Circadian Regulation of cAMP Response Element-mediated Gene Expression in the Suprachiasmatic Nuclei*

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Karl Obrietan‡, Soren Impey, Dave Smith, Jaime Athos, and Daniel R. Storm§
From the Department of Pharmacology, University of Washington, Seattle, Washington 98195-7280

A program of stringently-regulated gene expression is thought to be a fundamental component of the circadian clock. Although recent work has implicated a role for E-box-dependent transcription in circadian rhythmicity, the contribution of other enhancer elements has yet to be assessed. Here, we report that cells of the suprachiasmatic nuclei (SCN) exhibit a prominent circadian oscillation in cAMP response element (CRE)-mediated gene expression. Maximal reporter gene expression occurred from late-subjective night to mid-subjective day. Cycling of CRE-dependent transcription was not observed in other brain regions, including the suprachiasmatic nucleus and perifornix cortex. Levels of the phospho-active form of the transcription factor CREB (P-CREB) varied as a function of circadian time. Peak P-CREB levels occurred during the mid- to late-subjective night. Furthermore, photic stimulation during the subjective night, but not during the subjective day, triggered a marked increase in CRE-mediated gene expression in the SCN. Reporter gene experiments showed that activation of the p44/42 mitogen-activated protein kinase signaling cascade is required for Ca²⁺-dependent stimulation of CRE-mediated transcription in the SCN. These findings reveal the CREB/CRE transciptional pathway to be circadian-regulated within the SCN, and raise the possibility that this pathway provides signaling information essential for normal clock function.

In mammals, the suprachiasmatic nuclei (SCN)³ of the hypothalamus contain a circadian oscillator that functions as the major biological clock (1–3). The biorhythm generated by the SCN allows an organism to predict and coordinate its daily physiological processes to an approximate 24-h period. If the SCN are lesioned, there is a loss of physiological and behavioral circadian rhythms (4, 5). The most effective regulator of the endogenous clock is light; endogenous clock rhythmicity is entrained to the environmental light cycle by photic cues conveyed from the eyes to the SCN via the retinohypothalamic tract (RHT) (6). For example, if an animal receives a light flash during the dark phase of the day/night light cycle, the circadian rhythm is reset, or phase-shifted (7). Light-induced phase-shifting results from the synaptic release of glutamate from the RHT onto the SCN (8–10). Entrainment of the clock by light is thought to involve changes in gene expression. In support of this, several studies have shown that immediate early gene induction is triggered by light (11–13).

Recent work has revealed important information about specific proteins and transcriptional events essential for circadian rhythmicity. For example, CLOCK and BMAL1 proteins heterodimerize to form a transcription factor that binds the E-box enhancer element, resulting in mper1 gene expression (14). In Drosophila, PER/TIM dimers negatively regulate CLOCK-dependent transcription (15), thus forming a negative feedback loop. Mutations of any of these genes disrupts circadian rhythmicity (16–21), suggesting that this transcriptional loop is essential for normal clock function. Given these results, it is likely that the E-box enhancer element regulates the rhythmic expression of other genes within the SCN. However, the transcriptional activation of several circadian-regulated genes (vasopressin, brain-derived neurotrophic factor, Fos; Refs. 22–24) requires complex interactions of several different classes of enhancer elements (25–27), suggesting the involvement of different transcription pathways in circadian gene regulation within the SCN. The elucidation of these pathways will provide valuable insight into the series of coordinated transcriptional events underlying circadian rhythmicity.

Ostensibly, transcriptional pathways that contribute to SCN rhythmicity should have the capacity to integrate signaling information from a variety of stimuli, as well as possess properties that allow for its stringent regulation. One candidate is the CREB/CRE transcriptional pathway. This pathway has been shown to be activated by multiple kinases, including protein kinase A (PKA), Ca²⁺/calmodulin-dependent kinase, and mitogen-activated protein kinase (MAPK) (28–31). The CREB/CRE transcriptional pathway also has the capacity to integrate the activation of multiple signaling pathways and the strength of signal into striking variations in downstream gene transcription (32–35). Furthermore, CRE-mediated transcription can be rapidly repressed through a myriad of mechanisms, including inducible early cAMP repressor induction, phosphatase activation, or as a result of CREB heterodimerization with inhibitory transcription factors (32, 36). These unique functional properties led us to explore whether this transcriptional pathway plays a role in circadian rhythmicity. Toward this end, we used a mouse CRE-β-galactosidase transgenic reporter strain to monitor CRE-mediated transcription in vivo.

