Lithium Leads to an Increased FRQ Protein Stability and to a Partial Loss of Temperature Compensation in the *Neurospora* Circadian Clock

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Abstract In many organisms, the presence of lithium leads to an increase of the circadian period length. In *Neurospora crassa*, it was earlier found that lithium results in a decrease of overall growth and increased circadian periods. In this article, the authors show that lithium leads to a reduction of FRQ degradation with elevated FRQ levels and to a partial loss of temperature compensation. At a concentration of 13 mM lithium, FRQ degradation is reduced by about 60% while, surprisingly, the activity of the 20S proteasome remains unaffected. Experiments and model calculations have shown that the stability of FRQ is dependent on its phosphorylation state and that increased FRQ protein stabilities lead to increased circadian periods, consistent with the observed increase of the period when lithium is present. Because in *Neurospora* the proteasome activity is unaffected by lithium concentrations that lead to significant FRQ stabilization, it appears that lithium acts as an inhibitor of kinases that affect phosphorylation of FRQ and other proteins. A competition between Li^+ and Mg^{2+} ions for Mg^{2+}-binding sites may be a mechanism to how certain kinases are inhibited by Li^+. A possible kinase in this respect is GSK-3, which in other organisms is known to be inhibited by lithium. The partial loss of temperature compensation in the presence of lithium can be understood as an increase in the overall activation energy of FRQ degradation. This increase in activation energy may be related to a reduction in FRQ phosphorylation so that more kinase activity, that is, higher temperature and longer times, is required to achieve the necessary amount of FRQ phosphorylation leading to turnover. Using a modified Goodwin oscillator as a semiquantitative model for the *Neurospora* clock, the effects of lithium can be described by adding lithium inhibitory terms of FRQ degradation to the model.

Key words *Neurospora crassa*, circadian rhythm, lithium, oscillatory feedback loops, FRQ protein stability, temperature compensation

Circadian rhythms play important roles in the daily and seasonal adaptation of organisms to their environments (Dunlap et al., 2004). An interesting property of circadian rhythms is that their period is homeostatically regulated against environmental influences such as temperature, pH, or nutrition (Pittendrigh and Caldarola, 1973). Despite this homeostatic regulation, certain substances such as D_2O

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(Pittendrigh et al., 1973), protein synthesis inhibitors (Feldman, 1967; Goodenough et al., 1981; Jacklet, 1980; Khalsa et al., 1992), creatine (Roenneberg et al., 1988), kinase inhibitors (Comolli et al., 1994; Liu et al., 2000), phosphatase inhibitors (Comolli et al., 1996), or Li^+ ions can affect the circadian period and induce phase shifts (Dunlap et al., 1980; Johnsson et al., 1981; Taylor et al., 1982). The period lengthening effect by Li^+ ions was observed in many organisms (Engelmann, 1987; Iwahana et al., 2004; Lakin-Thomas, 1993), including humans (Johnsson et al., 1979). Lithium compounds also play a significant pharmaceutical role in the treatment of bipolar disorder (Keck, 2004), which was tentatively related to lithium's period-lengthening effects in humans.

While the mechanisms for some of the inhibitors can now be understood as a result of affecting clock protein synthesis or degradation within a transcriptional-translational circadian pacemaker (Dunlap and Feldman, 1988; Liu et al., 2000; Ruoff et al., 2005), the influence of Li^+ ions on the clock is not well understood.

In *Neurospora*, the clock gene *frequency* (*frq*) was found to be part of the circadian pacemaker by forming a negative feedback loop, in which the FRQ protein inhibits its own transcription (Dunlap and Loros, 2004). Because FRQ stability is one of the factors that determines circadian period and temperature compensation in this organism (Liu et al., 2000; Ruoff et al., 2005), we wished to investigate the influence of Li^+ ions on the FRQ protein stability. Consistent with the increased period lengths (Lakin-Thomas, 1993), we observed an increased stability of FRQ in the presence of lithium. Apparently due to the increased FRQ stability, the oscillator has partially lost temperature compensation similarly as found for frq^7 (Dunlap and Feldman, 1988; Gardner and Feldman, 1981) and frq^{S513I} (Ruoff et al., 2005).

MATERIALS AND METHODS

Growth Tube Experiments

Growth tubes were prepared as previously described (Ruoff et al., 2000) with 1X Vogel's medium (Vogel, 1956), 0.2% glucose, 0.17% arginine, and 1.5% agar. The tubes were inoculated with conidia of *bda* and transferred to incubators (darkness) directly after inoculation at different but constant temperatures (±0.5 °C). The growth rate was determined from undisturbed growth between 2 markings of the growth front. Dependent on the temperature, the time between these markings varied between approximately 3 and 4 days (30 °C) and 5 and 6 days (20 °C-25 °C). The average period length was determined by measuring the distance between 2 distant conidiation peaks and dividing the distance by the growth speed and the number of cycles between the peaks. In general, 6 growth tube experiments were run in parallel. Lithium was either supplied as Li_3-citrate, which replaced an equimolar amount of Na_3-citrate in Vogel's solution, or LiCl was simply added to the medium. No significant difference between these 2 methods was observed.

