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Ca^{2+}/cAMP Response Element-binding Protein (CREB)-dependent Activation of *Per1* Is Required for Light-induced Signaling in the Suprachiasmatic Nucleus Circadian Clock*

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Light is a prominent stimulus that synchronizes endogenous circadian rhythmicity to environmental light/dark cycles. Nocturnal light elevates mRNA of the *Period1* (*Per1*) gene and induces long term state changes, expressed as phase shifts of circadian rhythms. The cellular mechanism for *Per1* elevation and light-induced phase advance in the suprachiasmatic nucleus (SCN), a process initiated primarily by glutamatergic neurotransmission from the retinohypothalamic tract, was examined. Glutamate (GLU)-induced phase advances in the rat SCN were blocked by antisense oligodeoxynucleotide (ODN) against *Per1* and Ca^{2+}/cAMP response element (CRE)-decoy ODN. CRE-decoy ODN also blocked light-induced phase advances *in vivo*. Furthermore, the CRE-decoy blocked GLU-induced accumulation of *Per1* mRNA. Thus, Ca^{2+}/cAMP response element-binding protein (CREB) and *Per1* are integral components of the pathway transducing light-stimulated GLU neurotransmission into phase advance of the circadian clock.

Mammalian circadian rhythmicity is generated by endogenous alternations in transcription/translation of putative clock genes within the suprachiasmatic nucleus (SCN)[1] of the basal hypothalamus. As a projection site of the retinohypothalamic tract, the SCN is poised to respond to retinal light information, mediated primarily by glutamatergic (GLU) neurotransmission, to assure time-of-day congruence between the endogenous pacemaker and the external environment. The mechanisms by which the SCN decodes and processes light information are complex and change as the biochemical clock states progress through their 24-h cycle (1). Light resets the clock throughout the night via glutamatergic-*N*-methyl-D-aspartate receptor-mediated Ca^{2+} influx, which activates nitric-oxide synthase to liberate nitric oxide (NO) (2). At this point, the light signaling pathway diverges. In the early night, the light-induced state change, which delays subsequent rhythms, proceeds through NO-dependent activation of a neuronal ryanodine receptor. Light-induced state changes in the late night are independent of ryanodine receptor activation, but require activation of protein kinase G (PKG) (3–5).

The discovery of several specific genes associated with circadian rhythmicity, including *Period* (*Per*) and *Timeless* (*Tim*) (for review, see Ref. 6), raises questions regarding the mechanisms that interface nocturnal light signals with the molecular clockwork. Throughout the night, light stimuli sufficient to cause long term state changes, or phase shifts, of circadian rhythms of rodent wheel running correlate with increased phosphorylation of the transcription factor, Ca^{2+}/cAMP response element-binding protein (CREB) (7, 8), activation of Ca^{2+}/cAMP response element (CRE)-mediated transcription (9), and a rise in *Per1* mRNA (10–15). This investigation was undertaken to determine whether CRE-mediated activation of Per1 is required for light/GLU-induced phase resetting of the SCN clock. We hypothesized that the GLU-induced phase advance requires activation of CRE and elevation of *Per1* mRNA. Therefore, we examined the ability of an oligodeoxynucleotide (ODN) decoy (CRE-decoy) to 1) sequester CREB and inhibit CRE-mediated transcription, 2) inhibit GLU-induced phase advances of SCN electrical activity and light-induced phase advances of wheel running activity, and 3) block GLU-induced up-regulation of *Per1* levels.

EXPERIMENTAL PROCEDURES

Animals and Circadian Time—Long-Evans rats (6–12 weeks old) from our inbred line were used for all *in vitro* experiments. After greater than 35 generations of inbreeding, this line surpasses the requirements for genetic homogeneity, resulting in low variation for physiological experiments. Rats were entrained to a daily cycle of 12-h light:12-h dark and provided food and water *ad libitum*. Because the rat SCN generates stable 24-h rhythms of electrical activity when maintained *in vitro* over the 2–3 days of experimentation, *in vitro* clock time was determined from the lighting cycle in the donor colony. Circadian time 0 (CT 0) is designated as the time of lights on in the donor colony; subjective day is CT 0–12. Subjective night (CT 12–24) corresponds to the dark portion of the donor's cycle.

For *in vivo* behavioral experiments, male B6129PF1/J mice obtained from The Jackson Laboratory (Bar Harbor, ME) at ~4 weeks of age were placed in individual cages (27 × 21 × 14 cm) equipped with running wheels (12-cm diameter). Mice were entrained to a 12-h light: 12-h dark (12:12 light-dark; 100-lux white light) cycle and then released into constant darkness.

