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Calcium channel genes associated with bipolar disorder modulate lithium's amplification of circadian rhythms

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ABSTRACT

Bipolar disorder (BD) is associated with mood episodes and low amplitude circadian rhythms. Previously, we demonstrated that fibroblasts grown from BD patients show weaker amplification of circadian rhythms by lithium compared to control cells. Since calcium signals impact upon the circadian clock, and L-type calcium channels (LTCC) have emerged as genetic risk factors for BD, we examined whether loss of function in LTCCs accounts for the attenuated response to lithium in BD cells. We used fluorescent dyes to measure Ca^{2+} changes in BD and control fibroblasts after lithium treatment, and bioluminescent reporters to measure Per2::luc rhythms in fibroblasts from BD patients, human controls, and mice while pharmacologically or genetically manipulating calcium channels. Longitudinal expression of LTCC genes (*CACNA1C*, *CACNA1D* and *CACNB3*) was then measured over 12–24 h in BD and control cells. Our results indicate that independently of LTCCs, lithium stimulated intracellular Ca^{2+} less effectively in BD vs. control fibroblasts. In longitudinal studies, pharmacological inhibition of LTCCs or knockdown of *CACNA1A*, *CACNA1C*, *CACNA1D* and *CACNB3* altered circadian rhythm amplitude. Diltiazem and knockdown of *CACNA1C* or *CACNA1D* eliminated lithium's ability to amplify rhythms. Knockdown of *CACNA1A* or *CACNB3* altered baseline rhythms, but did not affect rhythm amplification by lithium. In human fibroblasts, *CACNA1C* genotype predicted the amplitude response to lithium, and the expression profiles of *CACNA1C*, *CACNA1D* and *CACNB3* were altered in BD vs. controls. We conclude that in cells from BD patients, calcium signaling is abnormal, and that LTCCs underlie the failure of lithium to amplify circadian rhythms.

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1. Introduction

Bipolar Disorder (BD) is a common mood disorder defined by alternating periods of depression and mania/hypomania, leading to disability and elevated risk of suicide (Oquendo et al., 2010). It is estimated that BD is 70–85% heritable, suggesting a genetic basis for the disorder (McGuffin et al., 2003). Other hallmarks of BD include disrupted behavioral rhythms, with inappropriate timing of activity, sleep, and appetite, indicating that circadian rhythm abnormalities may underlie BD (McCarthy and Welsh, 2012). Additional evidence linking circadian rhythms to BD comes from lithium. Lithium corrects the mood, activity and sleep disturbances in BD, and alters circadian rhythms. In humans (Kripke et al., 1979) and animals (Kripke and Wyborney, 1980; Welsh and Moore-Ede, 1990), lithium lengthens the rhythm period; and in cells, increases rhythm amplitude (Li et al., 2012). A set of ~20 "clock genes" (e.g. *PER1/2*, *CLOCK* and *ARNTL*) form the core of the circadian clock (Partch et al., 2014). Since BD is largely attributed to genetic factors, clock genes have been studied as susceptibility loci for BD. We previously identified circadian rhythm abnormalities in skin fibroblasts from BD patients. In particular, BD cells showed diminished capacity to amplify cellular circadian rhythms in response to lithium, with individual variation partly explained by a polymorphism in *GSK3B* (McCarthy et al., 2013). However, the mechanism by which lithium differentially increases amplitude in BD remains incompletely characterized. Since low rhythm amplitude may be one of the key rhythm abnormalities associated with BD (Jones et al., 2005; Gonzalez et al., 2014; McKenna et al., 2014), the

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cellular basis of amplitude modulation warrants investigation.

Ca^{2+} and the circadian clock have bidirectional interactions in neurons and peripheral cells (Noguchi et al., 2012; Ikeda, 2014; Schmutz et al., 2014). Hence, failure of lithium to amplify circadian rhythms in BD could be caused by altered Ca^{2+} clock inputs, or by clock outputs that perturb Ca^{2+} rhythms. Acutely, lithium activates intracellular Ca^{2+} release by inhibiting inositol monophosphatases, and stimulating inositol 1,4,5 tris-phosphate (IP3). Chronic lithium has the opposite effect, decreasing basal and stimulated intracellular Ca^{2+}, possibly by depleting cells of myo-inositol (Chen and Hertz, 1996; Yan et al., 2013). Lithium also inhibits glycogen synthase kinase β (GSK3B), a post-translational regulator of both LTCCs (Li and Sarna, 2011) and the circadian clock (Harada et al., 2005; Iitaka et al., 2005; Yin et al., 2006; Sahar et al., 2010).