Shaking Cultures and Western Blotting Analysis

Twenty-five milliliters of LL medium (2% sucrose in 1X Vogel's medium, with or without lithium) was inoculated with 200 µL conidial suspension (≈10^8 conidia/L) of the *bda* strain. Cultures were shaken for 24 h (25 °C) under continuous light (LL) (light intensity ≈ 2.5 W/m^2). When FRQ protein stability was tested, cultures were transferred to darkness (DD) and mycelium was harvested in 2-h time intervals for periods up to 12 h. The mycelium was then wrapped in Al foil, rapidly frozen in liquid nitrogen, and stored at –70 °C. Western blotting of FRQ was performed as described earlier (Ruoff et al., 2005).

Proteasome Activity

The 20S proteasome activity was determined in cell extracts using CHEMICON's 20S Proteasome Activity Assay Kit (www.chemicon.com, Cat. No. APT280) as described by the manufacturer. The assay is based on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC. Extracts from shaking cultures were prepared with the supplied assay buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 0.5% NP-40, and 0.01% SDS (w/v)). Total assay volume was 100 µL consisting of X µL extract, 10 µL of supplied proteasome substrate, and (90–X) µL assay buffer. When testing LiCl (5-20 mM) or lactacystin (0.094 µg/µL) as inhibitors, 10 µL of inhibitor, X µL of extract, and (80–X) µL assay buffer were preincubated for 15 min at room temperature before 10 µL of proteasome substrate was added. The assay was incubated for 2 h at 37 °C. After incubation, the free AMC fluorescence was measured at 440 nm (exciting the sample at 380 nm). The proteasome activity is

Table 1. Parameter Values Used in the Model Calculations

Rate/Dissociation Constant	*Value*	E_i *or* ΔH^0
k_1	0.3 h^{-1}	190 kJ/mol
k_2	0.3 h^{-1}	190 kJ/mol
k_3	0.3 h^{-1}	190 kJ/mol
k_4	0.27 h^{-1}	30 kJ/mol
k_5	0.2 h^{-1}	30 kJ/mol[a]
k_6	0.2 h^{-1}	30 kJ/mol[a]
K_I (equation 1)	1.77×10^6 mM	50 kJ/mol
n (equation 1)	6.1	—

NOTE: A detailed description of the model in the absence of lithium has been given previously (Ruoff et al., 2005). All rate constants are defined for frq^+ (wild-type) at T_{ref} = 292 K. Initial concentrations and other model properties are as described earlier.
a. E_5 and E_6 values for frq^+ in the absence of lithium. At 10 mM Li^+ or higher, these values were increased to 50 kJ/mol.

reported as emission intensity in arbitrary units (a.u.) when comparing samples using the same extract, or as emission intensity per milligram total protein (in a.u.) when different extracts are compared.

Model Calculations

As earlier described (Ruoff et al., 2005), the model calculations were performed with a temperature sensitive Goodwin-type oscillator (Goodwin, 1965). In all calculations, the rate constant values for frq^+ were used (Table 1). The influence of Li^+ ions is incorporated into the model by assuming that Li^+ reduces the FRQ degradation rate constants k_5 and k_6, possibly by inhibiting FRQ-phosphorylating protein kinases (*PK*) due to the rapid equilibrium:

$$(PK \cdot \mathrm{Li}_n^+)^{n+} \leftrightarrows PK + nLi^+ \qquad \text{(R1)}$$

Reaction R1 may be part of a competition between Li^+ and Mg^{2+} ions for Mg^{2+}-binding sites as a possible mechanism for Li^+ effects in cells (Mota de Freitas et al., 2006). The relative reduction of *PK* activity due to Li^+ binding and the accompanied decrease in the FRQ degradation rate constant k is described by the ratio α_{PK}:

$$\alpha_{PK} = \frac{[PK]}{[PK]_0} = \frac{[PK]}{[PK + (PK \cdot Li_n^+)^{n+}]} = \frac{K_I}{K_I + [Li^+]^n} \qquad (1)$$

where K_I is the dissociation constant of reaction R1 with $k = k_5 = k_6 = k_0 \cdot \alpha_{PK}$. Rate constant k_0 is the value of $k_5 = k_6$ in the absence of lithium. Because intracellular lithium concentrations are not known, external lithium concentrations were used in the calculations. The K_I and n values (Table 1) were determined by fitting the calculated period length at different lithium concentrations to the experimental values (Fig. 1B) using K_I and n as adjustable parameters.

