Journal of Psychopharmacology

http://jop.sagepub.com/

Lithium Differentially Affects Clock Gene Expression in Serum-Shocked nih-3t3 Cells

Teresa M Osland, Johan Fernø, Bjarte Håvik, Ivar Heuch, Peter Ruoff, Ole Didrik Lærum and Vidar M Steen

J Psychopharmacol published online 13 September 2010
DOI: 10.1177/0269881110379508

The online version of this article can be found at:
http://jop.sagepub.com/content/early/2010/09/10/0269881110379508

Published by:
SAGE
http://www.sagepublications.com

On behalf of:

British Association for Psychopharmacology

Original Paper

Lithium differentially affects clock gene expression in serum-shocked NIH-3T3 cells

Teresa M Osland[1,2], Johan Fernø[1,2], Bjarte Håvik[1,2], Ivar Heuch[3], Peter Ruoff[4], Ole Didrik Lærum[5,6] and Vidar M Steen[1,2]

Journal of Psychopharmacology
0(0) 1–10

DOI: 10.1177/0269881110379508
jop.sagepub.com

Abstract

Bipolar disorder has been associated with disturbances in circadian rhythms. Lithium is frequently used in the long-term treatment of bipolar disorder, and has been shown to prolong such rhythms in animals and humans. To examine whether lithium affects the expression of genes regulating the circadian clock, cultured NIH-3T3 cells were synchronized by serum-shocking, and the relative expression of the clock genes Period1 (*Per1*), Period2 (*Per2*), Period3 (*Per3*), Cryptochrome1 (*Cry1*), Cryptochrome2 (*Cry2*), Brain and muscle aryl hydrocarbon nuclear translocator-like 1 (*Bmal1*), Circadian locomotor output cycles kaput (*Clock*), *Rev-Erb-α* (*Nr1d1*), RAR-related orphan receptor α (*Ror-α*), Glycogen synthase kinase-3β (*Gsk-3β*), Casein kinase 1−ε (*CK1-ε*; *Csnk1ε*), E4 binding protein 4 (*E4BP4*; *Nfil-3*) and albumin D-binding protein (*Dbp*) was examined for three consecutive days in the presence of lithium (20 mM) or vehicle (20 mM NaCl). We found that lithium significantly increased the expression of *Per2* and *Cry1*, whereas *Per3*, *Cry2*, *Bmal1*, *E4BP4* and *Rev-Erb-α* expression was reduced. We also found that lithium prolonged the period of *Per2*. Taken together, these effects on clock gene expression may be relevant for the effects of lithium on biological rhythms and could also give new leads to further explore its mood-stabilizing actions in the treatment of bipolar disorder.

Keywords

Circadian rhythms, clock gene, lithium, gene expression, bipolar disorder

Introduction

Bipolar disorder is a severe chronic affective disorder, where patients experience mood extremes with alternating periods of mania and depression. Both environmental and genetic factors are thought to be involved in the aetiology of the condition, with a complex mode of inheritance. Interestingly, bipolar disorder has been associated with disturbances in circadian rhythms, particularly alterations in sleep pattern (Hallonquist et al., 1986; Kasper and Wehr, 1992; Klemfuss, 1992; Manji and Lenox, 2000; McClung, 2007). Disturbances of the sleep–wake cycle often precede onset of mania or depression, and several studies have demonstrated that the circadian rhythm in bipolar patients is shortened, although there have also been negative findings (see Lamont et al., 2007, for a review). Bipolar patients may also have abnormal cycling of body temperature, blood pressure and melatonin secretion (Atkinson et al., 1975; Kripke et al., 1978); see McClung (2007) for a review.

Circadian rhythms are oscillations characterized by having a period length of around 24 h (for reviews on circadian rhythms, see Dardente and Cermakian, 2007; Ko and Takahashi, 2006; Reppert and Weaver, 2002; and Takahashi et al., 2008). These endogenously generated rhythms persist in the absence of external stimuli, are constant over a temperature range and may be entrained by cues. A population of neurons in the suprachiasmatic nucleus of the hypothalamus constitutes the pacemaker of the mammalian circadian clock; see Moore et al. (2002) for a review. Circadian rhythms are also found in peripheral organs such as lungs, kidney, liver and skin. The circadian clocks are cell-autonomous, and even certain cell cultures oscillate (Balsalobre et al., 1998; Yoo et al., 2004); for a review, see Welsh et al. (2010).

The circadian clock is regulated by a set of clock genes that are functionally linked together by several positive and negative feedback loops. This system generates stable oscillations in the levels of mRNAs and proteins of the clock genes. The main feedback loop consists of the proteins Circadian

[1]Dr Einar Martens Research Group for Biological Psychiatry and Bergen Mental Health Research Center, Department of Clinical Medicine, University of Bergen, Norway.
[2]Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, Helse Bergen HF, Norway.
[3]Department of Mathematics, University of Bergen, Norway.
[4]Centre for Organelle Research, University of Stavanger, Norway.
[5]Section of Pathology, The Gade Institute, University of Bergen, Norway.
[6]Department of Pathology, Haukeland University Hospital, Helse Bergen HF, Norway.

Corresponding author:
Professor Vidar M Steen, Center for Medical Genetics and Molecular Medicine, Laboratory Building, Haukeland University Hospital, N-5021, Bergen, Norway
Email: vidar.martin.steen@helse-bergen.no

locomotor output cycles kaput (Clock) and Brain and muscle aryl hydrocarbon nuclear translocator-like 1 (Bmal1), which together form a heterodimer that promotes the transcription of the genes Period1 (*Per1*), Period2 (*Per2*), Period3 (*Per3*), Cryptochrome1 (*Cry1*) and Cryptochrome2 (*Cry2*). Following translation in the cytoplasm, the PER and Cry proteins dimerize and enter the nucleus where the Per–Cry complex inhibits the actions of the Clock–Bmal1 dimer, thus indirectly repressing their own transcription via a negative feedback loop.

Several studies have examined the molecular and genetic aspects of circadian rhythms in bipolar disorder. In cultured fibroblasts from bipolar patients, the expression of *Bmal1*, albumin D-binding protein (*Dbp*) and *Rev-Erb-α* oscillated with reduced amplitudes as compared with healthy controls (Yang et al., 2009). Genetic studies have examined variants in circadian genes including *Per2*, *Per3*, *Bmal1*, *Clock* and *Nr1d1* as potential susceptibility factors for bipolar disorder, and moderate evidence of association has been reported, although some studies were inconclusive or negative (Kishi et al., 2009; Mansour et al., 2006, 2009; Nievergelt et al., 2006; Shi et al., 2008).

