Current Biology, Vol. 13, 725–733, April 29, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S0960-9822(03)00252-5

cGMP-Dependent Protein Kinase II Modulates *mPer1* and *mPer2* Gene Induction and Influences Phase Shifts of the Circadian Clock

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Summary

Background: In mammals, the master circadian clock that drives many biochemical, physiological, and behavioral rhythms is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. Generation and maintenance of circadian rhythmicity rely on complex interlocked transcriptional/translational feedback loops involving a set of clock genes. Among the molecular components driving the mammalian circadian clock are the *Period* 1 and 2 (*mPer1* and *mPer2*) genes. Because the periodicity of the clock is not exactly 24 hr, it has to be adjusted periodically. The major stimulus for adjustment (resetting) of the clock is nocturnal light. It evokes activation of signaling pathways in the SCN that ultimately lead to expression of *mPer1* and *mPer2* genes conveying adjustment of the clock.

Results: We show that mice deficient in cGMP-dependent protein kinase II (cGKII, also known as PKGII), despite regular retinal function, are defective in resetting the circadian clock, as assessed by changes in the onset of wheel running activity after a light pulse. At the molecular level, light induction of *mPer2* in the SCN is strongly reduced in the early period of the night, whereas *mPer1* induction is elevated in *cGKII*-deficient mice. Additionally, we show that light induction of *cfos* and light-dependent phosphorylation of CREB at serine 133 are not affected in these animals.

Conclusions: cGKII plays a role in the clock-resetting mechanism. In particular, the ability to delay clock phase is affected in *cGKII*-deficient mice. It seems that the signaling pathway involving cGKII influences in an opposite manner the light-induced induction of *mPer1* and *mPer2* genes and thereby influences the direction of a phase shift of the circadian clock.

Introduction

The periodic succession of night and day has influenced life on earth for millions of years and has lead to the "internalization" of this change in the form of the circadian clock. Its main function is to organize the time course of biochemical, physiological, and behavioral processes, thereby optimizing an organism's performance in anticipating changing environmental conditions. The molecular basis of circadian rhythms is built of interlocking transcriptional and translational feedback loops displaying a period of about 24 hr. Because this period length is not exactly 24 hr, the clock needs to be reset periodically to serve as a reliable predictor of solar time. The dominant regulator of this entrainment is light [1]. Light is perceived by the retina and elicits glutamate release at the synapses of the retinohypothalamic tract (RHT), which are in direct contact with the suprachiasmatic nuclei (SCN) harboring the circadian clock pacemaker. Glutamate activates ionotropic glutamate receptors in the SCN, and this in turn triggers an influx of Ca^{2+} [2–4] in SCN neurons. This ultimately leads to the activation of several signal transduction pathways and evokes chromatin modifications [5], modification of clock proteins [6], and activation of immediate early genes, such as *cfos*, NGFI-A [7, 8], and the clock genes *mPer1* and *mPer2* [9–11]. The behavioral response induced by a nocturnal light pulse (or glutamate) depends on the clock's temporal state: light perceived during the early night elicits phase delays, whereas light perceived during the late night evokes phase advances [12].

Several signal transduction pathways seem to be involved in the clock's resetting mechanism (reviewed in [13]). Among these pathways are the cyclic AMP-responsive element binding protein (CREB) [3, 14, 15] and cyclic AMP-responsive element (CRE)-dependent transcription [16]. A role of cyclic GMP in this mechanism has also been suggested. Carbachol, a pharmacological agent, upregulates cGMP and activates cGMP-dependent protein kinases (cGKs, also known as PKGs) in SCN slice cultures, and this upregulation leads to phase shifts [17, 18]. Additionally, cGKs seem to be involved in the processing of cholinergic stimuli and can affect transcriptional activation [19, 20]. In mammals, two cGK genes, cGKI and cGKII, have been identified that are expressed in the CNS [21]. However, genetic evidence that cGKs participate in the entrainment mechanism of the mammalian circadian clock in vivo is still lacking.

Here, we show that disruption of the *cGKII* gene in mice leads to abnormal resetting of the circadian clock. A light pulse in the early night leads to smaller delays of clock phase. This is accompanied by a strong reduction of *mPer2* and an increase of *mPer1* expression. Our results suggest a role of cGKII in modulating *mPer1* and *mPer2* gene induction, which affects the clock-resetting mechanism.

Results

cGKII Is Expressed in the SCN, and *cGKII*$^{-/-}$ Mice Display a Normal Circadian Rhythm

In order to investigate potential functions of cGKII in the brain, we performed in situ hybridization experiments