**Experimental Procedures**

*This paper is available on line at http://www.jbc.org*
dark (L/D) cycle for at least 7 days before being transferred to constant darkness (D/D). Light intensity during L was ~400 lux. Food and water were available ad libitum throughout the experiment. Circadian activity rhythms were monitored using motion sensors connected to VitalView software (Mini-Mitter Co., Sunriver, OR).

For immunohistochemical analysis, the CRE/β-galactosidase rhythmicity, mice (n = 3–6/time point) kept in D/D for 6 days were sacrificed at 4-h intervals over a 24-h period. For the Western analysis of CRE/β-galactosidase rhythmicity, mice (4/time point) kept in D/D for 6 days were sacrificed at 6-h intervals over a 24-h period. For photic stimulation experiments, animals were exposed to light (400 lux) at CT 16.5, CT 22.5, and CT 23.5. Animals were then returned to constant darkness. Eight hours after light treatment, animals were sacrificed and brain slices were processed for β-galactosidase immunoreactivity.

**Tissue Collection**—Cervical dislocation followed by decapitation allowed for the rapid removal of the brain, which was immediately placed in ice-cold Dulbecco’s modified Eagle’s medium (pH 7.4) and cut into 400-μm coronal sections with a vibratome. For P-CREB experiments, animals were sacrificed and their brains were removed under dim red illumination < 10 lux. For immunohistochemistry, sections were placed in a 6% formaldehyde/phosphate-buffered saline (PBS) solution for 4–6 h at room temperature. Sections were then cryoprotected with 30% sucrose for at least 12 h. Thin (35–40 μm) sections were cut through the SCN using a sliding microtome. For Western analysis, 400-μm coronal brain slices were quick-frozen onto glass coverslips. The SCN and lateral hypothalamic areas were then excised with the use of a dissecting microscope. Tissue was stored at ~70 °C.

**Immunohistochemistry**—For β-galactosidase immunolabeling, free-floating sections were blocked for 2 h in 1% normal goat serum and 10% bovine serum albumin in PBS with 0.1% Triton X-100 (PBST). After blocking, sections were incubated overnight at 4 °C with an affinity-purified polyclonal β-galactosidase antibody raised in rabbit (1:1000 final dilution, Cappel) in PBST, and 2.5% bovine serum albumin. The tissue was then incubated for 6 h with a lissamine-rodamine-conjugated secondary antibody raised in goat and directed against rabbit IgG (2 μg/ml final dilution, Jackson Laboratories) in PBST containing 2.5% bovine serum albumin. To ensure minimal immunolabeling variability for endogenous rhythm experiments, all samples were processed concurrently.

For immunolabeling against the Ser-133 phosphorylated form of CREB, free-floating sections were initially blocked as described above, except that PBST also contained NaF (1 mM). Tissue was then incubated overnight with phospho-specific CREB antibody (1:500, New England Biolabs). On Western blots, this affinity-purified antibody specifically recognized the phosphorylated forms of CREB-related proteins ATM-1 and CREM. The tissue was then incubated for 6 h with a fluorescein-conjugated secondary antibody in goat and directed against rabbit IgG (2 μg/ml final dilution, Jackson Laboratories) in PBST containing 2.5% bovine serum albumin. To ensure minimal immunolabeling variability for endogenous rhythm experiments, all samples were processed concurrently.

For Western analysis, the region containing the SCN was excised from postnatal day 1 rat brain. Using the optic chiasm for orientation, an ~1-mm cube of tissue was removed. Tissue was washed, triturated, and plated as described above.

**Transfections**—SCN cells (1 × 10⁶ cells/well) were transfected with DORPER (Roche Molecular Biochemicals). On day 5 in culture, cells were treated with a complex of 0.6 μg of DNA and 6 μg of DOPSER, as described by the manufacturer, in 100 μl of minimal essential medium. After 6 h, the DNA-DORPER complex was replaced with conditioned tissue culture medium. In some experiments pcDNA3.1-LacZ (Invitrogen) was added (4 ng/well) for transfection efficiency normalization. Transfection efficiency typically did not vary by more than 10% for different transfections. The following plasmids have been described previously: dominant-negative MEK S222A (37), dominant-negative PKA, and the CRE-luciferase construct (38).