Behavioral Wheel Running Activity Data Acquisition—Circadian rhythms of behavior were assessed by monitoring the wheel-running activity of individually housed mice as described previously (16). Activity was conveyed to a Pentium III computer (Micron, Inc.) equipped with Clocklab Acquisition software (David Ferster, Northwestern Uni-

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[1] The abbreviations used are: SCN, suprachiasmatic nucleus; GLU, glutamate; PKG, protein kinase G; PKA, protein kinase A; CRE, Ca^{2+}/cAMP response element; CREB, Ca^{2+}/cAMP response element-binding protein; ODN, oligodeoxynucleotide; CT, circadian time; CREM, Ca^{2+}/cAMP response element modulator; MAPK, mitogen-activated protein kinase; CaMK, calmodulin kinase; ANOVA, analysis of variance.

versity) running in LabView (National Instruments, Inc., Austin, TX). Rhythms were plotted in an actogram with Clocklab Analysis software (David Ferster, Northwestern University) running in Matlab (Mathworks Co., Natick, MA).

Intra-SCN Cannula Implantation and Injection—A guide cannula (26 gauge; 11.2 mm total length; Plastics One, Inc., Roanoke, VA) was stereotaxically implanted unilaterally into the SCN under deep anesthesia (60 mg/kg sodium pentobarbital) using a stereotaxic apparatus (Stoelting, Wood Dale, IL). Surgical coordinates from bregma were −0.3-mm anterior-posterior, −0.1-mm medial-lateral, and −1.8-mm dorsal-ventral (from the surface of dura). The cannula was secured with small machine screws (Small Parts, Inc., Miami Lakes, FL) inserted into the skull and cranioplastic cement (Plastics One, Inc., Roanoke, VA). Following establishment of a well defined, free-running rhythm for at least 10 days, pharmacological manipulations were performed under dim (<1 lux) red illumination. A 10-μl Hamilton syringe (Hamilton Co., Reno, NV) fitted with a 1.5-cm piece of polyethylene tubing and a 33-gauge infusion cannula (Plastics One, Inc., Roanoke, VA) was employed to administer 0.3 μl of 100 μM CRE-decoy ODN (tgacgtcatgacgtcatgacgtca) or a 100 μM concentration of a mismatched sequence of the same nucleotide bases, CRE-mis (tgtggtcatgtggtcatgtggtca) through the guide cannula and into the SCN at CT 21.75. CRE-mis and certain CRE-decoy injections were followed 15 min later (CT 22) by 15-min, 20-lux light pulses. Injection sites were verified histologically, and data from only those animals in which the tip of the injection cannula penetrated the dorsal border of the SCN were used for analyses.

Isolation of SCN 2.2 Nuclear Extract and Electromobility Shift Assay—Nuclear extracts were isolated as described (17). Briefly, confluent SCN 2.2 cells were scraped in wash solution (15 mM HEPES, pH 7.2, 250 mM sucrose, 60 mM KCl, 10 mM NaCl, 1 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 2 mM $NaPP_i$, 5 μM mycrocystin-LR] and centrifuged at 2000 × *g* for 10 min. Pellets were resuspended in cell lysis solution (10 mM HEPES, pH 7.2, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 5 μM microcystin-LR, 2 mM NaF, 2 mM $NaPP_i$) and centrifuged at 4000 × *g* for 10 min to isolate nuclei. The pellet was resuspended in nuclei lysis solution (100 mM HEPES, pH 7.2, 1.5 mM $MgCl_2$, 1 mM EDTA, 800 mM NaCl, 2 mM NaF, 2 mM $NaPP_i$, 1 mM phenylmethylsulfonyl fluoride, 5 μM microcystin-LR, 25% glycerol and centrifuged at 14,000 × *g* for 30 min to pellet debris. Supernatant was stored at −20 °C until use. The electromobility of the CRE-decoy was adapted from (18). 5 μg of nuclear extract from SCN 2.2 cells transfected with 75 nM CRE-decoy or CRE-mis using Effectene (Qiagen) was incubated in 100 ng/μl poly(dI-dC), 300 nM dithiothreitol, 12 mM Tris pH 8.0, 2 mM $MgCl_2$, 60 mM KCl, 120 nM EDTA, pH 8.0, 12.5% glycerol for 30 min at 4 °C. 10,000 cpm of ^{32}P-end-labeled CRE-decoy was added and incubated at 37 °C for 10 min. The samples were separated by 8% PAGE and exposed on a phosphorimager screen.

