The CRTC1-SIK1 Pathway Regulates Entrainment of the Circadian Clock

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SUMMARY

Retinal photoreceptors entrain the circadian system to the solar day. This photic resetting involves cAMP response element binding protein (CREB)-mediated upregulation of Per genes within individual cells of the suprachiasmatic nuclei (SCN). Our detailed understanding of this pathway is poor, and it remains unclear why entrainment to a new time zone takes several days. By analyzing the light-regulated transcriptome of the SCN, we have identified a key role for salt inducible kinase 1 (SIK1) and CREB-regulated transcription coactivator 1 (CRTC1) in clock re-setting. An entrainment stimulus causes CRTC1 to coactivate CREB, inducing the expression of Per1 and Sik1. SIK1 then inhibits further shifts of the clock by phosphorylation and deactivation of CRTC1. Knockdown of Sik1 within the SCN results in increased behavioral phase shifts and rapid re-entrainment following experimental jet lag. Thus SIK1 provides negative feedback, acting to suppress the effects of light on the clock. This pathway provides a potential target for the regulation of circadian rhythms.

INTRODUCTION

There has been remarkable progress in our understanding of the complex intracellular mechanisms that generate and regulate circadian rhythms across multiple species. In mammals, the molecular clock arises from a transcriptional-translational-feedback loop, consisting of the transcription factors CLOCK and BMAL1 that drive the expression of their regulators, PERIOD and CRYPTOCHROME (Reppert and Weaver, 2002). These elements form part of an autoregulatory-feedback loop, the period of which is approximately 24 hr. Most cells of the body appear to possess such a molecular oscillator (Albrecht, 2012; Balsalobre et al., 1998), and these autonomous clocks are synchronized to each other and the external environment primarily by a circadian pacemaker within the suprachiasmatic nuclei (SCN) (Ralph et al., 1990). The SCN is in-turn aligned (entrained) to the solar day by exposure to light at dawn and dusk, detected by photoreceptors located exclusively within the retina of the eye. Photosensitive retinal ganglion cells (pRGCs), utilizing the photopigment melanopsin (OPN4), provide the primary light input to the SCN, exemplified by the fact that mice or human subjects lacking all their rods and cones still possess a full cohort of circadian responses to light (Freedman et al., 1999; Zaidi et al., 2007). Although rod and cone photoreceptors are not required for entrainment, they can partially compensate for the loss of melanopsin in Opn4−/− mice (Hattar et al., 2003). In addition, a growing body of evidence demonstrates that rods, and the different classes of cone photoreceptor, can complement the role of the pRGCs in circadian entrainment by expanding the irradiance range of the response and in the detection of light transitions (Lall et al., 2010; van Oosterhout et al., 2012).

There remains, however, only a very basic understanding of the molecular mechanisms whereby cellular oscillators within the SCN are entrained to environmental light. This can be summarized as follows: light activation of pRGCs causes the release of the neurotransmitters glutamate and PACAP (Hannibal et al., 2000) at the terminals of the retinohypothalamic tract (RHT) that projects directly to the SCN. Neurotransmitter release results in a Ca2+ influx, which activates “phosphorelay” signaling pathways. A number of different kinases have been implicated in these pathways, including PKA, PKG, CaMK, and MAPK, although their relative roles remain unclear (Meijer and Schwartz, 2003). All these pathways are thought to converge on the cAMP response element binding protein (CREB), which is activated by phosphorylation at Ser133 (Sinty et al., 1993) and Ser142 (Gau et al., 2002). Phosphorylated CREB then binds to cAMP response elements (CRE) in the promoters of light-regulated
The expression of both transcription coactivator 1 (CRTC1) to coactivate CREB, inducing light) that an entraining stimulus causes CREB-regulated transcription (Porterfield et al., 2007) all contain a highly conserved CRE. Significantly, the clock genes Per1 and Per2 also contain conserved CREs, which mediate their light induction (Albrecht et al., 1997; Schwartz et al., 2011). Elevated PER1 and PER2 adjust the molecular-feedback loop of the circadian oscillator to the light/dark (LD) cycle.

This linear description of light-induced Per gene induction cannot explain a key feature of light-regulated circadian behavior. Namely, that circadian entrainment is slow, taking multiple days to adjust to an advanced or delayed LD cycle. In most mammals, including jet-lagged humans, behavioral shifts are limited to approximately 1 hr (one time zone) per day (Aschoff, 1981). Furthermore, at a molecular level, the effect of light on Per gene induction is limited. For example, under extended illumination, Per1 mRNA light-induction peaks after ~1 hr and then returns to baseline (Vitaterna et al., 2006), indicating CREB transcription is arrested one hour after it is induced. Mirroring this molecular response, high-irradiance light pulses greater than 1 hr cease to have a phase-shifting effect upon the murine clock. This saturation of the response suggests the presence of an unidentified negative-feedback mechanism that effectively limits the magnitude of phase-shifting responses to light within the SCN. Thus, entrainment is a gradual process requiring repeated shifting stimuli over multiple days. Our aims in this study were to understand the molecular mechanisms that limit the effects of entrainment stimuli on the clock.