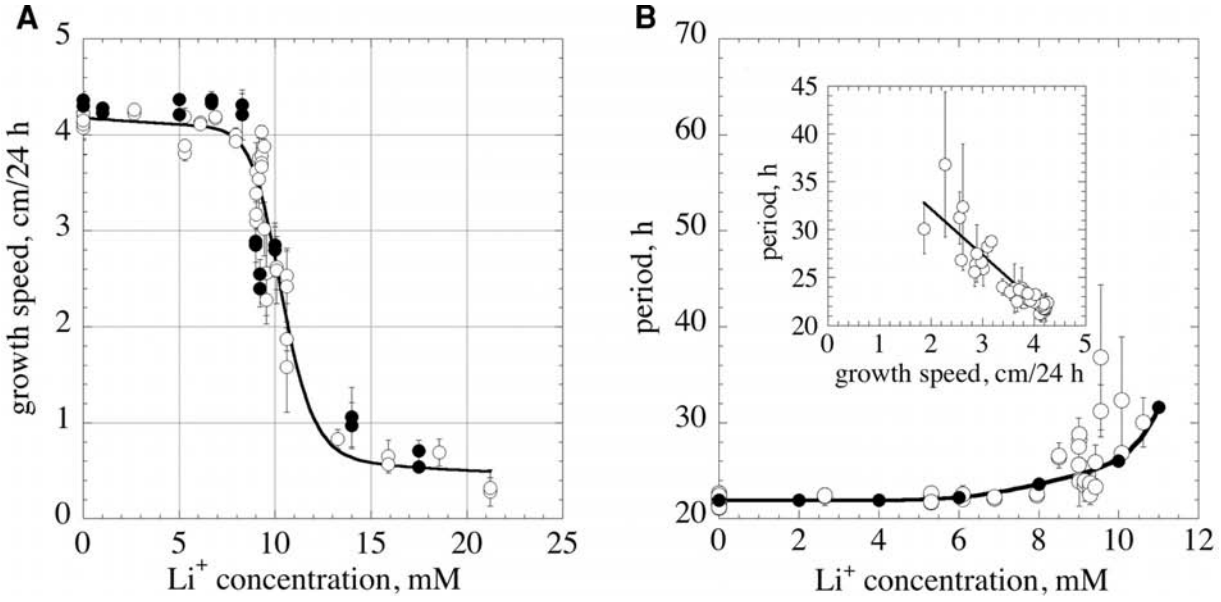

Figure 1. Growth speeds and period lengths. (A) Experimental growth speed at 25 °C as a function of Li^+ concentration. Open dots refer to experiments in which Na_3-citrate in Vogel's solution is replaced by an equimolar amount of Li_3-citrate. Solid dots refer to experiments in which LiCl was added to the medium. (B) Open dots: experimental period lengths at 25 °C as a function of Li^+ concentration in which Na_3-citrate in Vogel's solution is replaced by an equimolar amount of Li_3-citrate. Solid dots are computed results using a Goodwin-type model (Table 1) at 25 °C. Inset: correlation between period length (Fig. 1B) and growth rate (Fig. 1A, open circles).

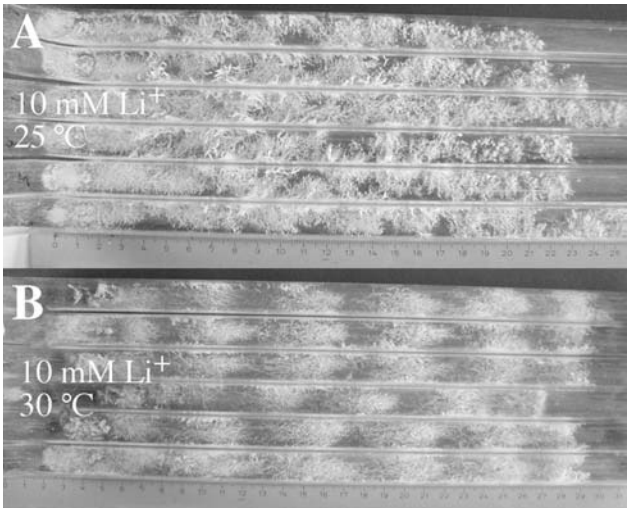

Figure 2. Influence of temperature. Higher temperatures diminish/reverse the effect of Li^+ ions on *Neurospora's* circadian rhythm by increasing growth rate and decreasing the period length. (A) 10 mM Li^+, 25 °C, growth rate 2.6 ± 0.3 cm/24 h, period 27.9 ± 4.0 h (n = 6). (B) 10 mM Li^+, 30.0 °C, growth rate 4.81 ± 0.85 cm/24 h, period 21.7 ± 0.5 h (n = 6). See also Figure 6B.

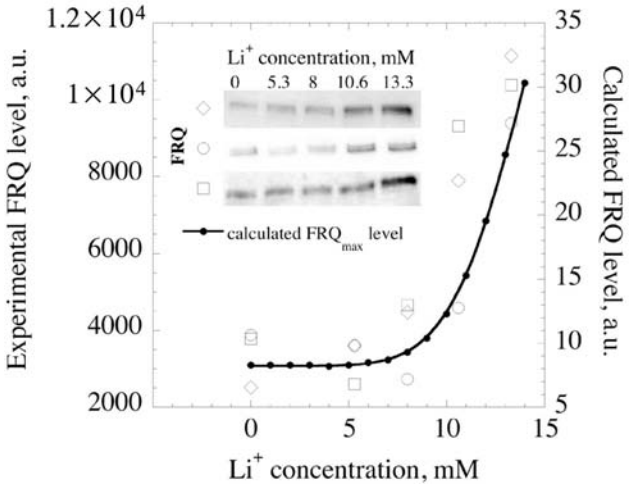

Figure 3. FRQ levels. Experimental (n = 3) and calculated FRQ levels as a function of Li^+ concentration at 25 °C. Samples of shaking cultures were taken directly after the LL→DD transfer. The densitometric determined values of the 3 Western blots indicated by the open circles, squares, and diamonds (insets) are plotted. Solid dots show calculated total FRQ levels after 24 h in light (i.e., no inhibition of *frq* transcription by FRQ protein). a.u. = arbitrary unit.