Luciferase Assay—Culture extracts were prepared by resuspending frozen SCN cultures stably transfected with a CRE-luciferase construct (45) with cell culture lysis buffer (Promega) at ~25 μl of cell culture lysis buffer/cm^2 cell culture surface area. Samples were centrifuged (2000 × *g* for 5 min) and supernatant collected for luciferase assay. Luciferase assays were performed using the Luciferase Assay System (Promega) and measured on an MLX microtiter plate luminometer (Dynex Technologies) by luc-50. Protein concentration of samples was determined by BCA protein assay kit (Pierce) against bovine serum albumin standards in cell culture lysis buffer. Relative luciferase activity/μg of protein was determined for each culture.

Preparation and Treatment of Brain Slices—Coronal brain slices (500 μm) were prepared at least 2 h prior to the onset of the dark phase of the light:dark cycle to avoid phase shifts during preparation (19). Slices maintained at 37 °C (95% 0_2, 5% $C0_2$) in a Hatton-style brain slice chamber were continuously perfused with minimal salts (Earle's balanced salt solution, pH 7.25) and glucose (24 mM). Perifusion was stopped during treatment. GLU (10 mM, 10 min) was applied by microdrop (1 μl) to the top of each slice. For antisense experiments, antisense sequence (αODN) against the 5′ start site of *Per1* (taggggaccactcatgtct), *Per2* (tatccattcatgtcg), or *Tim* (acaagtccatacacc) was applied at a concentration of 10 μM, 2 h prior to the time of GLU treatment by replacing the bath with medium containing the experimental reagent.

Single Unit Recordings of SCN Neuronal Activity—The phase-shifting effects of stimuli applied to SCN slices *in vitro* were assessed using the standard extracellular single unit recording technique (19). Spontaneous firing rates of individual neurons were grouped into 2-h running averages using 15-min lags. The time-of-peak for each experiment was determined by visual inspection of a plot of 2-h running averages for the symmetrically highest point. A characteristic sinusoidal pattern of change, such that activity was low at night and peaked near circadian time 7 (CT 7, 7 h after the onset of light in the donor colony) was observed in vehicle-treated control slices. Phase shifts were determined by comparing the mean time-of-peak from treatment groups to vehicle-treated controls. Certain recordings were performed with the experimenter blind to the treatment conditions.

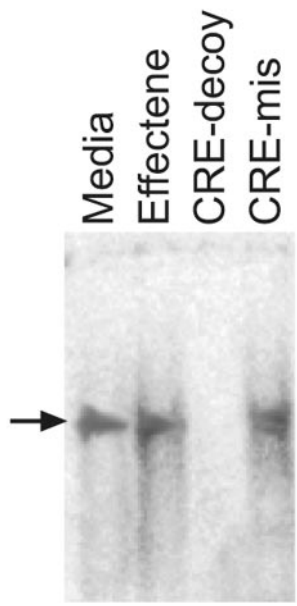

FIG. 1. **CRE-decoy blocks binding at CRE sites in SCN 2.2 cells.** Electromobility shift assay of a CRE probe incubated with nuclear extracts of SCN 2.2 cells non-transfected (*lane 1*) or transfected with the transfection reaction reagent, Effectene (*lane 2*), 75 nM CRE-decoy (*lane 3*), or 75 nM CRE-mis (*lane 4*). The *arrow* indicates the retarded mobility of the CRE probe due to binding of CREB. This DNA-protein interaction is absent in the SCN 2.2 cells transfected with the CRE-decoy.

In Situ Hybridization—Sixty minutes following the initiation of GLU treatment, slices were fixed overnight at 4 °C in 4% paraformaldehyde, followed by cryoprotection in 20% sucrose. Hybridization was performed on 10-μm sections using digoxygenin-labeled riboprobes (cRNA), as described previously (16, 20). Analysis of *mPer1*-positive cells was made in a midcaudal section of the SCN by an individual blind to the experimental design and identity of the samples.

