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# DISINHIBITION OF THE EXTRACELLULAR-SIGNAL-REGULATED KINASE RESTORES THE AMPLIFICATION OF CIRCADIAN RHYTHMS BY LITHIUM IN CELLS FROM BIPOLAR DISORDER PATIENTS

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## ABSTRACT:

Bipolar disorder (BD) is characterized by depression, mania, and circadian rhythm abnormalities. Lithium, a treatment for BD stabilizes mood and increases circadian rhythm amplitude. However, in fibroblasts grown from BD patients, lithium has weak effects on rhythm amplitude compared to healthy controls. To understand the mechanism by which lithium differentially affects rhythm amplitude in BD cells, we investigated the extracellular-signal-regulated kinase (ERK) and related signaling molecules linked to BD and circadian rhythms. In fibroblasts from BD patients, controls and mice, we assessed the contribution of the ERK pathway to lithium-induced circadian rhythm amplification. Protein analyses revealed low phospho-ERK1/2 content in fibroblasts from BD patients vs. controls. Pharmacological inhibition of ERK1/2 by PD98059 attenuated the rhythm amplification effect of lithium, while inhibition of two related kinases, c-Jun N-terminal kinase (JNK), and P38 did not. Knockdown of the transcription factors CREB and EGR-1, downstream effectors of ERK1/2, reduced baseline rhythm amplitude, but did not alter rhythm amplification by lithium. In contrast, ELK-1 knockdown amplified rhythms, an effect that was not increased further by the addition of lithium, suggesting this transcription factor may regulate the effect of lithium on amplitude. Augmentation of ERK1/2 signaling through DUSP6 knockdown sensitized NIH3T3 cells to rhythm amplification by lithium. In BD fibroblasts, DUSP6 knockdown reversed the BD rhythm phenotype, restoring the ability of lithium to increase amplitude in these cells. We conclude that the inability of lithium to regulate circadian rhythms in BD may reflect reduced ERK activity, and signaling through ELK-1.


## INTRODUCTION

Bipolar disorder (BD) is a psychiatric illness characterized by recurrent mood episodes of depression and mania; and disruptions in daily cycles of sleep and activity. Actigraphic studies of BD suggest low amplitude circadian rhythms may be central to these disturbances. Loss of rhythm amplitude has been associated with mood relapse (Ankers and Jones, 2009), and is associated with clinical features of mania, including activity, sleep, and disordered thought (Gonzalez et al., 2014). The occurrence of rhythm disturbances are not limited to symptomatic periods. Between mood episodes, euthymic subjects have more variable rhythms, with less daytime activity, more nighttime activity, and reduced sleep compared to controls (Jones et al., 2005; McKenna et al., 2014), suggesting that rhythm disturbances are a stable trait marker of BD. The mood stabilizer lithium treats BD symptoms, and has effects on circadian rhythms in cells, including increases in amplitude (Johansson et al., 2011; Li et al., 2012; McCarthy et al., 2013). Because skin fibroblasts contain cell autonomous circadian clocks, they can be used to study rhythms and their molecular mechanisms in clinical samples, including those from BD patients (Liu et al., 2007; McCarthy et al., 2013). Based on this fact, we found in previous studies that in fibroblasts from healthy controls, lithium increases amplitude, but generally fails to increase it in cells derived from BD patients (McCarthy et al., 2013), suggesting the bipolar clock may be less responsive to input signals stimulated by this drug. Among the inputs known to affect circadian rhythms are calcium signals from L-type channels (Kim et al., 2005), and the protein kinase GSK3B (Iitaka et al., 2005),

two molecules with links to BD. The mood stabilizing actions of lithium have been attributed to GSK3B inhibition (Klein and Melton, 1996), and genetic variants in *CACNA1C*, an L-type calcium channel gene have been strongly associated with disease risk for BD (PGC-BD, 2011). We have shown more recently that genetic variants in *GSK3B* (McCarthy et al., 2013) and *CACNA1C* (McCarthy et al., 2015) predict rhythm amplification by lithium in BD cell lines, suggesting overlap across BD susceptibility genes, regulators of circadian rhythms, and targets of lithium. However, how these molecular links are affected in BD remain incompletely described. Among the key molecular inputs to the circadian clock are the extracellular-signal-regulated kinases (ERK). Reports from postmortem brain indicate that there are decreased levels of ERK1/2 protein in BD (Dwivedi et al., 2001; Yuan et al., 2010). In suprachiasmatic nucleus (SCN) neurons, light evoked calcium signals are propagated into the nucleus to re-set the phase of the clock through ERK1/2 dependent processes (Dziema et al., 2003). Animal studies suggest that lithium may engage ERK as well to alter Per2 expression by activating the transcription factor EGR-1 (Kim et al., 2013). Importantly, both ERK1/2, and DUSP6, a negative regulator of ERK1/2, have been implicated in BD (Kim et al., 2012; Lee et al., 2006; Seifuddin et al., 2013; Yuan et al., 2010). Therefore, ERK signaling may be important for understanding circadian rhythm abnormalities in BD as they pertain to lithium. However, the implications for circadian rhythms have not yet been fully evaluated in clinical samples from BD patients or using the most advanced techniques for measuring rhythms. Therefore, the signal transduction pathways and nuclear targets engaged by lithium to regulate circadian rhythms remain incompletely characterized.

Presently, we describe the results of experiments implicating the ERK pathway in lithium's amplification of circadian rhythms in fibroblasts grown from patients with BD, healthy controls, and mice. Using a sensitive reporter gene assay, we distinguish the roles of other MAPKs (P38 and JNK) from ERK, and characterize the role of transcriptional regulators, ARNTL, EGR-1, CREB and ELK-1 on modulating signals from lithium to the circadian clock component PER2. Finally, we identify DUSP6 as a regulator of lithium's actions on the circadian clock, presenting evidence of pharmacological enhancement of lithium's effect on circadian rhythm amplitude.

## EXPERIMENTAL PROCEDURES

**Human Subjects.** Skin biopsies were obtained from BD (type I) patients who consented to research while hospitalized or participating in a clinical trial. Additional details regarding the sample have been reported previously (McCarthy et al., 2015; McCarthy et al., 2013). Use of human subjects was conducted in accordance with all pertinent regulations and approved by the VASDHS IRB.

**Bioluminescent Reporter Genes.** Fibroblasts were transduced as described previously with the *Per2::luc* lentiviral reporter gene. For siRNA transfection experiments in human fibroblasts, a modified *Per2::luc* lentiviral reporter containing blasticidin resistance gene was used to stably express the Per2::luc reporter, allowing selection and expansion of cell lines that expressed the reporter (Liu et al., 2007). NIH3T3 cells were stably

transfected with the Per2::luc reporter gene (NIH3T3$^{P2L}$) as described previously (McCarthy et al., 2015; Meng et al., 2008).