Here, we show both in vitro (via serum shock) and in vivo (via light) that an entraining stimulus causes CREB-regulated transcription coactivator 1 (CRTC1) to coactivate CREB, inducing the expression of both Per1 and Sik1. SIK1 then inhibits further expression of Per1 by phosphorylation of and deactivation of CRTC1. Critically, we show that knockdown of Sik1 within the SCN results in extended light-induced phase shifts and an enhanced rate of re-entrainment to an advanced LD cycle. As SIK1 acts to suppress the effects of light on the clock, this pathway provides a pharmacological target for the treatment of disturbed circadian rhythms, including jet lag, shift-work, and illnesses that disturb circadian timing.

RESULTS

Nocturnal Light Regulates the Expression of 536 Transcripts in the SCN

In order to identify signaling pathways involved in photic input, we examined the light-regulated transcriptome of the wild-type (Opn4+/+) SCN across a period of nocturnal light exposure. We collected SCN from mice that received a nocturnal light pulse delivered 4 hr after activity onset (circadian time/CT16) over a 2 hr time course and identified 536 genes as light-responsive in the wild-type SCN using Affymetrix whole-genome mouse exon arrays (Figure 1A and Figure S1 and Table S1 available online). Surprisingly, the majority (81%, 436 genes) of transcripts were downregulated in response to light, with a smaller number of genes showing upregulation (19%, 100 genes). These included the acutely upregulated immediate early genes (IEGs) previously shown to respond to light in the SCN, including Fos (Lupi et al., 2006), Nr4a1, and Eg1 (Porterfield et al., 2007), clock genes (Per1), and clock-controlled genes (Ror2). In addition, we confirmed Rad51 (Porterfield et al., 2007), a GTPase modulating calcium-binding proteins such as CAMKII, which has an identified role in photic resetting (Fukushima et al., 1997) and Dusp1, a MAP kinase phosphatase, characterized as light-induced and clock-controlled in the SCN (Doi et al., 2007).

Among the genes we identified were Dusp4 and Sik1, which were found to cluster with Per1 (Figure 1A). Sik1 encodes salt inducible kinase 1, a Ser/Thr protein kinase of the AMP-dependent protein kinases (AMPK) family of which AMPK rhythmically phosphorylates Cry1 (Lamia et al., 2009) and Dusp4 encodes a dual Ser/Thr and Tyr phosphatase selectively controlling ERK (Cagnol and Rivard, 2013). The downregulated transcripts include Tac1, which encodes substance P precursor, known to have a role in phase shifting (Kim et al., 2001); Sim1, a transcription factor of the bHLH-PAS family that also includes Clock; Plekho1, which shows homology with casein kinase and a number of receptors including the serotonin receptor Htr7; Lactrophilin (Lphn3); and the glycine receptors Gira1 and Gira2.

To gain insight into the pathways represented by these transcripts, we performed a gene-enrichment analysis based on gene ontology (GO) terms (Figure 1B). One category of interest that is significantly responsive to light includes a number of transcripts relating to synaptic remodeling and plasticity, which were upregulated at a late stage, and included Rasgrf1, Grit, and Ntn1.

A number of previous studies have highlighted the central role of CREB in phase shifting (Gau et al., 2002; Ginty et al., 1993) and the promoters of many previously identified light-responsive genes in the SCN including Fos, Per1, Nr4a1, and Dusp1 contain conserved CREs (Porterfield et al., 2007; Travnickova-Bendova et al., 2002). We overlaid our data with those of Zhang et al. (2005) to identify transcripts containing CRE sites in their promoters. We found that of the genes upregulated more than 1.5-fold, there was significant enrichment of those containing CRE sites near the TATA box (Figure 1C). This correlation implies that CREB drives the high-magnitude gene induction that underlies phase-shifting responses in the SCN. We provide a detailed characterization of CREB target genes that are induced by light in the SCN (Table S2). Analysis of individual clusters of the Opn4+/+ SCN light-induced transcriptome showed that in addition to CREB, binding sites for SP1 and KROX (in transcripts upregulated at 120 min) and TAL1, HNFalpha3, and WT1 (transcripts showing early repression followed by upregulation) were enriched.