RESULTS

CRE-decoy Blocks the Light/GLU-induced Phase Advance—The CRE-decoy is a synthetic single-stranded ODN that self-hybridizes to form duplex/hairpins (18). Theoretically, the CRE-decoy will sequester phospho-CREB, preventing its binding and activation of genes containing CRE in their promoters. CRE-decoy and CRE-mis ODN were used to determine whether activation of CRE sites is required for GLU-induced phase advance of the SCN neuronal firing rate rhythm. Two initial experiments were performed to demonstrate that CRE-decoy acts as predicted. The CRE-decoy (75 nM) blocked CRE-CREB binding in SCN 2.2 cells (Fig. 1) and inhibited reporter activity in SCN 2.2 cells stably transfected with a luciferase construct driven by 8 tandem repeats of the CRE-palindrome (Fig. 2). CRE-mis did not block CRE-CREB binding or alter CRE-driven luciferase activity. These data are in agreement with a previous study by Park *et al.* (18) demonstrating that the CRE-decoy can penetrate cells, bind CREB, and prevent transcriptional activation from CRE sites.

To determine whether CRE-mediated transcription is required for GLU-induced phase resetting within the SCN, CRE-decoy (1 μM) was applied to SCN slices prior to stimulation with GLU. Spontaneous SCN neuronal activity peaked near CT 7 in control slices (Fig. 3*a*, mean time-of-peak = CT 6.78 + 0.10, n = 8). CRE-decoy did not alter the timing of the SCN electrical activity rhythm when applied alone (Fig. 3, *b* and *f*, mean

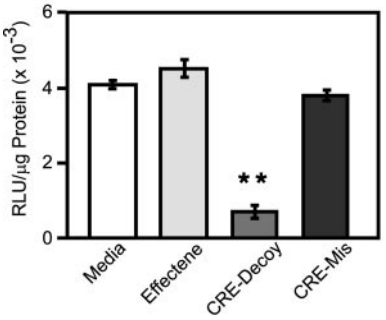

FIG. 2. **CRE-activated transcription is inhibited by the addition of the CRE-decoy.** Luciferase activity was quantitated from a SCN 2.2 cell line stably transfected with a luciferase construct driven by eight CRE sites (45). The relative light units (*RLU*) normalized to protein concentrations of cells transfected with just the transfection reagent, Effectene, or CRE-mis were similar that of the non-transfected cells (media). However, the cells transfected with the CRE-decoy demonstrated a significant inhibition in CRE-activated luciferase activity. ** indicates statistically significant differences ($p < 0.01$) as determined by ANOVA with Tukey's *post hoc* analysis.

time-of-peak CT 6.25 + 0.14, $n = 3$). Application of GLU to SCN slices at CT 20 caused a phase advance of the SCN electrical activity rhythm (Fig. 3, *c* and *f*, mean time-of-peak = CT 3.41 + 0.18, $n = 6$). CRE-decoy (Fig. 3, *d* and *f*, mean time-of-peak CT 6.50 + 0.25, $n = 3$), but not CRE-mis (Fig. 3, *e* and *f*, mean time-of-peak CT 3.47 + 0.12, $n = 3$), blocked the GLU-induced phase advance.

To determine whether CRE-mediated transcription is required for the phase resetting that occurs in response to light signals, CRE-decoy (100 μM) was injected into the SCN 15 min before exposure to a light pulse at CT 22. Neither CRE-decoy nor CRE-mis caused a phase shift of activity rhythms when injected alone (Fig. 4). Light pulses caused a characteristic 1.1-h phase advance. Phase resetting effects of light were blocked in the presence of the CRE-decoy. CRE-mis had no effect on light-induced phase shifts. These data suggest that transcriptional activation at CRE sites is required for the light-induced phase advance of circadian behavioral patterns.