**Cell Culture.** Human fibroblasts were grown from frozen cryovials to confluence in 100 mm plates in standard culture medium [DMEM with 10% fetal bovine serum (FBS), glutamine, and antibiotics (penicillin, streptomycin, and amphotericin). NIH3T3$^{P2L}$ cells were grown with hygromycin to select for cells expressing the Per2:luc transgene. Imaging studies of human cells were conducted using ~1.2 x $10^6$ cells in 35 mm plates. For NIH3T3$^{P2L}$ imaging, cells were dispersed into 24 well plates at ~2 x $10^5$ cells / well.

**Protein Analyses.** In order to understand the mechanism of lithium on rhythm amplitude in BD, we selected fibroblast lines that exemplified the BD vs. control differences reported in our previous studies. Four control cell lines that demonstrated lithium-dependent rhythm amplification and four BD cells lines that failed to show rhythm amplification after lithium were selected for western blot. Details of the cell line donors (Table S1) and sample rhythm traces (Figure S1) are provided. Protein solutions were made from frozen cell pellets using ice cold lysis buffer specially designed for assessment phospho-proteins (R&D Systems). Protein concentration was determined using the Pierce 660 nm Protein Assay (Life Technologies). Samples were denaturated at 95°C for 8 min and kept on ice for 8 min. Protein (12 ug) was loaded on 10% acrylamide gels and separated at 40 mA. After transfer to PVDF membranes (Roche) for 2 hr at 200 mA, membranes were blocked with 5% milk. Total ERK1/2 (Cell Signaling #4695), and phospho-ERK (Cell signaling #4370) were quantified using antibodies diluted 1:1000 in 5% BSA. B-tubulin immunoreactivity was measured as a loading control (Cell signaling #2146). For analyses, all data were adjusted for differences in B-tubulin. Phospho-ERK1/2 was then expressed as a fraction of total ERK1/2. An independent protein analysis was conducted in additional cell lines that were selected only on the basis of BD diagnosis, without regard to rhythm parameters (Table S1). Cell lysis and determination of total protein content was conducted as described for western blots. Prior to assay, all samples were diluted to ~150 µg/ml total protein. Following the manufacturers protocol, a colorimetric assay, the SimpleStep enzyme-linked immunosorbent assay (ELISA) Kit (Abcam, ab176660) was used to measure total and phospho-ERK1/2 content. Standards for ERK and phospho-ERK were run simultaneously in parallel. To conduct the assay, samples were incubated in antibody solution for 2 hours with gentle shaking at room temperature, followed by three washes. After washing, TMB horseradish peroxidase substrate was added to each well and incubated for 15 minutes. Optical density was recorded at 450 nm using a plate reader (Biotek Cytation 3).

**Drugs.** The drugs PD98059, ML3403, and SU3327 were purchased from Tocris Biosciences. Lithium chloride was purchased from Sigma. Drugs were dissolved in sterile water or ethanol as concentrated solutions, and diluted to the desired concentrations with the appropriate vehicle control. For rhythm experiments, lithium was applied 48 hr prior to rhythm recording and was present continuously throughout the recording procedure. When other drugs were used in conjunction with lithium, they were also present 48 hr before, and maintained continuously during the recording procedure.

**SiRNA Transfection.** Knockdown experiments in NIH3T3$^{P2L}$ cells and human fibroblasts were conducted using a kit according to the manufacturer's protocol (GE Healthcare). Three independent BD cell lines distinct from those used in protein experiments were selected based on their inability in previous rhythm analyses to amplify rhythms after treatment with lithium. Briefly, 2 x $10^4$ cells were distributed into 24 well plates. After 24 hr, siRNA and transfection reagent were mixed with medium and incubated for 20 min at room temperature. The transfection mixture was then added to the cells with medium, and incubated 48 hrs at 37°C. To maximize knockdown, siRNA pools (SMARTpool) that bind four distinct sites within a transcript were used. The siRNA pools used were *Arntl (*also called *Bmal1,* M-040483-01-0005), *Dusp6 (mouse: m-04005-01-0005; human: M-003964-01-0005)* and negative controls that do not interact with any known transcript (D-001206-14-05).

**Gene Expression.** For verification of siRNA knockdown of *DUSP6*, human fibroblast cultures were grown in parallel in 35 mm dishes and treated with negative control or *DUSP6* siRNA as described above. *GAPDH* was used as a non-rhythmic housekeeping control (Schmittgen and Livak, 2008). Dishes were collected 48 hr or 72 hr later. After collection, RNA was prepared using a Qiagen RNeasy kit, following the manufacturer's instructions. cDNA was synthesized using a kit (Applied Biosystems). Taqman RT-PCR was conducted using a BioRad CFX384 thermocycler with primers for human *DUSP6* (Hs00169257_m1) and *GAPDH* (Applied Biosystems).

**Statistical Analyses.** Statistical analyses were conducted using GraphPad Prism (San Diego, USA) using $p<0.05$ as the threshold for statistical significance. Two group analyses were performed using a two tailed t-test. Analyses of three or more conditions were performed using a one-way analysis of variance with post-tests.

## RESULTS

**Phospho-ERK1/2 is reduced in fibroblasts from BD patients.** Due to its association with BD and circadian rhythms (Coogan and Piggins, 2003; Dziema et al., 2003), phospho-ERK1/2 protein levels were examined by western blot in fibroblasts from BD patients and controls under baseline conditions or after 48 hr of lithium (1mM) treatment. This analysis revealed that lithium treatment of the cells did not cause a major effect on ERK1/2 phosphorylation, but that p-ERK1/2 is significantly (~30%) lower in BD cells that fail to amplify rhythms after lithium vs. control cells that do amplify rhythms (Figure 1A). We conducted a similar analysis of ERK1/2 in a larger, non-overlapping cohort of untreated BD and control cells using ELISA. Similar to the results observed in western blot assays, p-ERK1/2 levels were decreased by ~35% in BD cells compared to controls, a statistically significant difference (Figure 1B).