Lithium has been used for decades in the treatment of bipolar disorder, but its mechanisms of action remain uncertain. It has, however, been shown that lithium prolongs the circadian period of locomotor activity and body temperature rhythm in healthy individuals (Johnsson et al., 1983) and several other species, including mice (Iitaka et al., 2005) and fruit fly (Padiath et al., 2004). Lithium also dose-dependently prolongs the circadian period of firing rates in cultured mouse suprachiasmatic neurons (Abe et al., 2000). The prolongation of circadian biological rhythms by lithium has been linked to its effects on GSK-3β (Klein and Melton, 1996; Stambolic et al., 1996; Williams and Harwood, 2000). In line with this, lithium-independent overexpression of *Gsk-3β* (in NIH-3T3 cells) advanced the circadian phase, while lithium-mediated inhibition of GSK-3β prolonged the circadian period (Iitaka et al., 2005). Interestingly, these authors reported that lithium's inhibitory effect on GSK-3β might give rise to alterations in the expression of *Per2* in NIH-3T3 cells.

In the present study, we used serum-shocking of NIH-3T3 cells for entrainment and synchronization of circadian gene expression. We examined the effects of lithium on the expression of the clock genes *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1*, *Clock*, RAR-related orphan receptor α (*Ror-α*), nuclear receptor subfamily 1, group D, member 1 (*Rev-Erb-α Nr1d1*), *Gsk-3β*, Casein kinase 1, ε (*CK1-ε; Csnk1ε*), E4 binding protein 4 (*E4BP4; Nfil-3*) and *Dbp* for three consecutive days.

Materials and methods

Cell cultures

NIH-3T3 cells (murine fibroblasts) were grown in monolayer in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with glucose (4.5 g/L), L-glutamine (4 mM) and 10% heat-inactivated calf serum (GIBCO). Cells were plated out 5 days prior to the experiment on 12-well plates (1×10^5 cells/well) (Techno Plastic Products), reaching confluence after 2–3 days ($1–2 \times 10^5$ cells/well). The medium was not changed until the serum shock was initiated. At time zero, the cells were exposed to serum-rich medium (50% DMEM with L-glutamine as described above and 50% horse serum (GIBCO)) for 2 h. Subsequently, each well was washed twice with 500 μL tepid DMEM without serum. The cells were then grown in DMEM supplemented with 1% bovine serum albumin (Sigma-Aldrich) and 5, 10 or 20 mM lithium chloride, or 20 mM of sodium chloride (vehicle; control) for various periods of time. Cells were harvested every 4 h for 76 h. The cells were incubated at 37°C with 5% CO_2 in darkness and exposed to light only during medium renewal. A dose–response study with 5, 10 and 20 mM LiCl was done with $n = 3$ replicates, whereas the time-course experiments (20 mM LiCl) were performed with $n = 6$ replicates.

RNA isolation and real-time PCR

At the end of lithium/vehicle exposure, cells were washed with tepid phosphate buffered saline (PBS) and lysed with nucleic acid purification lysis buffer (Applied Biosystems). Total RNA was extracted and purified on an ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer's protocol. We measured the amount of RNA and controlled the quality of the samples on a NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies), before storage at −80°C. Reverse transcription of total RNA to cDNA was performed with TaqMan RT reagents (Applied Biosystems) following the manufacturer's protocol on a MasterCycler Gradient thermal cycler (Eppendorf). Relative gene expression for each sample was measured in triplicates by qRT-PCR on a 384-well ABI Prism 7900 HT sequence detector system (Applied Biosystems). A standard curve with six serial two-fold dilutions and a non-template control was included in each run, using SYBR-green (Applied Biosystems) for detection with the primers listed in Supplementary Table 1, or 2× master mix with Assay-on-Demand (Applied Biosystems). The qRT-PCR data were normalized relative to *Gapdh* and subsequently evaluated with the comparative Ct method using the SDS 2.3 program (Applied Biosystems). Similar results were obtained when using *Beta-actin* or *Beta-2-microglobulin* for normalization. The normalized gene expression values were set to 1 at time zero for all clock genes.

Flow cytometry

We used flow cytometry to assess whether there were cells undergoing active cell division after the serum shock. Cell cultures were washed once in ice-cold PBS and trypsinated before centrifugation at 200 *g* for 5 min. The cells were resuspended in PBS (2×10^6 cells/mL PBS) and fixed in an equal volume of absolute ethanol. Samples were stored at 4°C until flow cytometric analysis was performed. The cells were then centrifugated, washed and stained with a Triton X-100 solution with propidium iodide (2 μg/mL, Sigma-Aldrich). The fraction of cells in resting phase (G_1 and G_0) versus active phase (S, G_2 and M) was calculated by analysis with a Calibur flow cytometer (Becton Dickinson).

Table 1. Observed maximal and minimal relative expression values following the serum shock throughout the time course (76 h) for lithium- and vehicle-exposed samples (20 mM LiCl or 20 mM NaCl). Mean values and standard errors of the mean (SEM) are given, and $n = 6$ in repeated experiments. In the vicinity of troughs the gene expression levels approached zero, and the values of the lithium- and vehicle-exposed samples were compressed together. We used two-way ANOVA (treatment, time) to examine the effects of lithium on gene expression, and the main effect of treatment (lithium versus vehicle) is given in the table

Clock gene	Time of peaks (hours)	Peak values, controls	Peak values, lithium	Percentage change of peak values by lithium, compared to controls	Time of troughs (hours)	Trough values, controls	Trough values, lithium	Percentage change of trough values by lithium, compared to controls	*p*-value (two-way ANOVA)
Per2	24	2.8 ± 0.2	3.9 ± 0.2	+39%	12	0.2 ± 0.03	0.3 ± 0.02	+50%	<0.0005
	48	2.0 ± 0.1	2.6 ± 0.3	+30%	36	0.2 ± 0.02	0.4 ± 0.04	+100%	
	72	1.2 ± 0.1	1.5 ± 0.2	+25%	60	0.3 ± 0.04	0.5 ± 0.07	+67%	
Per3	24	11.7 ± 1.2	8.7 ± 0.9	−26%	4	0.2 ± 0.03	0.2 ± 0.02	0%	0.002
	48	9.7 ± 0.9	6.7 ± 0.7	−32%	36	1.1 ± 0.2	0.5 ± 0.07	−55%	
	72	6.6 ± 0.6	4.0 ± 0.6	−39%	60	1.5 ± 0.2	0.9 ± 0.1	−40%	
Cry2	24	2.4 ± 0.2	2.4 ± 0.4	0%	8	0.4 ± 0.03	0.3 ± 0.04	−25%	0.02
	48	2.4 ± 0.2	2.5 ± 0.3	+5%	36	1.1 ± 0.1	1.2 ± 0.1	+9%	
	72	2.2 ± 0.2	2.2 ± 0.3	0%	60	1.7 ± 0.2	1.4 ± 0.2	−18%	
Bmal1	36	2.1 ± 0.3	1.6 ± 0.2	−24%	20	0.3 ± 0.05	0.2 ± 0.03	−33%	0.003
	56	1.9 ± 0.3	1.4 ± 0.3	−26%	48	0.7 ± 0.06	0.6 ± 0.04	−14%	
					72	0.7 ± 0.04	0.7 ± 0.05	0%	
Cry1	28	1.5 ± 0.09	2.1 ± 0.05	+40%	12	0.2 ± 0.008	0.2 ± 0.02	0%	<0.0005
	56	1.4 ± 0.06	1.8 ± 0.1	+22%	40	0.4 ± 0.02	0.5 ± 0.06	+ 25%	
					68	0.4 ± 0.02	0.5 ± 0.05	+25%	
E4BP	32	0.8 ± 0.08	0.4 ± 0.05	−50%	20	0.3 ± 0.05	0.2 ± 0.01	−33%	<0.0005
4	56	1.2 ± 0.05	0.8 ± 0.08	−33%	44	0.4 ± 0.03	0.3 ± 0.01	−25%	
					68	0.5 ± 0.04	0.3 ± 0.04	−40%	
Rev-	16	12.7 ± 0.8	8.9 ± 0.8	−30%	8	0.5 ± 0.04	0.3 ± 0.01	−40%	<0.0005
Erb-α	44	12.1 ± 1.0	8.6 ± 0.6	−29%	28	0.5 ± 0.07	0.5 ± 0.04	0%	
	68	10.3 ± 1.0	7.9 ± 0.7	−23%	56	1.9 ± 0.09	1.2 ± 0.1	−37%	
Dbp	20	206.2 ± 49.3	168.1 ± 53.4	−18%	8	0.2 ± 0.03	0.1 ± 0.03	−50%	Non-
	44	123.1 ± 39.3	56.2 ± 20.8	−54%	36	1.1 ± 0.3	0.8 ± 0.1	−27%	significant
	72	47.3 ± 15.5	28.8 ± 11.1	−39%	60	3.4 ± 0.8	1.4 ± 0.4	−59%	

