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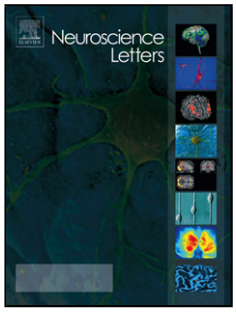

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Lithium effects on circadian rhythms in fibroblasts and suprachiasmatic nucleus slices from *Cry* knockout mice

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Lithium effects on cry knockout mice

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Highlights

- We tested whether CRY2 is involved in lithium's effects on PER2 circadian rhythms.
- In fibroblasts, lithium increased rhythm amplitude even in *Cry2*-/- cells.
- In SCN, lithium increased rhythm period even in *Cry*2-/- SCN.
- Amplitude-enhancing and period-lengthening effects of lithium are independent of CRY2.

ABSTRACT

Lithium is widely used as a treatment of bipolar disorder, a neuropsychiatric disorder associated with disrupted circadian rhythms. Lithium is known to lengthen period and increase amplitude of circadian rhythms. One possible pathway for these effects involves inhibition of glycogen synthase kinase-3β (GSK-3β), which regulates degradation of CRY2, a canonical clock protein determining circadian period. Although there is no evidence that GSK-3β directly phosphorylates CRY1, it is known to play important roles in regulating circadian period and phase. In this paper, we tested the hypothesis that lithium affects circadian rhythms through CRYs. We cultured fibroblasts and slices of the suprachiasmatic nucleus (SCN), the master circadian pacemaker of the brain, from *Cry1*-/-, *Cry2*-/-, or wild-type (WT) mice bearing the PER2::LUC circadian reporter. Lithium was applied in the culture medium, and circadian rhythms of PER2 expression were measured. In WT and *Cry2*-/- fibroblasts, 10 mM lithium increased PER2 expression and rhythm amplitude, but 1 mM lithium did not affect either period or amplitude. In non-rhythmic *Cry1*-/- fibroblasts, 10 mM lithium increased PER2 expression. In SCN slices, 1 mM lithium lengthened period ~1 h in all genotypes, but did not affect amplitude except in *Cry2*-/- SCN.

Thus, the amplitude-enhancing effect of lithium in WT fibroblasts was unaffected by *Cry2* knockout and occurred in the absence of period-lengthening, whereas the period-lengthening effect of lithium in WT SCN was unaffected by *Cry1* or *Cry2* knockout and occurred in the absence of rhythm amplification, suggesting that these two effects of lithium on circadian rhythms are independent of CRYs and of each other.

Keywords: Lithium, Cry, PER2, Circadian Rhythm, Suprachiasmatic Nucleus, Bipolar

INTRODUCTION

Bipolar disorder is a severe mood disorder that affects about 2.6% of the U.S. adult population [10]. Lithium is used as a standard treatment of bipolar disorder [22], but it has toxic side effects and is only effective in a subset of patients. It is important to clarify the target and pathways through which lithium affects behavior. Biochemically, one of lithium's main targets is GSK-3β, a serine/threonine kinase involved in pathological and protective cell signaling pathways [3, 14]. Because of the association between bipolar disorder and disrupted circadian rhythms, and the known rhythm amplitude-enhancing and period-lengthening effects of lithium, it has been suggested that lithium may stabilize mood via effects on the circadian clock [19].

In mammals, circadian rhythms of physiology and behavior are controlled by a master biological clock located in the brain, in the suprachiasmatic nucleus (SCN), which is required to synchronize cellular clocks throughout the body [20, 31]. A transcription-translation negative feedback loop underlies circadian oscillations in the SCN and other cells. This core loop involves activation of *Period* (*Per1, Per2, Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) gene transcription by a BMAL1/CLOCK heterodimer and delayed inhibition of this process by complexes containing PER and CRY proteins [20].

CRY1 and CRY2 are known to affect circadian period [28, 29]. Mice deficient in *Cry1* exhibit short period behavioral and SCN circadian rhythms, whereas mice deficient in *Cry2* exhibit long period rhythms [20]. In the absence of SCN coupling, however, *Cry1-/-* cells lose rhythmicity [16, 29]. GSK-3β phosphorylates several core clock proteins, including the CRY2 C-terminal, which regulates CRY2 degradation [2, 4, 12]. Thus, it is possible that CRY2 is involved in the mechanism by which lithium lengthens circadian period. Although there is no evidence that CRY1 is similarly phosphorylated by GSK-3β, it plays an important role in determining period and phase [5, 28, 29], so it could be indirectly involved in the pathway through which lithium affects circadian period. Interestingly, *Cry1-/-* mice exhibit reduced suppression by lithium of depression-like behavior, i.e. immobility in the forced swim test [26].