**Reporter Assays**—After removal of media, 150 μl of cell lysis buffer (0.2% Triton X-100, 4 mM ATP, 6 mM MgCl₂, 100 mM potassium phosphate, pH 7.8) was added to each well. Following one freeze/thaw cycle, luciferase activity was measured, as described in Ref. 39, and β-galactosidase activity was assayed, as described in Ref. 30, using a luminometer (Berthold).

**Western Blots**—Excised brain tissue was resuspended in 100 μl of buffer H (50 mM β-glycerophosphate, 15 mM EGTA, 0.1 mM Na₃VO₄, 1 mM dithiothreitol, 10 μg/ml aprotinin, 2 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and sonicated for 15 s. 100 μl of 5× sample buffer was then added, and the samples were heated to 90 °C for 10 min. Extracts were then vortexed (20 s) and centrifuged (8 min at 13,000 × g). 40 μl of extract was loaded onto an 8% SDS-PAGE gel and electrophoresed using standard procedures. For cultured SCN neurons, agonist-treated cells were lysed in hot (90 °C) 2.5× sample buffer (80 μl/dish). After vortexing and centrifugation (7 min at 13,000 × g), 30 μl of extract was loaded onto a 12% SDS-PAGE gel and electrophoresed using standard procedures. Once transblotted, membranes (Immobilon: Millipore) were blocked with 10% powdered milk in PBS. Membrane were then incubated overnight at 4 °C in PBST with primary rabbit antibody against β-galactosidase (1:500, 5Prime Inc.) or P-CREB (1:1000, New England Biolabs), or phospho-44/42 (P-ERK) specific antibody (1:1000 final dilution, New England Biolabs). Membranes were then treated with a goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (1:2000, Cappel). Immunoreactivity was developed using the Western-star alkaline phosphatase detection system (Tropix). A second cultured SCN cell membrane and the same β-galactosidase membrane were then probed with a monoclonal anti-CREB antibody (1:1000, Santa Cruz), and labeled with a rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase. The Renaissance chemiluminescence detection reagent (NEN over the digitized SCN image to determine mean fluorescent intensity. A basal level of background fluorescence was determined for each section by acquiring a mean fluorescent signal from the lateral hypothalamic area. This value was then subtracted from the value for the corresponding SCN. Imaging and data analysis were performed “blind.”
Life Science Products) was used to visualize immunoreactivity. Membranes were washed six times with a 5% milk/PBST solution after each antibody treatment.

RESULTS

Circadian Oscillation in CRE-mediated Gene Expression—If the CRE enhancer element plays a role in endogenous clock rhythmicity, then its regulation must fulfill several criteria. In the absence of photic cues, gene expression mediated by activation at the CRE must be regulated in a rhythmic manner. Furthermore, this rhythmicity should be observed within the SCN, and have a period close to 24 h. To address whether CRE-mediated gene expression shows circadian oscillations in the SCN, animals transgenic for the CRE-β-galactosidase reporter (33) were entrained to a 12-h L/D cycle, then placed in total darkness (D/D). Under this condition, circadian rhythmicity is controlled by the endogenous pacemaker. After 6 days in D/D, animals were sacrificed every 4 h over a 24-h cycle and coronal sections containing the SCN were labeled immunohistochemically for the expression of the reporter gene. Quantitation revealed a significant (analysis of variance: \( p < 0.001, F = 7.16 \)) circadian variation in CRE-mediated gene expression in the SCN (Fig. 1A). Maximal gene expression was observed from early- to mid-subjective day (Fig. 1B). Levels of the reporter gene dropped markedly from mid-subjective day to mid-subjective night, then rose during late-subjective night. Significant variations in CRE-mediated gene expression were not observed in other hypothalamic nuclei (supraoptic, Fig. 1C) or in other brain regions (piriform cortex and primary motor cortex, data not shown).

Circadian regulation of CRE-mediated transcription was also analyzed by Western blot. As above, transgenic mice were sacrificed over the circadian cycle. Within the SCN, the expression of the CRE-driven β-galactosidase reporter construct oscillated over the circadian cycle. Relatively high levels of reporter were observed during late-subjective night and early-subjective day (Fig. 2A). Reporter expression within the lateral hypothalamus did not significantly fluctuate over a 24-h period (Fig. 2B). Probing the same membranes revealed that levels of the transcription factor CREB did not significantly vary as a function of circadian time in either the SCN or the lateral hypothalamus. Together, these data reveal the presence of an endogenous, circadian, oscillation of CRE-dependent transcription localized to the SCN.

