010), and that variation in these genes is associated with circadian rhythm and psychiatric phenotypes in human behavior (Dong et al., 2011; Lavebratt et al., 2010; Archer et al., 2018; Jones et al., 2019). We reasoned that previously identified, functional polymorphisms in clock genes and *GSK3B* may also cause identifiable cellular circadian rhythm phenotypes and alter sensitivity to apoptosis (Table S2). The *PER1* variant rs3027178 was significantly associated with reduced *PER1* expression in fibroblasts from carriers of the minor G allele (Fig. 5A). In *Per2*-luc circadian rhythm assays conducted in human BD patient cells, *PER1* genotype had no significant association with amplitude, but homozygous carriers of the minor G allele in *PER1* trended towards longer period (26.2 h vs. 25.6 h, $p = 0.06$ Fig. 5B–C) and showed a significant difference in period shortening when the cells were tested *in vitro* with lithium 1 mM (homozygous minor alleles vs homozygous common/heterozygous: −0.5 h vs. 0.0 h, respectively, $p = 0.01$, Fig. 5D). The minor G allele was associated with decreased *PER1* expression and increased viability after STS treatment, similar to the effects of *Per1* knockdown and reduced expression in mouse cells (Fig. 5E).

The PER3 rs228729 variant marks a common variable number tandem repeat in PER3 protein that affects post-translational regulation. Therefore, it did not have any bearing on *PER3* expression in fibroblasts (Fig. 5F). In *Per2*-luc rhythm assays in human BD patient cells, the *PER3* variant rs228729 was significantly associated with rhythm amplitude (Fig. 5G) but was not associated with period either at baseline or after treatment of cells *in vitro* with lithium (Fig. 5H–I). After induction of apoptosis by STS, *PER3* genotype was significantly associated with viability, with cells homozygous for the T allele showing less viability (Fig. 5J), replicating a finding from the mouse study where the *PER3* siRNA manipulation resulting in higher amplitude was associated with greater cell death. *PER2* genotype did not predict viability in this small sample (Fig. S4A). The C allele of *GSK3B* SNP rs334558 associated with reduced gene expression (McCarthy et al., 2011), lithium response (Benedetti et al., 2005) and circadian rhythms (McCarthy et al., 2013) also predicted improved viability after STS (Fig. S4B). None of the genetic variants were associated with increased viability after treatment with lithium alone or STS + lithium.

4. Discussion

Gray matter loss in the brain is now a well-established finding in BD, but with unclear implications for diagnosis or disease course (Hibar et al., 2018, 2016). Establishing valid biomarkers that diagnose BD and predict lithium response would greatly facilitate effective and rapid treatment of mood disorders and open the door to identifying new mechanisms for improved therapeutic strategies. Accordingly, focusing on established brain abnormalities in BD holds promise for biomarker discovery. Previously reported markers of lithium response include components of the cell survival pathway and circadian clock (Anand et al., 2020; Beech et al., 2014; McCarthy et al., 2018). We examined these two processes in human and mouse cellular models of BD to identify overlap across the molecular pathways, assess their function and conduct a preliminary assessment of their predictive validity in determining lithium response.

4.1. Clock genes and apoptosis

In agreement with past studies of clock genes conducted in cancer cell lines (Sato et al., 2009, 2011), we found consistent evidence of a role for *PER1* and *PER3* in regulating apoptosis, affecting both initiation events (caspase activation) and endpoints (viability). We built on this previous work by studying primary cells from human BD patients and for the first time directly compared measures of apoptosis and circadian rhythms in the same samples. Our work marks an advance over past studies of cell death and lithium response in BD patient samples that ascertained lithium response retrospectively, and examined only spontaneous cell death without experimental induction of apoptosis and without measures caspase activity (Paul et al., 2020).

4.2. Network differences between Li-R and Li-Nr

The expression of clock gene and cell survival networks was distinctly co-regulated in fibroblasts from BD patients and controls. Numerous differences in co-expression patterns were observed within the clock network, the cell survival network and between networks. Notably, *BMAL1* a BD risk gene supported by evidence from GWAS was differentially co-expressed with other genes in two instances suggesting a possible pathological mechanism in BD (Mullins et al., 2021; Ruderfer et al., 2018). *BRCA1* co-expression was disrupted in four instances, making it the gene most dysregulated gene in our study. While evidence for *BRCA1* in BD is limited, familial mutations of this gene have been observed previously in BD case studies demonstrating circadian disruption and robust responses to lithium (Federoff and McCarthy, 2021). Organization of the BD gene networks was heterogeneous and differed significantly between Li-R and Li-NR. In one interesting example, a strong inverse correlation in *BRCA1-NR1D1* expression was identified in Li-NR. Notably Rev-Erbα protein encoded from *NR1D1* is regulated by lithium and variants in the gene have been associated previously with lithium response (McCarthy et al., 2011; Yin et al., 2006). Overall, coordination across networks was stronger in Li-NR cells. In the context of desynchronized circadian rhythms, strong coupling of the circadian clock to the cell cycle has been reported (Feillet et al., 2014). We have reported previously that circadian rhythms are weaker and desynchronized in BD, especially in Li-NR cells compared to controls (Mishra et al., 2021; Nudell et al., 2019). Therefore, high levels of correlation