Data analysis and modelling

To analyse the qRT-PCR data, we applied two different methods, each describing different aspects of the oscillations. Fold-changes and ratios of maximal versus minimal values were calculated based on the observed data. We then assessed whether lithium made the oscillations differ significantly from the control situation by performing two-way analysis of variance (ANOVA) (SPSS, IBM), with a main effect for treatment (lithium versus vehicle), a main effect for time and an interaction term between the two. Dunnett's post-hoc test was performed to identify the differences for the dose–response study. As an alternative approach, we used a squared sine function as a mathematical approximation of our data to evaluate the period, amplitude and damping of the circadian rhythms, as previously described (Christensen et al., 2004). Data from the qRT-PCR were processed using the programs perl (www.perl.org) and gnuplot (www.gnuplot.info) to fit the circadian rhythms to the function $f(t)$ of the form $f(t) = \lambda_1 + \lambda_2 \exp(-\lambda_3 t)\sin^2[(\pi\ t/p) + \phi]$, where t is time after the serum shock, λ_1 the overall gene expression level (offset), λ_2 the amplitude, λ_3 the damping, p the period, and ϕ is the circadian phase. The offset describes the displacement from zero of the mean value of the oscillation. A large offset indicated that the gene was highly expressed. A positive damping rate (λ_3) indicated that the amplitude of the oscillation would be reduced over time, and eventually cease. A high value for λ_3 indicated rapid damping and quick return to baseline expression level. The circadian phase indicated the relative displacement in time of the oscillation. Differences between controls and lithium-treated samples were determined by calculating the corresponding Z-scores and p-values, and p-values less than 0.05 were considered statistically significant. We calculated the standard errors σ for the parameters by $\sigma = \sqrt{(\sigma_c^2 + \sigma_{li}^2)}$, where σ_c and σ_{li} were the asymptotic standard errors for each parameter found with $f(t)$ for the controls and lithium-treated samples, respectively.

The results of the two methods coincided for the majority of the genes examined. Discrepancies may result from the varying degree of how well the functions approximated the data, and we therefore based our conclusions primarily on the ANOVA results. The reduced Chi square statistic describes the goodness of fit of the mathematical model, and the closer this parameter was to unity, the less discrepancy there was between the data and the approximation $f(t)$.

It should be noted that the reduced Chi square for the approximations varied considerably, indicating a large variation in goodness of fit.

Results

Circadian gene expression in cultured NIH-3T3 cells

Following the serum shock, the mRNA level of *Per2*, *Per3*, *Bmal1*, *Cry1*, *Cry2*, *Rev-Erb-α*, *E4BP4* and *Dbp* showed robust circadian cycling in vehicle-exposed cells (Figure 1). A surge of transcription was seen for *Per2*, *Cry1*, *Rev-Erb-α*, *Bmal1* and *E4BP4*, with the first peak being evident after 2 h (*Per2*, *Cry1* and *Rev-Erb-α*) or 4 h (*Bmal1* and *E4BP4*). There was also a modest increase of *Cry2* expression during the initial hours. In contrast, the expression of *Per3* and *Dbp* remained low for 12 h before starting to oscillate. The amplitudes of the circadian cycles dampened gradually during the observation period of 76 h (exceeding three 24-h cycles).

In vehicle-treated cells, for *Per2*, mRNA peaks occurred at approximately 24, 48 and 72 h after the initial induction, and the reciprocal minimal values (troughs) of expression were evident at 12, 36 and 60 h (Figure 1 and Table 1). The ratio between the maximal peak and the preceding minimum level fell from $2.8/0.2 = 14.0$ to $1.2/0.3 = 4.0$ (71%) over two cycles during the experiment, demonstrating a marked damping over time. Approximations with $f(t)$ demonstrated that the amplitude of *Per2* cycling was 3.9 and the period length was 24.3 h (Supplementary Table 2). The circadian phase of both *Per3* and *Cry2* coincided with that of *Per2*, and the damping was also quite rapid (Figure 1 and Supplementary Table 2). As expected, the expression pattern of *Bmal1* was in antiphase with *Per2* (Figure 1 and Supplementary Table 2). Approximations with $f(t)$ gave an amplitude of 1.8 and a period length of 27.1 h, making *Bmal1* the gene with the longest estimated period.

The circadian phases of *Cry1* and *E4BP4* displayed peaks and troughs about 8 h later than *Per2* (Figure 1 and Table 1). Approximations with $f(t)$ for *Cry1* gave an amplitude of 1.7 and a period length of 27.0 h. Similar to *Bmal1*, *Cry1* had a considerably lower amplitude and longer period than *Per2*. Several parameters for the *E4BP4* oscillation were similar to those for *Per2*, as the approximations with $f(t)$ gave an amplitude of 4.5 and a period length of 24.4 h.

The maximal relative expression values of *Rev-Erb-α* and *Dbp* were much higher than those seen for the other genes (Figure 1 and Table 1). Their circadian expression phases were 8 and 4 h advanced, respectively, as compared with *Per2* (Figure 1). *Rev-Erb-α* had a substantially higher amplitude and a longer period than *Per2*, as approximations with $f(t)$ gave an amplitude of 7.7 and a period length of 25.6 h. The expression of *Dbp* remained low for 12 h before reaching peaks 20, 44 and 72 h after the serum shock (Figure 1 and Table 1). This robust oscillation was also reflected in the approximation, where $f(t)$ gave an amplitude of 44.5 and a period length of 25.0 h.