**ERK inhibition.** We next examined whether ERK is involved in rhythm amplification by lithium in NIH3T3$^{P2L}$ cells and fibroblasts from healthy human controls. (In BD cells, amplitude typically fails to increase in response to lithium, so BD cells were not included). Treatment of NIH3T3$^{P2L}$ cells with lithium (10 mM) caused a mean increase in rhythm amplitude of ~25-50%. To investigate the role of ERK, we used PD98059, an inhibitor of MEK (the upstream activator of ERK), treating the cells with this drug either alone or at the same time as

lithium. At 1 μM PD98059 had little effect on baseline rhythm amplitude. At 10 μM, PD98059 reduced rhythm amplitude. Both concentrations had a period lengthening effect. When administered to NIH3T3$^{P2L}$ cells simultaneously with lithium for 48 hr, PD98059 reduced the amplitude effect in a concentration dependent manner, fully suppressing the lithium-induced rhythm amplification at 1 μM and 10 μM (**Figure 2A, 2B**). In human fibroblasts, lithium increases amplitude at 1 mM. In human cells, the effect of PD98059 (10 μM) was strong, reducing baseline amplitude, and fully blocking the amplifying effects of lithium 1 mM (**Figure 2C, 2D**). Taken together, the results indicate that lithium and ERK both contribute to rhythm modulation and that ERK inhibition is sufficient to reverse the effect of lithium on amplitude.

**P38 and JNK inhibition.** ERK is a member of the mitogen activated protein kinase (MAPK) family that also includes JNK and P38. JNK and P38 have been linked to circadian rhythms (Chansard et al., 2007; Hayashi et al., 2003; Kim et al., 2013), and JNK genes contain variants that may be associated with BD (PGC-BD, 2011). Therefore, P38 and JNK might overlap with ERK in modulating amplitude in response to lithium. We tested the effects of selective P38 and JNK inhibitors on rhythms in the presence of lithium. When used alone, the P38 inhibitor, ML3403 nominally reduced amplitude at 1uM, with significant reductions of 50% at 5uM and 75% at 10uM **(Figure 3A).** However, when used in conjunction with lithium, mild P38 inhibition with 1uM ML3403 slightly increased lithium's effect, causing a significant amplitude increase compared to lithium alone (**Figure 3A, 3B**). Notably, ML3403 also had a powerful period lengthening effect, increasing it from 26.9 hr at baseline, to 28.3 hr at 1uM, and 47.4 hr at 10 uM. The JNK inhibitor, SU3327 potently decreased amplitude in a concentration dependent manner, with 97% suppression at 10uM (**Figure 3C**). However, mild inhibition of JNK with SU3327 1uM concurrent with lithium treatment did not block the rhythm amplification effect of lithium (**Figure 3C, 4D**). In contrast to ERK inhibition by PD98059, which decreased baseline amplitude and attenuated lithium-induced rhythm amplification, inhibition of either P38 or JNK decreased baseline amplitude, but either did nothing or *sensitized* the response to lithium. Therefore, while P38 and JNK may be important for constitutive regulation of rhythms, we conclude their activities oppose lithium-induced rhythm amplification rather than promoting it.

**ARNTL, CREB and EGR-1 knockdown.** PER2 expression is regulated by multiple transcription factors. In order to clarify the transcriptional mechanisms by which ERK modulates lithium's amplification of rhythms, we tested the effects of knocking down selected transcription factors with siRNAs. After knockdown of *ARNTL* (also called *BMAL1*), the primary driver of rhythmic PER2 expression, NIH3T3$^{P2L}$ cells became completely arrhythmic, indicating that the knockdown procedure was highly effective. Nonetheless, treatment with lithium still increased the non-rhythmic, mean expression level of Per2::luc when *ARNTL* expression was reduced (**Figure S2A**). Among the downstream targets of ERK1/2 are CREB and EGR-1. Inducible *PER2* expression has been attributed to CREB (Obrietan et al., 1999) and EGR-1 (Kim et al., 2013) activation, and EGR-1 has been implicated specifically in the action of lithium upon *PER2* (Kim et al., 2013). In our siRNA experiments, knockdown of either CREB (**Figure 4A, S2B**) or EGR-1 (**Figure 4B, S2C**) resulted in maintained rhythmic oscillations, but with large reductions in amplitude. EGR-1 knockdown had the additional effect of shortening

period. However, in both cases, after correcting for lower baseline expression, lithium was still able to induce increases in amplitude similar in proportion to those in cells with intact CREB and EGR-1, suggesting these factors are not essential for the inducible effects of lithium on rhythm amplitude. To address the possibility of redundancy in the signaling pathway, we conducted simultaneous knockdown of CREB and EGR-1 in the same cells **(Figure 4C)**. Even in this situation, lithium induced proportionally similar increases in amplitude over baseline, speaking against these factors playing interchangeable roles in PER2 induction.

**ELK-1 knockdown.** ELK-1 is a transcriptional activator of immediate response genes like C-FOS and EGR-1. Like CREB and EGR-1, ELK-1 is activated by phosphorylation by ERK (Besnard et al., 2011), and has been implicated in the circadian phase resetting response to light (Coogan and Piggins, 2003). Unlike knockdown of the transcriptional activators ARNTL, CREB, and EGR-1, knockdown of ELK-1 increased rhythm amplitude ~55% over baseline in NIH3T3$^{P2L}$ cells, and blocked any further increase in amplitude by lithium (**Figure 4D, S2D**). These data suggest that ELK-1, possibly acting through transcriptional disinhibition, plays an important role in the lithium-inducible signaling pathway that regulates rhythmic PER2 transcription.

**DUSP6 knockdown potentiates ERK.** In order to further examine the role of the ERK pathway as a lithium-induced input to the clock, and to understand its implications for BD, we used our newfound knowledge of the signaling pathway to develop a gain of function model. ERK1/2 signaling is terminated by MAP-kinase phosphatases (MKP) encoded by members of the DUSP family of genes. DUSP6 (MKP-3) has been associated with BD in genetic (Kim et al., 2012; Lee et al., 2006) and gene expression (Seifuddin et al., 2013) studies, suggesting that it may be linked to the pathophysiology of BD. Most MKPs inhibit multiple protein kinases including P38, JNK, and ERK, making their effects widespread and non-specific (Kondoh and Nishida, 2007). In contrast, the protein from *DUSP6* is selective for ERK1/2, suggesting that inhibition of DUSP6 expression may offer a way to selectively increase ERK activity. We first used NIH3T3$^{P2L}$ cells to determine if *DUSP6* knockdown could alter the response of rhythms to lithium. In NIH3T3$^{P2L}$ cells, lithium 1 mM on its own failed to increase amplitude. However, with simultaneous lithium and knockdown of *DUSP6*, the amplitude response was sensitized, and lithium 1 mM increased amplitude twofold, as much as treatment with lithium 10 mM (**Figure 5A, 5B**). *DUSP6* knockdown with a higher concentration of lithium (10 mM) did not produce any additional amplification (**Figure 5A**). *DUSP6* knockdown alone had little effect on amplitude, but shortened circadian period by ~0.7 hr. Using the same experimental approach, we next tested the hypothesis that with *DUSP6* knockdown, BD cells that fail to amplify rhythms in response to lithium at a therapeutic concentration (1 mM) could be converted into rhythm amplifying cells. Selecting three additional BD fibroblast cell lines that previously failed to amplify rhythms in response to lithium (Table S1), we established cell lines that stably expressed Per2::luc, and treated cells with *DUSP6* or negative control siRNA. Cells were then treated with lithium 1 mM or vehicle. The rhythms in cells treated with negative control siRNA amplified only minimally after lithium 1mM, failing to differ statistically from the vehicle treated condition (**Figure 5C, 5D**). In contrast, when *DUSP6* was knocked down, lithium 1mM amplified rhythms robustly; indicating BD cells were sensitized to the drug, thereby reversing the BD-associated phenotype of weak amplitude response to lithium (**Figure 5C, 5D**).