RESULTS

Growth Tube Experiments

With increasing extracellular Li levels, the growth rate shows a rapid decrease at Li concentrations between 8 and 13 mM (Fig. 1A). The period of the sporulation rhythm increases correspondingly within the same Li concentration range (Fig. 1B). The solid line in Figure 1B shows calculations with the Goodwin model when the optimized K_I and n values (equation 1, Table 1) were used. At Li concentrations of about 10 mM or higher, the sporulation rhythm becomes arrhythmic (data not shown).

Arrhythmicity at higher Li levels can be counteracted (i.e., robust oscillations can be restored) by increasing the temperature (Fig. 2). The period length decreases markedly with increasing temperature (Fig. 2, see also Fig. 6B), similar to the behaviors of frq^7 or frq^{S513I} (Ruoff et al., 2005), showing that the oscillator in the presence of Li has partly lost temperature compensation.

FRQ Levels and FRQ Stability

Relative FRQ protein levels were determined when part of the Na_3-citrate of Vogel's medium was replaced by an equimolar amount of Li_3-citrate. Figure 3 shows the increase of FRQ levels with increasing lithium concentrations after 24-h growth in LL.

The stability of FRQ at different Li concentrations was measured by transferring shaking cultures from LL to DD. During growth in LL, inhibition of *frq* transcription by FRQ is derepressed by light (Dunlap and Loros, 2004). After the LL→DD transfer, inhibition of *frq* transcription is again restored and FRQ decreases without being produced. The FRQ levels are fitted to exponential decay kinetics $FRQ = FRQ_0 \cdot \exp(-kt)$, where FRQ represents FRQ levels, k is the degradation rate constant, $k = k_5 = k_6$ (Table 1), and t is the time in DD conditions. From the determined k value, the FRQ half-life is calculated as $t_{1/2} = \ln 2/k$. This method was previously used to estimate FRQ-protein stabilities in frq^{S513I} and $frq^{\Delta PEST\text{-}1}$ mutants (Gorl et al., 2001; Liu et al., 2000; Ruoff et al., 2005). We have chosen not to use cycloheximide (CHX) in the determination of k because CHX can dramatically decrease protein degradation (Ruoff et al., 1999).

Although the Western blots showed a certain variability in their overall signal strengths (dependent on how many times the FRQ antibodies were used), the quantification shows that FRQ degradation is slowed down with increasing Li concentrations (Fig. 4). FRQ degradation rate constants from several independent

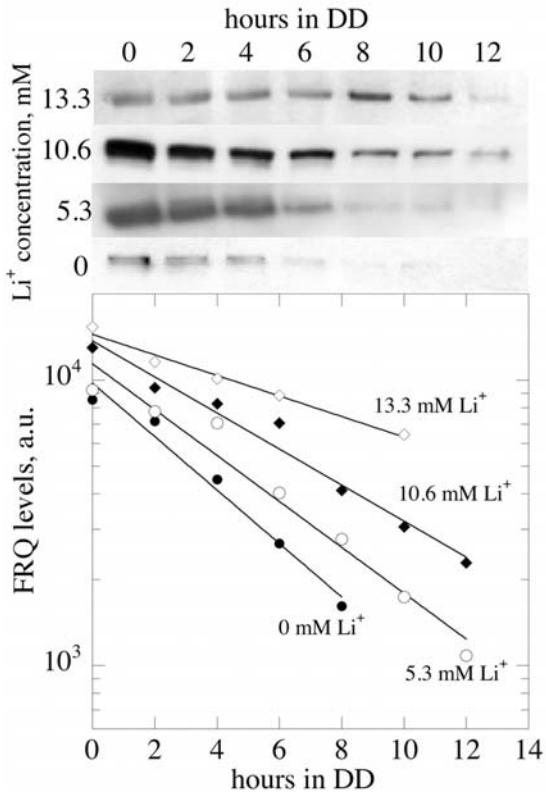

Figure 4. FRQ degradation rates. Western blots for FRQ at 25 °C after an LL to DD transfer at different Li^+ concentrations. Densitometric results are shown as semi-logarithmic plots below. Straight lines show exponential fits to the data where slopes represent 1st-order FRQ degradation rate constants. The shown blots give the following values: 0 mM Li^+, 0.22 h^{-1}; 5.3 mM Li^+, 0.18 h^{-1}; 10.6 mM Li^+, 0.15 h^{-1}; 13.3 mM Li^+, 0.08 h^{-1}. Other determined FRQ degradation rate constants from several independent experiments are shown in Figure 6A. Because the overall FRQ signal in the shown 13.3 mM Li blot turned out to be somewhat low, we multiplied the 13.3 mM data set by a constant. This was done to avoid a crossing between the different regression lines. Note that such a multiplication has no effect on the determined slope. a.u. = arbitrary unit.

experiments as a function of Li concentration are shown in Figure 6A.