The expression of *Per1*, *Clock*, *CK1-ε*, *Gsk-3β* and *Ror-α* did not display any obvious circadian oscillation (Figure 2). However, *Gsk-3β* and *Ror-α* mRNA levels increased over time by 40% and 300%, respectively, as compared with the level before the onset of the serum shock (Supplementary Table 3).

Dose-dependent effects of lithium

To explore the dose–response relationship of the proposed action of lithium on clock gene transcription (Iitaka et al., 2005), we investigated the effect of 5, 10 and 20 mM LiCl on *Per2* expression, and fold-changes were evaluated relative to vehicle (20 mM NaCl) at selected time points after serum-shocking (20, 24, 28, 32 and 36 h), see Table 2. We found a significant effect of lithium ($p = 0.02$, two-way ANOVA) over the examined time points, and Dunnett's post-hoc test showed a trend towards significance from 5 mM ($p = 0.4$) to 10 mM ($p = 0.08$) compared with the control groups, reaching significance with 20 mM LiCl ($p = 0.001$). In order to maximize the difference between drug- and vehicle-exposed cells, we chose to use 20 mM LiCl in the subsequent experiments (see below).

Effects of lithium on clock gene expression

We analysed the effect of lithium with two-way ANOVA and approximations with $f(t)$. The mathematical model was found to be more stringent than the ANOVA analysis, see Table 3. Two-way ANOVA showed that the expression of both *Per2* and *Cry1* was markedly increased by lithium ($p < 0.0005$) (Figure 1 and Table 1). In contrast, lithium reduced the expression of *Per3* ($p < 0.0005$), *Cry2* ($p = 0.02$), *Bmal1* ($p = 0.003$), *E4BP4* ($p < 0.0005$) and *Rev-Erb-α* ($p < 0.0005$) (Figure 1 and Table 1). Lithium had no significant effect on *Dbp*. With respect to the genes that did not display circadian oscillation, lithium reduced the expression of *Clock* ($p = 0.02$, two-way ANOVA) and *Ror-α* ($p < 0.0005$, two-way ANOVA) (Figure 2 and Supplementary Table 3). We found no significant effect of lithium on the expression of *Per1*, *CK1-ε* or *Gsk-3β*.

Approximations with $f(t)$ confirmed the up-regulation by lithium of *Per2* expression, as the amplitude was increased from 3.9 to 4.8 ($p = 0.004$) and the period was prolonged from 24.3 to 25.4 h ($p = 0.0006$) (Supplementary Table 2). For *Bmal1*, approximations with $f(t)$ demonstrated a trend towards prolongation of the period from 27.1 to 28.9 h ($p = 0.07$) (Supplementary Table 2), while no effect was seen on the amplitude. Applying $f(t)$, the amplitude of *Per3* was reduced by lithium from 14.2 to 8.1 ($p = 0.0001$), while for *E4BP4*, lithium decreased the amplitude ($p = 0.0009$) and overall expression level ($p = 0.0004$) (Supplementary Table 2). Furthermore, lithium significantly reduced the overall expression levels of *Cry2* ($p = 0.003$). For *Dbp* we observed a trend towards lower amplitude with lithium ($p = 0.08$), while the overall expression level was reduced ($p = 0.0008$). No significant alterations of the period length were seen for *Per3*, *E4BP4*, *Cry2* or *Dbp*, and no significant effect was found with $f(t)$ for *Cry1* or *Rev-Erb-α* (Supplementary Table 2); for an overview, see Table 3.

The effect of lithium on the cell cycle phase

Influence on cell division and growth could have been potential confounding factors for the observed oscillations in gene