In parallel experiments using RT-PCR to confirm effectiveness of the knockdown procedure, we determined that *DUSP6* expression was reduced by ~85% at 48 hr and ~78% at 72 hr after transfection of *DUSP6* siRNA, corresponding to the 0 hr and 24 hr time points of the rhythm recording period.

## DISCUSSION

We identified ERK1/2 as regulators of lithium's effect on circadian rhythm amplitude in fibroblasts from BD patients, and demonstrated that phospho-ERK1/2 is decreased in BD cells that show weak rhythm amplification to lithium. Genetic studies of BD and molecular studies of lithium's mechanism have pointed to ERK pathways previously. Decreased levels of ERK1/2 phosphorylation were reported postmortem in the prefrontal cortex of patients with BD (Yuan et al., 2010), and in both prefrontal cortex and hippocampus of suicide victims (Dwivedi et al., 2009). Accordingly, in genome wide association studies, MAPK1, the gene encoding ERK2, shows suggestive evidence of association with BD (PGC-BD, 2011). MAPK3, the gene encoding ERK1 is in a region of chromosome 16p11.2 with structural variation, reportedly duplicated in BD, recurrent depression and schizophrenia (Malhotra and Sebat, 2012). But since most human studies have been conducted using isolated DNA samples or in postmortem brain, the functional implications of abnormal ERK signaling for BD are not well understood, and it is not yet known what neuronal functions in living cells might be affected as a result of perturbed ERK signaling. From animal studies, ERK signaling is known to be important for the phase resetting response to light in the brain's primary circadian pacemaker, the SCN (Dziema et al., 2003). However, the role of ERK signaling for circadian clock function has not been studied previously in clinical samples. Our work demonstrates that in cells from BD patients, lithium's amplification of circadian rhythms is reduced by inhibition of ERK, and enhanced by potentiation of ERK signaling via knockdown of *DUSP6*, directly implicating ERK signaling in this process.

**ERK inputs to the clock.** We interpret our data as reflecting engagement of the ERK pathway by lithium to regulate rhythmic PER2 expression. However, similar results could conceivably arise through the convergence of independent signals from lithium and ERK1/2, to regulate rhythm amplitude through distinct, parallel mechanisms. For several reasons, we do not believe this to be the case. First, BD cells largely fail to respond to lithium amplification of rhythms, and phospho-ERK1/2 is decreased in BD cells. Furthermore, there is specificity among MAPKs in attenuating lithium effects. JNK and P38 inhibition modulate baseline amplitude in a similar manner to ERK, but only ERK inhibition reverses the amplitude increasing effect of lithium. Most convincingly, knockdown of *DUSP6*, an ERK1/2 specific MAPK-phosphatase sensitizes the lithium response, but has little effect by itself. In assessing the phospho-ERK1/2 content in BD cells, there are some limitations to consider. Our sample was small, and some samples were not randomly selected. Moreover, since control patients were not exposed to mood stabilizer medications, our data cannot distinguish between the long-term effects on cells of psychotropic medications and inherent biological factors related to BD. Similarly, some of our conclusions are based on findings from human control or mouse cell lines, and the ERK pathway may function distinctly in BD cells. Finally, we cannot yet determine if ERK1/2 are the primary sites of dysfunction in BD

along the signaling pathway. While ERK1/2 could be directly affected in BD, several other genes functioning upstream of ERK1/2 have been implicated in BD [e.g. *NTRK2/3* (Nurnberger et al., 2014; Smith et al., 2009) , *ADCY2* (Muhleisen et al., 2014), *CACNA1C* (McCarthy et al., 2015; PGC-BD, 2011). Given the polygenic nature of BD, and its phenotypic heterogeneity, it is possible that pathogenic variation could be located in one or more of these interconnected genes (**Figure S3**).

**Restoring the amplitude response to lithium in BD cells.** In behavioral studies of BD patients, weak (low amplitude) rhythms, characterized by indistinct boundaries between day/night activities are a hallmark of BD, both during clinically stable and symptomatic periods (Ankers and Jones, 2009; Gonzalez et al., 2014; Jones et al., 2005; McKenna et al., 2014). Therefore, low rhythm amplitude in cells may have implications for the abnormal activity patterns observed in BD. While the clinical and behavioral implications of low amplitude cellular rhythms after lithium remain to be defined, we have shown that the phenotype is reversible by inhibiting DUSP6, a negative regulator of ERK. These results are important for several reasons. First, they further implicate the ERK pathway in the pathophysiology of BD. Next, they underscore the importance of signaling networks, demonstrating that a biological process (e.g. circadian rhythms) can be altered at multiple points, and conversely how the effects on the ubiquitous ERK system may have pleiotropic effects on non-circadian functions. Finally, the results point to a potential new drug target, possibly allowing for the modification of ERK signaling in BD to modify rhythms. DUSP6 inhibitors have been developed as potential chemotherapeutic agents, but are not well characterized in central nervous system disorders like BD (Vogt et al., 2003).

**Incorporating ERK into the GSK3B model of lithium's action.** When considered with our previous results (McCarthy et al., 2013) and the literature, our results indicate that both the ERK1/2 pathway and GSK3B pathway are altered in BD cells and/or affected by lithium. Our previous work demonstrated that genetic variation in GSK3B is selectively associated with the amplitude response to lithium in BD patients (McCarthy et al., 2013), and selective pharmacological inhibition of GSK3B increases amplitude in a manner similar to lithium (Li et al., 2012). This suggests that both GSK3B and ERK1/2 are importantly involved in the amplitude increasing effect of lithium, with GSK3B acting at baseline to reduce baseline rhythm amplitude and ERK acting to increase it. Our results indicate that lithium induces rhythm amplification by shifting this balance, affecting both signaling systems. The effects of ERK and GSK3B may reflect convergence on clock proteins as post-translational modifiers, or interactions between them. It is known that ERK can work in concert with the protein kinases AKT and RSK to inhibit GSK3B (Doble and Woodgett, 2003; Mendoza et al., 2011), and while not essential for lithium inducibility, CREB is also dually phosphorylated in a functionally opposing manner by GSK3B and inputs from ERK. These are two additional points of convergence in modulation of amplitude by lithium. However, there is considerable complexity in the interaction of signal transduction mechanisms, and the means by which GSK3B and ERK interact to modulate PER2 expression are not yet clear.

