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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 (p-ERK) 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

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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. Published by Elsevier B.V.

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 is 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 the protein kinase GSK3B (Iitaka et al., 2005), and calcium signals from L-type channels (Kim 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 to alter PER2 expression by activating the transcription factor EGR-1 (Kim et al., 2013). Moreover, 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 role of ERK in circadian rhythms has 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, and present evidence that lithium's effect on circadian rhythm amplitude is modifiable and subject to enhancement.

2. Experimental procedures

2.1. 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, 2013). Use of human subjects was conducted in accordance with all pertinent regulations and approved by the VASDHS IRB.

2.2. Bioluminescent reporter genes

Fibroblasts were transduced as described previously with the *Per2::luc* lentiviral reporter gene. For mouse siRNA studies, NIH3T3 cells were stably transfected with the *Per2::luc* reporter gene ($NIH3T3^{P2L}$) under hygromycin selection as described previously (McCarthy et al., 2015; Meng et al., 2008). For siRNA experiments in human fibroblasts, a modified *Per2::luc* lentiviral reporter containing blasticidin resistance gene was used to stably express *Per2::luc*, allowing selection and expansion of cell lines that expressed the reporter (Liu et al., 2007).

2.3. 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.