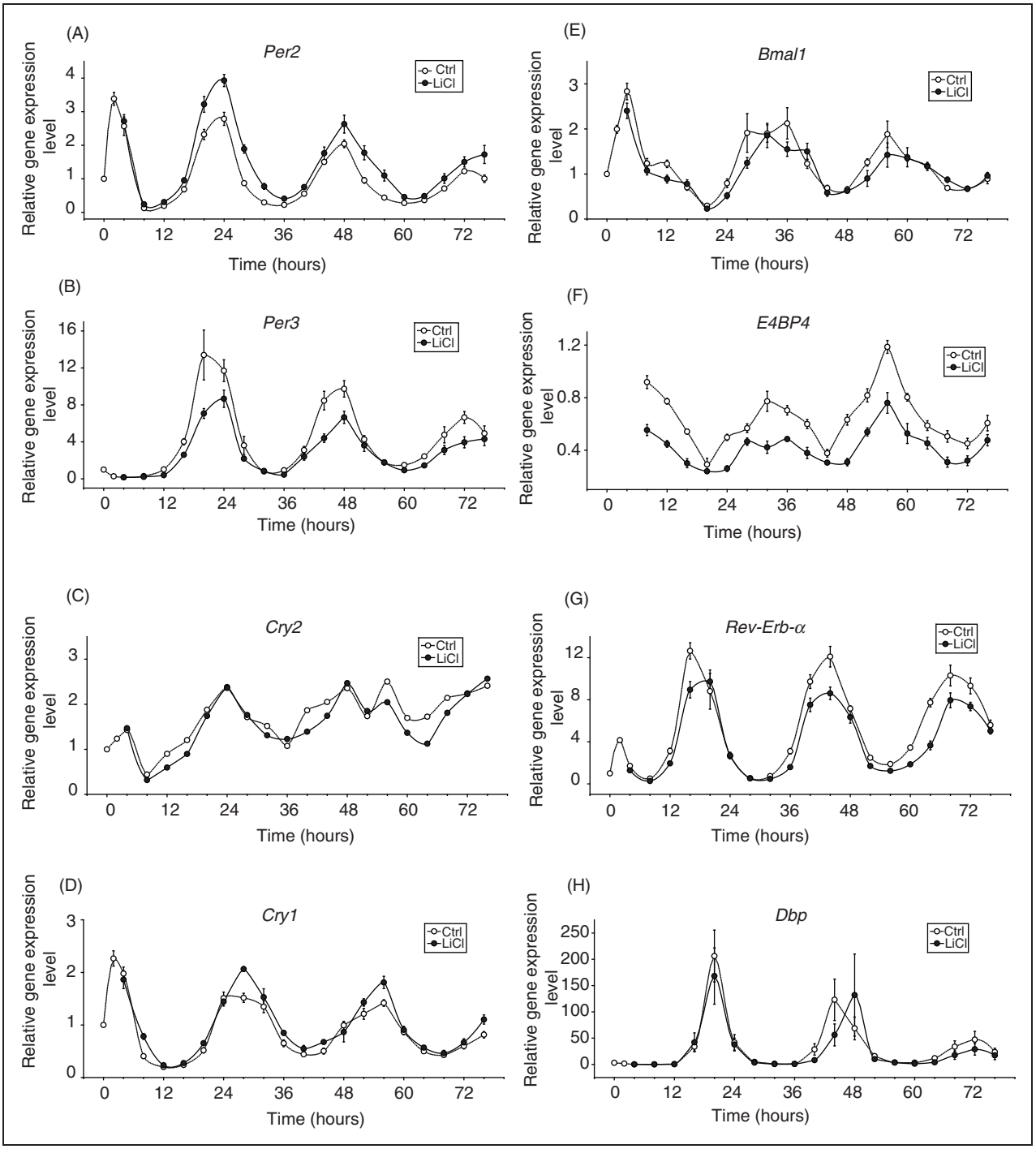

Figure 1. Circadian gene expression of *Per2, Per3, Cry2, Cry1, Bmal1, E4BP4, Rev-Erb-α* and *Dbp* in the absence and presence of lithium. The mRNA levels peak at different time points, and the oscillations are given with increasing phase delay compared with *Per2*. At time $t = 0$ the serum shock was initiated, at $t = 2$ h the cells were treated with 20 mM LiCl (filled symbols) or 20 mM NaCl (open symbols). The expression value of each given clock gene normalized to *Gapdh* at time zero (shortly before onset of the serum shock) was set to 1 for all genes. We used $n = 6$ in repeated experiments, and for each time point, means and SEM are shown. *E4BP4* levels were transiently increased following the serum shock (data not shown).

expression. We used flow cytometry to examine whether the serum shock interfered with these processes. The distribution of cells in the different cell phases was estimated in vehicle- and lithium-exposed samples, following a method described by Smaaland et al. (1991). We found no significant difference of the phase between the vehicle- and lithium-exposed samples during the first 27 h after the serum shock. Over 85% of the cells were in the quiescent G_0 phase, and less than 3.5% were in the G_2 interphase, irrespective of the presence or absence of lithium.

Table 2. Dose-dependent effects of lithium on *Per2* expression at selected time points. Gene expression values of *Per2* show a dose-response effect of lithium (5, 10 or 20 mM LiCl) versus controls (20 mM NaCl), with $n = 3$. Mean values and standard deviations are given

Time	20 hours	24 hours	28 hours	32 hours	36 hours
Control	4.1 ± 0.4	4.1 ± 1.8	1.1 ± 0.3	0.4 ± 0.1	0.5 ± 0.1
5 mM LiCl	4.6 ± 0.6	4.1 ± 0.9	2.1 ± 0.4	0.8 ± 0.8	0.6 ± 0.1
10 mM LiCl	4.4 ± 0.4	4.6 ± 1.6	2.4 ± 0.8	1.4 ± 0.6	0.7 ± 0.1
20 mM LiCl	4.7 ± 0.8	5.8 ± 1.9	3.0 ± 0.3	1.1 ± 0.2	0.8 ± 0.2

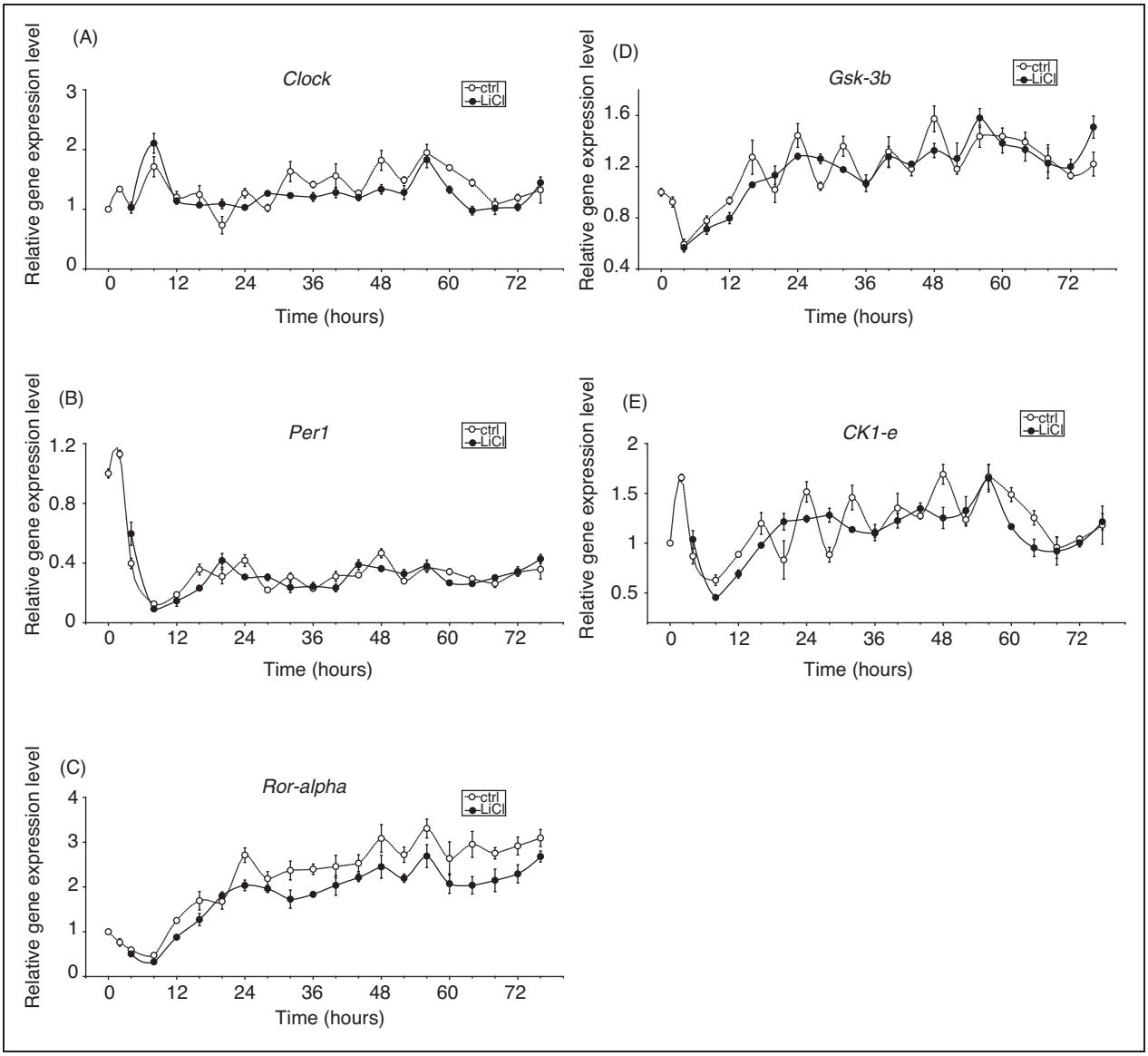

Figure 2. Observed gene expression of *Clock, Per1, Ror-α, Gsk-3β* and *CK1-ϵ*. There was no evident circadian oscillation of these genes. For *Clock, Per1, Ror-α* and *CK1-ϵ* $n = 3$, and for *Gsk-3β*, $n = 6$. For each time point, means and SEM are shown. At time $t = 0$ the serum shock was initiated, at $t = 2$ hours the cells were treated with 20 mM LiCl (filled symbols) or 20 mM NaCl (open symbols). The expression value of each given clock gene normalized to *Gapdh* at time zero (shortly before onset of the serum shock) was set to 1 for all genes.