between clock and cell survival networks in Li-NR may reflect desynchronization resulting in weak circadian rhythms and “hijacking” of the circadian clock by strong inputs (i.e. phase locking) from the adjacent cell cycle pathway. In controls and Li-R, clock gene expression may be more autonomous, and less influenced by adjacent pathways given the higher degree of internal synchronization within the circadian clock network. Our gene expression analysis was not well-powered to identify group differences for individual genes. We estimate we had 80% power to detect very large effects ($d \geq 1.8$), but may not have been able to identify effects of smaller magnitude.

4.3. Distinct apoptosis mechanisms in control and bipolar disorder cells

Lithium was protective in controlled apoptosis assays conducted in human fibroblasts. In BD samples specifically, lithium inhibited STS-induced caspase activity leading to a significant survival advantage vs. control cells. That lithium protection from apoptosis is enhanced in BD compared to control samples implies that lithium addresses a BD-specific cellular abnormality. In particular, it indicates caspase activity is regulated by lithium distinctly in BD cells. Caspases 3/7 are enzymes responsible for initiating apoptosis. They are activated by two distinct upstream mechanisms termed the intrinsic and extrinsic pathways (Elmore, 2007; Orrenius et al., 2003). The intrinsic pathway is triggered by calcium and activation of caspases 1/9 [61]. The extrinsic pathway is engaged by ligands such as TNFα that activate distinct upstream pathways mediated by caspase 8. Both intrinsic and extrinsic pathways converge upon mitochondrial BAX proteins that promote apoptosis. The anti-apoptosis effects of lithium require calcium (Kang et al., 2003), and we have observed in human fibroblasts that lithium causes less intracellular calcium activity in BD patient vs. control cells (McCarthy et al., 2015). In the present context, this may imply that following lithium treatment and the rise in calcium in control cell, the calcium-dependent intrinsic pathway is activated by caspase 1/9, whereas in BD cells, lithium triggers lower levels of calcium that may be sufficient to support lithium protection pathwats but with reduced activation of the intrinsic cell death pathway. In this way, a more favorable balance of lithium-stimulated calcium signaling might have the benefit of making BD cells less impacted by calcium-driven apoptosis signals and more protected by lithium. Calcium channel genes (e.g. *CACNA1C*) have been implicated in BD and show evidence of circadian disruption (McCarthy et al., 2015; Mullins et al., 2021). They may be plausible candidates to explain the differences presently observed in caspase activity and the anti-apoptotic effects of lithium. Extrinsic pathway or other caspase-independent factors may also be involved. For instance, TNFα has been linked with mood episodes in BD (Goldsmith et al., 2016) and lithium response (Guloksuz et al., 2012).

There was no detectable difference between Li-R and Li-NR in caspase activation or viability in the STS apoptosis assay. However, network analysis did reveal difference between these sub-groups. In *Per2*-luc circadian rhythm assays, additional group differences were observed in caspase activation and period that distinguished controls, Li-R and Li-NR. Taken together, these results indicate that there are detectable differences among control, Li-R and Li-NR in circadian clock and cell survival networks under conditions that do not strongly trigger apoptosis, but that these differences are overwhelmed in the context of a strong inducer of apoptosis like STS. Unfortunately, in the fibroblast model, use of STS was

essential to induce apoptosis in a controlled and lithium-reversible manner. However, gray matter loss in BD is subtle and only detectable when comparing large groups of patients and controls (Hibar et al., 2018, 2016). Accordingly, damage to the brain in BD likely emerges from persistently increased physiological dysregulation of mitochondria, calcium and glutamate transmission in neurons rather than focused toxic exposures (Mertens et al., 2015; Scotti-Muzzi et al., 2021). In this context, the differences in gene expression observed between Li-R and Li-NR cells at rest or treated with lithium may be more relevant than the STS model to the condition *in vivo*. These contexts may better reflect the physiological state of circadian and cell survival pathways in response to perturbed neurophysiological functions that and are known to be altered by lithium and may involve ATM, CHEK2 and P53 proteins (Kim and Thayer, 2009; Di Giovanni and Rathore, 2012; Herrup et al., 2013). Conversely, treatment with STS is a stronger apoptosis signal than neurons in the BD brain would typically encounter and might model only a limited set of circumstances.