Elevation of Per1 mRNA Is Required for GLU-induced Phase Advance—Alterations in the circadian timing of wheel-running activity and SCN firing rate rhythms represent behavioral and physiological changes associated with light at night. Likewise, induction of *Per1* has become a marker of the molecular response to nocturnal light/GLU. To determine whether mRNA for *Per1*, *Per2*, or *Tim* is required for GLU-induced phase advance, αODN surrounding the 5′ start site of each mRNA was applied to the SCN slice in the presence or absence of GLU (Fig. 5). Spontaneous SCN neuronal activity peaked near CT 7 in control slices (Fig. 5*a*, mean time-of-peak = CT 6.78 + 0.10, $n = 8$). GLU induced a ~3.5-h phase advance in the time-of-peak neuronal activity compared with controls (Fig. 5*b*, mean time-of-peak CT 3.42 + 0.14, $n = 6$). *Per1* αODN alone did not alter the time-of-peak (Fig. 5*c*, mean time-of-peak = CT 6.42 + 0.35, $n = 3$). Upon GLU stimulation, *Per1* αODN blocked the usual ~3.5-h phase advance (Fig. 5*d*, mean time-of-peak = 6.56 + 0.21, $n = 3$). A *Per1* sense ODN (Fig. 5*e*, mean time-of-peak = 3.75 + 0.12, $n = 3$), and a *Per1* ODN carrying three mismatched nucleotides (Fig. 5*f*, mean time-of peak = 3.53 + 0.35, $n = 3$) had no effect on GLU-induced phase advances. Thus, *Per1* mRNA induction is required for GLU-induced clock resetting in the late night. On the other hand, *Tim* αODN (Fig. 5*g*, mean time-of-peak CT 3.6 + 0.22, $n = 3$) or *Per2* (Fig. 5*g*, time-of-peak CT 3.11 + 0.75, $n = 3$) did not effect the GLU-induced phase advances.

CRE Activation Regulates GLU Induction of Per1—To determine whether the rise in *Per1* mRNA induced by GLU and required for the phase shift is mediated by CREB activation, SCN slices were treated and analyzed for *Per1* by *in situ* hybridization. The number of *Per1*-positive cells increased 400% by 60 min after GLU treatment at CT 20 (Fig. 6, $p < 0.01$), consistent with previous results (16). Whereas treatment with CRE-decoy alone did not affect *Per1* levels, the decoy blocked GLU induction of *Per1* (Fig. 6). These data suggest that activation of CREB-regulated transcription is required to induce *Per1* mRNA.

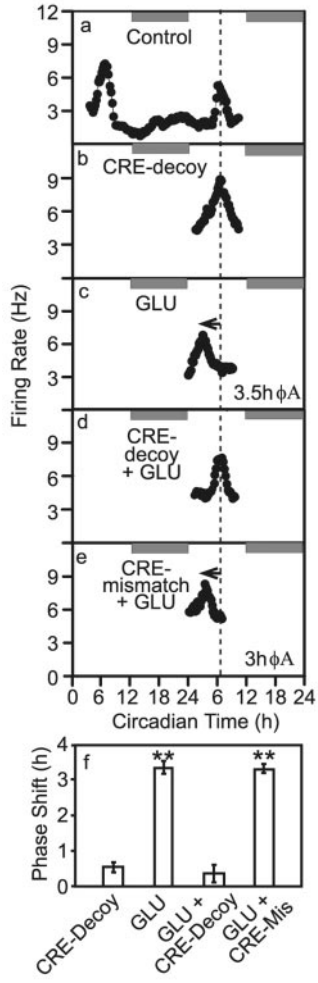

FIG. 3. **Inhibiting CRE activation blocks GLU-induced phase advances *in vitro*.** *a*, a representative control neuronal activity rhythm with a peak near CT 7 on days 1 and 2 *in vitro*. *b*, application of CRE-sense (1 μM) alone from CT 18–20 had no effect on the time-of-peak electrical activity *in vitro*. *c*, at CT 20, GLU (10 mM) induced a ~3.5-h phase advance in the SCN electrical activity rhythm. *d*, CRE-sense (1 μM) blocked the GLU-induced phase advance. *e*, CRE-mismatch did not block the GLU-induced phase advance. The *bar graph* of mean data ($n = 3$/condition) demonstrates samples treated with CRE-decoy are not significantly different from controls, and GLU treatment samples treated with CRE-mis are not significantly different from GLU treatment alone. Statistical treatments were the same as in Fig. 1.

DISCUSSION

Mechanisms coupling environmental light signals to molecular changes that lead to long term state changes within the circadian clock are emerging as complex and multifunctional. Nocturnal light resets the circadian clock through glutamatergic neurotransmission of retinohypothalamic tract origin (2, 21–24). Throughout the night, light/GLU phosphorylates CREB (7, 8), activates CRE-mediated transcription (9), and stimulates immediate early genes (25–28) and the clock gene