**Transcriptional control of amplitude by lithium.** Recent studies in mice using Western blot and PCR over a 12-24 hr time course concluded that ERK is involved in lithium's modulation of PER2 rhythms, but that EGR-1

is essential for increasing PER2 expression (Kim et al., 2013). Using a more sensitive method, more frequent sampling, and longer duration of data collection we found that while both CREB and EGR-1 do indeed have important roles in constitutive expression of PER2, amplification of rhythms by lithium was largely intact in the absence of these transcription factors. Instead, our data implicate ELK-1 as an important transcriptional regulator of PER2. A large-scale genomic study using ChIP-Seq identified ELK-1 binding on the PER2 promoter (Odrowaz and Sharrocks, 2012). Other studies have demonstrated that ELK-1 is activated by ERK in the SCN following a light pulse (Coogan and Piggins, 2003). However, we are unaware of any previous studies specifically describing the effects of ELK-1 on PER2 function. ELK-1 is often regarded as an inducible transcriptional activator of immediate early genes. However, in the unstimulated state, it acts as a transcriptional repressor (Besnard et al., 2011). This latter inhibitory action may be more salient over the relatively long circadian time course of the present study (hours to days). Therefore, one explanation for our findings is that the long-term transcriptional repressor activity of ELK-1 is attenuated by ERK1/2 signals, leading to disinhibition of PER2. However, it remains possible that the action of ELK-1 on PER2 is indirect, and mediated by the actions of ELK-1 on one or more additional genes. It is well known that ELK-1 stimulates EGR-1, another inducer of PER2. ELK-1 binding has also been found in NPAS2, CSNK1D, RORB, and RORC (Odrowaz and Sharrocks, 2012), all genes that could alter PER2 expression by affecting network interactions within the clock transcriptional-translational loop. While our data implicate ELK-1 as a terminal mediator of lithium's action on PER2, they do not necessarily implicate this transcription factor in BD since the observed rhythm phenotypes in BD cells can be explained by upstream regulators of ELK-1. Nonetheless, identifying the transcriptional targets of ELK-1 regulation could provide insight into which genes are involved in yielding the therapeutic effects of lithium. Therefore, further research into this gene in BD is warranted.

**Conclusions.** Loss of ERK1/2 activity could explain some of the circadian rhythm disturbances reported previously in BD, while engagement of ERK1/2 by lithium may be an important aspect of its therapeutic mechanism. Our work shows that ERK activity may be modifiable, and that the ability of lithium to modulate rhythms can be adjusted. Further studies are required to apply these findings to neurons, and behaviorally relevant phenotypes in BD like sleep and activity.

**Figure 1. ERK1/2 is differentially phosphorylated in BD and control cells after lithium.** A) Levels of phospho-ERK1/2 was measured by Western blot in BD and control fibroblasts either at baseline (untreated) or after 48hr of treatment with lithium (N = 4/group). * indicates that BD cells had less p-ERK compared to controls ($p<0.05$). B) Protein analysis was repeated using distinct methodology and a larger cohort of control (N=7) and BD (N=10) cells. * indicates that BD cells were again shown to have less p-ERK compared to controls ($p<0.005$). For both experiments, data are expressed as the ratio of P-ERK/total ERK normalized to the mean control values.

**Figure 2. Pharmacological inhibition of ERK signaling reduces rhythm amplification by lithium.** A) In NIH3T3$^{P2L}$ cells, lithium (10 mM) increases amplitude (n = 4-8 per group). Lithium no longer amplifies rhythm in the presence of PD98059 (1 or 10 µM), an upstream inhibitor of ERK. *One way ANOVA, with post-hoc test $p<0.0001$ B) Representative traces of NIH3T3$^{P2L}$ cells treated with lithium and PD98059 as indicated. C) PD98059 (10 µM) blocks the lithium amplifying effect of lithium (1 mM) in control fibroblasts (N = 6 per group). *indicates $p<0.001$ by One way ANOVA; in post-test, lithium is significantly higher than PD98059+lithium ($p<0.05$). D) Representative traces of a human fibroblast line treated with lithium and/or PD98059 as indicated. N.S. indicates not significantly different.

**Figure 3 P38 and JNK do not contribute to rhythm amplification by lithium.** A) In NIH3T3$^{P2L}$ cells the P38 inhibitor ML3430 reduces amplitude in a concentration dependent manner, but augments instead of interfering with lithium's ability to amplify rhythms. B) Representative traces of NIH3T3$^{P2L}$ cells treated with vehicle, lithium and/or ML3430 as indicated. C) In NIH3T3$^{P2L}$ cells the JNK inhibitor SU3327 reduces amplitude in a concentration dependent manner, but does not interfere with lithium's ability to increase amplitude. D) Representative traces of NIH3T3$^{P2L}$ cells treated with vehicle, lithium and/or SU3327 as indicated. N=3-6 per group * indicates $P<0.05$ by One-Way ANOVA with post-test.

**Figure 4. Knocking down transcriptional regulators of PER2 differentially affects rhythm amplification by lithium.** Knockdown of CREB (A) or EGR-1 (B) causes significant loss of rhythm amplitude, but does not affect the ability of lithium to amplify circadian rhythms. Lithium produces proportionally similar increases in rhythm amplitude with or without knockdown of either CREB or EGR-1. C) This remains true even when both transcription factors are knocked down, speaking against functional redundancy between CREB and EGR-1. D) However, when ELK-1 is knocked down, rhythm amplitude increases, and is not increased further by addition of lithium.

**Figure 5 Potentiating ERK by knockdown of DUSP6 sensitizes rhythms to the amplifying effect of lithium.** A) NIH3T3$^{P2L}$ cells typically show an amplitude response to lithium at 10 mM, but not at lower concentrations (1 mM). In the presence of DUSP6 siRNA, NIH3T3$^{P2L}$ cells respond to both concentrations, indicating a sensitized response to lithium. B) Representative traces of NIH3T3$^{P2L}$ cells treated with lithium 1 mM and/or DUSP6 siRNA as indicated. C) Many BD cells do not show an amplitude response to lithium. Three

representative BD lines were selected based on past non-response. Knockdown of DUSP6 converted the non-responsive BD cells into lithium responsive cells. D) Representative traces of non-responsive BD cells treated with lithium 1 mM and/or DUSP6 siRNA as indicated.