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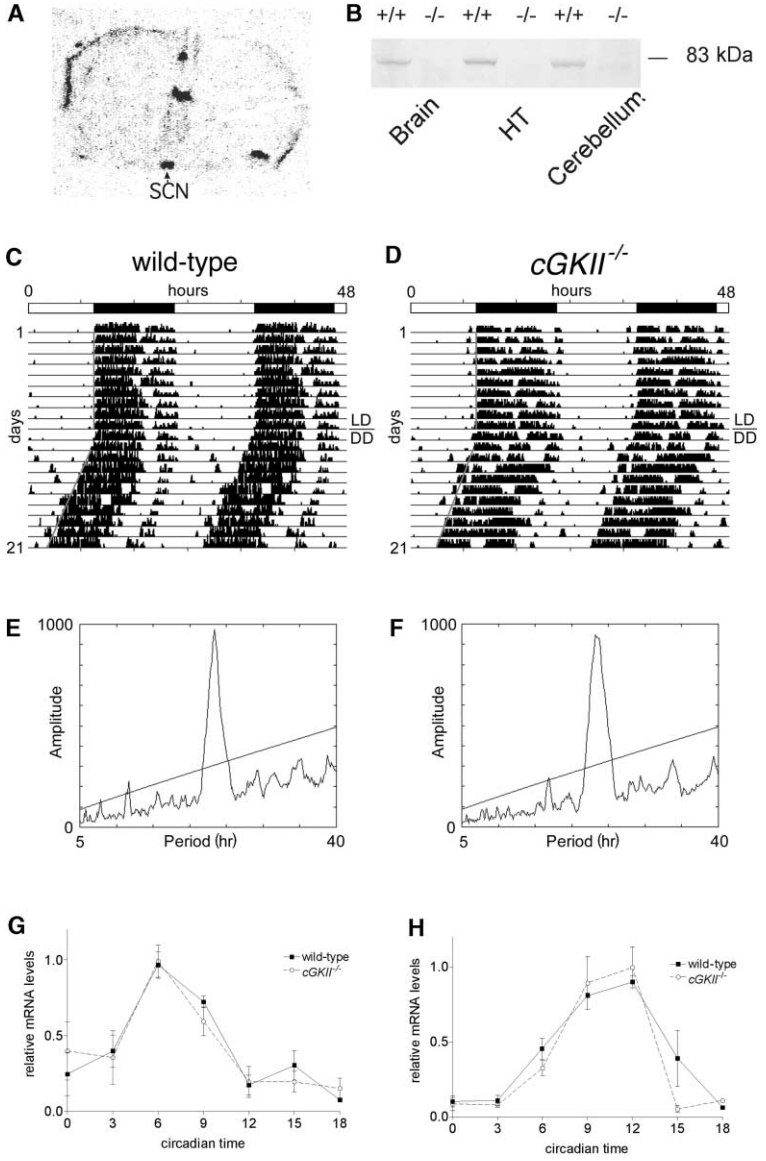

Figure 1. Expression of *cGKII* mRNA and Protein in Mouse Brain, Locomotor Activity Rhythms, and *mPer* Gene Expression in Wild-Type Animals and *cGKII*-Deficient Mice

(A) Analysis of *cGKII* mRNA expression in mouse brain by using mRNA in situ hybridization. The gene is expressed in the suprachiasmatic nuclei (SCN).

(B) Western blot analysis of cGKII protein expression in the entire brain without SCN (Brain), in the hypothalamic region (HT) including the SCN, and in the cerebellum of wild-type (+/+) and *cGKII*-deficient (−/−) mice.

(C and D) Activity records (actograms) of representative (C) wild-type and (D) *cGKII*-deficient mice are shown in double-plotted format. Each horizontal line represents two 24-hr periods; the second (right) 24-hr period in one line is repeated on the next line at the left half. Vertical bars represent periods of wheel running. Animals were initially kept in a 12 hr light:12 hr dark cycle (LD; black and white bars on top indicate night and day, respectively) and were subsequently released into constant darkness (DD). The gray lines mark the onsets of activity and illustrate the rhythmicity of both wild-type and mutant mice in DD.

(E and F) χ^2 periodogram analysis was performed for both groups of animals in DD. Plots represent data from 10 consecutive days starting 1 week after lights were turned off. The ascending line indicates a statistical significance of $p = 0.001$, as determined by the ClockLab program. In LD, all mice had a significant rhythm with a period (τ) of 24.0 hr.

(G) Expression profile of the *mPer1* gene in the SCN of wild-type (black squares) and *cGKII*-deficient mice (open circles).

(H) Expression profile of the *mPer2* gene in the SCN of wild-type (black squares) and *cGKII*-deficient mice (open circles). Error bars represent the SEM of three measurements.

to localize the *cGKII* gene transcript. We identified *cGKII* mRNA to be present in the suprachiasmatic nuclei (SCN) of the mouse brain (Figure 1A). In the next step, we tested whether the cGKII protein is present in the brain (without the ventral hypothalamic region containing the SCN), in the ventral hypothalamic region with SCN, and in the cerebellum. We detected the cGKII protein in the brain, in the hypothalamic region (including SCN), and in the cerebellum of wild-type, but not of *cGKII* knockout (*cGKII*$^{-/-}$), mice (Figure 1B). The presence of the *cGKII* mRNA and the cGKII protein in the SCN suggested that this protein might be involved in the regulation of the circadian clock. Therefore, we set out to measure wheel-running activity, which is a good behavioral test for the functionality of the circadian clock [22]. We found that *cGKII*$^{-/-}$ mice displayed circadian wheel-running activity comparable to wild-type mice (Figures 1C–1F). In constant darkness conditions (DD), the endogenous period length (τ) was 23.40 ± 0.13 hr (mean ± SD, n = 10) for the *cGKII*$^{-/-}$ mice and 23.41 ± 0.21 hr (n = 10) for the wild-type animals. The overall activities under light/dark (LD) conditions were 13,688 ± 2,457 (revolutions/day ± SD, n = 10) for wild-type animals and 10,195 ± 2,337 (n = 10) for *cGKII*$^{-/-}$ mice. Under dark/dark (DD) conditions, overall activities were 15,873 ± 3,345 (revolutions/day ± SD, n = 9) for wild-type mice and 11,351 ± 1,663 (n = 9) for *cGKII*$^{-/-}$ animals. These results show that the amount of activity was not significantly different between the two genotypes, although *cGKII*$^{-/-}$ mice have a tendency to be less active and the onset of activity seems to be less precise than in wild-type animals. Taken together, it appears that *cGKII*$^{-/-}$ mice have no defective circadian clock. This is supported by our finding that *mPer1* and *mPer2* gene expression is identical in wild-type and *cGKII*$^{-/-}$ mice (Figures 1G and 1H). This result also indicates that the clock phase is identical in both genotypes.

cGKII$^{-/-}$ Mice Are Defective in Resetting the Circadian Clock

Multiple intracellular signaling pathways are involved in synchronizing the circadian clock to the environment

