CaMKII is essential for the cellular clock and coupling between morning and evening behavioral rhythms

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Daily behavioral rhythms in mammals are governed by the central circadian clock, located in the suprachiasmatic nucleus (SCN). The behavioral rhythms persist even in constant darkness, with a stable activity time due to coupling between two oscillators that determine the morning and evening activities. Accumulating evidence supports a prerequisite role for Ca2+ in the robust oscillation of the SCN, yet the underlying molecular mechanism remains elusive. Here, we show that Ca2+/calmodulin-dependent protein kinase II (CaMKII) activity is essential for not only the cellular oscillation but also synchronization among oscillators in the SCN. A kinase-dead mutation in mouse CaMKIIα weakened the behavioral rhythmicity and elicited decoupling between the morning and evening activity rhythms, sometimes causing arrhythmicity. In the mutant SCN, the right and left nuclei showed uncoupled oscillations. Cellular and biochemical analyses revealed that Ca2+-calmodulin–CaMKII signaling contributes to activation of E-box-dependent gene expression through promoting dimerization of circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein 1 (BMAL1). These results demonstrate a dual role of CaMKII as a component of cell-autonomous clockwork and as a synchronizer integrating circadian behavioral activities.

[Keywords: circadian clock; CaMKII; morning and evening oscillators; coupling of oscillators; SCN; CLOCK/BMAL1]

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In mammals, the master circadian pacemaker in the hypothalamic suprachiasmatic nucleus [SCN] generates rest–activity cycles to synchronize with daily changes of environmental conditions [Reppert and Weaver 2002, Crocker and Sehgal 2010, Welsh et al. 2010]. Furthermore, in order to adapt to seasonal changes, the activity time, the period between the onset and offset of behavioral activities, is thought to be regulated by two independent clocks in the SCN, termed morning and evening oscillators [Pittendrigh and Daan 1976, de la Iglesia et al. 2000, Jagota et al. 2000, Inagaki et al. 2007, Helfrich-Förster 2009]. Decoupling of these oscillators is observed in some species. The golden hamster [Mesocricetus auratus] shows dramatic dissociation of morning and evening activity rhythms under constant light conditions [Pittendrigh and Daan 1976]. Similarly, the white-footed mouse [Peromyscus leucopus] or house mouse [Mus musculus] inbred CS strain shows spontaneous dissociation of morning and evening activity rhythms in constant darkness [DD] [Pittendrigh and Daan 1976, Abe et al. 1999]. Morning and evening oscillators are normally coupled to maintain the constant length of the activity time, although the mechanism of communication between the oscillators is left undetermined.

Robust behavioral rhythms are integrated by a neuronal network among individual SCN neurons communicating through neurotransmitters and electrical signaling [Yamaguchi et al. 2003, Aton and Herzog 2005, Maywood

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et al. 2007; Hastings et al. 2008). Behavioral rhythms are severely disrupted in mice carrying a null mutation in vasoactive intestinal peptide (VIP) receptor 2 (VPAC2), a receptor for VIP and pituitary adenylate cyclase-activating peptide (PACAP) (Harmar et al. 2002). Inhibition of the intracellular signaling (e.g., cyclic AMP or G-protein signaling) results in a prolonged free-running period (O’Neill et al. 2008; Doi et al. 2011), but importantly, the loss of function of the cyclic nucleotide/G-protein signaling pathway does not cause dissociation of morning and evening activity rhythms in C57BL/6 mice. Consistently, a modeling study suggests that the mechanism of communication between the oscillators governing morning and evening behaviors is distinct from the coupling mechanism among individual neurons [Indic et al. 2008].

The cell-autonomous circadian oscillation of both the SCN and the peripheral cells is generated by a transcription-based autoregulatory feedback mechanism (Schibler and Sassone-Corsi 2002, Kondratov et al. 2003). Basic helix–loop–helix transcription factors circadian locomotor output sequences cycle 1 (CLOCK) and brain and muscle Arnt-like protein 1 (BMAL1) form a heterodimer that activates transcription of Period [Per] and Cryptochrome [Cry] genes through their E-box enhancer elements. Translated PER and CRY proteins then translocate into the nucleus, where they interfere with CLOCK/BMAL1-dependent transcription and repress their own expression.