Here, we tested the effects of lithium on circadian rhythms of cultured fibroblasts and SCN from *Cry1*-/- or *Cry2*-/- mice, using the PER2::LUC reporter to assay real-time rhythms of clock gene expression.

MATERIALS AND METHODS

Animals

Generation of mPer2^{Luciferase} (PER2::LUC) knockin mice was described previously [34]. For this study, we used an alternative PER2::LUC mouse line incorporating an SV40 polyadenylation site to enhance expression levels [32]. The mice were developed at Northwestern University using the same methodology as the original strain of knockin mice [34]. Mice were backcrossed with C57BL/6J mice more than 10 generations and bred as homozygotes. *Cry1*-/- and *Cry2*-/- mice were generously donated by Dr. Akira Yasui at Erasmus University in the Netherlands [29]. *Cry1*-/-::PER2::LUC and *Cry2*-/-::PER2::LUC mice were generated by crossing PER2::LUC mice with *Cry1*-/- or *Cry2*-/- mice. In this paper, we refer to PER2::LUC, *Cry1*-/-::PER2::LUC, and *Cry2*-/-::PER2::LUC mice as wild-type (WT), *Cry1*-/-, and *Cry2*-/- mice, respectively. All mice were maintained as homozygous and housed in LD 12:12 light cycles (12 h light, 12 h dark) throughout gestation and from birth until used for experiments. Mouse studies were conducted in accordance with regulations of the Institutional Animal Care and Use Committee at University of California, San Diego.

Cell and SCN Culture

Primary tail fibroblasts and SCN slices were prepared from neonates (1-7 day old) of three types of mice (WT, *Cry1*-/-, and *Cry2*-/-) as described previously [21]. SCN slices were cultured in explant medium [pH 7.4; serum-free, 350 mg/l sodium bicarbonate, no phenol red (Corning 90-013-PB, Corning, NY), 10 mM HEPES, 25 U/ml penicillin, 25 µg/ml streptomycin, 2% B-27 (Life Technology 17504-

044)]. For luminescence recordings, fibroblasts and SCN slices were transferred to explant medium with 1 mM luciferin potassium salt (BioSynth L-8220). Lithium chloride (Sigma) was dissolved in dH_2O. Fibroblast cultures prepared in parallel were separated into 3 groups treated with 0, 1, or 10 mM lithium. SCN slices were first cultured in explant medium without lithium for 1-2 weeks, then treated with 1 mM lithium for a week.

Luminometry

For measuring luminescence rhythms from cultures, we placed the sealed 35 mm culture dishes into a luminometer (LumiCycle, Actimetrics, Inc., Wilmette, IL), inside a standard tissue culture incubator set at 35°C, dry, 0% CO_2. Luminescence from each dish was measured by a photomultiplier tube for 70 s at intervals of 10 min and recorded as counts/sec for about 7 - 30 days. Due to high initial transients of luminescence after medium change, the first 12 h of data were excluded from analysis. When average bioluminescence intensity for 0.5-5.5 d of recording did not exceed 40 counts/sec above the background level (10-50 counts/sec) or dropped to background level within 4 days, the cells or explants were considered damaged and were excluded from analysis.

Rhythm Data Analysis

Average PER2 expression was calculated during the interval from 0.5 d to 5.5 d after start of recording or medium change. For fibroblast data, average PER2 expression was normalized to a control sample run in parallel (or an average of several controls) (Fig. 1D). We computed rhythm parameters using LumiCycle Analysis (Actimetrics, Wilmette, IL, USA). To obtain period and amplitude, data were first detrended (by subtracting a 24 h running average) and smoothed (by averaging 10 adjacent points). Then, using the LM Fit (Sin) function, the best fit sine wave was obtained, and used to estimate circadian period and amplitude (Fig. 1E-F, Fig. 2F). Amplitudes of fibroblasts were normalized to the value of control samples run in parallel (Fig. 1A, 1E). Amplitudes and average PER2 expression of SCN were normalized to corresponding values of the same culture before lithium treatment (Fig. 2D-E). To count fibroblast

number, cells were washed with PBS and stained with DAPI (4',6-diamidino-2-phenylindole) (Vectashield mounting medium, Vector Laboratories, Burlingame, CA, USA). Numbers of nuclei were counted in 9 areas of 1 mm^2 and normalized to control samples run in parallel (Fig. 1G). Double plots of representative bioluminescence traces of SCN cultures were generated by ClockLab (Actimetrics) (Fig. 2 A-C). Each row was normalized to the maximum value over two days. Data in the first 0.5 d after medium change were excluded due to high transient PER2 expression.