To identify any genes from our screen that could play a direct role in the core circadian oscillator, we reviewed data obtained from bioluminescent recordings from a genome-wide RNAi screen on Per2-luc transfected U2OS cells (Zhang et al., 2009). Silencing of several of the genes caused significant changes in period length, which would indicate a role in regulating phase delays of the clock (Pendergast et al., 2010). Of particular interest were two kinases that caused period lengthening, Csnk2a1
period = 31.2 hr versus 25.2 for control), which is downregulated by light, is a serine threonine kinase. It is closely related to CsnkE, which is mutated in the Tau mutant hamster, which also shows period lengthening (Lowrey et al., 2000). Silencing of Sik1, which is upregulated by light in the SCN, in a manner similar to Per1 (Figure 1A) also shows period lengthening (28.0 hr).

**Opn4−/− Mice Show Attenuated Transcriptional Responses to Light in the SCN**

Melanopsin-expressing retinal ganglion cells provide the major retinal projection to the SCN (Güler et al., 2008). The endogenous light sensitivity of these cells conferred by melanopsin (OPN4) is important for circadian entrainment. Mice lacking melanopsin
oscillator, we used the immortalized mouse embryonic fibroblast (Opn4−/−) to test the effects of SIK1 inhibition on the molecular circadian clock models and the intact animal. In order to test the effects of SIK1 inhibition on the molecular circadian clock models and the intact animal, we used the immortalized mouse embryonic fibroblast (Opn4−/−) to test the effects of SIK1 inhibition on the molecular circadian clock models and the intact animal.

Identification of SIK1 as a Potential Repressor of Phase-Shifting Responses

As CREB transcription plays a critical role in photic entrainment (Ginty et al., 1993), and because SIK1 acts both as a repressor of CREB transcription (Katoh et al., 2006) and as a target of CREB regulation itself, Sik1 emerged as a strong candidate for an inducible repressor acting as a brake on photic input to the SCN. Sik1 functions by phosphorylating and thereby deactivating the CREB coactivator CRTC (CREB-regulated transcription coactivator), which exists in three isoforms, CRTC1–3. These isoforms exhibit a tissue-specific expression pattern and the dual requirement for CREB phosphorylation and the presence of CRTC provides the complexity and specificity required in the response to cAMP signaling. Indeed, the SIK/CRTC pathway can regulate CREB transcription in other cellular systems (Berdeaux et al., 2007; Kanyo et al., 2009). In these cases, Ca2+ and cAMP signaling triggers calcineurin-mediated dephosphorylation of CRTC (Altarejos and Montminy 2011; Screaton et al., 2004), and its nuclear translocation (Ch’ng et al., 2012). CRTC in turn stimulates CREB transcription of genes containing CRE, including Sik1. The resulting induction of Sik1 represses CREB transcription through the phosphorylation of CRTC. In such a manner, Sik1 could provide negative feedback on photic input to the SCN by inhibiting the light-induced, CREB-mediated expression of Per1.

To test this hypothesis, we investigated the function of the CRTC-SIK1 pathway in regulating the circadian clock. To this end, we studied the nuclear translocation of CRTC1; the resultant CREB-mediated Sik1 and Per1 induction; and the effect of Sik1 on the transcription of Per1 and on re-entrainment in both cellular clock models and the intact animal.

The CRTC-SIK1 Cascade Regulates CREB-Mediated Transcription in Phase Shifting In Vitro