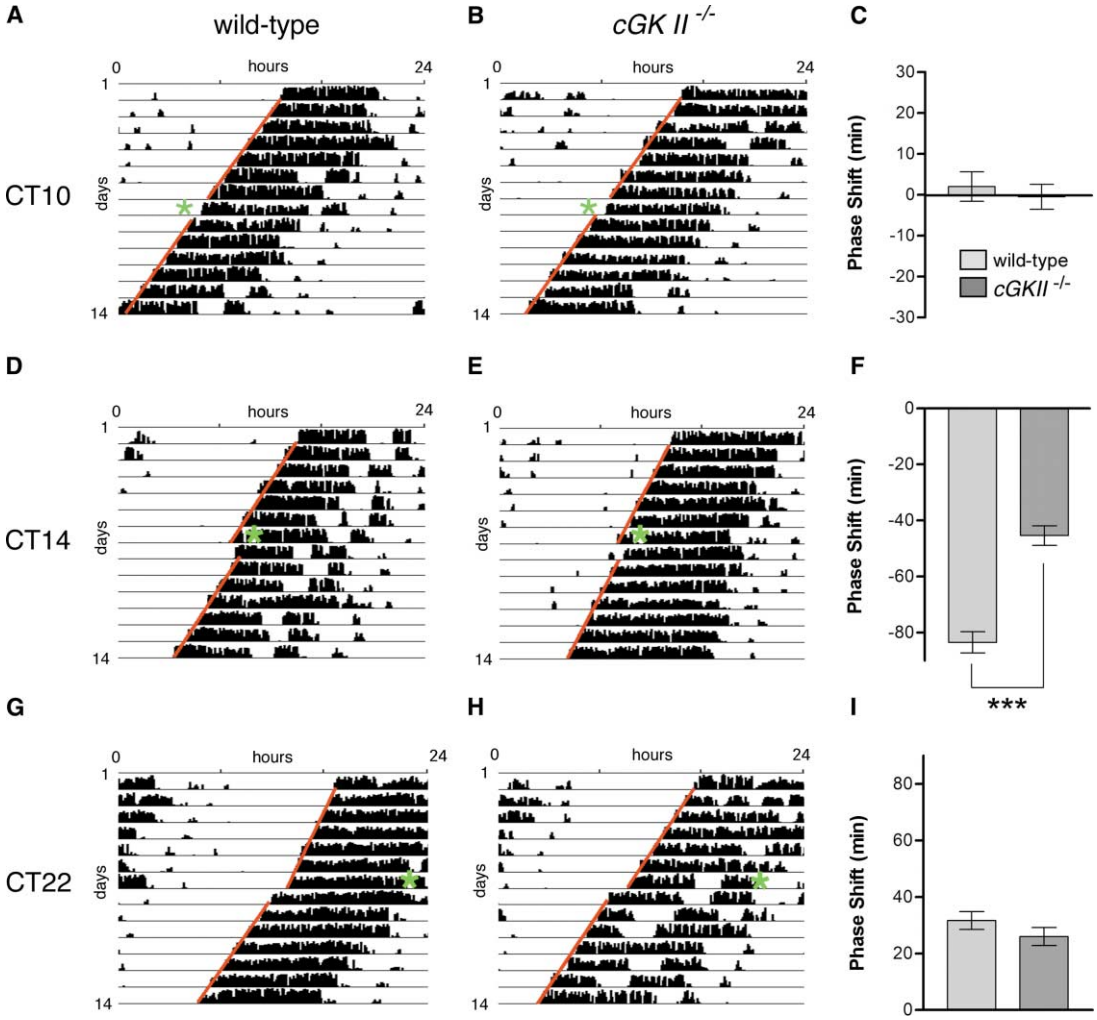

Figure 2. Impairment of Light-Induced Phase Shifts of Locomotor Activity in *cGKII*-Deficient Mice

(A–I) Shown are representative actograms of (A, D, and G) wild-type and (B, E, and H) *cGKII*-deficient mice in response to a 15-min light pulse at (A and B) CT10, (D and E) CT14, and (G and H) CT22. The timing of the light pulses is represented by green asterisks. Red lines indicate the onset of activity before and after the light treatment. The right panels show quantification of phase shifts after a light pulse at (C) CT10, (F) CT14, and (I) CT22. Significant differences (***$p < 0.001$) were determined with a student's t test. Data presented are mean ± SEM ($n = 10$ for all experiments).

[13]. We asked whether cGKII is involved in mediating light information to the clock in the SCN. Therefore, we applied light pulses to wild-type and *cGKII*$^{-/-}$ mice that were kept under constant darkness conditions and studied the resetting of the circadian rhythm. We found that a light pulse applied at circadian time (CT) 10, which corresponds to subjective day, did neither evoke a phase shift of the clock in wild-type nor in *cGKII*$^{-/-}$ mice (Figures 2A–2C). A light pulse applied at CT14 resulted in a phase delay of the clock by 83.5 ± 12.0 min ($n = 10$) in wild-type animals (Figure 2D). The phase shift in *cGKII*$^{-/-}$ mice was significantly smaller at 44.4 ± 11.4 min ($n = 10$) (Figures 2E and 2F). A light pulse given in the late subjective night at CT22 led to a similar phase advance in wild-type and *cGKII*$^{-/-}$ mice. The phase advances were 31.7 ± 9.5 min ($n = 9$) for wild-type and 26.0 ± 9.5 min ($n = 9$) for *cGKII*$^{-/-}$ mice (Figures 2G–2I). These results suggest that cGKII plays a role in the light-mediated signaling pathway that leads to phase delays.

mPer1 and *mPer2* genes have been implicated to be involved in phase advancing or phase delaying the circadian clock, respectively [10, 23]. In particular, it has been shown that the *mPer2* gene is significantly induced by light at CT14, but not at CT22, whereas *mPer1* mRNA expression is inducible throughout the subjective night [10, 11, 24]. Therefore, we tested whether the light inducibility of these two *Per* genes is altered in *cGKII*$^{-/-}$ mice. At CT14, we observed that light induction of *mPer1* transcription is higher in *cGKII*$^{-/-}$ mice compared to wild-type animals (Figure 3A; 5.6 ± 0.3, $n = 3$, versus 4.0 ± 0.6-fold relative to wild-type control, $n = 3$, $p = 0.016$). Interestingly, at CT14, *mPer2* is significantly less inducible by light in *cGKII*$^{-/-}$ mice compared to wild-type animals (Figure 3B, 1.6 ± 0.2, $n = 3$, versus 4.0 ± 0.9-fold, $n = 3$, $p = 0.0009$). At CT22, induction of transcription of *mPer1* by a light pulse is similar in wild-type and *cGKII*$^{-/-}$ mice (Figure 3C). Induction of *mPer2* transcription, however, is very low and is comparable between