In addition to the transcriptional regulation, dynamic fluctuation of ions, especially Ca$_2^+$, appears to be essential for circadian clockwork [Naito et al. 2005]. Individual neurons in a cultured SCN slice exhibit robust circadian fluctuation of intracellular Ca$_2^+$ concentration [Ikeda et al. 2003; Enoki et al. 2012], while chelating of intracellular Ca$_2^+$ abrogates rhythmic expression of clock genes [Lundkvist et al. 2005]. These studies led us to speculate that a Ca$_2^+$-dependent feedback system regulates the transcriptional rhythms and raise a fundamental question as to how the Ca$_2^+$ signaling is integrated into the transcriptional feedback mechanism. In the present study, we identified Ca$_2^+$/calmodulin-dependent protein kinase II (CaMKII) as an essential component of the cellular oscillation machinery. Furthermore, we found that CaMKII plays an important role for synchronization among individual neuronal clocks and for mutual coupling between the morning and evening oscillators that define the activity time of the circadian behaviors in mice.

**Results**

**CaMKII bolsters robust behavioral rhythms with organized coupling of morning and evening oscillators**

CaMKII is an important signal-transducing molecule that is activated by an increase of cytosolic Ca$_2^+$ (Colomer and Means 2007). CaMKII is encoded by four genes in mice: CamKIIa, CamKIIb, CamKIIy, and CamKIIb, among which CamKIIa and CamKIIb are expressed predominantly in the brain, while CamKIIy and CamKIIb are ubiquitously expressed [Tombes et al. 2003]. We confirmed that, when compared with the other isoforms, CaMKIIa is highly expressed in the mouse whole brain [Supplemental Fig. S1] as well as in the SCN neurons governing the behavioral rhythms [Nomura et al. 2003]. In order to characterize the role of CaMKII in the behavioral rhythms, we employed recently generated CaMKIIa knock-in mice, the CaMKIIa.K42R mutant, in which the kinase activity is completely lost, while the protein level is kept almost normal (Yamagata et al. 2009). Because CaMKII serves as a structural protein in addition to mediating phosphorylation signaling, the kinase-dead knock-in mice enable us to characterize the in vivo function of the kinase activity. This contrasts sharply with studies on CaMKIIa knockout mice with its protein deficiency. The total CaMKII activity in the forebrain of the knock-in mice is reduced to 30%–40% of that of wild-type mice [Yamagata et al. 2009].

The mutant mice showed no significant difference from wild type in total levels of wheel-running activities under 12 h:12 h light–dark cycles (LD) or DD conditions [Supplemental Fig. S2A] and in the dark to light activity ratio (Supplemental Fig. S2B). A $\chi^2$ periodogram analysis of the wheel running activity rhythms in DD revealed a prolonged free-running period in the mutant mice (Fig. 1A,B; Supplemental Fig.S2C; Supplemental Table S1). The robustness of the behavioral rhythms was assessed by the periodogram amplitude (Qp), a parameter that reflects the strength or regularity of a rhythm [Sokolov and Bushell 1978]. The mutant mice showed a progressive decline of the Qp value from the second through the fourth week [DD2w–DD4w] in DD (Fig. 1C), indicating a reduced stability of the mutant SCN clock when placed in DD. Indeed, three out of 13 mutant mice initially showing rhythmic behaviors eventually became arrhythmic [Fig. 1A, right, Supplemental Table S1]. These results demonstrate that CaMKIIa plays an essential role in mediating the high-amplitude and stable behavioral rhythms.

Here we found that the free-running period of the mutant mice assessed by their activity offset was significantly longer than that determined by their activity onset [Fig. 1A,D, Supplemental Table S1] and thereby caused a progressive lengthening of the activity time in DD (Fig. 1E). Furthermore, the temporal activity profile of the mutant mice was characterized by a marked increase of the morning activity [Fig. 1F, Supplemental Fig. S2D], whereas the mutant showed a relative decrease of the evening activity and the emergence of a third peak, termed the night peak, between the evening and morning peaks [Fig. 1F, Supplemental Fig. S2D]. In the mutant, the evening and morning peaks gradually became temporally decoupled, while the night peak stayed coherent with the evening peak [Fig. 1F,G]. These observations collectively demonstrate an essential role for CaMKIIa in tight coupling of the oscillators that govern morning and evening behavioral activities.