One necessary step proximal to the induction of CRE-mediated gene expression is the phosphorylation of CREB at Ser-133. Given the circadian oscillation in CRE-mediated transcription, it is controlled by the endogenous pacemaker. After 6 days in D/D, animals were sacrificed every 4 h over a 24-h cycle and coronal sections containing the SCN were labeled immunohistochemically for the expression of the reporter gene. Quantitation revealed a significant (analysis of variance: \( p < 0.001, F = 7.16 \)) circadian variation in CRE-mediated gene expression in the SCN (Fig. 1A). Maximal gene expression was observed from early- to mid-subjective day (Fig. 1B). Levels of the reporter gene dropped markedly from mid-subjective day to mid-subjective night, then rose during late-subjective night. Significant variations in CRE-mediated gene expression were not observed in other hypothalamic nuclei (supraoptic, Fig. 1C) or in other brain regions (piriform cortex and primary motor cortex, data not shown).

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FIG. 1. Circadian oscillations in CRE-mediated gene expression under free-running conditions. C57/BL6 mice transgenic for the CRE-β-galactosidase construct were initially entrained to a 12-h L/D cycle, then placed in constant darkness (D/D). After 6 days in D/D, animals were sacrificed every 4 h, and SCN-containing tissue was immunolabeled for the expression of β-galactosidase. A, color-coded confocal images of coronal sections through the central SCN at different CT during the subjective day and night. CT 0 defines the beginning of subjective day; CT 12 defines the beginning of subjective night. Dark red fluorescent hues correspond to weak reporter gene expression; yellow fluorescent hues correspond to strong reporter gene expression. The peak in CRE-mediated gene expression was observed at CT 6; minimal levels of reporter gene were observed at CT 18. B, graphical representation of the relative level of reporter gene in the SCN is shown for each time point. A minimum of three animals were analyzed for each time point. Error bars in B denote S.E. OC, optic chiasm; 3V, third ventricle.
stimulation to CRE-dependent transcription. To assess the contribution of the MAPK cascade, SCN neurons were cotransfected with the CRE-reporter and a dominant-negative interfering form of MEK, an upstream activator of MAPK. This completely inhibited K+- and NMDA-stimulated gene expression (Fig. 5E). Similar results were obtained with PD 98059, a specific inhibitor of MEK (data not shown). Forskolin-activation of CRE-dependent transcription was slightly reduced by specific inhibitor of MEK (data not shown). Forskolin-activation of CRE-dependent transcription was slightly reduced by specific inhibitor of MEK (data not shown).

B. Significant circadian variations in β-galactosidase or CREB were not observed in tissue from the lateral hypothalamus.

**DISCUSSION**

The results presented here show that the SCN exhibit a prominent circadian oscillation in CRE-mediated gene expression in dark-adapted animals. Furthermore, photic stimulation during the subjective night triggered CRE-dependent transcription, whereas light treatment during the subjective day was not effective. Transient transfection experiment revealed that the ERK/MAPK pathway activity is essential for Ca2+-dependent stimulation of CRE-mediated transcription in SCN cells. Together, these results provide the first evidence linking the CREB/CRE transcriptional pathway to endogenous timing mechanisms.

**Photic Stimulation of CRE-mediated Transcription**—There is a large body of evidence suggesting that the phase-shifting effects of light result from new protein synthesis. However, the enhancer elements mediating transcriptional activation have not been thoroughly characterized. Our data suggest that the CRE may play a central role in the ability of light to activate gene expression and, in turn, phase-shift the clock. In support of this, we show that light induces CRE-mediated gene expression. Additionally, the finding that a brief 5-min light treatment triggers a highly localized induction of CRE-mediated gene expression correlates with a duration of light shown to phase-shift overt activity rhythms (11). The general pattern of CRE-mediated gene expression, both temporally and anatomically, parallels the induction of several immediate early genes thought to be involved in phase-shifting the clock (11–13). Given that the promoters for these immediate early genes (including c-fos, junB, and NGFI-B) contain at least one CRE (48–50), it is reasonable to hypothesize that the CRE may play a central role in mediating the ability of light to trigger immediate early gene induction. Our results showing that light induces CRE-regulated gene expression in a phase-restricted manner are consistent with work showing that light-induced CREB phosphorylation is restricted to the subjective night (40). These results suggest that stringent regulation of the CREB/CRE transcriptional pathway during the day may be a critical element that confers the phase-restricted phase-shifting effects of light.