Proteasome Activities

Because FRQ is degraded by the proteasome (He and Liu, 2005), the observed decrease of FRQ degradation with increasing lithium concentrations (Fig. 4) may be related to a corresponding inhibition of the proteasome by lithium. To test this possibility, we have measured the 20S proteasome activity by using lactacystin as an inhibitor. Lactacystin is a compound isolated from *Streptomyces* that is used as a selective inhibitor of the 20S proteasome (Corey and Li, 1999; Fenteany and Schreiber, 1998; Omura et al., 1991). Lactacystin irreversibly alkylates subunit X of the 20S proteasome (Fenteany and Schreiber, 1998). Figure 5A shows a typical result of 20S proteasome activities in extracts of lithium-free grown mycelia when lithium or lactacystin was added to the extract. Note the strong inhibition by lactacystin, whereas lithium even at 20 mM did not significantly affect the proteasome activity. This is also the case when mycelia were grown in the presence of 13.3 mM lithium (black bars, Fig. 5B). This clearly shows that the approximately 60% reduction in the FRQ degradation rate constant at 13.3 mM lithium (Fig. 6A) cannot be accounted for by a corresponding reduction in the proteasome activity.

Model Calculations

The solid line in Figure 6A shows the calculated FRQ degradation rate constant $k = k_5 = k_6 = k_0 \cdot \alpha_{PK}$ using the optimized K_I and n values (Table 1). The partial loss of temperature compensation in the presence of Li can be modeled by including the temperature dependence of process R1 in the equations with an activation enthalpy of 50 kJ/mol (Table 1). In addition, due to the altered degradation properties of FRQ in the presence of Li, activation energies E_5 and E_6 are increased from 30 to 50 kJ/mol (Table 1). Figure 6B shows calculated period lengths (open symbols) in the presence (10 mM) or absence of Li together with corresponding experimental results (solid symbols).

DISCUSSION

The period-increasing effect of Li^+ ions on circadian rhythms was noted more than 30 years ago by the work of Engelmann, Johnsson, and coworkers (Engelmann, 1987; Johnsson et al., 1979). Lakin-Thomas (1993) studied the influence of Li on the *Neurospora* circadian clock and concluded that the period-increasing effect of Li is not related to possible effects on the inositol pathway.

Here we show that in the presence of Li, FRQ degradation is slowed down, FRQ levels are increased, and temperature compensation is partly lost (Figs. 3, 4, 6). This finding is consistent with results showing that a more stable FRQ protein leads to increased period lengths and also to a partial loss in temperature compensation (Liu et al., 2000; Ruoff et al., 2005).