Desynchronization of individual cellular rhythms and decoupling of oscillations between left and right nuclei in the SCN of CaMKIIa.K42R mice

The unique behavioral phenotype of CaMKIIa.K42R mice prompted us to investigate rhythms in pacemaker neurons
Roles of CaMKII in the circadian clock of the mouse SCN

Figure 1. CaMKII is essential for behavioral rhythms and coupling of morning and evening activities. (A) Representative wheel-running rhythm of CaMKIIaK42R mice. Because CaMKIlaK42R mice showed a temporal change in daily behavioral rhythm, the rhythm parameters were analyzed by using the period indicated as LD, DD1w, DD2w, DD3w, or DD4w. Three out of 13 mutant mice showed arrhythmic behavior, as observed in DD4w in the right panel. Wild-type [WT] littermates were used as control mice. (B) Prolonged period length in behavioral rhythm of CaMKIIaK42R mice. The period length of behavioral rhythm from DD1w to DD3w was analyzed by χ² periodogram by using ClockLab software (Actimetrics). Plotted are the period lengths of individual animals. Bars indicate mean with SEM. (*P < 0.05; **P < 0.01 versus wild type [Student’s t-test]). (C) Temporal reduction in periodogram amplitude (Qp) of behavioral rhythm in CaMKIIaK42R mutant mice. The mean value of the Qp in DD1w was set to 100. [*P < 0.05 versus wild type [Student’s t-test]]. (D) Prolonged period determined by onset or offset of wheel-running activity rhythm in CaMKIIaK42R mutant mice. Data shown are representative results [A], the mean (F), or mean with SEM [B–E,G] from 11 wild-type or 13 CaMKIIaK42R mice.

Individuals

Figure 1. CaMKII is essential for behavioral rhythms and coupling of morning and evening activities. (A) Representative wheel-running rhythm of CaMKIIaK42R mice. Because CaMKIlaK42R mice showed a temporal change in daily behavioral rhythm, the rhythm parameters were analyzed by using the period indicated as LD, DD1w, DD2w, DD3w, or DD4w. Three out of 13 mutant mice showed arrhythmic behavior, as observed in DD4w in the right panel. Wild-type [WT] littermates were used as control mice. (B) Prolonged period length in behavioral rhythm of CaMKIIaK42R mice. The period length of behavioral rhythm from DD1w to DD3w was analyzed by χ² periodogram by using ClockLab software (Actimetrics). Plotted are the period lengths of individual animals. Bars indicate mean with SEM. (*P < 0.05; **P < 0.01 versus wild type [Student’s t-test]). (C) Temporal reduction in periodogram amplitude (Qp) of behavioral rhythm in CaMKIIaK42R mutant mice. The mean value of the Qp in DD1w was set to 100. [*P < 0.05 versus wild type [Student’s t-test]]. (D) Prolonged period determined by onset or offset of wheel-running activity rhythm in CaMKIIaK42R mutant mice. Data shown are representative results [A], the mean (F), or mean with SEM [B–E,G] from 11 wild-type or 13 CaMKIIaK42R mice.

Pharmacological inhibition of CaMKII abolishes individual cellular oscillations and intercellular coupling among SCN neurons

The role of CaMKII in the SCN pacemaker neurons was examined by its pharmacological inhibition with a specific inhibitor, KN93. The robust circadian oscillation of the bioluminescence signals from the SCN slice of PER2::LUC mice was rapidly blunted by administration of 100 μM KN93 [Fig. 3A,B], and washout of the drug restored the gene expression rhythm [Fig. 3A]. Single-cell monitoring of the SCN neurons revealed that KN93 treatment progressively attenuated the peak amplitude of the individual cellular rhythms [Fig. 3C,D] and desynchronized them [Fig. 3C]. The desynchronization effect was verified by Rayleigh test, which demonstrated a decrease in the vectorial length of the peak phases in KN93-treated neurons and an increase in the standard deviation thereof [Fig. 3E]. On the other hand, KN92, an
We found that simultaneous siRNA-mediated knockdown of the ubiquitously expressed isoforms of the CaMKII family by knocking down the specific isoform. We then examined the contribution of CaMKII isoforms to the amplitude of the individual rhythm in NIH3T3 fibroblasts (Fig. 4C,D; Supplemental Fig. S4). In contrast, KN92 treatment had no significant effect on the amplitude of the bioluminescence rhythms in NIH3T3 fibroblasts (Supplemental Fig. S5; Wang and Simonson 1996; Tombes et al. 2003), stimulated expression of the E-box-regulated genes, such as Per1, Dec1, Dec2, Dbp, and Cry1, was markedly down-regulated throughout the day by treatment with 20 mM KN93 (Fig. 4E). Consistently, stable expression of CaMKIIα (Supplemental Fig. S5; Wang and Simonson 1996; Tombes et al. 2003), stimulated expression of the E-box-regulated genes, such as Per1 and Dbp, in NIH3T3 cells (Fig. 4F).