Table 3. Significant effects of lithium on circadian gene expression

	ANOVA	Approximations with *f(t)*		
Gene	Expression level	Amplitude	Period length	Offset
Per2	Increased	Increased	Increased	–
Cry1	Increased	–	–	–
Per3	Reduced	Reduced	–	–
Cry2	Reduced	–		Reduced
Bmal1	Reduced	–	Trend towards increase	–
E4BP4	Reduced	Reduced	–	Reduced
Rev-Erb-α	Reduced	–	–	–
Dbp	–	Trend towards reduction	–	Reduced
Clock	Reduced	Not applicable	Not applicable	Not applicable
Ror-α	Reduced	Not applicable	Not applicable	Not applicable

Discussion

The main finding in this study is that lithium differentially altered the amplitude of expression of several clock genes in serum-shocked cultured murine fibroblasts. The expression of *Per2* and *Cry1* was increased, while the expression of *Per3*, *Cry2*, *Bmal1*, *E4BP4* and *Rev-Erb-α* was reduced.

Phase relationships of the clock genes confirm the validity of the circadian model system

Serum-shocking of NIH-3T3 cells represents a useful way to study a peripheral mammalian clock in vitro (Fustin et al., 2009; Iitaka et al., 2005; Ohno et al., 2007). We investigated the expression pattern of 12 clock genes for 76 h after serum-shocking. The majority of the clock genes displayed robust circadian rhythms. We confirmed phase relationships that have been reported by others, such as the antiphase between *Per2* and *Bmal1* (Hirota and Fukada, 2004; Oishi et al., 1998; Sato et al., 2004) and between *E4BP4* and *Dbp* (Hastings and Herzog, 2004; Mitsui et al., 2001), the 4 h delayed phase of *Cry1* compared with *Per2* (Fustin et al., 2009), and the delay of approximately 4 h of the circadian phase of *Dbp* as compared with *Rev-Erb-α* (Balsalobre et al., 1998). The considerably higher relative peaks of *Per3*, *Rev-Erb-α* and *Dbp* are in agreement with in vivo studies (Lopez-Molina et al., 1997; Wuarin et al., 1992).

The circadian rhythms dampened markedly over time, which limited the available observation time in the model system. This has also been reported by others (Hirota and Fukada, 2004; Yamazaki et al., 2000), and is caused by lack of input and synchronization from a central oscillator, which in vivo is performed continuously by the pacemaker in the suprachiasmatic nucleus (Nagoshi et al., 2004; Welsh et al., 2004). To compensate for this limitation, we explored the usefulness of the approximations $f(t)$ to calculate parameters such as amplitude, period and damping rate. The squared sine function is a generally adopted approximation of circadian rhythms, but it is not ideal, as indicated by the large range in the goodness of fit for some genes (e.g. *Cry1* and *Dbp*), and therefore the numerical results must be interpreted with care.

Lithium differentially affects circadian gene expression

Lithium exposure altered the expression pattern of several clock genes, increasing the transcription of *Per2* and *Cry1*, while reducing the expression of *Per3*, *Cry2*, *Bmal1*, *E4BP4* and *Rev-Erb-α*. These differential effects may be reflective of both direct effects of lithium on the clock gene expression and compensatory mechanisms necessary to maintain circadian rhythms, made by other molecular components of the clock that are affected by lithium. Our results support the work of Iitaka et al., where they examined the effects of augmenting concentrations of lithium (5, 10 and 20 mM LiCl) on the expression of *Per2*, *Bmal1* and *Dbp*, and found a dose-dependent increase for *Per2* only (Iitaka et al., 2005).

Intriguingly, the observed lithium-related increase of *Per2* expression occurred without any preceding increase of *Clock* and *Bmal1*, the positive transcriptional regulators of *Per* and *Cry*. As an alternative mechanism of increased *Per2* expression, lithium has been shown to inhibit GSK-3β, and this effect has been linked to lithium-induced prolongation of circadian biological rhythms (Klein and Melton, 1996; Stambolic et al., 1996; Williams and Harwood, 2000). The level of *Gsk-3β* mRNA did not oscillate in our study, but augmented steadily over time, with no additional effect of lithium. However, it is still possible that lithium exerts its effect through GSK-3β, e.g. by affecting the circadian cycling of GSK-3β phosphorylation levels, as described by Iitaka et al. (2005). However, if the effect were mediated via GSK-3β, we would have expected cascade effects on several clock components, including *Cry2* and *Rev-Erb-α* (Harada et al., 2005). We did not see an increase in the transcription of *Cry2* by lithium, although we did find reduced *Rev-Erb-α* expression. *Rev-Erb-α* inhibits *Bmal1* transcription, but we did not observe increased *Bmal1* expression by lithium. This is in agreement with the findings of Iitaka et al. (2005), but contrasts with the findings by Yin et al. (2006). This discrepancy is puzzling, as all three studies had similar experimental conditions. On the other hand, other mechanisms could be involved, and it has been suggested that the lengthening effect of lithium is caused by other targets (Hirota et al., 2008).

Numerical calculations using the Goodwin oscillator suggested that increased stabilities in clock mRNA or clock protein lead to a longer period and vice versa

(Ruoff et al., 1996). Using a model of the mammalian circadian oscillator (Becker-Weimann et al., 2004), we found the same type of behaviour independent of whether a positive feedback was present or not (data not shown). This effect has also been experimentally observed for Neurospora (Liu et al., 2000; Ruoff et al., 2005), Drosophila (Harms et al., 2004) and mammals (Meng et al., 2008). As an example, recent results on the Neurospora circadian clock (Jolma et al., 2006) indicate that lithium stabilizes the FREQUENCY protein (a protein with similarities to the Period proteins in mammals) and leads to an increased period length.

Lithium, clock genes and bipolar disorder

Bipolar disorder in associated with disruption of circulation rhythms. In particular, sleep pattern is often disturbed preceding onset of mania or depression. Part of the bidirectional mood-stabilizing effect of lithium is proposed to be due to its actions on circadian rhythms (Kaladchibachi et al., 2007; for review, see McClung, 2007). It is therefore interesting that we identified several changes in clock gene expression by lithium on genes involved in downstream effects of the circadian clock, as they could be relevant for the molecular mechanisms involved in lithium's therapeutic action.