In our experiments conducted in human BD patient and control fibroblasts, caspase activation correlated with circadian period following lithium exposure. In agreement with many past studies, our knockdown experiments in mouse cells indicate that *Per1* and *Per3* participate in regulating period length, but also that have pleiotropic roles in regulating caspase activity and cell survival. This was revealed by knockdown experiments that caused partial (50–80%) loss of gene expression and rhythm disruption of similar magnitude to what might be induced by strong environmental changes (e.g., light exposures, jet lag) or genetic variation indicating the clock genes effects on cell survival may have physiological relevance *in vivo*. Reduced expression of *Per1* in mouse cells caused lower caspase activation and greater protection from apoptosis. Genetic variation in *PER1* is also associated with decreased expression, period shortening following lithium. These lines of evidence converge to suggest lithium shortening, perhaps through *PER1* may contribute to lower caspase activation and confer protection to cells against apoptosis. *PER3* serves largely opposite roles, wherein loss of expression favors caspase activation and cell death. Gene co-expression network maps that revealed distinct profiles of *PER1* and *PER3*. In BD cells, the co-regulation of *PER3* and *CHEK2* was stronger than in controls. Similarly, the correlation between *PER1* and *PER2* was much stronger in Li-NR vs Li-R. *PER1* is influenced by numerous environmental stimuli and overlaps in function with PER2 (Motzkus et al., 2000; Albrecht et al., 2001). Therefore, strong coordination of *PER1* with other genes may risk destabilizing networks by sensitizing cells to external signals and/or phase locking rhythms to other cellular events (Feillet et al., 2014). Our previous observation of increased *PER2* in neurons from Li-NR is consistent with this observation (Mishra et al., 2021). We found that *PER1* and *PER3* influence the baseline vulnerability to apoptosis in opposite directions, possibly by distinctly interacting with the cell death regulators such as *BAX, P53* and *BCL2* that have previously been shown to be rhythmically expressed (Magnone et al., 2014; Miki et al., 2013; Hua et al., 2006) and show correlations with circadian clock genes in our model.

4.4. Limitations

While the circadian clock and cell survival networks are highly conserved across cell types, the fibroblast model is limited in the context of studying cell death mechanisms in neurons.

Fibroblasts differ across several key features that make neurons especially vulnerable (e.g. comparatively low energetic requirements, lack of glutamate receptors) meaning that fibroblasts must be treated with potent agents to induce cell death that may be even more severe than the conditions promoting cellular stress in BD patient brains. Indeed, it is not clear that the decreased brain volume in BD reflects neuronal death or smaller neurons with less extensive dendritic processes (Gigante et al., 2011). Therefore, the evidence we provide from STS experiments indicating that lithium is protective distinctly in BD cells remains equivocal with regards to the lack differences between Li-R and Li-NR as the STS model may overwhelm subtle differences between groups. Future work should make use of neuronal cell types and cellular stressors to develop other models that approximate the conditions in the brain of BD patients (Mertens et al., 2015; Mishra et al., 2021). While we focused on the circadian clock system, many of our studies were conducted at a single time point, and could have missed differences only observable under dynamic conditions that involve circadian rhythms. Similarly, we focused on just a small set of genes in the circadian clock and cell survival networks, just a small fraction of both systems. Analysis of additional genes will be required to understand more about these networks and their interactions. Finally, many of our experiments were conducted in small samples, and it is possible that some experiments were underpowered to identify subtle effects.