In order to test the effects of Sik1 inhibition on the molecular oscillator, we used the immortalized mouse embryonic fibroblast cell line NIH 3T3 as an in vitro model of the circadian clock. These cells can be phase shifted using serum stimulation (Balsalobre et al., 1998). We found that CRTC1 translocated to the nucleus following a serum shock (Figure 2A) and Sik1, which contains a conserved CRE/ATF (Figure 2B top) was induced (Figure 2B bottom). Increased levels of CRTC phosphorylation measured in cell lysates collected before and after serum shock, were observed as a consequence of Sik1 induction (Figure 2C). In order to specifically inhibit Sik1 activity, we used a gene-specific RNAi-based approach (Figures 2D and S3A–S3D and Table 1). Lysates of Sik1 knockdown cells showed no increase in CRTC phosphorylation after serum shock (Figure S3E). Cells transfected with siRNA against Sik1 (siSik1) showed severely attenuated induction of Sik1 resulting from serum treatment (Figure 2D and Table 1) with no significant change over time. To test the proposed role of Sik1 and Crtc in phase shifting of the clock, Per1 induction was assayed following silencing of Sik1 and Crtc isoforms expressed in NIH 3T3 cells (Crtc1 and Crtc3, Figure S3B). Significant changes of Per1 levels over the duration of the experiment were apparent after treatment with nontargeting control (siNT) and siSik1 (Figure 2E and Table 1). However, knockdown of Sik1 resulted in an increase in the duration and levels of Per1 induction as measured by a significant increase in the area under the curve for Sik1 (Figure 2G, Tables 1 and 2). In order to test that Sik1 acts via CRTC to attenuate CREB-mediated transcription of Per1, we assessed the effect of cosilencing Crtc1 and Crtc3 (siCrtc) on serum-shock-induced Per1 expression. Knockdown of Crtc attenuated the induction of Per1 (Figures 2F and 2G, Tables 1 and 2) with no significant difference over time after serum treatment. This result supports the proposed model whereby the effects of Sik1 are mediated through CRTC. Crucially, this result also demonstrates that CRTC is an important CREB coactivator required in the phase-shifting response. Circcadian expression of Per1 is regulated by multiple promoters, including the E box; therefore, we confirmed this requirement for CRTC in the phase-shifting response by examining the induction of two other light/serum-responsive genes (Egr1 and Nr4a1) that contain CREs in their promoters (as identified previously [Zhang et al., 2005]) and show a robust response to phase-shifting stimuli. These transcripts showed increased induction in cells treated with siSik1 and attenuation with siCrtc (Figures 2H, 2I, S2A, and S2B). We verified the attenuated induction of Fos, Per1, and Egr1 in the Opn4−/− SCN using qPCR. Analysis of transcription factor binding site (TFBS) showed that those transcripts with the greatest attenuation in Opn4−/− mice, including Fos, Nr4a1 and Per1, were enriched for WT1, a repressor of Egr1 (Tables S3 and S4). Our results indicate that in Opn4−/− mice, transcriptional responses in the SCN are broadly attenuated, mirroring the attenuated Fos induction and circadian responses to light previously observed. These results suggest that in the absence of melanopsin, there is an overall reduction in signal amplitude reaching the SCN via the RHT.

Pharmacological Inhibition of SIK1 Results in Augmented Phase-Shifting Responses

Findings from the in vitro study using RNAi in fibroblasts were further supported by data from the direct pharmacological inhibition of Sik1 by indirubin-3′-monoxime (I3M) (Hashimoto et al., 2008) (Figure 3A), which produced both an increase in the level and the duration of serum-induced Per1 and Per2 expression in NIH 3T3 cells following a serum shock (Figures 3B and 3C). In order to quantify the phase shift induced, we used PER2::LUC mouse embryonic fibroblasts (MEFs) in a double-serum treatment experiment (Balsalobre et al., 1998). The first serum shock was used to synchronize the cells and the second to allow precise quantification of the phase shift. We observed a 10.90 ± 1.02 hr phase delay of the PER2::LUC rhythm in I3M-treated cells,
compared with 7.59 ± 1.00 hr delay in the control, a difference of 3.3 hr (Figures 3D and 3E). In order to assess the effect of I3M on the SCN, Per1:luc mice were exposed to light at ZT14 for 10 min, and the SCN was removed and maintained in culture. The rhythms of SCN treated with I3M peaked approximately 1 hr later than DMSO-treated controls (Figure 3F). It should be noted that I3M is a nonspecific inhibitor of SIK1, and although directly inhibiting SIK1 (Figure 3A), its targets also include the kinase GSK3B, which has a role in the circadian clock (Hashimoto et al., 2008).

Nevertheless, these results using I3M and those using Sik1-specific RNAi are consistent (Figure 2). Collectively, these data show that inhibition of SIK1 increases the magnitude of phase delays in the molecular clock.