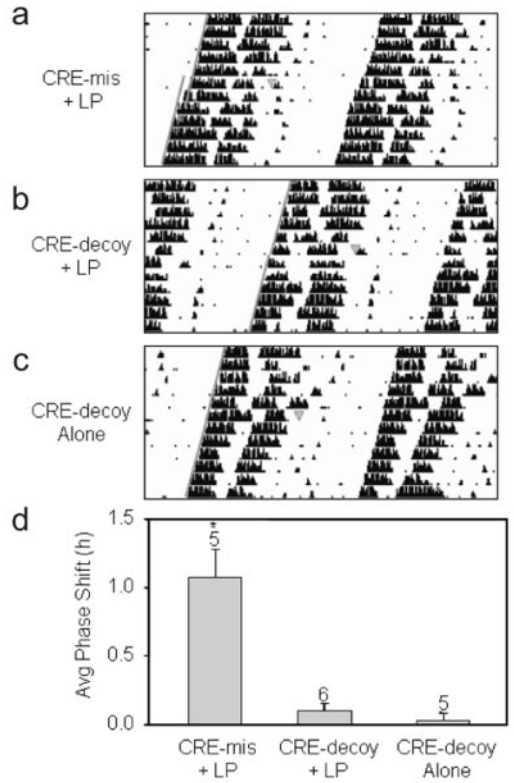

FIG. 4. **Inhibiting CRE activation blocks light-induced phase advances *in vivo*.** *a–c*, representative double-plotted actograms of wheel-running activity patterns of 2 days in tandem/line. Using activity onset as phase marker, the data depict unaffected light-induced phase shift following CRE-mis treatment (*a*), lack of light-induced phase advance following CRE-decoy treatment (*b*), and unaffected wheel-running rhythm following CRE-decoy treatment without light pulse (*c*). Each *horizontal line* represents 48-h of wheel-running activity with the second 24 h of each line redrawn as the first 24 h on the next line. *Vertical marks* represent 6-min bins of activity plotted relative to the maximum activity of the animals for the duration of the record. *Diagonal lines* are drawn to ease visualization of phase shifts. *d*, summary *bar graph* depicting magnitudes of responses following the three conditions as indicated. CRE-decoy + light pulse and CRE-decoy alone do not induce significant changes in phase of these activity rhythms. * = $p < 0.05$; ▽ = time of light pulse.

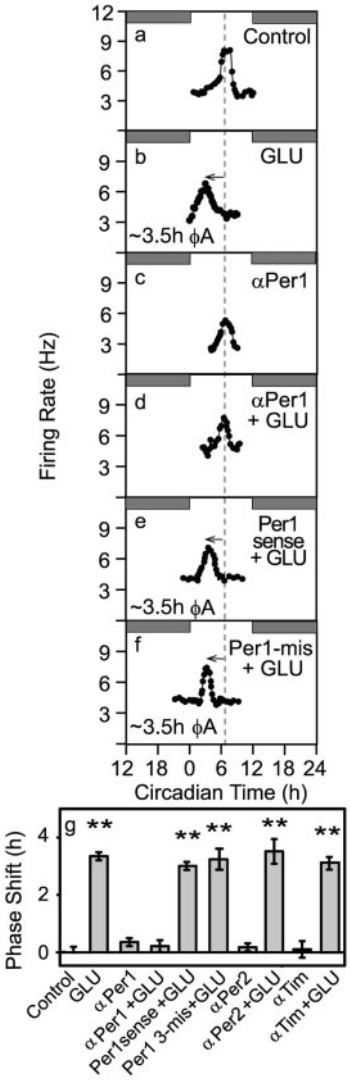

FIG. 5. ***Per1* is required for GLU to induce phase shifts *in vitro*.** *a*, the spontaneous electrical activity rhythm peaks near CT 7 in controls. *b*, at CT 20, GLU (10 mM, 10 min) advanced the electrical activity rhythm by ~3.5 h. *c*, *Per1* αODN (10 μM) applied from CT 18–20 had no effect on the time-of-peak electrical activity. *d*, *Per1* αODN blocked the GLU-induced phase advance at CT 20. *e*, *Per* sense ODN (10 μM) applied from CT 18–20 had no effect on the GLU-induced advance in time-of-peak electrical activity. *f*, *Per* missense ODN did not block the GLU-induced phase advance at CT 20. *g*, summary of the effects of *Per1*, *Per2*, and *Tim* antisense ODNs on GLU-induced phase advances at CT 20. ** indicates statistically significant differences ($p < 0.01$) as determined by ANOVA with Tukey's *post hoc* analysis.

Per1 (10–15). Our data provide definitive evidence that CRE-regulated activation of *mPer1* mRNA is required for light/GLU-induced phase advances during the late subjective night as predicted from the studies of Travnickova-Benova *et al.* on transfected JEG3 cells (29).