5. Conclusions

In a BD patient-derived cellular model of lithium protection, we provide new evidence that coordination between the circadian clock and cell survival pathways is distinct in BD and sometimes distinguishes Li-R from Li-NR. Moreover, common variation affecting the expression of circadian clock genes (especially *PER1* and *PER3*) may be sufficient to have effects on cell survival in BD patient cells. Differences between Li-R and Li-NR were not observed in every assay, and did not differ in the extent to which lithium protects cells from apoptosis. Therefore, how the organization of circadian clock and cell death pathways relate to lithium response in BD was not fully ascertained in the present study, but these preliminary results indicate the question should be studied further. Future studies may benefit from the use of patient derived neuronal cells, sensitive measures of cell death mechanisms and/or larger samples to provide good statistical power.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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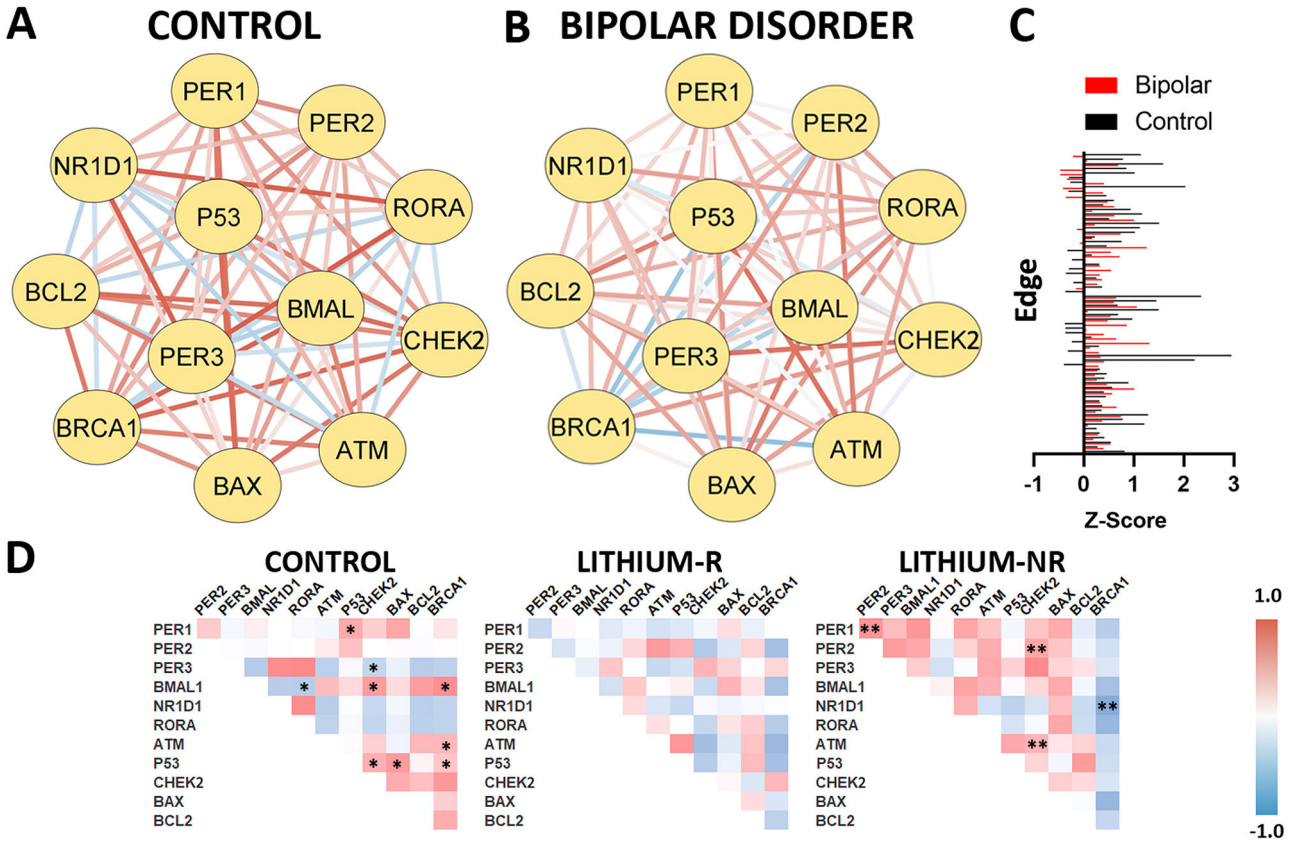

Fig. 1.

Circadian clock and cell survival gene expression networks in BD patient and control fibroblasts. Gene expression of 6 clock genes and 6 apoptosis genes was measured in fibroblasts from controls and Li-R/Li-NR BD patients. (A) Using the pairwise correlation between genes (nodes) as a proxy for connection (edges), network maps were constructed to examine expression patterns across A) control ($N = 10$) and (B) BD ($N = 13$) cells. Strength of correlations is shown by color (scale lower right: red indicates high, blue indicates low). (C) Mean edge strength differed significantly in control (black) and BD (red) expression networks (mean edge strength $Z = 0.55 \pm 0.08$ control vs 0.33 ± 0.04, $p<0.05$). (D) Examination of specific edge-node relationships was conducted post-hoc and compared between control and BD groups (Li-R and Li-NR). Relationships that differed significantly between BD and controls after FDR correction are indicated (*). Similar analysis was conducted to examine specific edge-node differences between Li-R and Li-NR. Relationships that differed significantly between Li-R and Li-NR after FDR correction are indicated (**). Overall configuration between Li-R and Li-NR was significantly different ($p<0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