We explored the role of CaMKII in the transcriptional feedback loops by examining expression profiles of the clock genes in the presence of KN93. Chronic treatment of Rat-1 cells with 10 or 20 μM KN93 dose-dependently weakened the rhythmicities of all of the clock genes examined (Fig. 4E). Among them, expression of E-box-regulated genes, including Per1–3, Dec1, Dec2, Dbp, and Cry1, was markedly down-regulated throughout the day by treatment with 20 μM KN93 (Fig. 4E). Consistently, stable expression of CaMKIIα (1–290), a constitutively active form of CaMKIIα (Supplemental Fig. S5; Wang and Simonson 1996; Tombes et al. 2003), stimulated expression of the E-box-regulated genes, such as Per1 and Dbp, in NIH3T3 cells (Fig. 4F).

We then examined the role of upstream regulators of CaMKII signaling in the cellular clockwork. Chelating of intracellular Ca2+ by administration of BAPTA-AM reduced the amplitude of the cellular rhythm in Rat-1 cells (Fig. 5A,B). The treatment attenuated E-box-dependent gene expression in Rat-1 cells (Fig. 5C), as was observed for the effect of KN93 (Fig. 4E). Similarly, application of calmidazolium, a specific inhibitor of calmodulin, reduced the amplitude of the cellular rhythm (Fig. 5D,E) and E-box-dependent gene expression (Fig. 5F). These results together indicate that Ca2+-calmodulin–CaMKII signaling provides important input to the cell-autonomous circadian oscillator through activating E-box-mediated transcription.

CaMKII is essential for normal oscillation of cellular clocks

We focused on the role of CaMKII in the intracellular oscillation mechanism by using cultured Rat-1 fibroblasts, which show prominent circadian rhythms in gene expression with no significant intercellular connection (Rosbash 1998; Kon et al. 2008). Bioluminescence rhythms of Rat-1 cells expressing the Bmal1-luc reporter, which drives rhythmic expression of LUC from the Bmal1 promoter (Kon et al. 2008), were significantly impaired in the presence of 20 μM KN93 (Fig. 4A,B, Supplemental Fig. S3). In contrast, KN92 treatment had no significant effect on the amplitude of the cellular clock (Fig. 4A,B, Supplemental Fig. S3A). We then examined the contribution of CaMKII isoforms by knocking down the specific isoform. We found that simultaneous siRNA-mediated knockdown of the ubiquitously expressed isoforms CamkIIy (si1 or si2) and CamkIbα (si3 or si4) markedly attenuated the amplitude of the Bmal1-luc rhythms in NIH3T3 fibroblasts (Fig. 4C,D, Supplemental Fig. S4).
CaMKII facilitates heterodimerization of CLOCK and BMAL1

The intense effects of Ca\(^{2+}\)/CaMKII inhibition on the E-box-dependent gene expression led us to investigate whether CaMKII functionally interacts with E-box activators. Coimmunoprecipitation experiments indicated that CaMKII interacts with CLOCK in the nuclear extracts of mouse liver prepared at Zeitgeber time (ZT) 6 or ZT18 (Fig. 6A). Transient expression of CLOCK and BMAL1 with CaMKII(1–290) increased the relative abundance of the up-shifted band of CLOCK that represents the phosphorylated form (Fig. 6B, left; Supplemental Fig. S6) while having no appreciable effect on BMAL1 phosphorylation (Fig. 6B, right). We focused on the phosphorylation states of endogenous CLOCK and CLOCK-associated BMAL1 in synchronized Rat-1 fibroblasts. Phosphorylation of CLOCK and BMAL1 showed a dynamic circadian variation.

Figure 3. CaMKII is essential for cellular oscillation and intercellular coupling among neuronal clocks in the circadian pacemaker. (A) Effect of CaMKII inhibitor on bioluminescence rhythm at the SCN tissue level. The SCN slices were prepared from PER2\(^{TLUC}\) mice, and bioluminescence intensity from the slice culture was recorded. The CaMKII inhibitor was applied from day 4.5 to day 12 during recording of the rhythm. (B) Decrease in PER2 peak expression under the CaMKII inhibitor at the tissue level. First peak expression levels of PER2\(^{TLUC}\) during the drug treatment was set to 100% in each experiment. (*) \(P < 0.05\) versus DMSO-treated LUC. Data shown are representative results (A) or the mean with SEM (B) from six independent experiments. (C) Effect of the CaMKII inhibitor on bioluminescence rhythm at the single-cell level. SCN slices were prepared from PER2\(^{TLUC}\) knock-in mice, and the CaMKII inhibitor was applied from the fourth to eighth days during recording of individual cellular rhythms. (D) Decrease in PER2 peak expression under the CaMKII inhibitor at the single-cell level. First peak expression level of PER2\(^{TLUC}\) during the drug treatment was set to 100% in each experiment. (*) \(P < 0.05\) versus DMSO-treated LUC. Data shown are representative results of four independent experiments.