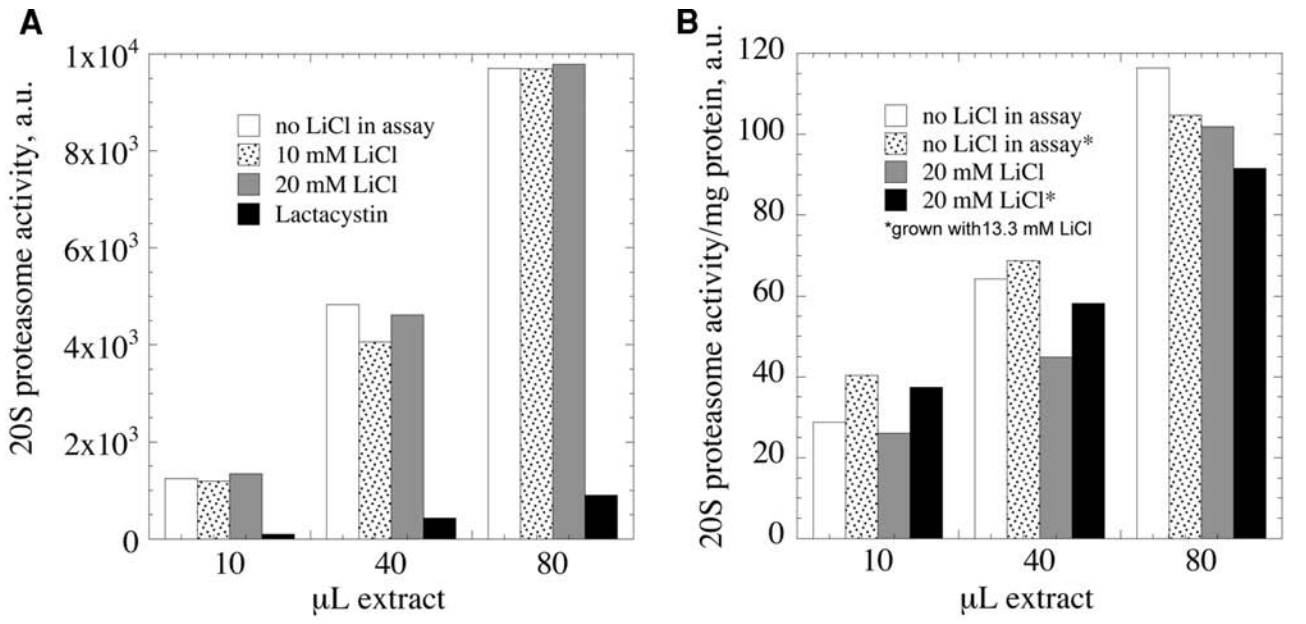

Figure 5. Proteasome activities. (A) 20S proteasome activity measured with different amounts of the same extract. No difference in proteasome activity is observed in the presence or absence of lithium in the assay, while lactacystin shows a marked inhibition of proteasome activity. One representative experiment out of 3 is shown. (B) 20S proteasome activity measured with 2 different extracts, one where mycelium was grown without LiCl and the other where 13.3 mM LiCl was present. Also, here, no significant influence of Li^+ concentration on the proteasome activity is observed. a.u. = arbitrary unit.

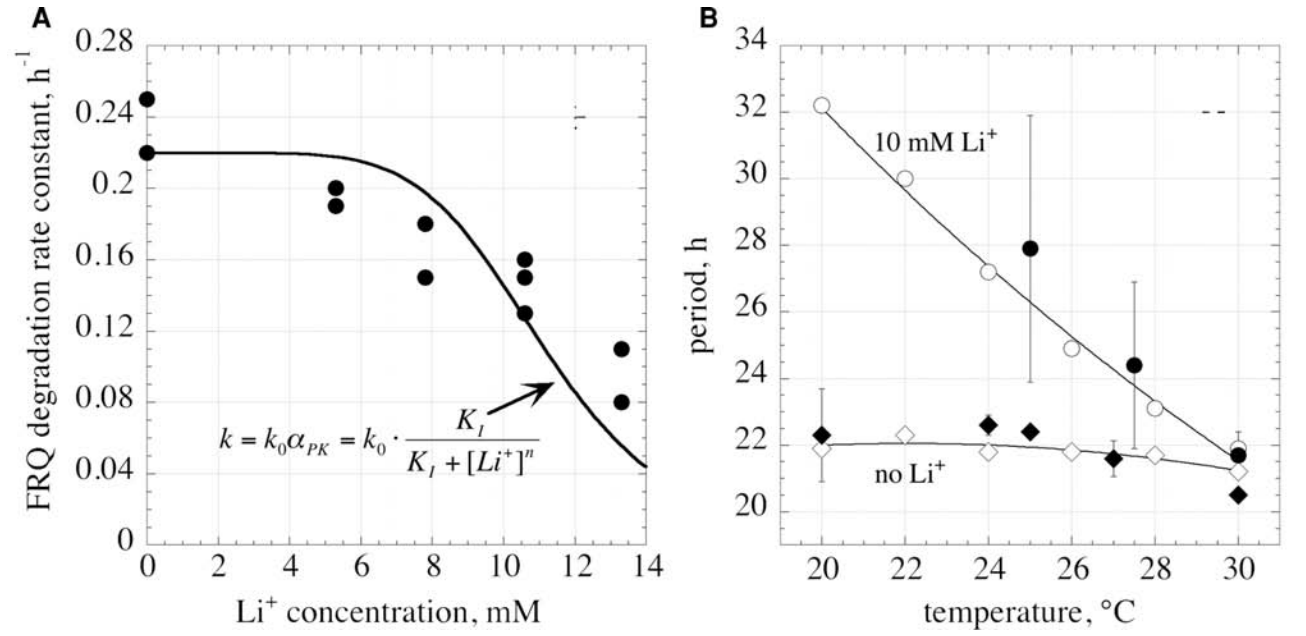

Figure 6. Experimental results versus model calculations. (A) Solid black dots show experimentally determined FRQ degradation rate constants k from several independent experiments. Solid line shows the theoretical $k = k_0 \cdot \alpha_{PK}$ (see also equation 1) using the optimized values of K_I (2.5×10^6 mM) and n (6.1). (B) Partial loss of temperature compensation in the presence of 10 mM Li^+ ions. Solid dots show experimental results. Open circles show the calculated period as a function of temperature. Parameter values are as in Table 1, but E_5 and E_6 values are increased to 50 kJ/mol. Solid and open diamonds show experimental and calculated temperature-compensated period lengths, respectively, in the absence of Li^+ (Ruoff et al., 2005).