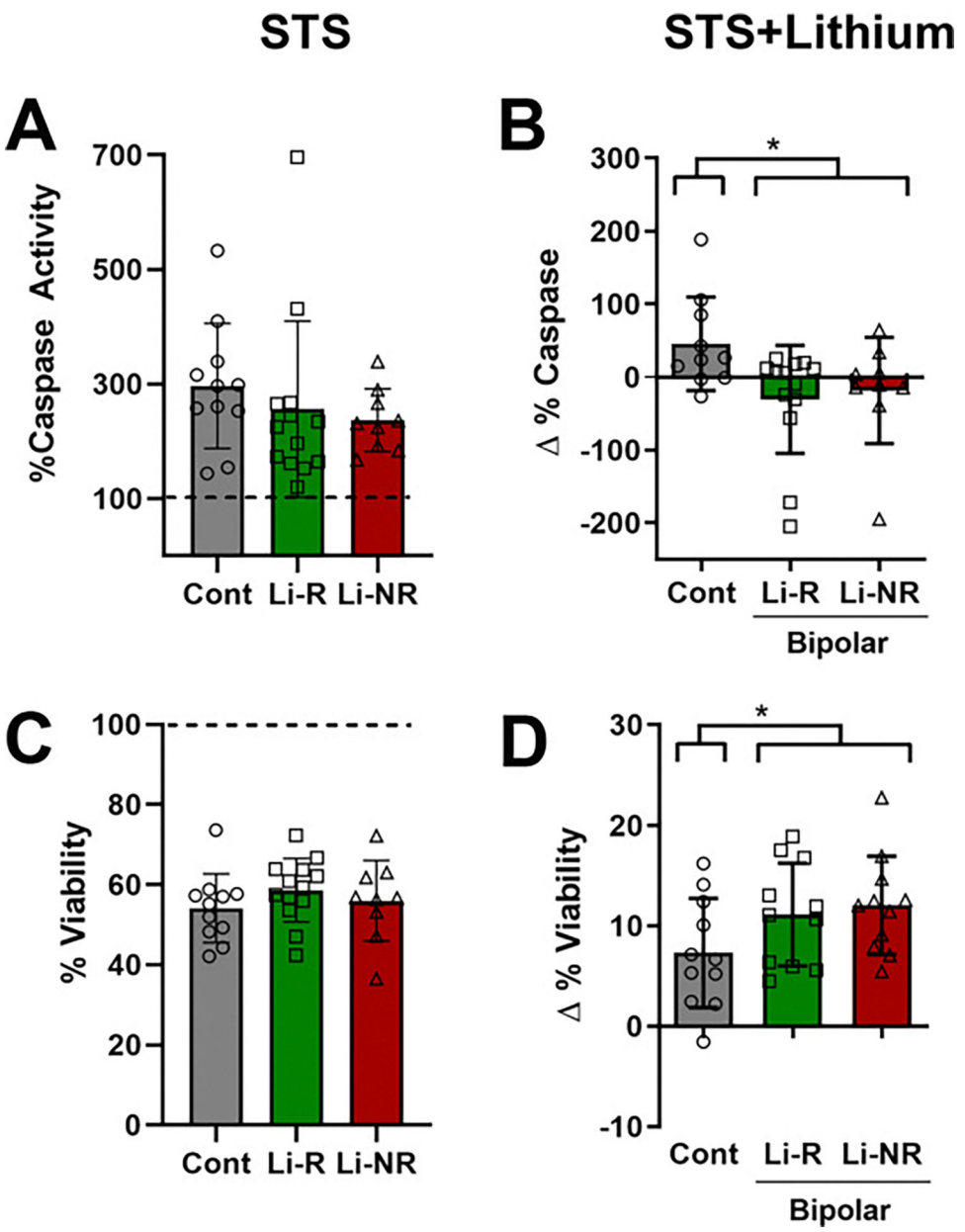

Fig. 2.

Staurosporine-induced apoptosis and viability in BD and control fibroblasts. (A) Treatment of fibroblasts with STS 62.5 nM for 18 h caused a 252 ± 16% increase in caspase 3/7 activity (mean across all groups) compared to vehicle (dashed line). One-way ANOVA revealed a significant effect of drug (p<0.0001), but changes in caspase activity were similar across control (gray), Li-R (green) and Li-NR (red) cells. (B) Compared to STS treatment alone, co-treatment of cells *in vitro* with lithium (10 mM) and STS increased caspase activity an additional 45 ± 20% in controls to 352 ± 41% (n = 10), but decreased caspase activity by 25% ± 15 in BD (n = 22) to 223 ± 16%. Two-way ANOVA revealed significant effects of drug (p<0.0001), BD vs. control (p<0.05) and drug x BD interaction (p<0.001), indicating the lithium had distinct effects on apoptosis in BD and controls (n = 10). The

decrease in caspase activity was similar in Li-R (30 ± 20%, $n = 13$) and L-NR (18 ± 24%, $n = 9$). (C) 48 h after treatment with STS, viability decreased to 55.9 ± 1.6% vs. vehicle (dashed line). One-way ANOVA revealed a significant effect of STS ($p<0.0001$), but viability was similar across control, Li-R and Li-NR cells. (D) Co-treatment of fibroblasts with lithium and STS significantly increased viability compared to STS alone. The increase in viability was greater in BD (12.0 ± 1.0%) vs. control (7.1 ± 1.6%) and was significantly different between groups (Two-way ANOVA $p<0.05$, post-hoc test BD vs control $p<0.03$). The increase in viability from lithium was similar in Li-R (green, $N = 13$) and Li-NR (red, $N = 9$). For all results, values are expressed as mean ± standard error of the mean (SEM). Error bars in graphs indicate SEM. * indicates significant group difference BD vs control $p<0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