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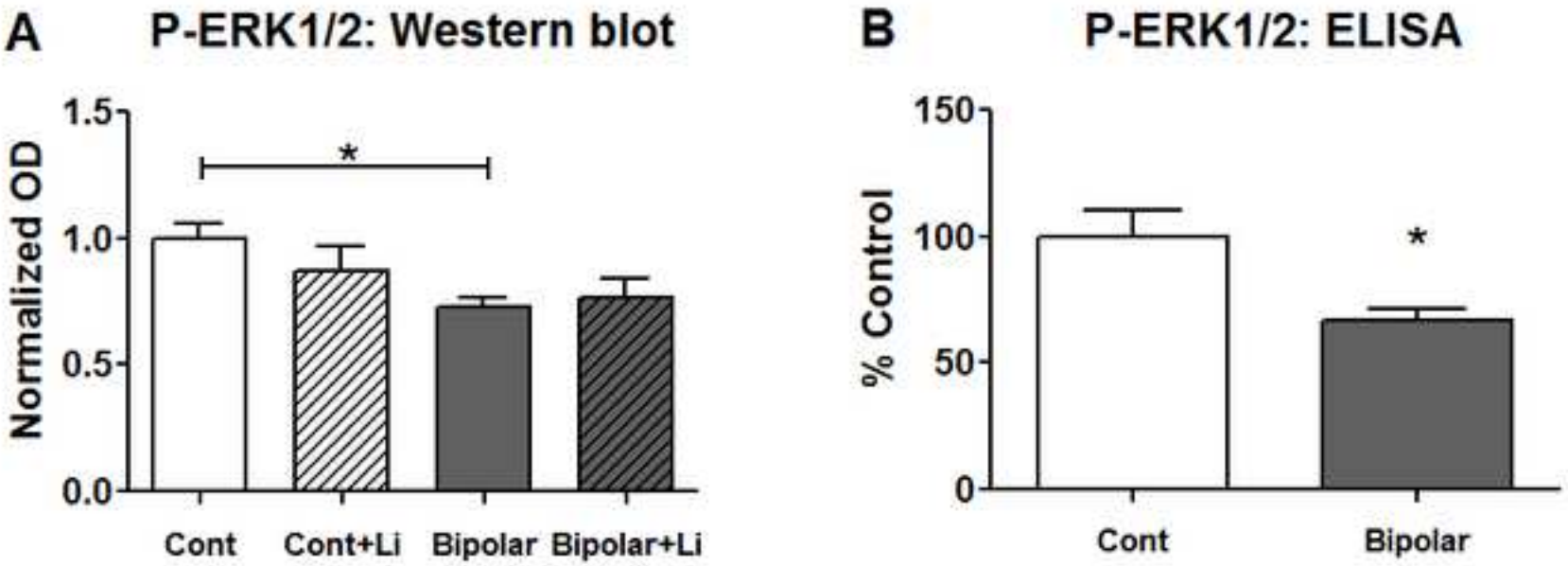

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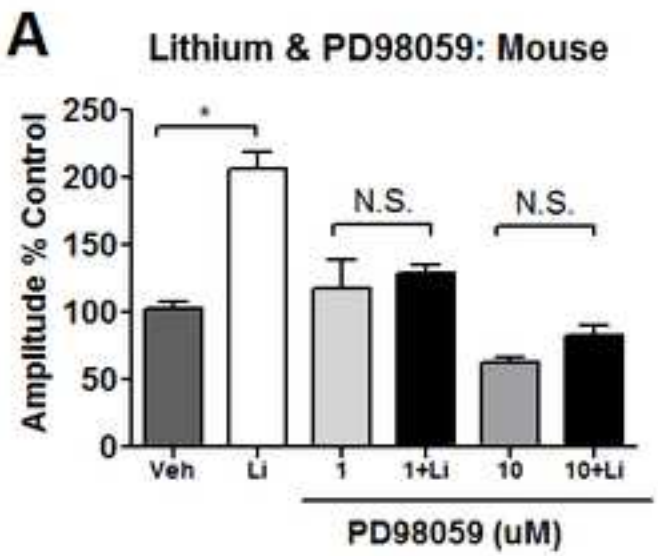