Genome wide association studies indicate that clock gene variants are only weakly associated with BD (McCarthy et al., 2012; Byrne et al., 2014). In contrast, L-type Ca^{2+} channels (LTCCs) have been identified as important BD susceptibility genes (PGC-BD, 2011). Among Ca^{2+} channel genes, *CACNA1C* is most strongly implicated, with the C allele at the G/C polymorphism rs4765913 conferring risk for BD. Suggestive associations in *CACNA1D*, *CACNB3* have also been identified (PGC-BD, 2011; Nurnberger et al., 2014). Calcium channels are classified according to their pore-forming α1 subunit, while accessory β and α2δ subunits are shared across multiple channels. *CACNA1C* and *CACNA1D* encode the α1 subunits for two LTCCs, Cav1.2 and Cav1.3, whereas *CACNB3* encodes a β3 subunit. Previous studies have examined Ca^{2+} signaling in BD cells (Hahn et al., 2005; Chen et al., 2014), but few have linked abnormalities to particular LTCCs identified as genetic risk factors. Among investigations of *CACNA1C*, there is conflicting evidence as to whether BD-associated variation causes gain or loss of function. A recent study using human induced neurons homozygous for the variant risk allele reported that *CACNA1C* expression was increased, with corresponding increases in electrical activity (Yoshimizu et al., 2015). A previous post-mortem brain study came to the opposite conclusion, reporting decreased *CACNA1C* expression associated with the BD risk allele (Gershon et al., 2014). Therefore, BD-associated variants in LTCCs remain incompletely characterized in cellular models of BD.

We examined the hypothesis that the weak amplitude response to lithium in BD cells could be explained by loss of function in LTCCs, resulting in weak Ca^{2+} inputs to the circadian clock. We found that fibroblasts from BD patients were sufficient to model some aspects of LTCC function as they relate to circadian rhythms. Using molecular reporters to study Ca^{2+} and circadian rhythms in these cells, we investigated genetic and molecular connections among LTCCs, lithium and the clock, and determined that lithium engages Ca^{2+}. Over the short term, the intracellular Ca^{2+} response is blunted in BD cells independently of LTCCs. Over hours-days, LTCCs regulate cellular circadian rhythms, and *CACNA1C* is involved with the lithium-induced amplification of rhythms. Finally, some LTCCs appear to be clock controlled genes, with altered features of expression in BD.

2. Methods

2.1. Human subjects

Punch biopsies were obtained from the skin over the deltoid from BD (type I) patients who consented to research while hospitalized, or participating in a lithium clinical trial. Demographic characteristics of donors are shown in Table S1. Controls and BD donors did not differ in mean age or sex distribution. The majority of donors were Caucasian, and most BD patients (94%) were on medication at the time of biopsy. Use of human subjects was conducted in accordance with all pertinent regulations and approved by the VASDHS IRB.

2.2. Cell culture

Human fibroblasts were grown from frozen cryovials to confluence in 100 mm plates in standard culture media [DMEM with 10% fetal boving serum (FBS), glutamine 2 mM and antibiotics (penicillin, streptomycin, and amphotericin)]. In order to control for differences in donors' medication history, all cells were passaged a minimum of four times before use. *Per2* expression reflects network activity across the circadian clock (Welsh et al., 2005). Therefore, human fibroblasts were transduced with the *Per2*::*luc* lentiviral reporter gene to assess circadian rhythms as described previously (McCarthy et al., 2013). In order to facilitate screening of drugs and siRNAs in a cell type comparable to human fibroblasts, we developed a mouse fibroblast line that stably expressed the Per2::luc reporter ($NIH3T3^{P2L}$), using a construct described previously (Meng et al., 2008). $NIH3T3^{P2L}$ cells were grown under hygromycin selection to enrich Per2::luc expression. Luminometer studies of human cells were conducted using ~1.2×10^6 cells in 35 mm plates. For $NIH3T3^{P2L}$ luminometer studies, cells were dispersed into 24 well plates at ~2×10^5 cells/well.

2.3. Drugs

Diltiazem, verapamil, and 2-aminoethoxydiphenylborane (2-APB), were purchased from Tocris Biosciences. Lithium chloride was purchased from Sigma. Drugs were dissolved in sterile water or DMSO. Prior to drug studies, cells were distributed into multiple smaller plates, and treated in parallel, under identical conditions, allowing for within sample matching. To ensure even application, concentrated lithium (1000×) was added to growth media and distributed from a common drug solution into each culture plate. Additional drugs were handled in a similar manner, alone or with lithium. Vehicle controls for solvents were used when indicated.

2.4. Calcium fluorescence imaging

Ca^{2+} imaging was performed using a Fluo-4 NW calcium assay kit (Life Technologies) following the manufacturer's protocol. In brief, fibroblasts were distributed into 96 well plates and loaded with cell permeable fluo-4 for 30 min at 37 °C, followed by incubation at 25 °C for 30 min. Baseline fluorescence level was measured over 5 s using a BioTek Cytation3 reader. Fluorescence was then measured at 0.25 s intervals for 90 s. Background was determined in wells containing no cells. Individual wells were analyzed singly in series with lithium added to cells to a final concentration of 1 mM. Change in fluorescence were read immediately afterwards. Stimulation was determined by % increase from baseline to the smoothed peak. Relative increases in signal were determined by subtracting the background fluorescence, and calculating the fold change from baseline. Experiments were conducted over four runs (n = 4–8 cell lines/experiment) using control (N = 9) and BD (N = 10) cells. Data were normalized to reduce variability across experiments, and analyzed using a two-tailed T-test.

2.5. Luminometry

Human fibroblast rhythms were recorded from 35 mm plates over 5 days with a luminometer (Actimetrics) as described previously (McCarthy et al., 2013). $NIH3T3^{P2L}$ rhythms were measured using 4 × 24 well plate format luminometer (Actimetrics) over 5–7