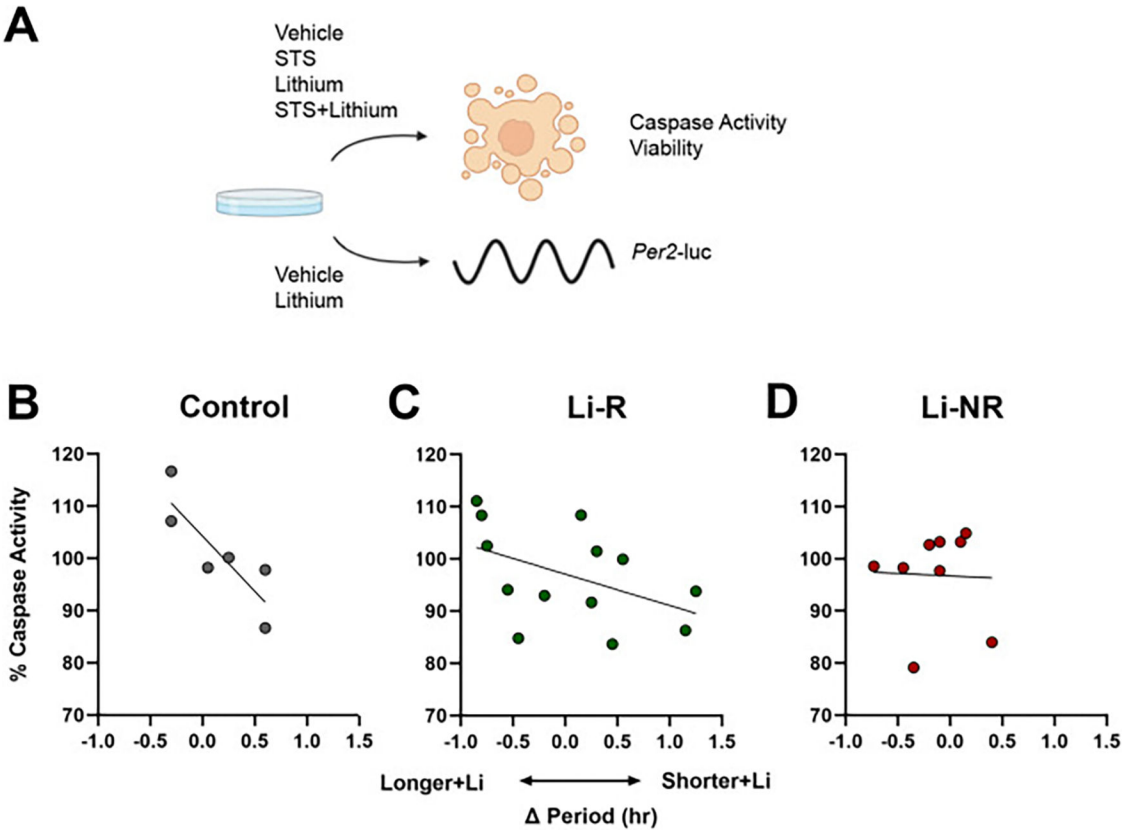

Fig. 3.

Relationship between circadian period and caspase activity following lithium treatment differs by BD diagnosis and lithium response. (A) Parallel studies of fibroblast cultures were conducted under different drug conditions to measure caspase activity and viability (vehicle, STS, lithium and STS + lithium) and circadian rhythms (vehicle and lithium). Caspase and viability were assessed for correlation with three circadian rhythm parameters (period, amplitude and phase) estimated from rhythm measurements collected from the same cell line. Caspase activity was measured in fibroblasts from control (gray, $n = 5$) and Li-R (green, $n = 13$) and Li-NR (red, $n = 9$). Caspase activity following lithium treatment *in vitro* showed overall correlation with the period shortening effects of lithium on circadian rhythms ($r=-0.4$, $p<0.05$ for all samples, $n = 27$). However, this relationship differed by group. Correlation was strongest in (B) controls ($r=-0.85$, $p<0.05$), moderate in (C) Li-R ($r=-0.46$, $p = 0.11$) and absent in (D) Li-NR ($r=-0.03$, $p = 0.93$). X axis indicates Δ period expressed as (baseline-lithium). Circadian rhythm effects of lithium were evaluated at 1 mM, the concentration that best distinguishes BD and control samples and corresponds to therapeutic drug levels (McCarthy et al., 2013). Survival effects of lithium on fibroblasts were measured at 10 mM which showed protectively properties against apoptosis. Lithium 1 mM was ineffective at protecting cells from STS in fibroblasts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