**Signaling Pathways**—A role for Ca2+ in photic entrainment of the clock has been suggested by the finding that light-induced phase-shifts require NMDA receptor activation (8). Given the evidence identifying a transcriptional component to light-induced phase shifts, we assessed the signaling mechanisms that couple Ca2+ to gene expression in the SCN. In primary cultures of SCN neurons, we found that increasing cytosolic Ca2+, either through high K+ or NMDA administration, resulted in enhanced CRE-dependent transcription. Interestingly, besides glutamate, RHT nerve terminals also express PACAP (51), a peptide capable of stimu-
Lating cAMP production. Light-induced release of transmitters capable of stimulating Ca\(^{2+}\) and cAMP pathways may be important for robust activation of the CREB/CRE transcriptional pathway in the SCN. Along these lines, we have observed that modest stimulation of cAMP signaling pathways that, alone, was unable to increase CRE-mediated transcription, potently augmented Ca\(^{2+}\)-dependent CRE-mediated transcription in the SCN.\(^2\)

CRE-mediated gene expression is regulated by a variety of cellular stimuli acting through a number of different kinase

\(^2\)K. Obrietan and D. R. Storm, unpublished observation.
Our work shows that Ca\textsuperscript{2+} stimulation of CRE-mediated transcription was dependent upon activation of the MAPK signaling pathway. Cotransfection with a dominant-negative interfering form of MEK or treatment with the MEK inhibitor PD 98059 blocked Ca\textsuperscript{2+}-stimulated gene expression. Coupling of Ca\textsuperscript{2+} to activation of the MAPK cascade has been shown to be dependent upon an enhancement of Ras-GTPase catalytic activity (52). Ca\textsuperscript{2+}-dependent Ras activation is triggered by a variety of signaling intermediates, including calmodulin kinases (42, 53), Src (54), Ras-GRF (55), and the epidermal growth factor receptor (56). The MAPK pathway gains access to the nucleus via the activation-dependent nuclear translocation of ERK. ERK has been shown to trigger CREB phosphorylation through activation of RSK 1, 2, and 3, all of which are CREB kinases (57). A requirement for MAPK activity was also revealed by the observation that Ca\textsuperscript{2+}-induced CREB phosphorylation was attenuated by the MEK inhibitor PD 98059. In addition, forskolin-stimulated CREB phosphorylation was reduced by PD 98059, indicating that the cAMP-dependent signaling pathway acts, in part, via activation of the MAPK cascade. Along these lines, cAMP has been shown to activate the MAPK cascade in hippocampal and cortical neurons (31).

Within the SCN, elevated cytosolic Ca\textsuperscript{2+} has been shown to trigger CREB phosphorylation through a mechanism requiring the production of nitric oxide (58). Recently, Ca\textsuperscript{2+}-dependent nitric oxide activation was shown to elicit ERK phosphorylation in neuronal cultures (59), thus providing a pathway by which light-induced nitric oxide production could trigger sequential MAPK activation. Although other transcription factors may be activated by increased cytosolic Ca\textsuperscript{2+}, it is intriguing to note that nitric oxide synthetase antagonism blocks both glutamate-induced CREB-phosphorylation and glutamate-induced phase-shifts (58, 60). This suggests a strong correlation between clock phase-shifting and the activation of a transcriptional pathway involved in triggering CRE-mediated gene expression. Taken together, the results presented here provide a mechanism by which glutamate receptor stimulation leads to CRE-dependent transcription.

**Endogenous Rhythmicity**—Rhythmic transcription appears to be central to maintaining circadian timekeeping. For example, a mutated form of the putative transcription factor CLOCK abolishes circadian activity rhythms under D/D conditions (16). Our data show that CRE-mediated gene expression is regulated in a circadian manner under free-running conditions. Levels of reporter protein began to rise during the late subjective night and peaked during mid-subjective day. Given the approximately 6 h between transcription and maximal reporter expression, one may deduce that induction of CRE-mediated gene expression is restricted to the subjective night, and possibly the early subjective morning. This result indicates that the phase-dependent regulation of endogenous CRE-mediated gene rhythmicity overlaps with the phase dependence of light inducible CRE-mediated gene expression, suggesting that a similar signaling mechanism may govern both processes.