CaMKII is essential for cellular oscillation and intercellular coupling among neuronal clocks in the circadian pacemaker. (A) Effect of CaMKII inhibitor on bioluminescence rhythm at the tissue level. The SCN slices were prepared from PER2\(^{TLUC}\) mice, and bioluminescence intensity from the slice culture was recorded. The CaMKII inhibitor was applied from day 4.5 to day 12 during recording of the rhythm. (B) Decrease in PER2 peak expression under the CaMKII inhibitor at the tissue level. First peak expression levels of PER2\(^{TLUC}\) during the drug treatment was set to 100% in each experiment. (*) \(P < 0.05\) versus DMSO-treated LUC. Data shown are representative results (A) or the mean with SEM (B) from six independent experiments. (C) Effect of the CaMKII inhibitor on bioluminescence rhythm at the single-cell level. SCN slices were prepared from PER2\(^{TLUC}\) knock-in mice, and the CaMKII inhibitor was applied from the fourth to eighth days during recording of individual cellular rhythms. (D) Decrease in PER2 peak expression under the CaMKII inhibitor at the single-cell level. First peak expression level of PER2\(^{TLUC}\) during the drug treatment was set to 100% in each experiment. (*) \(P < 0.05\) versus DMSO-treated LUC. Data shown are representative results of four independent experiments.
as revealed by alteration in relative intensities of the three CLOCK bands [hypershifted, shifted, and nonshifted] (Yoshitane et al. 2009) and the two BMAL1 bands [shifted and nonshifted] (Fig. 6C, lanes 1–4). The rhythmic phosphorylation of CLOCK and BMAL1 was abrogated in the presence of KN93 (Fig. 6C, lanes 5–12). Densitometric analysis verified that the inhibitor treatment down-regulated CLOCK phosphorylation and up-regulated the

Figure 5. Intracellular Ca\textsuperscript{2+} and calmodulin are important for cellular oscillation and E-box-dependent expression of clock genes. [A] Effect of BAPTA-AM on the Rat-1 cellular rhythms. [B] Effect of BAPTA-AM on the amplitude of the cellular rhythms. [\textsuperscript{*}] \( P < 0.5 \times 10^{-3} \); [\textsuperscript{**}] \( P < 0.1 \times 10^{-7} \) (Student’s t-test). Relative amplitudes were calculated as described in the Materials and Methods. [C] Effect of BAPTA-AM on clock gene expression. [D] Effect of calmodulin inhibitor on the Rat-1 cellular rhythms. [E] Effect of calmodulin inhibitor on the amplitude of the cellular rhythms. [\textsuperscript{*}] \( P < 0.5 \times 10^{-3} \) (Student’s t-test). Shown are representative data [A,D] or mean with SEM [B, E] from three independent experiments. [F] Effect of the calmodulin inhibitor on clock gene expression. The mRNA signals obtained by RT–PCR analysis were normalized by those of Tbp mRNA. [C,F] Data are mean with SD from two independent experiments.

Figure 6. CaMKII promotes phosphorylation of CLOCK and interaction of CLOCK with BMAL1. [A] Association of CLOCK and CaMKII in vivo. The lysates were prepared from the liver sampled at the indicated time points and used for immunoprecipitation by anti-CLOCK (positive control), anti-CaMKIIa/d or anti-Flag [negative control] antibody, followed by immunoblotting by anti-CLOCK antibody. [B] Effect of CaMKII overexpression on mobility shift of CLOCK and BMAL1. In HEK293 cells, CLOCK and BMAL1 were expressed with the constitutively active form of CaMKII or LacZ. The top panel shows representative raw data, and the bottom panel shows mean with SEM from three [left panel] or six [right panel] independent experiments. [\textsuperscript{*}] \( P < 0.005 \) (Student’s t-test). [C] Effect of CaMKII inhibitor on heterodimers of CLOCK and BMAL1. Rat-1 cells were cultured as described in the legend for Figure 4 and sampled at the indicated time points after the rhythm induction by dexamethasone. The cell lysates were used for immunoprecipitation by anti-CLOCK antibody, and the precipitates were immunoblotted by anti-CLOCK [top panel] or anti-BMAL1 [bottom panel] antibody. [D] Ratio of the up-shifted form of CLOCK and BMAL1 under CaMKII inhibition. The ratio means the up-shifted band intensity per total band intensity of CLOCK [left panel] or BMAL1 [right panel]. [E] Decreased interaction of CLOCK with BMAL1 under CaMKII inhibition. Representative data [A,C] or the mean with SEM [D,E] from three independent experiments are shown.
relative phosphorylation level of BMAL1 across the day (Fig. 6D). Notably, KN93 treatment reduced the protein amounts of BMAL1 that was kept associated with CLOCK [Fig. 6E], while total BMAL1 proteins showed no measurable decrease (Supplemental Fig. S7). Together, these results suggest that CaMKII-mediated phosphorylation of CLOCK facilitates its interaction with BMAL1 and potentiates E-box-dependent gene expression.