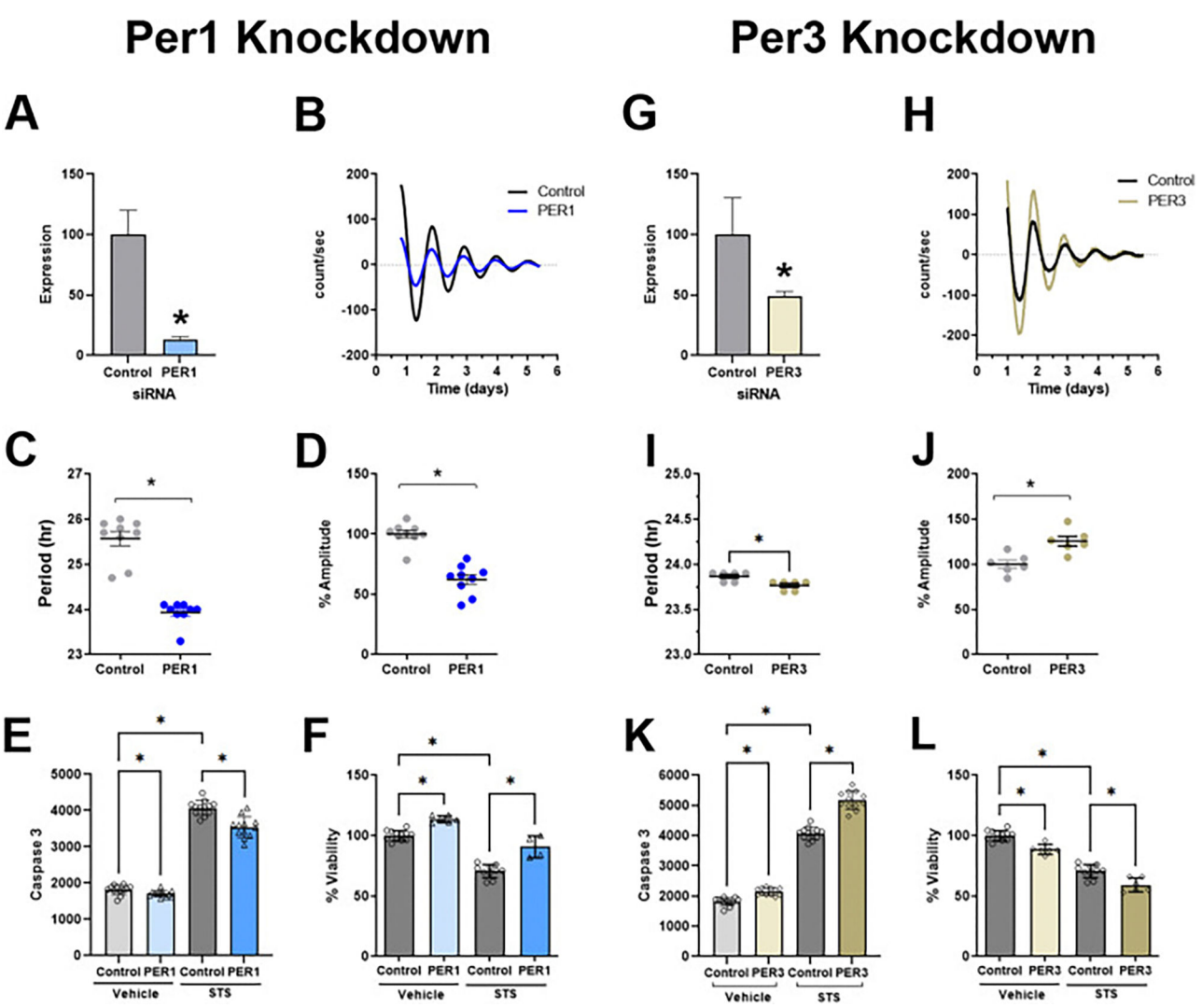

Fig. 4.

Causal connections between circadian clock genes and apoptosis. Gene knockdown with siRNA caused (A) reduced expression of *Per1* by 80% (normalized to control, $p<0.002$, $n = 3–6$). (B) *Per1* knockdown caused significant effects on cellular circadian rhythms including (C) shorter period (one-way ANOVA $p<0.05$, $n = 8$/ group) and (D) decreased amplitude (one way ANOVA $p<0.05$, $n = 8$/ group). *Per1* knockdown caused (E) decrease in caspase activity (one-way ANOVA $p<0.0001$, $n = 6$/group) and (F) increased viability both at baseline and after STS-induced apoptosis (one-way ANOVA $p<0.0001$, $n = 6$/group). Gene knockdown with siRNA (G) significantly reduced *Per3* expression by 50% (normalized to control, $p<0.05$, $n = 3–6$) and (H) caused significant effects on cellular circadian rhythms including (I) a modest lengthening effect on period (one way ANOVA $p<0.05$, $n = 6$/group) and (J) increased amplitude (one way ANOVA $p<0.05$, $n = 6$/group) *Per3* knockdown caused (K) increase in caspase activity (one-way ANOVA $p<0.0001$, $n = 12$/group) and (L) decreased viability both at baseline and after STS-induced apoptosis (one-way ANOVA $p<0.0001$, $n = 12$/group). * Indicates $p<0.05$.