It is unclear why the light-evoked stimulation of CRE-mediated gene expression was greater than the peak in reporter expression resulting from endogenous pacemaker activity. Possible explanations may include more robust activation of signaling pathways by light or synergism between signaling pathways that are activated by light and the endogenous clock. Interestingly, we recently reported that light triggers MAPK activation in the SCN, and that MAPK activity is regulated in a circadian manner in the SCN under D/D conditions (61). Recent work performed in Drosophila has revealed a robust circadian oscillation in CRE-mediated gene expression and an
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interdependence between rhythmic CRE-dependent transcription and period oscillations, indicating that the CRE transcriptional pathway is a component of the circadian clock (74). Further work in mammalian systems may reveal a similar interaction between the CRE transcriptional pathway and period homolog rhythmicity.

Circadian variations in the phosphorylation state of the transcription factor CREB at Ser-133 were also observed. The peak in the P-CREB rhythm preceded the reporter gene peak by approximately 6 h, an expected time lag for transcriptional activation and maximal protein expression. The circadian P-CREB rhythm does not result from CREB oscillations, since levels of CREB in the SCN were stable at subjective day versus night time points. This result suggests that circadian oscillations in P-CREB result from circadian fluctuations in the activity state of CREB kinases or phosphatases.

It is unclear how rhythmic CRE-mediated gene expression is maintained under free-running conditions. However there are several plausible explanations. Given that extracellular membrane receptor-mediated signaling events regulate CRE-mediated gene expression, one may expect to observe circadian changes in the level of extracellular transmitters capable of eliciting CRE-mediated gene expression. In support of this idea, circadian variations in the concentrations of excitatory amino acids have been observed within the region of the SCN under free-running conditions, and in slice preparations (62–64). Circadian oscillations in CRE-dependent transcription also may be a result of an inherent rhythmic transcriptional program of SCN pacemaker cells. Another possibility is that the amount or ratio of CREB heterodimerization partners within the SCN varies over the circadian cycle. In support of this idea, CREM-deficient mice do not express circadian locomotor activity.

The circadian expression of a variety of genes within the SCN may result from circadian CRE-dependent transcription. For example, the promoters for several peptides that show circadian oscillations at the mRNA or protein level in the SCN, including vasopressin, somatostatin, and, during development, vasoactive intestinal peptide (22, 65, 66), contain one or more CREs (67–69). Interestingly, these peptides have the capacity to both modulate CRE-dependent transcription, either positively or negatively (70–72), and to alter rhythmicity when added to the SCN (47, 73). Conceivably, the circadian expression of these proteins could be generated by temporally overlapping feedback loops that either activate or inhibit CRE-mediated transcription. Based on the results presented here, we propose that the CRE transcriptional pathway plays an important role in orchestrating the series of transcriptional events essential for both endogenous clock rhythmicity and the ability of light to phase-shift the clock.

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REFERENCES

1. Hastings, M. H., Best, J. D., Ebling, F. J., Maywood, E. S., McNulty, S., Schurov, I., Selvage, D., Sloper, P., and Smith, K. L. (1996) Brain Res. 111, 147–174
2. Miller, J. D., Morin, L. P., Schwartz, W. J., and Moore, R. Y. (1996) Sleep 19, 614–667
3. van den Pol, A. N., and Dudek F. E. (1993) Neuron 9, 793–811
4. Moore, R. Y., and Eichler, V. B. (1972) Brain Res. 42, 201–206
5. Stephan, F. K., and Zucker, I. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1583–1586
6. Moore, R. Y., and Lenn, N. J. (1972) J. Comp. Physiol. 146, 1–14
7. Daan, S., and Pittendrigh, C. S. (1976) J. Comp. Physiol. 106, 253–266

P. Sassone Corsi, personal communication.
14. Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998) Science 280, 1599–1603

15. Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steves, T. D. L., Wacht, C. J., Takahashi, J. S., and Kay, S. A. (1998) Science 280, 719–725

16. Vitaterna, M. H., King, D. P., Chang, A. M., Kornhauser, J. M., Lowrey, P. L., McDonald, J. D., Dove, W. F., Pinto, L. H., Turek, F. W., and Takahashi, J. S. (1994) Science 264, 1237–1240