CaMKII as a state variable of the circadian oscillator

In the fundamental concept of circadian oscillators, components underlying the rhythm generation should change rhythmically in the amount and/or the activity, which collectively define the state of the oscillator (Aronson et al. 1994; Yamaguchi et al. 2003; Dunlap et al. 2004; O’Neill et al. 2008). CaMKII activity exhibits a circadian variation in a phase close to that of E-box-dependent transcription rhythms (Agostino et al. 2004). We asked whether acute CaMKII inhibition starting and ending at various times of the day affects the phase of the clock in Rat-1 cells stably expressing *Bmal1-luc*. During a 48-h application of 20 μM KN93, the cellular rhythms were almost completely lost, and removal of the drug (at circadian time [CT] 0, 3, 6, 9, 12, 15, 18, or 21) resumed the oscillation starting at a unique phase irrespective of the time at which the drug was added (Fig. 7A); that is, the cellular clocks were always reset to approximately CT4, a time close to the trough of E-box-dependent gene expression [here we defined CT0 as the peak time of *Bmal1-luc*-derived bioluminescence signals] (Fig. 7B). These results indicate that inhibition of CaMKII activity resets the phase of the oscillators to the trough level of E-box gene expression.

Discussion

CaMKII appears to regulate the circadian clock at multiple levels. Previously, CaMKII was shown to play an important role in the photic input pathway and rhythmic gene expression of clock genes (Yokota et al. 2001; Lundkvist et al. 2005; Harrisingh et al. 2007; Welsh et al. 2010). Photic signal induces a glutamate-mediated cytoplasmic Ca\(^{2+}\) increase in the SCN neurons. Activated CaM/CaMKII then phosphorylates and activates a transcription factor, cAMP response element-binding protein (CREB), which rapidly increases transcription of *Per1* to induce phase shifts of the clock (Nomura et al. 2003). The present study identifies a novel pathway in which CaMKII activates CLOCK/BMAL1-mediated E-box-dependent gene expression, which is important for the cell-autonomous circadian oscillation [Figs. 4–7]. Intracellular Ca\(^{2+}\) concentration in the rodent SCN shows a circadian change (Ikeda et al. 2003; Enoki et al. 2012), suggesting that cytosolic Ca\(^{2+}\) rhythm is integrated into the cell-autonomous oscillation system. Consistently, intracellular Ca\(^{2+}\) concentration increases in a phase similar to or slightly advanced from that of the activation of CaMKII and E-box-dependent transcription (at CT4–CT8) (Ikeda et al. 2003; Agostino et al. 2004; Welsh et al. 2010). Chelating of intracellular Ca\(^{2+}\) or inhibition of calmodulin blunted cell-autonomous circadian gene expression rhythms even in the nonneuronal cultured cells (Fig. 5). Based on these observations, we propose a novel model in which CaMKII signaling couples Ca\(^{2+}\) dynamics with the E-box-dependent transcriptional feedback mechanism and thereby stabilizes self-sustaining cellular oscillation (Fig. 7C).
In animal behavioral rhythms, dissociation of morning and evening activity rhythms was previously described in several species, and the phenomenon led to an idea that the morning and evening activities are independently regulated by two oscillators [Pittendrigh and Daan 1976]. Pioneering studies on the behavioral rhythms of Drosophila clearly demonstrated that the morning and evening activities are independently regulated by different clock neurons communicating with each other in the brain [Grégoire et al. 2004; Stoleru et al. 2004, 2005]. In mammals, multiple oscillating cell groups are observed within the SCN (de la Iglesia et al. 2000; Jagota et al. 2000; Inagaki et al. 2007; Helfrich-Förster 2009). When the morning and evening activity rhythms are dissociated in golden hamsters, the left and right SCN show different phases in clock gene expression rhythm [de la Iglesia et al. 2000]. Unilateral lesion of the SCN abolished the splitting and established a single rhythm of behavioral activity [Pickard and Turek 1982]. In normal conditions, the left and right SCN are strongly connected with each other even in isolated culture [Michel et al. 2013], and the functional connection is important for coupling of their free-running rhythms, as observed in wild-type SCN slices over 10 d [Fig. 2]. In the present study, genetic ablation of CaMKIIa activity in C57BL/6 mice resulted in dissociation of the morning and evening activity rhythms in behavior [Fig. 1] and caused decoupling of oscillations between the left and right SCN [Fig. 2]. These studies support the idea that the decoupling of the free-running period between the left and right SCN underlies dissociation of the morning and evening activity rhythms in the behavior. Importantly, dissociation of the behavioral rhythms is not observed in normal C57BL/6, a strain that shows robust behavioral rhythmicity, whereas the other strains, such as C3, exhibit an unstable behavioral rhythm with spontaneous rhythm splitting in DD [Abe et al. 1999]. Because loss of CaMKIIa activity induced splitting-like behavior in DD and decreased the amplitude of the rhythm [Fig. 1], CaMKII-mediated tight coupling between the morning and evening oscillators may minimize the dissociation of behavioral activities in C57BL/6 mice and confer robustness of the rhythmicity.