RESULTS

Lithium increases circadian rhythm amplitude in WT and Cry2-/- *fibroblasts, and PER2 expression in all genotypes.*

To study whether CRYs are involved in the pathway by which lithium affects circadian rhythms of fibroblasts, we cultured mouse fibroblasts of three genotypes, WT, *Cry1*-/- and *Cry2*-/-. Fibroblasts were used as a representative of non-neuronal peripheral cells. A clinically therapeutic concentration of lithium (1 mM) did not significantly affect circadian rhythm parameters (average PER2 expression, amplitude, and period) in fibroblasts, regardless of genotype (Fig. 1A-F). Although not compatible with therapeutic use, a higher concentration of lithium (10 mM) was also tested to clarify effects on circadian rhythms. A higher concentration of lithium (10 mM) significantly increased average PER2 expression 2- to 3-fold in all genotypes (2.7 ± 0.3, 2.1 ± 0.1, 2.9 ± 0.2 fold ± SEM in WT, *Cry1*-/-, and *Cry2*-/-, respectively, n = 5 - 7) and increased amplitude about 2.5-fold in WT and *Cry2*-/- cells (2.4 ± 0.1, 2.5 ± 0.4 fold ± SEM in WT and *Cry2*-/-, respectively, n = 5 – 6). There was no significant effect on period (Fig. 1F). Amplitude and period of *Cry1*-/- were not assessed because there were no PER2 rhythms in *Cry*1-/- fibroblasts (Fig. 1B).

To test whether the increase of average PER2 expression is caused by an increase in number of cells or increased PER2 expression in single cells, we counted numbers of cells cultured with 0 or 10 mM lithium on the 2nd day after lithium treatment, when PER2 expression is the highest (Fig. 1A-C). There was no

significant difference in number of cells during 10 mM lithium treatment compared to parallel untreated controls (Fig. 1G).

Lithium lengthens circadian period in WT, Cry1-/-*, and* Cry2-/- *SCN slices.*

To test whether CRYs are involved in the pathway that lithium affects circadian rhythms of the SCN, we cultured neonatal mouse SCN slices of three genotypes: WT, *Cry1*-/-, and *Cry2*-/-. Before lithium treatment, *Cry1*-/- and *Cry2*-/- SCN slices showed shorter (22.8 ± 0.3 h, mean ± SEM, n = 8) and longer period (25.9 ± 0.4 h, mean ± SEM, n = 6), respectively, compared to WT (24.1 ± 0.2 h, mean ± SEM, n = 10), as reported previously [16]. At a clinical concentration (1 mM), lithium significantly lengthened the period of all three genotypes by ~1 h (WT, 25.1 ± 0.3 h, n = 10; *Cry1*-/-, 23.5 ± 0.3 h, n = 8; *Cry2*-/-, 27.0 ±0.2 h, n = 6, mean ± SEM) (Fig. 2D). Amplitude was reduced ~26% in *Cry2*-/- SCN compared to before treatment, but there were no significant differences in other genotypes (Fig. 2E). About 22% statistically significant reduction was observed in average PER2 expression of *Cry2*-/- SCN compared to before treatment, but there were no significant differences in average PER2 expression in other genotypes (Fig. 2D).

DISCUSSION

Lithium is thought to exert many of its biological effects by inhibiting GSK-3β [11], which is known to phosphorylate several core clock proteins directly, including BMAL1 [24], CLOCK [27], CRY2 [12], PER2 [9], and REVERBα [30, 33]. In this paper, we tested the possibility that lithium affects circadian rhythms through CRYs, which play important roles in setting circadian amplitude and period [15, 28, 29]. We found that the typical lithium effects we observed in WT fibroblasts (increase of PER2 expression and amplitude) and SCN (period lengthening), are also observed in *Cry1*-/- or *Cry2*-/- mouse tissues.

In previous studies, lithium increased amplitude and lengthened period of circadian rhythms in mouse lung fibroblasts and human skin fibroblasts [1, 8, 18]. In this paper, we found that 10 mM lithium increases amplitude in mouse tail fibroblasts prepared from neonates, but period lengthening was not

observed, suggesting that sensitivity to lithium may vary across tissues, species, and developmental stages. The increases in PER2 expression and rhythm amplitude were observed in *Cry1-/-* and *Cry2-/-* fibroblasts as well as WT fibroblasts, suggesting that the CRYs are not involved in the pathway by which lithium acts on circadian rhythm amplitude.