Inhibition of CRE-mediated transcription by an exogenous CRE sequence (CRE-decoy) that binds CREB, thereby outcompeting its binding to endogenous CRE, conclusively demonstrates that CREB/CRE-activated transcriptional events are required for the light/GLU-induced phase advance. These results extend the findings of a previous study that defined a requirement for CREB phosphorylation on Ser^{142} for light-induced phase shifts (30) by demonstrating that CRE-activated transcriptional events are also required.

The presence of CRE-elements (31) capable of binding CREB and responding to forskolin and EGF (29) in the *Per1* promoter has suggested that one critical function of light/GLU-induced CRE-mediated transcription is generation of new *Per1* mRNA. Elevation of *Per1* mRNA in response to light is a hallmark of the response of the mammalian molecular clockworks to light. Induction of *Per1* is required for light/GLU-induced phase shifts in both early (32) and late night (Fig. 5). Blockage of GLU-induced *Per1* accumulation in the presence of the CRE-decoy provides compelling evidence that CRE-mediated transcription contributes to increased levels of *Per1*. Whereas our data do not exclude a role for ATF-1 or ATF-2 interaction with CRE elements, a previous study indicates that ATF-1 and ATF-2 do not bind the CRE-element in the Per1 promoter (29). We also did not examine the role of CREM in the *Per1* promoter. However, because CREM inhibits CRE-mediated transcription, it is unlikely that induction of *Per1* mRNA is mediated by CREM.

Our data designate *Per1* as an integral component of the input pathway for light/GLU signaling and reveal a critical distinction between induction of *Per1* and *Per2*/*Tim*. ODN against *Per2* and *Tim* had no effect on GLU-induced phase shifts when applied in a 2-h window before and during the stimulus. *Per2*, which also contains a canonical CRE within its promoter (29), may also be induced by light (14, 33). Our data suggest that *Per2* is not required for light/GLU-induced phase

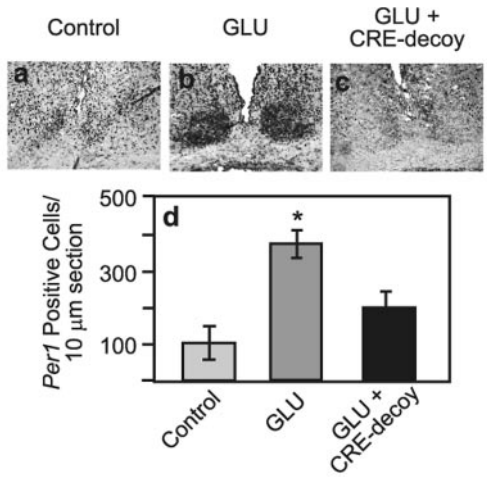

FIG. 6. **Effects of inhibiting CRE activation on GLU-induced *Per1* mRNA levels in the SCN at CT 20.** *a*, a digoxygenin-labeled cRNA probe detected low basal levels of *Per1* mRNA in control sections. *b*, GLU significantly elevated *Per1* mRNA 60 min after GLU treatment at CT 20. *c*, inhibition of CRE-mediated transcription blocked GLU-induced *Per1* at CT 20. *d*, for quantitation, positive cells were counted from one SCN in a single, midcaudal section by an experimenter blind to the treatment. *Bars* represent mean ± S.E. of four to six independent experiments. * represents statistically significant differences compared with control values at the same circadian time as determined by ANOVA ($p < 0.01$) with Tukey's *post hoc* analysis.

advance. CREB-142 knock-out mice (30), which show increased *Per2* despite a severely attenuated phase-shifting reponse to light, are consistent with our data and suggest different roles for *Per1 versus Per2*. Because elevation of *Tim* by light is restricted to early night (20), it is unlikely that *Tim* mRNA plays a role in the late night. We hypothesize that any light/GLU-induced alteration in *Per2* or *Tim* is a consequence of the reestablishment of the new circadian phase, which happens in less than 1 h in response to the phase-shifting event (34).