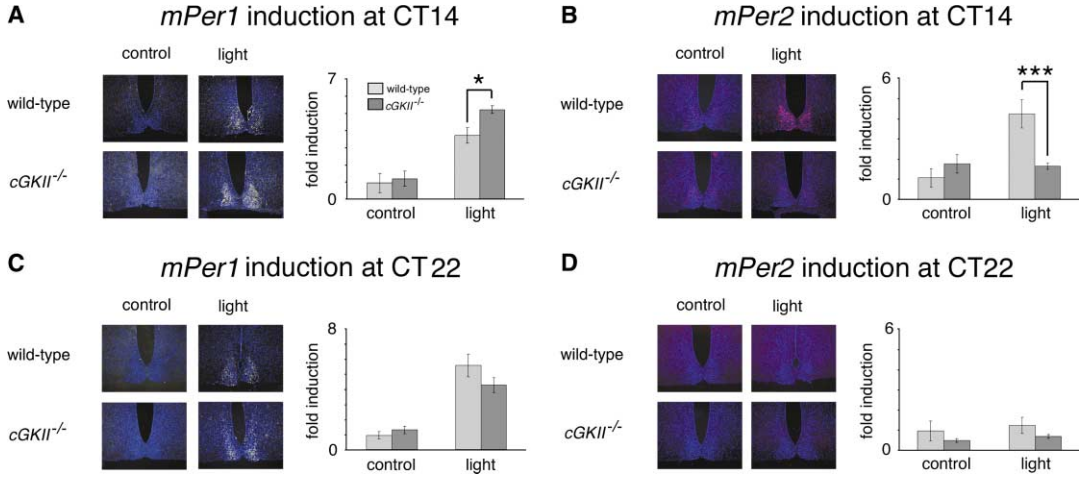

Figure 3. Light Inducibility of *mPer2*, but Not *mPer1*, Is Reduced in *cGKII*-Deficient Mice

Analysis of *mPer1* and *mPer2* expression in the SCN by in situ hybridization.

(A) Induction of *mPer1* expression by a 15-min light pulse applied at CT14.

(B) Induction of *mPer2* expression after a pulse at CT14.

(C) *mPer1* induction after a pulse at CT22.

(D) *mPer2* induction after pulse at CT22.

Animals were sacrificed at CT15 and 23, respectively. The left panels show representative micrographs. The location of each section was determined by Hoechst dye staining (blue). The in situ signal is given in yellow (*mPer1*) or in red (*mPer2*). The data shown in the quantifications are means ± SEM of three different experiments each. Significant differences (*$p < 0.05$, ***$p < 0.001$) were determined by a student's t test.

wild-type and *cGKII*$^{-/-}$ mice (Figure 3D). These results indicate that cGKII plays a specific role in the signaling pathway that is important for delaying clock phase. It seems that *mPer2* expression is particularly affected.

cGKII Is Not Acting through Phosphorylation of CREB at Serine 133 to Regulate *mPer2* Gene Induction

Phosphorylation of the transcription factor cAMP-responsive element binding protein (CREB) has been shown to play a role in the induction of the *Per1* gene [15, 25–27]. In contrast, the *Per2* gene, which also contains a CRE in its promoter, is less responsive to CREB. To test whether cGKII is acting via phosphorylation of CREB, we performed immunohistochemical experiments to detect phosphorylation of CREB at serine 133 in wild-type and *cGKII*$^{-/-}$ brain sections. We found that at CT14, as well as at CT22, CREB protein is expressed at similar levels in both wild-type and *cGKII*$^{-/-}$ mice (Figures 4A and 4B). After a 15-min light pulse at CT14 or at CT22, CREB is equally phosphorylated in both genotypes (Figures 4A and 4B). These experiments indicate that the light-dependent signaling pathway, which is responsible for *Per2* gene induction and acts through cGKII, does not involve CREB phosphorylation at serine 133.

Induction of the Immediate Early Gene *cfos* Is Not Affected in *cGKII*$^{-/-}$ Mice

A light pulse applied in the dark period not only induces *mPer* genes but also induces the immediate early gene *cfos* [8]. To elucidate whether the mechanism for *mPer* gene induction and *cfos* induction by a nocturnal light pulse is comparable, we investigated whether *cfos* inducibility is altered in *cGKII*$^{-/-}$ mice. We found that at CT14, cFos protein is induced to a similar extent in wild-type and *cGKII*$^{-/-}$ mice (Figure 4C). The same was observed at CT22 (Figure 4D), indicating that light-mediated induction of cFos is independent from cGKII and is regulated by a mechanism different from the one regulating *mPer2* induction.

The Retina Reacts Normally to Light in *cGKII*$^{-/-}$ Mice

To investigate whether a defect in retinal transmission is the cause for the observed resetting defect in *cGKII*$^{-/-}$ mice, we performed an electroretinographic analysis of wild-type and *cGKII*$^{-/-}$ mice (Figure 4E). Under scotopic as well as under photopic conditions, the electroretinograms of both genotypes are comparable, indicating that the retina in *cGKII*$^{-/-}$ mice is reacting normally to light. Additionally, histology and CREB phosporylation at serine 133 in the retina were not different between wild-type and *cGKII*$^{-/-}$mice (data not shown). Hence, we conclude that a retinal defect is unlikely to be the cause of the resetting defect observed in *cGKII*$^{-/-}$ mice.