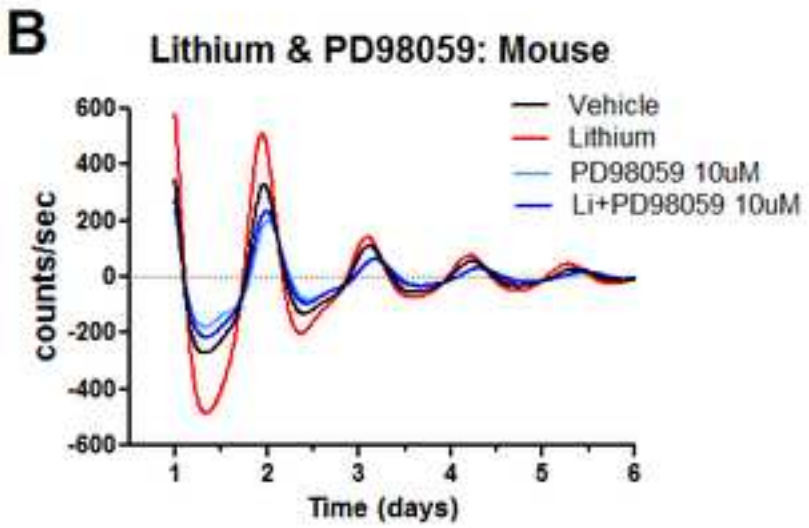


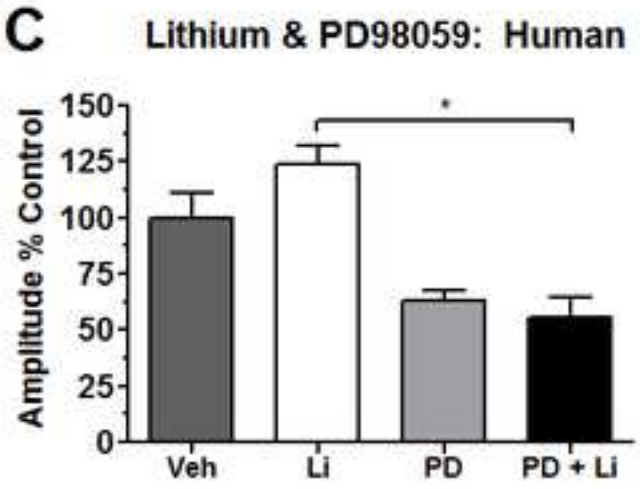


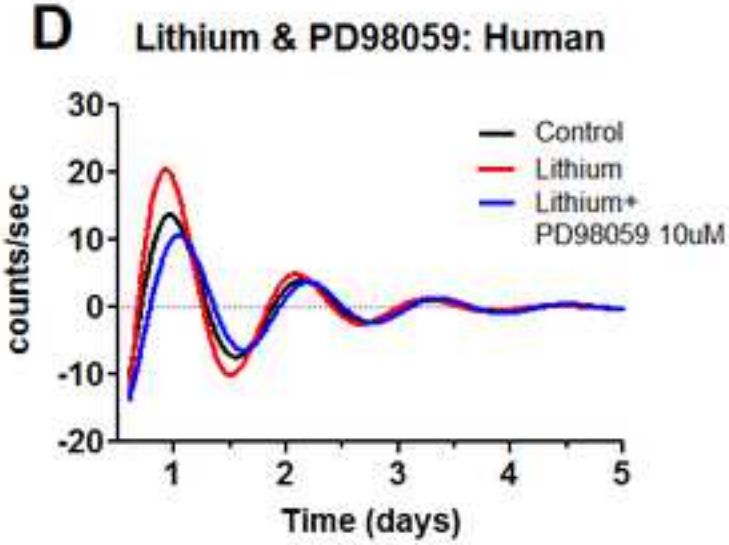

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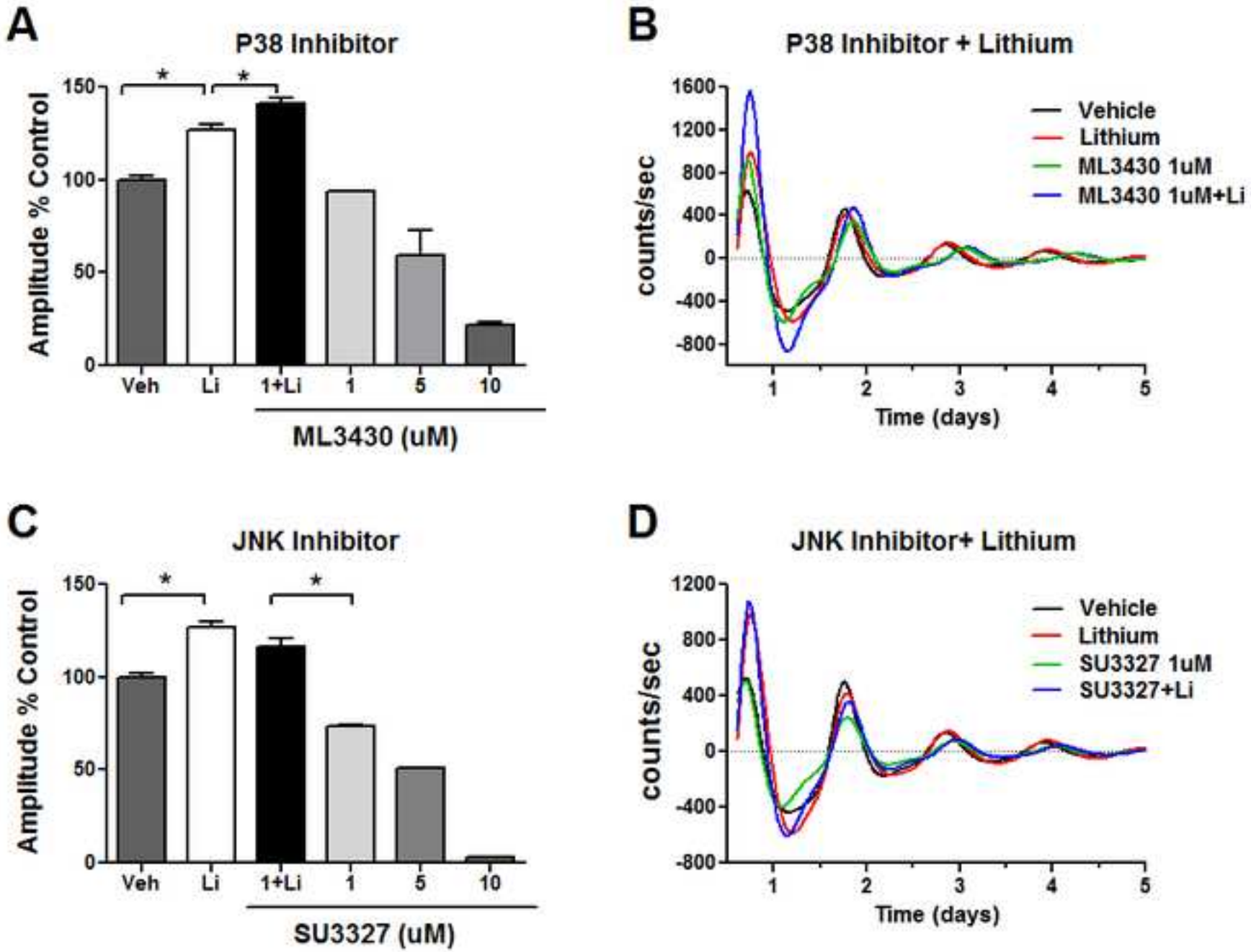