Figure 2. The Role of Sik1 in Phase-Shifting Responses in NIH 3T3 Embryonic Fibroblasts
(A) CRTC1 shows nuclear translocation after serum shock (before serum, top; after 10 min serum, bottom).
(B and C) Bioinformatic analysis of the Sik1 promoter region showing conservation of the CRE across several mammalian species (B, top). Sik1 was induced after serum shock (B, bottom, n = 4), leading to (C) increased phosphorylation of CRTC. A CRTC peptide is phosphorylated by purified SIK1, measured by the incorporation of 32P from 32P ATP (+SIK1). When incubated with cell lysates (lysate, 0 min), serum shock (lysate, 120 min) increased significantly phosphorylation of CRTC (p = 0.006, Student’s t test, n = 8). Experiments containing no lysate are shown as a negative control (− lysate). Representative blots are shown (left).
(D) Sik1 induction following serum shock was attenuated significantly following silencing of Sik1 or Crtc compared with nontargeting control siRNA (siNT: p = 1.5 × 10−6, siSik1: p = 4.1 × 10−5, siCrtc: p = 0.01, n = 4, area under curve [AUC] analysis shown in Figure S3).
(E) Sik1 silencing enhanced Per1 induction following serum shock (siNT: p = 2.2 × 10−5, siSik1: p = 9.3 × 10−5).
(F) Following Crtc silencing, no significant induction of Per1 expression was observed (siCrtc: p = 0.06). AUC analysis (normalized to NT control) for Per1 expression following silencing of Sik1 showed a significant increase (p = 0.00047 siSik1 versus siNT, Student’s t test), not seen with Crtc silencing (p = 0.799 siCRTC versus siNT, Students’ t test).
(G) Normalised data for Per1 induction following Sik1 and Crtc knockdown as measured by Area Under Curve (AUC) from (E) and (F).
(H and I) Egr1 (H) and I) Nr4a1, both CRE-regulated transcripts, show enhanced expression with Sik1 silencing and attenuated or no induction with Crtc silencing. AUC analysis shown in Figure S3. All mRNA levels normalized to housekeeping controls and r = 0, change over time analyzed by one-way ANOVA, *** = p < 0.001, * = p < 0.05, n.s. = p > 0.05. Error bars = SEM; n = 4. See also Figure S3 and Tables 1 and 2.

Phase-Shifting Stimuli Induce CRTC1 Nuclear Translocation and Sik1 Induction in the SCN
Of the different CRTC isoforms, CRTC1 is the most abundantly expressed in the SCN (Figure 4A). Therefore, we investigated
whether phase-shifting stimuli trigger the nuclear translocation of CRTC1 in the SCN. Application of a serum shock to dissociated SCN cells in culture resulted in the translocation of CRTC1 from the nucleus to cytoplasm within 10 min (Figure 4B) and an up-regulation of SIK1 in the SCN at 120 min after the light pulse (Figure 4C). We verified the expression patterns of SIK1 in the SCN seen on the exon arrays using qPCR (Figure 4D). SIK1 was light-induced with a peak mRNA expression at 60 min following both a CT16 phase-delaying and CT22 phase-advancing light pulse in wild-type mice, confirming a role for SIK1 in both phase advances as well as phase delays. Further, SIK1 induction is higher at CT22 when compared with CT16. In agreement with a role of SIK1 in negative feedback, Egr1 and Nr4a1 expression were found to be correspondingly lower at CT22.

**Table 1. Induction over Time**

| siNT  | siSik1 | siCrtc |
|-------|--------|--------|
| Sik1  | ↑↑     | x      | ↑      |
| p = 0.0000015 | p = 0.41 | p = 0.00979 |
| Per1  | ↑↑     | ↑↑     | x      |
| p = 0.000022 | p = 0.000093 | p = 0.06 |
| Egr1  | ↑↑     | ↑↑     | x      |
| p = 1.2 × 10^-10 | p = 4.2 × 10^-14 | p = 0.18 |
| Nr4a1 | ↑↑     | ↑↑     | ↑      |
| p = 0.000143 | p = 0.0000057 | p = 0.00755 |

Induction as measured by change in expression levels by one-way ANOVA across time. Expression levels of indicated transcripts in NIH 3T3 cells treated with siSik1, siCrtc, or siNT were analyzed for change over a 3 hr time course (Figure 2D–2I), p value calculated by one-way ANOVA, indicated in small print in each cell, n = 4. Number of ↑ indicates expression levels, with ↑↑ indicating control levels, ↑↑↑ indicates increase compared to control, ↑ indicates decreased induction compared to control and x indicating no significant induction.

**Table 2. Total Expression Levels Relative to siNT**

| siNT  | siSik1 | siCrtc |
|-------|--------|--------|
| Sik1  | ↑      |       |
| p = 0.0078 | p = 7.7 × 10^-7 |
| Per1  | ↑      | No change |
| p = 0.00047 | p = 0.799 |
| Egr1  | ↑      | No change |
| p = 0.00064 | p = 0.38 |
| Nr4a1 | ↑      |       |
| p = 0.046 | p = 0.075 |

Expression as measured by area under curve from Figures 2D–2F, 2H, and 2I. p value from t test comparing siSik1/siCrtc with siNT. Total expression over the three hour time course calculated from area under curve (AUC) from Figures 2D–2I and S3F–S3H, relative to siNT, p value from t test comparing AUC values from each treatment (siSik1 or siCrtc) relative to siNT. ↑ indicates increased AUC and therefore increased expression compared to control, ↓ indicates decreased AUC and therefore decreased expression compared to control.