We found that the main effects of lithium exposure were on the amplitude and expression level of oscillation, and not on the circadian period, with the exception of *Per2*. This is highly interesting in light of the recent findings by Yang and et al. who investigated the expression of clock genes in cultured fibroblasts from 12 patients with bipolar disorder and 12 healthy controls (Yang et al., 2009). Cells were serum-shocked to induce oscillations and gene expression was examined for 72 h. They found no differences in the period length between patients and controls, but demonstrated that the amplitude of peak expression for *Bmal1*, *Rew-Erb-α* and *Dbp* was reduced in bipolar patients. This corresponds well with our results, as we have shown that lithium altered the amplitude of several clock genes, but apparently affected the period length of only one gene, *Per2*. It should also be noted that genetic case-control studies have provided moderate evidence of association between bipolar disorder and variants in *Per2*, *Per3*, *Bmal1*, *Clock* and *Rev-Erb-α* (Kripke et al., 2009; Mansour et al., 2006; Nievergelt et al., 2006), but larger studies are required for verification.

Possible limitations of the study

There is a risk that the serum shock may influence the cell cycle as well as the circadian clock. This possibility was argued against over a decade ago by (Balsalobre et al., 1998) in their model system with Rat-1 fibroblasts using the following arguments: 1) the generation time of the cells was 15 h (differing considerably from 24 h); 2) the cell medium used was serum-free and should thus prohibit cell division; and 3) the addition of an inhibitor of DNA replication did not affect their results. We further investigated the possible influence of the serum shock on cell division or growth in our model system by performing flow cytometry. In support of the conclusions of Balsalobre et al. (1998), where they found that cell cycle progression was not relevant for the oscillating gene expression, we found the vast majority of the cells to be in a quiescent phase throughout the first 27 h following the induction, and there was no significant difference between lithium- and vehicle-treated cells.

Lithium has a narrow therapeutic window, with serum concentration 0.6–1.2 mM (Muller-Oerlinghausen et al., 2002). Low doses are ineffective, whereas many patients may experience toxic effects when the serum concentration exceeds 1.5–2 mM. However, drug doses in vitro are commonly much higher than therapeutically relevant serum concentrations (Iitaka et al., 2005; Kaladchibachi et al., 2007; Wu et al., 2007). Flow cytometry demonstrated that the cell cycle remained unaffected by 20 mM LiCl. Furthermore, we observed both up- and down-regulation of different clock genes by lithium, while the rhythmicity in oscillating genes was preserved, thereby excluding a general toxic effect of the drug in the cultured cells.

We analysed our data with ANOVA and approximations with $f(t)$. The mathematical model was noticeably more stringent than ANOVA. Effects of lithium that were significant with ANOVA were not always supported by corresponding findings using approximations with $f(t)$, while vice versa, all significant changes found by $f(t)$ were confirmed by significant effects using ANOVA. The discrepancies could be due to the low goodness of fit of the function $f(t)$. Moreover, approximating with $f(t)$, alteration of period length by lithium was found only for *Per2*. It is possible that the period of other clock genes was also changed, but that this was masked by an overly stringent model, low goodness of fit, too few replicates ($n = 6$), or by missing the true peaks, due to the limited number of harvesting time points (every 4 h); moreover, it could be caused by the limitation in observation time. Lithium triggered mechanisms in the circadian clock, as made evident by the effects on the amplitude, but it could be that prolonged period (in general) is a secondary effect, and perhaps a consequence of changes in the amplitude, which takes longer to achieve.

Concluding remarks

We showed that lithium differentially altered the amplitude of circadian expression of several clock genes in serum-shocked cultured murine fibroblasts. The peak amplitude of *Per2* and *Cry1* was increased, while the maximal amplitude of *Per3*, *Cry2*, *Bmal1*, *E4BP4* and *Rev-Erb-α* was reduced. Interestingly, only the period of *Per2* was significantly changed. These differential effects on clock gene expression may be relevant for the effects of lithium on biological rhythms and could also give new leads to further explore its mood-stabilizing actions in the treatment of bipolar disorder.

Acknowledgements

We are grateful to Raymond Lygre and Åsne Mokleiv at The Gade Institute, Department of Pathology, Haukeland University Hospital, for technical assistance with flow cytometry.

Funding

This study was supported by the Research Council of Norway and Dr Einar Martens Fund.

References

Abe M, Herzog ED and Block GD (2000) Lithium lengthens the circadian period of individual suprachiasmatic nucleus neurons. *Neuroreport* 11: 3261–3264.

Atkinson M, Kripke DF and Wolf SR (1975) Autorhythmometry in manic-depressives. *Chronobiologia* 2: 325–335.

Balsalobre A, Damiola F and Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 93: 929–937.

Becker-Weimann S, Wolf J, Herzel H and Kramer A (2004) Modeling feedback loops of the Mammalian circadian oscillator. *Biophys J* 87: 3023–3034.

Christensen MK, Falkeid G, Loros JJ, Dunlap JC, Lillo C and Ruoff P (2004) A nitrate-induced frq-less oscillator in Neurospora crassa. *J Biol Rhythms* 19: 280–286.

Dardente H and Cermakian N (2007) Molecular circadian rhythms in central and peripheral clocks in mammals. *Chronobiol Int* 24: 195–213.

Fustin JM, O'Neill JS, Hastings MH, Hazlerigg DG and Dardente H (2009) Cry1 circadian phase in vitro: wrapped up with an E-box. *J Biol Rhythms* 24: 16–24.

Hallonquist JD, Goldberg MA and Brandes JS (1986) Affective disorders and circadian rhythms. *Can J Psychiatry* 31: 259–272.

Harada Y, Sakai M, Kurabayashi N, Hirota T and Fukada Y (2005) Ser-557-phosphorylated mCRY2 is degraded upon synergistic phosphorylation by glycogen synthase kinase-3 beta. *J Biol Chem* 280: 31714–31721.

Harms E, Kivimae S, Young MW and Saez L (2004) Posttranscriptional and posttranslational regulation of clock genes. *J Biol Rhythms* 19: 361–373.

Hastings MH and Herzog ED (2004) Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. *J Biol Rhythms* 19: 400–413.

Hirota T and Fukada Y (2004) Resetting mechanism of central and peripheral circadian clocks in mammals. *Zoolog Sci* 21: 359–368.

Hirota T, Lewis WG, Liu AC, Lee JW, Schultz PG and Kay SA (2008) A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3beta. *Proc Natl Acad Sci U S A* 105: 20746–20751.

Iitaka C, Miyazaki K, Akaike T and Ishida N (2005) A role for glycogen synthase kinase-3beta in the mammalian circadian clock. *J Biol Chem* 280: 29397–29402.

Johnsson A, Engelmann W, Pflug B and Klemke W (1983) Period lengthening of human circadian rhythms by lithium carbonate, a prophylactic for depressive disorders. *Int J Chronobiol* 8: 129–147.

Jolma IW, Falkeid G, Bamerni M and Ruoff P (2006) Lithium leads to an increased FRQ protein stability and to a partial loss of temperature compensation in the Neurospora circadian clock. *J Biol Rhythms* 21: 327–334.