FRQ is a central clock element, and its stability is highly regulated by PEST-like sequences (Gorl et al., 2001) and multiple phosphorylation sites (Liu et al., 2000), which are part of *Neurospora's* temperature compensation mechanism (Ruoff et al., 2005). The protein kinases CKII and CaMK-1 and the phosphatase PP1 have been shown to determine FRQ stability and are necessary for the normal operation of *Neurospora's* circadian clock (Yang et al., 2003; Yang et al., 2004). There is also evidence that FRQ degradation requires FWD1, a F-box/WD-40 repeat-containing protein, which is considered to be necessary for the ubiquitination of FRQ and its degradation via the ubiquitin-proteasome pathway (He and Liu, 2005). FRQ turnover is also regulated by the COP9 signalosome that controls the FWD1 ubiquitin ligase complex (He et al., 2005).

The observation that lithium slows down FRQ degradation (Fig. 4) without significantly reducing proteasome activity (Fig. 5) was somewhat surprising because for mammalian WEHI-3B$^+$ cells, a distinct inhibition of the 20S proteasome by LiCl was observed (Rice and Sartorelli, 2001). In view of this result, one may speculate that the influence of lithium on FRQ stability is due to an altered FRQ phosphorylation status rather than to an effect on the proteasome. One known target of lithium is glycogen synthase kinase-3 (GSK-3) in which lithium specifically acts as an inhibitor (Klein and Melton, 1996; Mota de Freitas et al., 2006; Ryves and Harwood, 2001). GSK-3 homologs have been found in all eukaryotes including plants (Jonak and Hirt, 2002) and play a role in the fly (Martinek et al., 2001; Padiath et al., 2004) and mammalian circadian clock (Iitaka et al., 2005; Iwahana et al., 2004; Yin et al., 2006).

In *Neurospora*, 1 or several kinases (such as GSK-3) may show decreased activities due to lithium inhibition, which would diminish degradation-specific phosphorylation sites in FRQ. As a result, more kinase activity, that is, higher temperatures and longer times, is required to achieve the same amount of FRQ phosphorylation leading to turnover. The restoration of a robust conidiation rhythm in the presence of lithium at higher temperatures (Fig. 2) may be due to an increased dissociation between lithium and its targets, as suggested in the model by the temperature dependence of reaction R1 (Table 1).

Recently, several *Neurospora gsk*-3 knockout strains (heterokaryon transformants) were produced, but no significant change of the period was observed. However, it appears that a homokaryon *gsk-3*^{ko} strain is probably lethal, and it is difficult to draw conclusions from the heterokaryon *gsk-3* transformants (H. Colot, J. Loros, J. Dunlap, personal communication, February 2006).

Despite the simplicity of the Goodwin-type oscillator, the influence of lithium on the *Neurospora* clock can be semiquantitatively described by this model when adding lithium-inhibiting terms of FRQ degradation to the model, indicating that the increase of the period and the partial loss of temperature compensation can be understood as part of the FRQ negative feedback loop (Dunlap and Loros, 2004).

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REFERENCES

Comolli J, Taylor W, and Hastings JW (1994) An inhibitor of protein phosphorylation stops the circadian oscillator and blocks light-induced phase shifting in *Gonyaulax polyedra*. J Biol Rhythms 9:13-26.

Comolli J, Taylor W, Rehman J, and Hastings JW (1996) Inhibitors of serine/threonine phosphoprotein phosphatases alter circadian properties in *Gonyaulax polyedra*. Plant Physiol 111:285-291.

Corey EJ and Li WD (1999) Total synthesis and biological activity of lactacystin, omuralide and analogs. Chem Pharm Bull (Tokyo) 47:1-10.

Dunlap JC and Feldman JF (1988) On the role of protein synthesis in the circadian clock of *Neurospora crassa*. Proc Natl Acad Sci U S A 85:1096-1100.

Dunlap JC and Loros JJ (2004) The *Neurospora* circadian system. J Biol Rhythms 19:414-424.

Dunlap JC, Loros JJ, and DeCoursey PJ (2004) *Biological Timekeeping*. Sunderland (UK): Sinauer Associates.

Dunlap JC, Taylor W, and Hastings JW (1980) The effects of protein synthesis inhibitors on the *Gonyaulax* clock. I. Phase-shifting effects of cycloheximide. J Comp Physiol 138:1-8.

Engelmann W (1987) Effects of lithium salts on circadian rhythms. In *Chronobiology and Psychiatric Disorders*, Halaris A, ed, pp 263-289. Amsterdam: Elsevier.

Feldman JF (1967) Lengthening the period of a biological clock in *Euglena* by cycloheximide, an inhibitor of protein synthesis. Proc Natl Acad Sci U S A 57:1080-1087.

Fenteany G and Schreiber SL (1998) Lactacystin, proteasome function, and cell fate. J Biol Chem 273:8545-8548.

Gardner GF and Feldman JF (1981) Temperature compensation of circadian periodicity in clock mutants of *Neurospora crassa*. Plant Physiol 68:1244-1248.

Goodenough JE, Bruce VG, and Carter A (1981) The effects of inhibitors affecting protein synthesis and membrane activity on the *Chlamydomonas Reinhardii* phototactic rhythm. Biol Bull 161:371-381.

Goodwin BC (1965) Oscillatory behavior in enzymatic control processes. Adv Enzyme Regul 3:425-438.

Gorl M, Merrow M, Huttner B, Johnson J, Roenneberg T, and Brunner M (2001) A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*. EMBO J 20:7074-7084.