The intercellular coupling of neuronal clocks in the SCN is established by multiple signaling such as neurotransmitters, CaMP signaling, and electrical synapses [Welsh et al. 2010]. Inhibition of these molecules results in changes in the free-running period or attenuation of rhythmicities of the behavioral rhythms [Harmar et al. 2002; Long et al. 2005; O’Neill et al. 2008; Doi et al. 2011], the phenotypes similar to what we observed for the CaMKIIaK42R mutant in the present study [Figs. 1, 2]. Interestingly, decoupling of the morning and evening activity rhythms is unique to the CaMKIIa mutant among a wide variety of genetically engineered mice [Fig. 1], raising the possibility that CaMKII signaling plays a major role in functional coupling of the morning and evening oscillators in mice. Because the phase of the cellular clock is tightly regulated by the activity levels of CaMKII [Fig. 7], Ca2+-calmodulin–CaMKII signaling may serve as the core machinery of the cellular clocks that adjust their phases in response to changes in cytosolic Ca2+ level regulated by intercellular communications and environmental signals. This idea is consistent with the previously reported role of CaMKII signaling involved in light entrainment of the circadian clock [Yokota et al. 2001].

Abnormalities in CaMKII are found in patients with several neurological disorders with disrupted clock function, including schizophrenia, bipolar disorders, and Alzheimer’s disease [Colomer and Means 2007; Schibler and Sassone-Corsi 2002]. Because several psychotropic drugs such as fluvoxamine and desipramine or lithium modulate CaMKII activity [Celano et al. 2003; Sasaki and Sassone-Corsi 2002], recovery of normal clock function through the pharmacological action on CaMKII may prove effective for the treatment of complicated neuronal disorders. Here we demonstrate that CaMKII activity regulates multiple parameters of the circadian clock, such as period length [Figs. 1, 2], oscillation amplitude [Figs. 1–4], phase [Fig. 7], and the interneuronal coupling [Figs. 2, 3]. The present study illuminates CaMKII as a druggable target for innovative therapy of disorders that associate with clock dysfunction.

Materials and methods

Real-time monitoring of circadian rhythms of cultured cells

Real-time monitoring of cellular circadian gene expression was performed using Rat-1 or NIH3T3 cells expressing a luciferase reporter under the control of the upstream region of the Bmal1 gene [Kon et al. 2008]. Briefly, the cells were plated on 35-mm dishes (1.0 × 106 cells per dish) and cultured at 37°C under 5% CO2 in phenol red-free DMEM [Sigma-Aldrich, catalog no. D2902] supplemented with 10% FBS (Biowest), 3.5 mg/mL glucose, 3.7 mg/mL NaHCO3, 50 U/mL penicillin, and 50 μg/mL streptomycin. Twenty-four hours after the plating, the cells were treated with 0.1 μM desmethylthionine for 2 h, and the medium was replaced with a recording medium [DMEM supplemented with 10% FBS, 3.5 mg/mL glucose, 25 U/mL penicillin, 25 μg/mL streptomycin, 0.1 mM luciferin, 10 mM HEPES-NaOH at pH 7.0]. The bioluminescence signals from the cells were continually recorded at 37°C under air with a dish-type bioluminescence detector, Kronos [ATTO, AB-2500] or LumiCycle [Actimetrics]. For normalization of the bioluminescence levels, raw data were divided by the mean for bioluminescence activities across the 7-d recording. The normalized rhythms were detrended by subtracting 24-h centered moving averages, and the areas under the curves (arbitrary units) were used for calculating the relative amplitudes of the rhythms [DeBruyne et al. 2007].