Discussion

The clock in the SCN encompasses a dynamic system of regulatory mechanisms that respond differentially to light. During the dark phase, the clock exhibits phase-resetting sensitivity to a number of stimuli, including light and its neurotransmitter glutamate [13]. This sensitivity is gated, and the SCN are nonresponsive to these signals during the subjective day [2]. In the early hours of the night, light and glutamate induce phase delays, whereas, in the late portion of the night phase, advances of circadian rhythms are observed.

To investigate the role of the cGKs in the resetting mechanism of the circadian clock, we first studied the

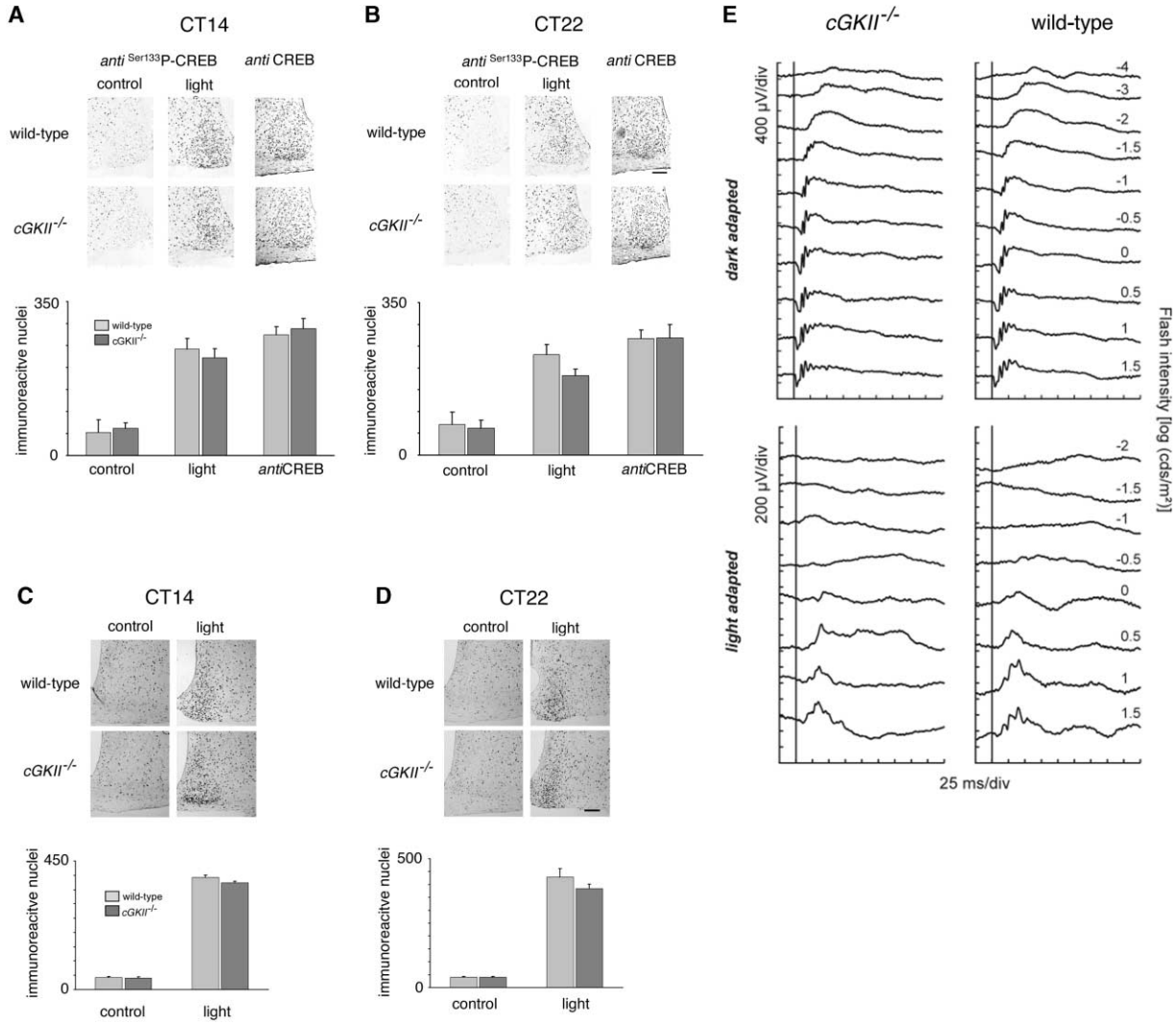

Figure 4. Light-Induced CREB Phosphorylation and cFos Expression in the SCN and Electroretinogramms from Wild-Type and *cGKII*-Deficient Mice

(A) Immunohistochemical analysis of ^{Ser133}P-CREB in the SCN of wild-type (upper row) and *cGKII*-deficient mice (lower row). Shown are representative micrographs of SCN sections immunostained for ^{Ser133}P-CREB with (light) or without light administration (control) at CT14. As a control, immunostainings of CREB for both wild-type and *cGKII*$^{-/-}$ animals are shown for both CT14 and CT22 in the right panels. The diagram shows numbers of ^{Ser133}P-CREB immunoreactive nuclei in the SCN with (light) or without light exposure (control) for wild-type (light gray bar) and mutant (dark gray bar) animals.

(B) Immunohistochemical analysis of ^{Ser133}P-CREB in the SCN of wild-type (upper row) and *cGKII*-deficient mice (lower row) at CT22. Data presented are mean ± SD of three animals each.

(C) cFos protein induction by light in the SCN of wild-type (upper row) and *cGKII*-deficient mice (lower row). Shown are representative micrographs of SCN sections immunostained for cFos with (light) or without light administration (control) at CT14.

(D) cFos protein induction by light in the SCN of wild-type (upper row) and *cGKII*-deficient mice (lower row) at CT22. The diagram shows numbers of cFos immunoreactive nuclei in the SCN with (light) or without light exposure (control) for wild-type (light gray bar) and mutant (dark gray bar) animals. Data presented are mean ± SEM of three animals.