Kaladchibachi SA, Doble B, Anthopoulos N, Woodgett JR and Manoukian AS (2007) Glycogen synthase kinase 3, circadian rhythms, and bipolar disorder: a molecular link in the therapeutic action of lithium. *J Circadian Rhythms* 5: 3.

Kasper S and Wehr TA (1992) The role of sleep and wakefulness in the genesis of depression and mania. *Encephale* 18(Spec No 1): 45–50.

Kishi T, Kitajima T, Tsunoka T, Ikeda M, Yamanouchi Y, Kinoshita Y, et al. (2009) Genetic association analysis of serotonin 2A receptor gene (HTR2A) with bipolar disorder and major depressive disorder in the Japanese population. *Neurosci Res* 64: 231–234.

Klein PS and Melton DA (1996) A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A* 93: 8455–8459.

Klemfuss H (1992) Rhythms and the pharmacology of lithium. *Pharmacol Ther* 56: 53–78.

Ko CH and Takahashi JS (2006) Molecular components of the mammalian circadian clock. *Hum Mol Genet* 15(Spec No 2): R271–R277.

Kripke DF, Mullaney DJ, Atkinson M and Wolf S (1978) Circadian rhythm disorders in manic-depressives. *Biol Psychiatry* 13: 335–351.

Kripke DF, Nievergelt CM, Joo E, Shekhtman T and Kelsoe JR (2009) Circadian polymorphisms associated with affective disorders. *J Circadian Rhythms* 7: 2.

Lamont EW, Legault-Coutu D, Cermakian N and Boivin DB (2007) The role of circadian clock genes in mental disorders. *Dialogues Clin Neurosci* 9: 333–342.

Liu Y, Loros J and Dunlap JC (2000) Phosphorylation of the Neurospora clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock. *Proc Natl Acad Sci U S A* 97: 234–239.

Lopez-Molina L, Conquet F, Dubois-Dauphin M and Schibler U (1997) The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J* 16: 6762–6771.

Manji HK and Lenox RH (2000) The nature of bipolar disorder. *J Clin Psychiatry* 61(Supp 13): 42–57.

Mansour HA, Talkowski ME, Wood J, Chowdari KV, McClain L, Prasad K, et al. (2009) Association study of 21 circadian genes with bipolar I disorder, schizoaffective disorder, and schizophrenia. *Bipolar Disord* 11: 701–710.

Mansour HA, Wood J, Logue T, Chowdari KV, Dayal M, Kupfer DJ, et al. (2006) Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia. *Genes Brain Behav* 5: 150–157.

McClung CA (2007) Circadian genes, rhythms and the biology of mood disorders. *Pharmacol Ther* 114: 222–232.

Meng QJ, Logunova L, Maywood ES, Gallego M, Lebiecki J, Brown TM, et al. (2008) Setting clock speed in mammals: the CK1 epsilon tau mutation in mice accelerates circadian pacemakers by selectively destabilizing PERIOD proteins. *Neuron* 58: 78–88.

Mitsui S, Yamaguchi S, Matsuo T, Ishida Y and Okamura H (2001) Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. *Genes Dev* 15: 995–1006.

Moore RY, Speh JC and Leak RK (2002) Suprachiasmatic nucleus organization. *Cell Tissue Res* 309: 89–98.

Muller-Oerlinghausen B, Berghofer A and Bauer M (2002) Bipolar disorder. *Lancet* 359: 241–247.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F and Schibler U (2004) Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* 119: 693–705.

Nievergelt CM, Kripke DF, Barrett TB, Burg E, Remick RA, Sadovnick AD, et al. (2006) Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder. *Am J Med Genet B Neuropsychiatr Genet* 141B: 234–241.

Ohno T, Onishi Y and Ishida N (2007) The negative transcription factor E4BP4 is associated with circadian clock protein PERIOD2. *Biochem Biophys Res Commun* 354: 1010–1015.

Oishi K, Sakamoto K, Okada T, Nagase T and Ishida N (1998) Antiphase circadian expression between BMAL1 and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. *Biochem Biophys Res Commun* 253: 199–203.

Padiath QS, Paranjpe D, Jain S and Sharma VK (2004) Glycogen synthase kinase 3beta as a likely target for the action of lithium on circadian clocks. *Chronobiol Int* 21: 43–55.

Reppert SM and Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* 418: 935–941.

Ruoff P, Loros JJ and Dunlap JC (2005) The relationship between FRQ-protein stability and temperature compensation in the

Neurospora circadian clock. *Proc Natl Acad Sci USA* 102: 17681–17686.

Ruoff P, Mohsenzadeh S and Rensing L (1996) Circadian rhythms and protein turnover: the effect of temperature on the period lengths of clock mutants simulated by the Goodwin oscillator. *Naturwissenschaften* 83: 514–517.

Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, et al. (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* 43: 527–537.

Shi J, Wittke-Thompson JK, Badner JA, Hattori E, Potash JB, Willour VL, et al. (2008) Clock genes may influence bipolar disorder susceptibility and dysfunctional circadian rhythm. *Am J Med Genet B Neuropsychiatr Genet* 147B: 1047–1055.

Smaaland R, Laerum OD, Lote K, Sletvold O, Sothern RB and Bjerknes R (1991) DNA synthesis in human bone marrow is circadian stage dependent. *Blood* 77: 2603–2611.

Stambolic V, Ruel L and Woodgett JR (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr Biol* 6: 1664–1668.

Takahashi JS, Hong HK, Ko CH and McDearmon EL (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet* 9: 764–775.

Welsh DK, Takahashi JS and Kay SA (2010) Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol* 72: 551–577.

Welsh DK, Yoo SH, Liu AC, Takahashi JS and Kay SA (2004) Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr Biol* 14: 2289–2295.

Williams RS and Harwood AJ (2000) Lithium therapy and signal transduction. *Trends Pharmacol Sci* 21: 61–64.

Wu X, Zvonic S, Floyd ZE, Kilroy G, Goh BC, Hernandez TL, et al. (2007) Induction of circadian gene expression in human subcutaneous adipose-derived stem cells. *Obesity (Silver Spring)* 15: 2560–2570.

Wuarin J, Falvey E, Lavery D, Talbot D, Schmidt E, Ossipow V, et al. (1992) The role of the transcriptional activator protein DBP in circadian liver gene expression. *J Cell Sci (Suppl)* 16: 123–127.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, et al. (2000) Resetting central and peripheral circadian oscillators in transgenic rats. *Science* 288: 682–685.

Yang S, Van, Dongen HP, Wang K, Berrettini W and Bucan M (2009) Assessment of circadian function in fibroblasts of patients with bipolar disorder. *Mol Psychiatry* 14: 143–155.

Yin L, Wang J, Klein PS and Lazar MA (2006) Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. *Science* 311: 1002–1005.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, et al. (2004) PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A* 101: 5339–5346.