DISCUSSION

The circadian system is entrained to environmental zeitgebers (time givers), which allow the appropriate alignment of internal and external time. However, the adjustment of the clock to such zeitgebers is rarely instantaneous, often occurring over repeated 24 hr cycles. What limits the effects of entraining stimuli on the circadian system represents a key, yet largely unanswered question. This issue provides the focus for the work presented here. We have identified >500 genes that are light regulated within the SCN. From these, we defined a key role for the CRTC-SIK1 pathway in regulating CREB-mediated clock...
Figure 3. Pharmacological Inhibition of SIK1 by I3M Causes Enhanced Phase Shifting

(A) Incorporation of 32P from 32P ATP into CRTC peptide by purified SIK1, SIK1 + 20 μM I3M (SIK1+I3M) and SIK1 + 100 nM Staurosporine (SIK1+Stau, staurosporine being a broad-spectrum kinase inhibitor); I3M directly inhibits SIK1.

(B and C) Period1 and 2 induction following 30 min serum treatment in PER2::LUC MEFs treated with indirubin-3’-monoxime (I3M) in DMSO or DMSO alone for the duration of the experiment. Relative gene expression of (B) per1 and (C) per2 normalized to GAPDH.

(D) Representative baseline detrended bioluminescence recordings from PER2::LUC MEFs treated with a single (orange, first red arrow) or second serum shock 10 hr later (second red arrow) in the presence of I3M (red) or DMSO alone (green), with I3M creating a larger phase shift than the DMSO-treated controls. Timing of I3M or DMSO treatment is shown by the horizontal gray bar. The first serum pulse is given to synchronize the cells and the second allows for phase shift from synchronized conditions, allowing precise quantification of the phase shift.

(E) Time of peak bioluminescence determined from data as shown in (C). Data are mean ± SEM first and second peaks after the second serum pulse. Cells treated with I3M peak significantly later than DMSO-treated controls (one-way ANOVA; p < 0.01, with post hoc t tests, *p < 0.05).

(F) Zeitgeber time of second peak of bioluminescence from SCN collected from light-pulsed per1::luc mice treated with I3M versus DMSO-treated (DMSO) controls (*t test, p < 0.05). Error bars = SEM.

gene expression. Our data show both in vitro and in vivo that CRTC1 translocates to the nucleus after phase-shifting stimuli and acts as a coactivator of CREB-driven transcription of Sik1 and Per1. Following in vitro Crtc silencing, induction of Per1 and other CREB-mediated genes is greatly attenuated. Elevated Per1 aligns the transcriptional-translational-feedback loop to the zeitgeber stimulus (Albrecht, 2012; Albrecht et al., 1997). We also show that Sik1 phosphorylates and deactivates CRTC with an appropriate time delay, which effectively blocks further CRTC-driven Per1 transcription. Critically, we show that in vivo RNAi knockdown of Sik1 within the SCN greatly enhances light-induced behavioral phase shifts and re-entrainment to LD cycles. Collectively, these data provide empirical evidence for a molecular mechanism that limits the impact of an entraining stimulus on the circadian system.

It is worth emphasizing that the Sik1-CRTC regulation of CREB-mediated gene expression we describe within the context of the circadian system has also been demonstrated in several nonclock pathways. For example, in skeletal myocytes, Sik1 functions as a Ca2+-responsive CREB-regulated HDAC kinase (Berdeaux et al., 2007) and a Ca2+-induced regulator of Na+ transport in kidney cells (Sjöström et al., 2007). In hippocampal neurons, CRTC1 functions as a sensor of stimulation, by traveling from the synapse to the nucleus in response to activation, triggering CREB mediated transcription and thereby regulating hippocampal plasticity (Ch‘ng et al., 2012). An appreciation of the role of CRTC1-Sik1 regulation in these noncircadian pathways very much supports our interpretation of how this signaling pathway functions within the molecular clock.

The same regulatory processes that induce Per1 also induce Sik1. Following the induction of these genes, phosphorylation then plays a key, yet distinct, role in the molecular clockwork. The phosphorylation of core clock proteins is a major post-translational event whereby delays are introduced into the transcriptional-translational-feedback loop to generate a ~24 hr oscillation. Several proteins have been described in this process, including FBXL3, an F box protein that targets phosphorylated CRY for degradation (Godinho et al., 2007); CSNK1e, in the Tau mutant hamster, which phosphorylates PER1, CRY1, and BMAL1 (Lowrey et al., 2000) and NEMO/NLK that phosphorylates PER to initiate a time-delay phosphorylation circuit (Chiu et al., 2011). In contrast to these kinases, Sik1 does not target the core clock proteins to control their degradation; rather, it attenuates the induction of clock genes in response to entraining stimuli, functioning as a negative-feedback mechanism to deactivate the molecular pathways that are activated following a phase-shifting stimulus.