(E) Electrophysiological characteristics of *cGKII*-deficient mice. Calibration marks: vertical: 400 µV/div (dark adapted), 200 µV/div (light adapted); horizontal: 25 ms/div. Top panels: dark-adapted (scotopic) intensity series of a *cGKII*-deficient (left) and a wild-type mouse (right). Bottom panels: light-adapted (photopic) intensity series of a *cGKII*-deficient (left) and a wild-type mouse (right). No apparent differences can be observed between the two genotypes.

expression pattern of cGKII. Our experiments indicate that cGKII is expressed in the murine SCN (Figures 1A and 1B). The presence of cGKII in the SCN is in line with the concept that cGMP-dependent signaling cascades play an important role in entrainment of the circadian clock [13]. Therefore, we tested *cGKII*-deficient mice for a defective circadian system. Under a 12 hr light:12 hr dark cycle, as well as under constant darkness conditions, *cGKII*$^{-/-}$ mice display a normal circadian rhythm with a period length that is comparable to wild-type mice (Figures 1C–1F); this finding indicates that cGMP signaling mediated via cGKII is not required for normal

clock function. This is supported by the finding that *mPer1* and *mPer2* gene expression is similar between wild-type and *cGKII*$^{-/-}$ mice (Figures 1G and 1H). However, our experiments indicate that the cGKII signaling pathway plays an important role in the resetting mechanism of the circadian clock. A light pulse in the early portion of the night at CT14 elicits strong phase delays of the clock in wild-type animals, whereas, in *cGKII*$^{-/-}$ mice, such a phase delay is reduced (Figures 2D–2F). This reduction does not have its roots in an altered phase of the clock in *cGKII*$^{-/-}$ mice because the expression patterns of *mPer1* and *mPer2* and the clock period are similar in *cGKII*$^{-/-}$ and wild-type mice (Figures 1C–1H). Interestingly, phase advances are not altered in *cGKII*$^{-/-}$ mice compared to wild-type animals (Figures 2G–2I). These findings indicate that *cGKII*$^{-/-}$ plays a prominent role in the signaling mechanism that leads to phase delays. This is in contrast to studies placing the cGKs into the signaling pathway for phase advances [28, 29]. The discrepancy, however, might have its roots in experimental differences. First, many studies have been performed in SCN slice cultures. These could display different sensitivity to pharmacological agents compared to SCN in live animals. Second, most of the studies to date have been performed by using pharmacological agents that block cGK activity but don't discriminate between cGKI and cGKII. This might lead to observations that are dominated by cGKI, because it might have a higher affinity for these agents or may be more abundant than cGKII, thereby masking cGKII function. Only limited data are available on the expression of cGKI in mammalian SCN. There is evidence that cGKI is expressed in the rat SCN [30], whereas only cGKII, but no cGKI protein, was found in the hamster SCN [20].

To investigate target genes of the signaling pathway mediated by cGKII, we performed in situ hybridization experiments. We found that *mPer1* gene induction is increased in *cGKII*$^{-/-}$ mice compared to wild-type animals when a light pulse was given at CT14 (Figure 3A). In contrast, light-induced *mPer2* gene expression in the SCN was strongly reduced in *cGKII*$^{-/-}$ mice (Figure 3B). Moreover, a light pulse applied at CT22 does not alter *mPer1* and *mPer2* gene expression in *cGKII*$^{-/-}$ mice compared to wild-type animals (Figures 3C and 3D). The strong reduction of *mPer2* inducibility at CT14 seems to be correlated with a reduced ability to delay the phase of the clock (Figure 2). This parallels previous findings describing *mPer2* mutant mice to be unable to delay clock phase [23]. In contrast to *mPer2* mutant mice, *cGKII*$^{-/-}$ mice display phase delays. The reason for this is probably an increase of light-induced *mPer1* expression at CT14 (Figure 3A). This might compensate for the consequences of reduced induction of *mPer2* expression resulting in partial rescue of phase delays (Figure 2F). In support of this notion is evidence that *mPer1* can play a role in the phase delay mechanism [31]. The complete inability of *mPer2* mutant mice (having cGKII) to delay clock phase is probably reinforced by the reduction of *mPer1* expression ([32], U.A. unpublished data). It seems that in *mPer2* mutant mice, cGKII is attenuating light-induced *mPer1* expression. This would explain the complete loss of phase delays observed in *mPer2* mutant mice [23]. Taken together, cGKII is necessary for

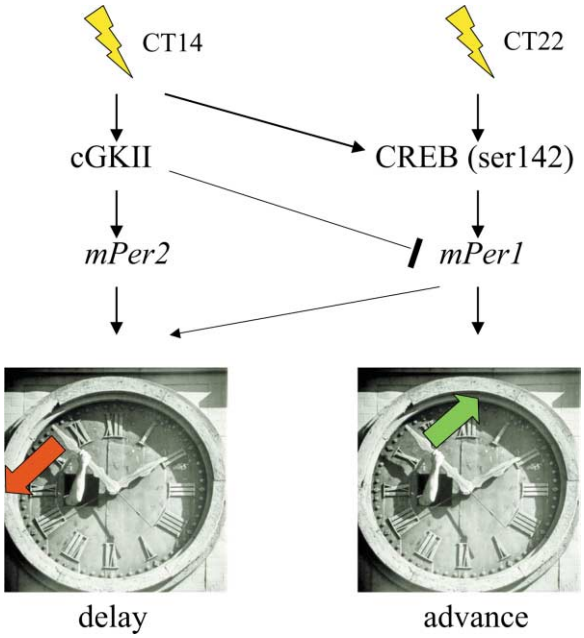

Figure 5. Model for a Possible Molecular Mechanism Regulating the Resetting of the Circadian Clock

Light pulses at CT14 and CT22 start a signaling cascade involving cGKII and CREB phosphorylation, respectively. The differential activation of signaling cascades differentiates between the induction of the clock genes *mPer1* and *mPer2* and leads to phase advances or phase delays.

mPer2 gene induction at CT14, whereas it has an inhibitory effect on *mPer1* induction, either influencing kinetics or absolute amounts of induction. Hence, cGKII appears to affect light-induced expression of *mPer1* and *mPer2* at CT14 in an opposite manner.