These data raise a fundamental question with regard to the role of *Per1* within the molecular clockwork. Is *Per1* part of the core clock mechanism or exclusively a component of an input or entrainment pathway? The absence of free-running circadian rhythms in mice lacking *Per2*, *Cry1*, and *Cry2* has established those genes as core elements in the mammalian clock (35–37). The data from *Per1* knock-out mice are less definitive. *Per1* knock-outs generated by two separate groups of investigators display rhythmicity, albeit with a shortened free-running period (38, 39). *Per1* knock-outs from a third group have disrupted rhythms only after an extended period in constant darkness (40). Thus, aggregate data argue for the placing of *Per1* on an input pathway leading from light/GLU. Interestingly, the overall behavior of *Per1* in the SCN is remarkably similar to that of several immediate early genes, especially the *fos* and *jun* families (26, 37, 41–43). The mRNAs for several *fos* and *jun* family members oscillate with a peak immediately proceeding that of *Per1*. Light responsiveness reveals additional similarity: nocturnal light causes rapid, transient fluctuations in *Per1* and *fos* and *jun* family members (11–15, 31, 37, 41), possibly via common CRE-elements in their promoters (13, 31, 33).

Cellular mechanisms that couple the light/GLU signal to activation of CREB and, subsequently, induction of *Per1* remain unclear. A number of kinases are known to phosphorylate CREB and activate CRE-mediated transcription. A recent study suggests that activation of cAMP/PKA and MAPK pathways can activate CRE-mediated induction of *Per1* in transfected human choriocarcinoma JEG3 cells (29). Caution must be employed when equating signal transduction in transfected cells to native SCN neurons that may or may not contain the same innate signaling elements and networks. Whereas our data clearly indicate that light/GLU phase shifts and *Per1* induction are completely blocked in the absence of CREB-activated transcription in the SCN, activation of PKA and MAPK are insufficient to account for these data. The MAPK pathway is activated by nocturnal light signals, but inhibition of MAPK only partially blocks light/GLU-induced phase shifts (16, 44). cAMP is elevated after nocturnal GLU stimulus, and inhibition of PKA blocks GLU-induced phase delays, but exogenous activation of PKA does not reset the clock at night; inhibition of PKA actually augments light/GLU-phase advances (16, 46). On the other hand, phosphorylation of CREB on Ser^{142}, a site identified as a substrate for only casein kinase II (47) and CaMKII (48–50), is required for light-induced phase shifting (30). However, inhibition of CaMK only attenuates light-induced phase shifts (51–53); effects of casein kinase II on CREB activation in the SCN and phase-shifts are unknown.

Thus, many of these signaling pathways cannot by themselves account fully for the transduction of the light response to the transcriptional apparatus. However, Ca^{2+} pathways that act via NO are entirely necessary. A Ca^{2+}-exclusive activation mechanism may provide a basis for neuron-specific/Ca^{2+}-selective induction of gene programs (54), such as light/GLU-induced phase shifting in the SCN. GLU, the neurotransmitter that conveys the light signal to the SCN, activates *N*-methyl-D-aspartate receptors to increase intracellular Ca^{2+} and eventually to release of nitric oxide. Nitric-oxide synthase inhibitors fully block light/GLU-induced phase shifts (2). In early night, GLU-induced NO release leads to ryanodine receptor-mediated Ca^{2+}-induced Ca^{2+} release, a step limited to and necessary for phase delay (5). In late night, GLU-induced NO release activates the cGMP/PKG signaling cascade, a pathway also requiring Ca^{2+} activation. Unlike inhibition of MAPK or CaMK, PKG inhibition *completely* blocks light/GLU-induced phase advances (3–5). Understanding PKG, MAPK, CaMK, and CREB signaling will elucidate their targets and interrelationships. Thus, signaling that can activate CREB and induce phase shifts in the SCN is complex, and the present data are incomplete.

Overall, our demonstration of critical roles for CRE activation and *Per1* production emphasize the roles these elements play in transforming the light/GLU signal into broad transcriptional activation of many genes (55). Many promoters contain CRE sites. Why should the CREs of *Per1* be the first activated and the gatekeepers of the genomic response to light? How do the multiplicity of signals that impinge on the *Per1* promoter not only activate it, but do so in a way that permits decoding of the light signal so that intensity and time-of-day are integrated in an adaptive response? The aggregate physiological data emerging from analysis of the integrated physiological response, phase-shifting in native tissue, argue for a central role for NO/Ca^{2+}-signaling via CREB activation of *Per1*. While multiple kinases have been identified as modulatory to CREB (PKA, MAPK, CaMK), their effects do not explain why CREB activation is essential. Nor do we know if additional regulatory elements in the *Per1* promoter are contributory. Thus, a multiplicity of intracellular signals, each activated by the impinging light signal, are required to activate the transcriptosome leading to elevation of *Per1* and, ultimately, resetting of the circadian clock. The importance of each of these signals remains the topic of future investigation.

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