But why limit the impact of an entraining stimulus on the clock? Almost all physiological systems benefit from buffering mechanisms that prevent sudden and large changes, and in this regard...
the circadian system is no exception. This would be particularly important for the clock because it has to maintain internal synchrony between the multiple cell-autonomous circadian oscillators throughout the body. If sudden and large shifts are imposed upon one part of the circadian network, such as the SCN, internal desynchrony among the cellular clocks of the body will result. Under such circumstances normal physiology will be severely compromised (Albrecht, 2012). By limiting the phase-shifting effects of light and other clock-resetting stimuli, such as food (Stokkan et al., 2001) or activity (Mrosovsky and Salmon, 1987) the circadian system would be shielded from such disruption. Such a buffering mechanism in the natural world would protect the clock from abnormal Zeitgeber stimuli. In addition, limiting the impact of light on the SCN would be useful in nonequatorial latitudes during the equinoxes when the time of dawn and dusk, along with activity and food availability, moves very rapidly. With a rapidly changing twilight transition, the circadian system has to maintain a balance between entrain-ment and the prevention of internal de-synchrony. Under these conditions, CRTC1-SIK1-driven inhibition of Per transcription may help achieve this compromise.

Sleep and circadian rhythms disruption (SCRD) is endemic within the 24/7 economies of the developed and developing industrialized societies (Foster and Wulff, 2005). In addition, SCRD is a common feature of both neuropsychiatric and neurodegenerative disease (Wulff et al., 2010). In shift-work, jet lag, and brain abnormalities in general, SCRD is associated with chronic diseases such as cancer, metabolic disorders, cardiovascular disease, immune dysfunction, and impaired cognition (Hastings et al., 2003). Currently, there are very few options available to stabilize or adjust the circadian axis and sleep timing in individuals with SCRD. However, the discovery of the molecular mechanisms that generate and regulate the circadian system provides targets for the development of therapeutic agents that regulate internal time. Indeed, there have been recent efforts in this direction, including the use of small molecule activators of cryptochrome (Hirota et al., 2012) and an inhibitor of CSNK1E (Meng et al., 2010). Such modulators act on the central clock to lengthen or shorten period. An alternative approach would be to shift the clock to an appropriate phase. As SIK1 acts to suppress the effects on light on the clock, this pathway presents a tractable target.

**EXPERIMENTAL PROCEDURES**

**Animals**

Oprn
-/- mice (Hattar et al., 2002; Lucas et al., 2003) were maintained on a C57Bl/6_129Sv background as heterozygous breeders. For SCN sample collection and RNA extraction, see Extended Experimental Procedures.
Affymetrix Whole-Genome Exon Array Experiments
Sense probes were prepared for hybridization from 300 ng of total RNA for Opn4+/+ shams SCN (n = 6), LP30, LP60, and LP120 (n = 4) and Opn4-/- shams (n = 4), LP30, LP60, and LP120 (n = 3) using the GeneChip WT Sense Target Labeling kit (Affymetrix, Santa Clara, CA, USA) and hybridized overnight to GeneChip Mouse Exon 1.0 ST Arrays (Affymetrix Santa Clara, CA, USA). Arrays were washed, stained, and scanned according to manufacturer’s guidelines.

GeneChip Data Analysis
CEL file data were extracted, log transformed and quantile normalized with Expression Console (v1.1) software from Affymetrix using the RMA-sketch algorithm at the exon-core probe set level including the detection above background (DABG) p value for each probe set. Exon array quality control was assessed using Expression Console (V1.1) and any outliers removed. Subsequent analysis steps were carried out in Microsoft Excel 2007. Probe sets were filtered by DABG p value for each array sequentially, discarding data for probe

Figure 5. In Vivo Knockdown of Sik1 Results in Enhanced Phase Shifting and Rapid Re-Entrainment in a Jet-Lag Protocol
(A) C57Bl/6 mice were housed under a 12:12 hr LD cycle before siRNA ICV injection into the 3V (indicated by red asterisk on actograms). Ninety-six hours postinjection, the mice were given a 30 min light pulse (red arrow) at CT14.5, then placed in DD to enable phase-shift magnitude to be determined. Representative actograms from light-pulsed animals receiving siNT (siNT Light, top) or siSik1 (siSik1 Light, bottom) shown. Actograms are enlarged around the day of the light pulse for clarity (right).