Real-time monitoring of SCN rhythms

Bioluminescence signals from SCN cultures were recorded as described previously [Inagaki et al. 2007]. The coronal SCN slices (100 μm thick) at the middle of the rostro–caudal axis were prepared from PER2::LUC mice [Yoo et al. 2004] using a micro-slicer at ZT6–ZT10, and the bioluminescence signals from the slices were recorded by LumiCycle [Actimetrics]. Single-cell analysis was performed using Cellgraph [ATTO] equipped with a highly sensitive cryogenic CCD camera [ORCA-II ER or ImagaEM, Hamamatsu Photonics]. Individual cellular rhythms were analyzed using Aquacosmos software [Hamamatsu Photonics], and
distribution of the phases was analyzed by circular statistics software [Oriana, Kovach Computing Services]. The bioluminescence signals were recorded after the slice was set on the apparatus [at time 0]. In experiments using a drug, the medium was replaced with a medium containing KN92, KN93, or DMSO 96 h after the setting.

RT–PCR analysis and RNAi
Total RNA was prepared from cultured cells or mouse tissues using TRIzol reagent [Invitrogen] according to the manufacturer's protocol. RT–PCR analysis was performed as described previously [Kon et al. 2008]. PCR primers used were as follows: for CamkIIy, 5'-AATCATTAAGATCACAGAACAC-3' and 5'-TCTCTCTCTCTGACTGACTGGT-3'; and for CamkIIb, 5'-GA TGACTTTCACAGATTCTAC-3' and 5'-TTCTGCCCTTTGAT-3'. Using siDirect (http://design.RNAi.jp), siRNAs were designed as follows: CamkIIy si1, 5'-CAGUGACAGAUAUCUGAAGA-3'; CamkIIy si2, 5'-GCCUUAUGCCACUACUCUACU-3'; CamkIIb si3, 5'-AAGACAUAGUGCCAAGAGAU-3'; CamkIIb si4, 5'-GUAGACUCCUGUUGAGAAUUU-3'; control siA, 5'-CUCGCCGACGACCGUUAUC-3'; and control siB, 5'-GAUCCUGGAGCAGCGCUAGC-3'. One day after plating NIH3T3 cells on a 35-mm dish [1.0 x 10^4 cells per dish], siRNA [250 pmol each] was transfected to the cells using Lipofectamine 2000 [Invitrogen].

Immunoprecipitation and immunoblotting analyses
Immunoprecipitation was performed as described previously [Yoshitane et al. 2009]. Briefly, anti-CLOCK antibody (Sigma-Aldrich, M2, as a control) was added to the mouse liver nuclear extracts [Fig. 6A] or the cultured cell lysates [Yoshitane et al. 2009]. Briefly, anti-CLOCK antibody (CLNT1), Immunoprecipitation was performed as described previously [Celano et al. 2012]. Immunoprecipitation and immunoblotting analyses were designed as follows: CamkIIy si1, 5'-CAGUGACAGAUAUCUGAAGA-3'; CamkIIy si2, 5'-GCCUUAUGCCACUACUCUACU-3'; CamkIIb si3, 5'-AAGACAUAGUGCCAAGAGAU-3'; CamkIIb si4, 5'-GUAGACUCCUGUUGAGAAUUU-3'; control siA, 5'-CUCGCCGACGACCGUUAUC-3'; and control siB, 5'-GAUCCUGGAGCAGCGCUAGC-3'. One day after plating NIH3T3 cells on a 35-mm dish [1.0 x 10^4 cells per dish], siRNA [250 pmol each] was transfected to the cells using Lipofectamine 2000 [Invitrogen].

Animal experiments
The animal experiments were conducted in accordance with the guidelines of the University of Tokyo. CaMKIIyK42R knock-in mice were generated as described previously [Yamaqata et al. 2009]. The mice [C57BL/6 background, male, 10 wk old] were housed individually at 23°C in cages [13 x 23 x 15 cm] equipped with a running wheel [diameter 10 cm] with food and water available ad libitum. Wheel-running rhythms were monitored under DD conditions after entrainment in 12 h:12 h LD cycles for at least 2 wk. The number of wheel revolutions was collected every minute into a computer system. Behavioral data were analyzed by using ClockLab software [Actimetrics], and the onset and offset of the daily activity were defined as described in the legend for Figure 1.

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