He Q, Cheng P, and Liu Y (2005) The COP9 signalosome regulates the *Neurospora* circadian clock by controlling the stability of the SCFFWD-1 complex. Genes Dev 19:1518-1531.

He Q and Liu Y (2005) Degradation of the *Neurospora* circadian clock protein FREQUENCY through the ubiquitin-proteasome pathway. Biochem Soc Trans 33:953-956.

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.

Iwahana E, Akiyama M, Miyakawa K, Uchida A, Kasahara J, Fukunaga K, Hamada T, and Shibata S (2004) Effect of lithium on the circadian rhythms of locomotor activity and glycogen synthase kinase-3 protein expression in the mouse suprachiasmatic nuclei. Eur J Neurosci 19:2281-2287.

Jacklet JW (1980) Circadian rhythm from the eye of *Aplysia*: temperature compensation of the effects of protein synthesis inhibitors. J Exp Biol 84:1-15.

Johnsson A, Johnsen PI, Rinnan T, and Skrove D (1981) Basic properties of the circadian leaf movements of *Oxalis regnellii*, and period change due to lithium ions. Physiol Plant 53:361-367.

Johnsson A, Pflug B, Engelmann W, and Klemke W (1979) Effect of lithium carbonate on circadian periodicity in humans. Pharmakopsychiatr Neuropsychopharmakol 12:423-425.

Jonak C and Hirt H (2002) Glycogen synthase kinase 3/SHAGGY-like kinases in plants: an emerging family with novel functions. Trends Plant Sci 7:457-461.

Keck PE Jr (2004) Defining and improving response to treatment in patients with bipolar disorder. J Clin Psychiatry 65(Suppl 15):25-29.

Khalsa SB, Whitmore D, and Block GD (1992) Stopping the circadian pacemaker with inhibitors of protein synthesis. Proc Natl Acad Sci U S A 89:10862-10866.

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.

Lakin-Thomas PL (1993) Evidence against a direct role for inositol phosphate metabolism in the circadian oscillator and the blue-light signal transduction pathway in *Neurospora crassa*. Biochem J 292(Pt 3):813-818.

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.

Martinek S, Inonog S, Manoukian AS, and Young MW (2001) A role for the segment polarity gene shaggy/GSK-3 in the *Drosophila* circadian clock. Cell 105: 769-779.

Mota de Freitas D, Castro MM, and Geraldes CF (2006) Is competition between Li^+ and Mg^{2+} the underlying theme in the proposed mechanisms for the pharmacological action of lithium salts in bipolar disorder? Acc Chem Res 39:283-291.

Omura S, Fujimoto T, Otoguro K, Matsuzaki K, Moriguchi R, Tanaka H, and Sasaki Y (1991) Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells. J Antibiot (Tokyo) 44:113-116.

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.

Pittendrigh CS and Caldarola PC (1973) General homeostasis of the frequency of circadian oscillations. Proc Natl Acad Sci U S A 70:2697-2701.

Pittendrigh CS, Caldarola PC, and Cosbey ES (1973) A differential effect of heavy water on temperature-dependent and temperature-compensated aspects of circadian system of *Drosophila pseudoobscura*. Proc Natl Acad Sci U S A 70:2037-2041.

Rice AM and Sartorelli AC (2001) Inhibition of 20 S and 26 S proteasome activity by lithium chloride: impact on the differentiation of leukemia cells by all-trans retinoic acid. J Biol Chem 276:42722-42727.

Roenneberg T, Nakamura H, and Hastings JW (1988) Creatine accelerates the circadian clock in a unicellular alga. Nature 334:432-434.

Ruoff P, Behzadi A, Hauglid M, Vinsjevik M, and Havas H (2000) pH homeostasis of the circadian sporulation rhythm in clock mutants of *Neurospora crassa*. Chronobiol Int 17:733-750.

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 U S A 102:17681-17686.

Ruoff P, Vinsjevik M, Mohsenzadeh S, and Rensing L (1999) The Goodwin model: simulating the effect of cycloheximide and heat shock on the sporulation rhythm of *Neurospora crassa*. J Theor Biol 196:483-494.

Ryves WJ and Harwood AJ (2001) Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. Biochem Biophys Res Commun 280:720-725.

Taylor WR, Dunlap JC, and Hastings JW (1982) Inhibitors of protein synthesis on 80S ribosomes phase shift the *Gonyaulax* clock. J Exp Biol 97:121-136.

Vogel HA (1956) A convenient growth medium for *Neurospora* (medium N). Microbiol Gen Bull 15:42-43.

Yang Y, Cheng P, He Q, Wang L, and Liu Y (2003) Phosphorylation of FREQUENCY protein by casein kinase II is necessary for the function of the *Neurospora* circadian clock. Mol Cell Biol 23:6221-6228.

Yang Y, He Q, Cheng P, Wrage P, Yarden O, and Liu Y (2004) Distinct roles for PP1 and PP2A in the *Neurospora* circadian clock. Genes Dev 18:255-260.

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