Consistent with several previous studies [1, 8, 11], we observed period lengthening by lithium in cultured WT SCN. Although *Cry1-/-* and *Cry2-/-* SCN have shorter and longer period than WT in the absence of lithium, a clinical concentration of lithium lengthened the period of *Cry1-/-* and *Cry2-/-* SCN equally, by about 1 h. This suggests that CRYs are not involved in the pathway by which lithium acts on circadian rhythm period.

An important limitation of our study is that, due to partial redundancy of CRY1 and CRY2 function [5], we cannot eliminate the possibility that lithium might affect circadian rhythms by acting on either CRY1 or CRY2. That is, lithium could act on CRY2 in *Cry1-/-* cells, and on CRY1 in *Cry2-/-* cells. However, we consider this unlikely, because lithium's effects are so similar in *Cry1-/-* and *Cry2-/-* cells. Moreover, it is impossible to test this using *Cry1-/-;Cry2-/-* cells, because they are non-rhythmic [16].

Lithium affected WT fibroblasts and SCN differently. First, 1 mM lithium lengthened the period of the SCN, but even 10 mM lithium had little effect on period of fibroblasts. This suggests that the signaling pathway by which lithium lengthen period is more sensitive in SCN neurons than in fibroblasts. Second, 10 mM lithium increased PER2 expression and amplitude in fibroblasts, but 1 mM lithium reduced PER2 expression and amplitude of *Cry2-/-* SCN, whereas 10 mM lithium reduced PER2 expression and amplitude of WT SCN irreversibly, presumably by damaging neurons (data not shown).

In previous studies, GSK-3β inhibition has produced inconsistent effects on cellular circadian rhythms, either shortening or lengthening period (or delaying phase) [6, 7, 9]. Also, lithium may affect circadian rhythms through signaling pathways other than GSK-3β. For example, chronic treatment of astrocytes with lithium causes a pronounced inhibition of K^+-mediated increase in intracellular Ca^{2+}, which is known to affect circadian period and other rhythm properties [17, 21, 25]. Further investigations are necessary to fully understand the signaling pathways through which lithium affects circadian rhythms.

Clock gene mutant mice show various mood- or anxiety-related behavioral phenotypes [13]. For example, lithium reduces immobility time in the forced swim test in WT mice but not in *Cry1-/-* mice, and striatal dopamine level is significantly lower in *Cry1-/-* than in WT mice [26]. *Clock* mutant mice show mania-like behavior, which is partly rescued by lithium or induction of functional CLOCK in the ventral tegmental area [23]. To understand the effects of lithium on psychiatric disorders, it will be important to consider how lithium affects mood-related functions in specific brain regions, as well as the specific signaling pathways through which lithium affects circadian rhythms and behavior.

Finally, the amplitude-enhancing and period-lengthening effects of lithium seem to be independent. In fibroblasts, lithium produced a 2- to 3-fold increase of PER2 expression and rhythm amplitude, but no lengthening of period. In SCN, on the other hand, lithium lengthened period but did not increase PER2 expression or rhythm amplitude. These results suggest that the biochemical pathway by which lithium lengthens period is independent from that by which it increases PER2 expression and rhythm amplitude.

Conclusions

In this paper, we show that lithium affects circadian rhythms by at least two different pathways, rhythm amplification and period lengthening, both independent of CRYs. Further studies delineating the specific biochemical mechanisms by which lithium affects circadian rhythms in cells may suggest possible mechanisms for its mood-stabilizing effects.

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Figure legends

Figure 1

Effects of lithium on *Cry1-/-* and *Cry2-/-* mouse fibroblasts. (A-C) Representative recordings of PER2::LUC bioluminescence of WT (A), *Cry1-/-* (B), and *Cry2-/-* (C) fibroblasts, cultured in 0, 1, and 10 mM lithium. (D-F) Average PER2 expression (D), PER2::LUC rhythm amplitude (E), and PER2::LUC rhythm period (F) of fibroblasts from WT, *Cry1-/-*, and *Cry2-/-* mice, cultured in 0, 1, 10 mM lithium. (G) Number of fibroblasts cultured in 0, 1, 10 mM lithium. For D, G & E, data were normalized to 0 mM lithium. Error bars are SEM, numbers in columns are numbers of cultures. Statistical significance was tested by Kruskal-Wallis test followed by Mann-Whitney *U*-test with Bonferroni correction (**$p < 0.01$).