(B) Phase-shifting responses to light are significantly larger following knockdown of Sik1 in the SCN (siSik1) when compared to nontargeting siRNA (siNT) controls (98 min versus 59 min, p = 0.036, Student’s t test, n = 5). Error bars = SEM. See also Figure S4.

(C) C57Bl/6 mice were housed under a 12:12 hr LD cycle before siRNA ICV injection into the 3V (indicated by red asterisk). Forty-eight hours postinjection, the LD cycle was advanced by 6 hr and 10 days after the first shift, the LD cycle was advanced 6 hr again. Faster re-entrainment was observed with the Sik1 knockdown (siSik1) mice. Three actograms are displayed for each treatment (siNT or siSik1), showing that regardless of activity levels, Sik1 knockdown accelerates re-entrainment.

(D) Phase relative to new LD cycle (second shift) plotted against days after the shift in cycle. Day before shift indicated as 0 (before the dotted line). ** = p < 0.01, * = p < 0.05 Student’s t test, phase of siSik1 versus siNT-treated animals on each day, n = 6 for siNT, n = 11 for siSik1. Representative actograms enlarged around the area plotted in the graph are indicated. Error bars = SEM. See also Figures S4 and S5.

1108 Cell 154, 1100–1111, August 29, 2013 ©2013 Elsevier Inc.
sets for which \( p \geq 0.05 \). Only probe sets called as “present” on all arrays \( (n = 18) \) were further analyzed (110,026 probe sets) by one-way ANOVA and filtered by \( p \) value \( (p \leq 0.05) \) (corresponding to 4,384 probe sets that respond to light). Genes that were light-responsive were identified by significant probe set enrichment. Probe set enrichment was evaluated by comparing the number of significant probe sets for an individual gene to (1) number of probe sets on the array for that gene, (2) number of probe sets significantly changing, and (3) the total number of probe sets on the array based upon a binomial distribution. Finally, probe sets were filtered by probe set enrichment \( p \) value of \( p < 0.05 \) (1,417 probe sets corresponding to 536 genes with the false-positive rate determined to be 0.02). The same probe sets were subsequently analyzed in the \( \text{Opr}^4/C \) data to assess attenuation. Refer to extended experimental procedures for hierarchical clustering, functional pathway analysis and TFBS prediction.

For qPCR, primer sequences, western blotting, immunostaining, microscopy, CRTC phosphorylation assay, and i3M-based studies, see Supplemental Information.

**RNAi Studies**

siRNAs were synthesized as siGENOME (Dharmacon) for in vitro experiments, siSTABLE (Dharmacon) or custom synthesized by Roche for in vivo work. The siRNAs supplied by Roche were moderately stabilized for in vivo use and tagged with a cholesterol conjugate to aid transfection. The sequences are as described in Supplemental Information.

**In Vitro RNAi**

NIH 3T3 cells (mouse embryonic fibroblast cell line) were cultured in DMEM supplemented with 10% FBS at 37°C, 5% CO₂. Where required, cells were transfected with 10 nM siRNA using Lipofectamine RNAmax (Invitrogen, Life Technologies) according to manufacturer’s instructions. The cells were maintained for at least 24 hr in 1% FBS in DMEM at confluence. At 0 min, the cells were synchronized by application of 50% horse serum (Balsalobre et al., 1998; Duffield et al., 2002) returned to 1% FBS in DMEM after 30 min (serum shock treatment). RNA was isolated from cells at 30 min intervals over 3 hr. RNA was extracted using the RNeasy mini kit (Qiagen) and qPCR was performed as discussed in supplementary material to measure knockdown/gene expression.

**In Vivo RNAi**

siRNA complexes with Invivofectamine (Invitrogen) prepared according to manufacturer’s instructions were delivered using microinjection into the 3V with stereotaxic equipment with coordinates (Paxinos and Franklin, 2008) as manufacturer's instructions were delivered using microinjection into the 3V siRNA complexed with Invivofectamine (Invitrogen) prepared according to manufacturer’s instructions. The cells were maintained for at least 24 hr in 1% FBS in DMEM at confluence. At 0 min, the cells were synchronized by application of 50% horse serum (Balsalobre et al., 1998; Duffield et al., 2002) returned to 1% FBS in DMEM after 30 min (serum shock treatment). RNA was isolated from cells at 30 min intervals over 3 hr. RNA was extracted using the RNeasy mini kit (Qiagen) and qPCR was performed as discussed in supplementary material to measure knockdown/gene expression.

**ACCESSION NUMBERS**

The ArrayExpress accession number for the GeneChip data reported in this paper is E-MEXP-3933.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.004.

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