There is evidence that the signal transduction pathways responsible for *mPer1* and *mPer2* gene induction are different. A mutation in the phosphorylation site of CREB at serine 142 has been shown to abolish light-induced *mPer1*, but not *mPer2*, expression at CT14 [15]. To investigate whether cGKII acts through CREB to induce *mPer* gene expression, we tested phosphorylation of CREB in *cGKII*$^{-/-}$ mice. Because a mutation in the phosphorylation site at serine 142 of CREB does not alter *mPer2* gene induction at CT14 [15], this phosphorylation site is probably not affected in *cGKII*$^{-/-}$ mice. Therefore, we tested whether we could detect a difference between wild-type and *cGKII*$^{-/-}$ mice in the phosphorylation of CREB at serine 133 (Figures 4A and 4B). Interestingly, no differences could be observed. These and previous findings indicate that cGKII probably doesn't act through a mechanism involving phosphorylation of CREB and acts neither at serine 133 nor at serine 142.

Per genes are not the only light-inducible genes in the SCN. For example, *cfos* gene expression is induced in the SCN upon application of a nocturnal light pulse [8]. It has been suggested that cGKs are involved in the activation of *cfos* [33]. Therefore, we tested *cfos* inducibility in *cGKII*$^{-/-}$ mice. Our results show that wild-type and *cGKII*$^{-/-}$ mice display similar amounts of light-

induced cFos protein at CT14 and CT22 (Figures 4C and 4D). Therefore, we conclude that cGKII is not required for *cfos* induction. The normal *cfos* induction in *cGKII*$^{-/-}$ mice indicates that the retinae in these mice are probably not defective. This finding is complemented by the electrophysiological analysis that yielded normal results in *cGKII*$^{-/-}$ mice (Figure 4E).

Based on our findings, we propose a resetting mechanism of the mammalian circadian clock as depicted in Figure 5. A light pulse presented at CT14 elicits activation of cGKII in the SCN neuron. This induces the *mPer2* gene by an unknown mechanism ultimately contributing to delay clock phase. At the same time, activation of *cGKII* limits induction of the *mPer1* gene, which is activated via the phosphorylation of CREB at serine 142 [15]. Hence, light-induced *mPer1* expression at CT14 is positively and negatively regulated by ^{Ser142}P-CREB and cGKII, respectively. A lack of cGKII leads to a loss of *mPer2* gene activation and a stronger activation of *mPer1*. The stronger activation of *mPer1* compensates partially for the lack of *mPer2* induction and thus prevents a complete loss of phase delays. A loss of normal *mPer2* function, however, does not affect the limitation of *mPer1* activation via cGKII, and, hence, *mPer1* cannot contribute to delay clock phase. This leads to a complete loss of phase delays in *mPer2* mutant mice [23]. A light pulse applied at CT22 leads to CREB phosphorylation at serine 142 that is necessary for *mPer1* gene induction and an advance of clock phase. This scenario is consistent with the data presented here and previous findings [15, 23, 31].

Conclusions

Taken together, it seems that cGKII plays an important role in the light-resetting mechanism of the circadian clock. We propose that cGKII is gating the light information received by an SCN neuron at CT14 by activating *mPer2* and attenuating *mPer1* gene expression. Our findings illustrate a possible molecular mechanism for understanding the resetting mechanism of the circadian clock.

Experimental Procedures

Animals

The *cGK II* knockout (*cGKII*$^{-/-}$) mice on a 129/Sv background were generated by homologous recombination [34]. Heterozygous *cGK II*$^{+/-}$ animals were backcrossed to C57Bl/6 for nine generations, and the resulting progeny were mated to each other, to obtain *cGK II*$^{+/+}$ (wild-type) and *cGKII*$^{-/-}$ mice on a C57Bl/6 background. From these mice, we used 2- to 6-month-old males for all experiments. Locomotor activity monitoring was performed as described [22, 35]. Briefly, for free-running analysis, animals were entrained to a 12 hr light:12 hr dark (LD) cycle for 10 days before release into constant darkness (DD). Circadian period (τ) was determined by calculating a regression line for the onsets of activity of 10 days of stable rhythmicity in DD by using the ClockLab software (Actimetrics). For phase shifts, animals were kept in DD for at least 10 days before the experiment. The endogenous period (τ) was determined by extrapolating a regression line for the onsets of activity of 10 days of stable rhythmicity, and circadian times were calculated for each animal (CT) for the following day. Animals were exposed to a 15-min light pulse (400 Lux, see [22, 35]) at the depicted circadian time points (CT10, CT14, or CT22) and were subsequently returned into DD. Phase shifts were determined as the time difference between regression lines of activity onsets before and after light application. On the first 2 days after the light administration, the animals were thought to be in transition and were therefore omitted from the evaluation.