Figure 2

Effects of lithium on *Cry1-/-* and *Cry2-/-* mouse SCN. (A-C) Representative recordings of PER2::LUC bioluminescence of WT (A), *Cry1-/-* (B), and *Cry2-/-* (C) SCN slices cultured in 0 and 1 mM lithium. Bioluminescence was normalized to the maxima of two consecutive days and double plotted. Gray lines are regression lines fitted to times of daily maxima. Periods estimated by regression line are shown on the right of the graph, *x*-axis is time of day (h), *y*-axis is days after start of recording. Average PER2 expression (D), amplitude of PER2::LUC rhythms (E), and period of PER2::LUC rhythms (F) of WT, *Cry1-/-*, and *Cry2-/-* SCN slices cultured in 0 and 1 mM lithium. In F, black diamonds and open circles indicate periods of individual cultures and their means, respectively. Statistical significance was tested by paired *t*-test (*$p < 0.05$, **$p < 0.01$). Error bars are SEM, numbers in columns are numbers of cultures.

FIG. 1

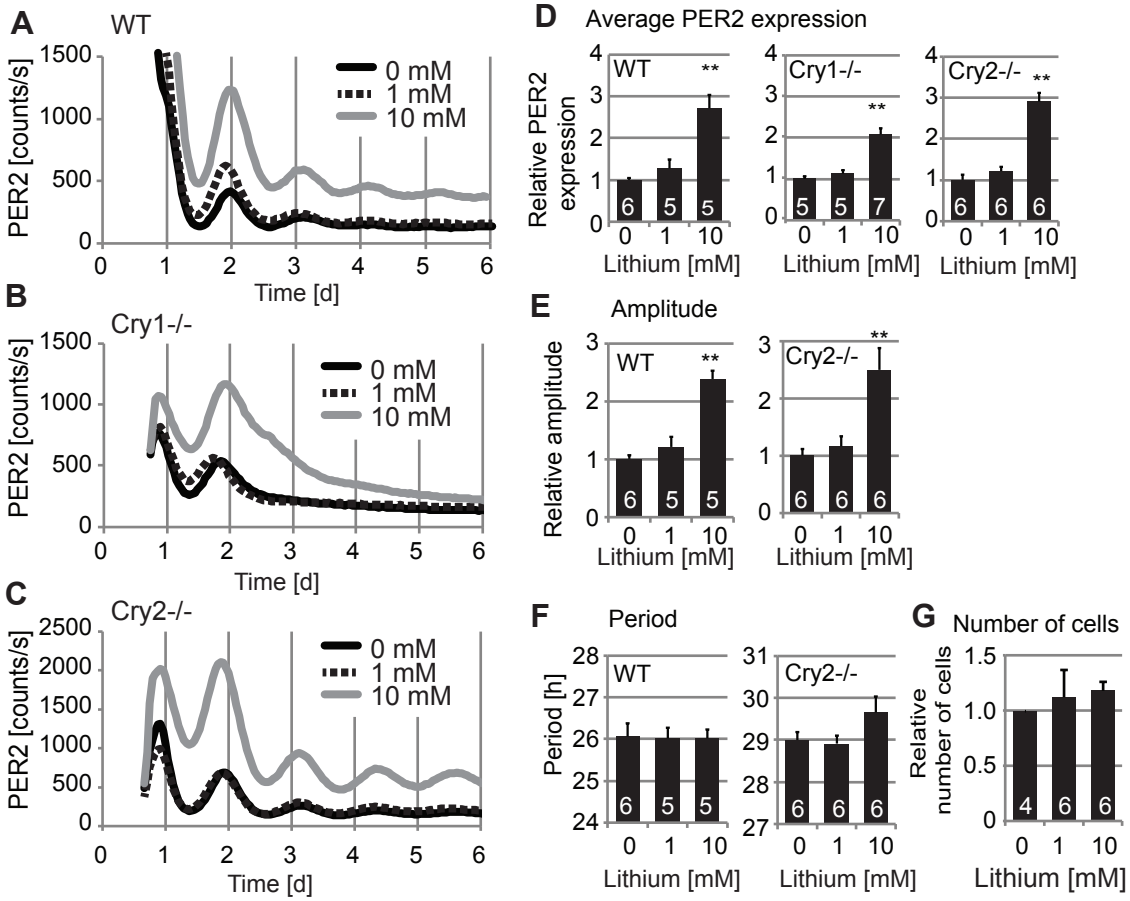

FIG. 2