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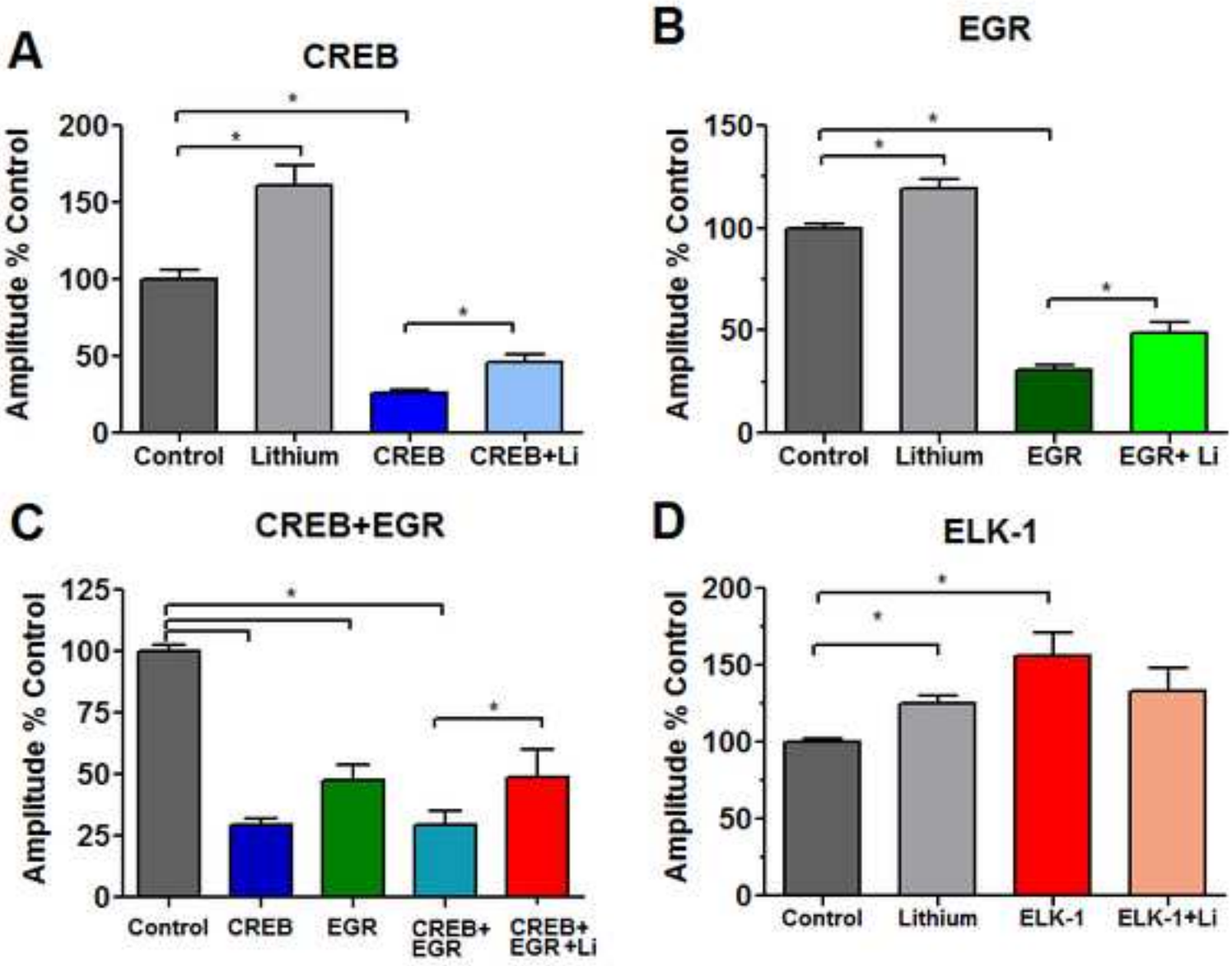

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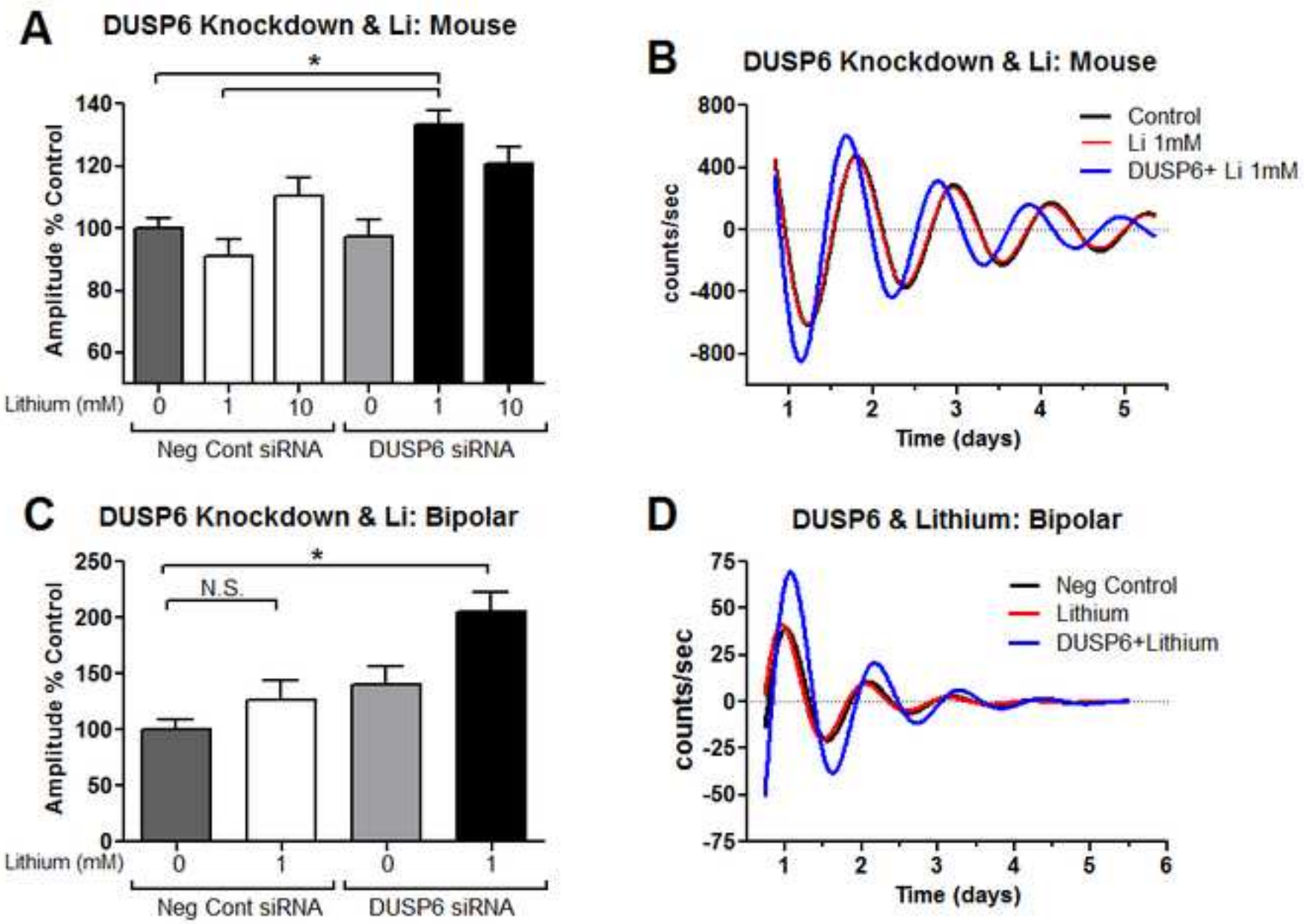

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Michael J. McCarthy: Designed and conducted experiments, analyzed data, wrote the manuscript

Heather Wei: Conducted experiments, analyzed data, edited manuscript

Dominic Landgraf: Conducted experiments, analyzed data, edited manuscript

Melissa J. Le Roux: Conducted experiments, analyzed data

David K. Welsh: Analyzed data, edited manuscript

## CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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