In Situ Hybridization

For Figure 1A, adult C57Bl/6 mice were anaesthesized with sodium pentobarbital and were perfused with 50 ml ice-cold PBS (pH 7.4). The brain was removed and quickly frozen in isopentane cooled in a dry ice/ethanol bath. Sections (20 μm thick) were cut in a cryostat (Leica, Jung CM 3000), thaw-mounted onto Super Frost Plus-slides (Menzel-Gläser), fixed with 4% paraformaldehyde in PBS (pH 7.4), and acetylated with 0.25% acetic anhydride (Merck) in 0.1 M triethanolamine (pH 8.0) for 10 min. The slides were dehydrated in several changes of ethanol, isopropanol, and xylene, dried, and then prehybridized for 2 hr at 42°C in hybridization buffer (10 mM Tris [pH 8.0], 0.3 M NaCl, 1 mM EDTA, 1× Denhardt's solution, 10% dextran, 50% deionized formamide, 50 mM DTT). For in situ hybridization, a cDNA of *cGKII* from mouse brain ([36]; nt 960–1740) was cloned into the PAL1-Vector, radio-labeled with [^{35}S]-UTP (Amersham) by using the reverse transcription kit (Stratagene Europe), and incubated with prehybridized sections at a concentration of 5×10^6 cpm/ml hybridization buffer for 16 hr at 55°C. After hybridization, the slides were washed two times in 2× SSC, 1 mM DTT, 1 mM EDTA, incubated in RNase A (20 μg/ml, Life Technologies) for 30 min at room temperature, and then washed twice in 2× SSC, 1 mM DTT, 1 mM EDTA. The sections were then washed at high stringency in 0.1× SSC, 1 mM DTT, 1 mM EDTA for 2 hr at 75°C, dehydrated, dried, and exposed to BioMax MR (Kodak) film for 6 days. The slides were then dipped in autoradiography emulsion NTB-2 (Kodak), exposed for 1–8 weeks, and developed in D-19 developer (Kodak). Sections were lightly counterstained with haematoxyline/eosin or toluidine blue and were examined with bright- and dark-field illumination to identify cerebral structures. Photomicrographs were taken in 20×- or 40×-fold magnification by using a Kodak Select 400ASA-film.

To determine *mPer1* and *mPer2* gene expression in wild-type and *cGKII*$^{-/-}$ mice (Figures 1G and 1H), animals were kept for 10 days in DD with running wheels to determine the period length of each animal. Mice were sacrificed in darkness (CT0, 3, 6, 9, 12, 15, and 18) by using a night vision apparatus (rigel 3200, rigel optics). For light induction experiments, animals were kept in DD for at least 10 days before the experiment. Light pulses were applied as described above, and animals were sacrificed at CT15 or CT23 by cervical dislocation under dim red light (15W) to avoid light influence on *Per* gene expression. Control animals were kept in DD and were sacrificed at CT15 or CT23 without a previous light exposure. Brains were removed and processed for in situ hybridization as described [37]. The probes for *mPer1* and *mPer2* were as described [10]. Quantification was performed by densitometric analysis of autoradiograph films (Amersham Hyperfilm MP) by using the NIH Image program after conversion into the relative optical densities by the ^{14}C-autoradiographic microscale (Amersham Biosciences). Data from the SCN were normalized with respect to the signal intensities in an equal area of the lateral hypothalamus. Three sections per SCN were analyzed. Relative induction values were calculated by defining the wild-type control value of each experiment as 1.

Immunoblot Analysis

Brain proteins were separated on 9% SDS-PAGE and were transferred to PVDF membranes. Membranes were blocked with 5% skim milk in Tris buffer (10 mM Tris/HCl, 150 mM NaCl [pH 8.0]) containing 0.05% Tween 20 (TBST), and were then incubated with 1:25 rabbit anti-cGKII antibody (a polyclonal antibody against the amino terminus of the cGKII protein, cgkII-NT-ab1) for 1 hr at room temperature. The membranes then were washed in TBST and incubated with 1:5000 goat anti-rabbit IgG-AP (Dianova) for 1 hr at room temperature. The membrane was washed repeatedly, and cGKII was detected by using the AP reaction with BCIP/NBT.

Immunohistochemistry

Animals were sacrificed by cervical dislocation, and brain slices were prepared as described for in situ hybridization. Sections were boiled in 0.01 M sodium citrate (pH 6) for 10 min to unmask hidden antigen epitopes and were processed for immunohistochemical detection by using the Vectastain Elite ABC system (Vector Labora-

tories) and diaminobenzidine with nickel amplification as chromogenic substrate. Immunostained sections were inspected with an Axioplan microscope (Carl Zeiss), and the area of the SCN was determined by comparison to Nissl-stained parallel sections. Semiquantitative analysis for ^{Ser133}P-CREB immunoreactivity in the SCN was performed by using NIH Image program. Images were digitized; background staining was used to define a lower threshold. Within the whole area of the SCN, all cell nuclei exceeding the threshold value were marked. Three sections of the intermediate aspect of the SCN were chosen at random for further analysis. Values presented are the mean of three different experiments ± SEM. Primary antibodies against CREB, phosphorylated at the residue serine 133 (Cell Signaling Technology; order number 9191S), and cFos protein (Oncogene, order number PC38T-20UL) were used at a dilution of 1:1,000 and 1:3,000, respectively.

Electroretinography (ERG)

ERGs were obtained as previously reported [38]. Briefly, mice were anesthesized following dark adaptation overnight with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg), and the pupils were dilated. The ERG equipment consisted of a Ganzfeld bowl, a DC amplifier, and a PC-based control and recording unit (Multiliner Vision; Jaeger-Toennies). Band-pass filter cutoff frequencies were 0.1 and 3000 Hz. Single flash recordings were obtained both under dark-adapted (scotopic) and subsequently light-adapted (photopic) conditions. Light adaptation was achieved with a background illumination of 30 cd/m^2 starting 10 min before photopic recordings. Single flash stimulus intensities were increased from 10^{-4} cd*s/m^2 to 25 cd*s/m^2 and were divided into ten steps of 0.5 and 1 log cd*s/m^2. Ten responses were averaged with an interstimulus interval (ISI) of either 5 s or 17 s (for 1, 3, 10, 25 cd*s/m^2).

Acknowledgments

We would like to thank A. Streit, E. Maronde for comments on the manuscript, and G. Pendyala for technical assistance. This study was supported by the Swiss National Science Foundation, the Novartis Foundation, the European Community and the Swiss Office for Education and Research (Braintime project QLRT-2001-01829), the State of Fribourg (U.A.), and the Deutsche Forschungsgemeinschaft (F.H. and M.W.S.).

Received: November 12, 2002
Revised: March 18, 2003
Accepted: March 19, 2003
Published: April 29, 2003

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