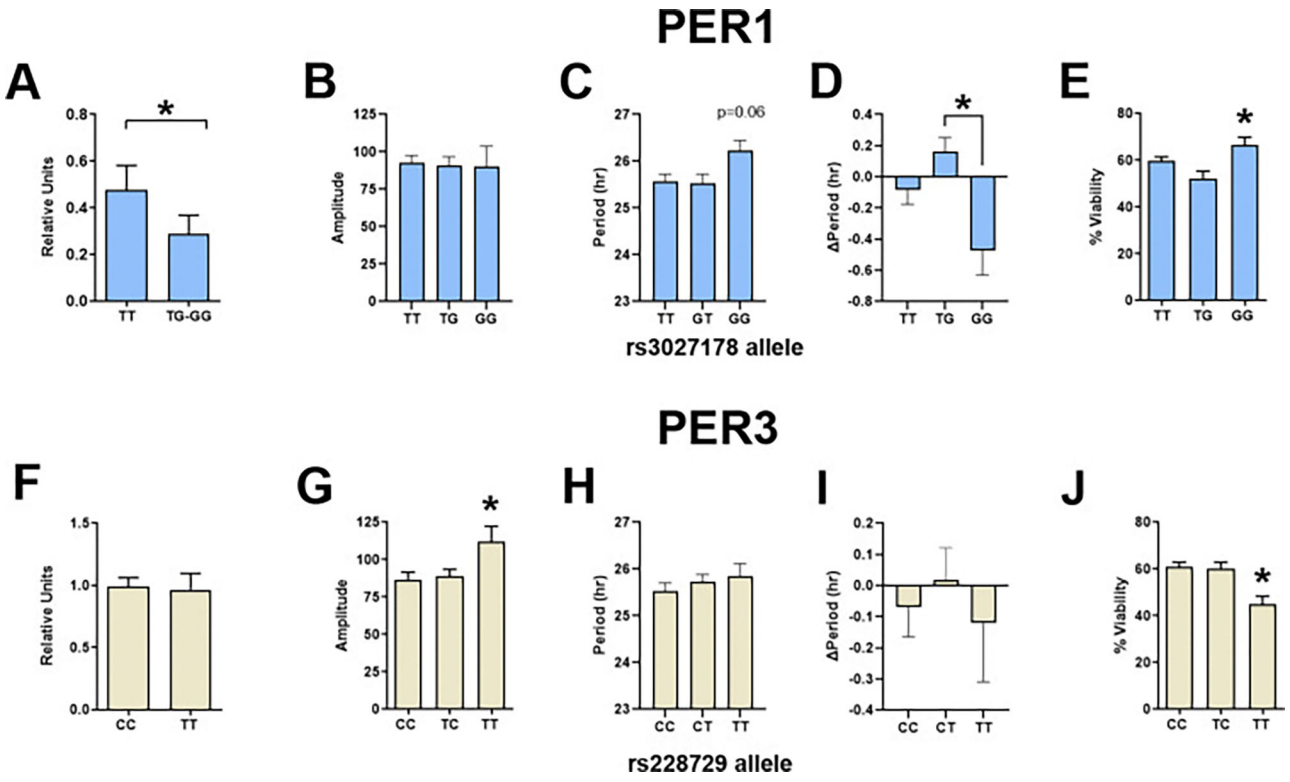

Fig. 5.

Common genetic variants in PER1 and PER3 are associated with BD fibroblast sensitivity to apoptosis. The *PER1* SNP rs3027178 was associated with significantly increased PER1 expression in fibroblasts with T/T alleles (*N* = 7) compared to combined sample of T/G (*n* = 5) and G/G (*n* = 1) alleles using a dominant genetic model (T-test <0.005). In *Per2-luc* rhythm assays in BD patient fibroblasts, *PER1* genotype had (B) no effect on amplitude but was associated with (C) a trend towards longer period at baseline and (D) a significantly greater period-shortening effect of lithium (1 mM) treatment *in vitro*. For *PER1* rhythm assays samples size were T/T (*n* = 35), T/G (*n* = 21) and G/G (*n* = 7). (E) *PER1* genotype was associated with viability after STS treatment as fibroblasts with the G/G allele (*n* = 3) were significantly more viable than fibroblasts with T/T (*n* = 10) or T/G (*n* = 9) alleles. (F) The SNP rs228729 (*PER3* VNTR) was not associated with any difference in *PER3* expression but the T/T genotype was associated with (G) higher amplitude in BD patient fibroblasts. In the same cells, period did not differ by *PER3* genotype (H) at baseline or (I) in response to lithium treatment *in vitro*. For *PER3* rhythm assays sample sizes were T/T (*n* = 11), C/C (*n* = 28) and T/C (*n* = 24). (J) *PER3* genotype was associated with viability: the T/T genotype (*n* = 4) was significantly less viable compared to cells with C/C (*n* = 9) or T/C (*n* = 8) alleles.