17. Konopka, R. J., and Benzer, S. (1971) Science 171, 255–262

18. Kyriacou, C. P., and Hall, J. C. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6729–6733

19. Konopka, R. J., and Benzer, S. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 2112–2116

20. Sehgal, A., Price, J. I., Man, B., and Young, M. W. (1994) Science 263, 1603–1606

21. Allada, R., White, N. E., So, W. V., Hall, J. C., and Rosbash, M. (1998) Cell 93, 791–804

22. Kalsbeek, A., Bijls, R., Engelmann, M., Wotjak, C., and Landgraf, R. (1995) Brain Res. 692, 75–82

23. Prosser, R. A., Macdonald, E. S., and Heller, H. C. (1994) Mol. Brain Res. 25, 151–156

24. Liang, F. Q., Walline, R., and Earnest, D. J. (1998) Neuron 24, 2112–2116

25. Robertson, L. M., Kerppola, T. K., Vendrell, M., Luk, D., Smeyne, R. J., Deloulme, J. C., Chan, G., and Storm, D. R. (1998) Neuron 24, 273–280

26. Xing, J., Kornhauser, J. M., Xia, Z., Thiele, E. A., and Greenberg, M. E. (1998) Neuron 24, 693–700

27. Iwasaki, Y., Oiso, Y., Saito, H., and Majzoub, J. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 709–716

28. Xing, J., Konopka, R. J., Xia, Z., Tiele, E. A., and Greenberg, M. E. (1998) Mol. Cell. Biol. 18, 1466–1455

29. Farnsworth, C. L., Freshney, N. W., Rosen, L. B., Ghosh, A., Greenberg, M. E., and Feig, L. A. (1995) Nature 376, 524–527

30. Rosen, L. B., and Greenberg, M. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1113–1118

31. Impey, S., Obrietan, K., and Storm, D. R. (1998) Nat. Neurosci. 1, R579–R585

32. Obrietan, K., Impey, S., and Storm, D. R. (1998) Neuron 21, 869–883

33. Bito, H., Deisseroth, K., and Tsien, R. W. (1996) Cell 87, 1203–1214

34. Impiey, S., Mark, S., Villacres, E. C., Poser, S., Chavkin, C., and Storm, D. R. (1996) Neuron 16, 973–982

35. Sheng, M., McFadden, K., and Greenberg, M. E. (1990) Neuron 4, 571–782

36. Tan, Y., Low, K. G., Boccia, C., Grossman, J., and Comb, M. J. (1994) Mol. Cell. Biol. 14, 7546–7556

37. Foulkes, N. S., and Sassone-Corsi, P. (1996) Biophys. Acta 1308, F101–F121

38. Seger, R., Seger, D., Reska, A. A., Munar, E. S., Eldar-Finkelman, H., Dobrowolska, G., Jensen, A., Campbell, J. S., Fischer, E. H., and Krebs, E. G. (1994) J. Biol. Chem. 269, 25699–25709

39. Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994) Mol. Cell. Biol. 14, 6107–6116

40. de Wet, J. R., Wood, K. V., Deluca, M., Helinski, D. R., and Subramaniam, S. (1997) Mol. Cell. Biol. 7, 725–737

41. Brindel, P., Nakajima, T., and Montminy, M. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10521–10525

42. Konopka, R. J., and Benzer, S. (1971) Science 175, 1133–1144

43. Schwaninger, M., Blume, R., Kruger, M., Lux, G., Oetjen, E., and Kneple, W. (1995) J. Biol. Chem. 270, 4224–4235

44. Schwaninger, M., Blume, R., Kruger, M., Lux, G., Oetjen, E., and Kneple, W. (1995) J. Biol. Chem. 270, 4224–4235

45. Deeb, J. P., Chen, D., Weber, E. T., Fainman, L. E., Rea, M. A., and Gillette, M. E. (1994) Science 266, 1713–1717

46. Obrietan, K., Impey, S., and Storm, D. R. (1998) Nat. Neurosci. 1, 693–700

47. Glass, J. D., Hauser, E. L., Blank, J. L., Selim, M., and Rea, M. A. (1993) Am. J. Physiol. 265, R504–R511

48. Honma, S., Katsuno, Y., Shinohara, K., Abe, H., and Honma, K. (1996) Am. J. Physiol. 271, R579–R585

49. Ban, Y., Shigeyoshi, Y., and Okamura, H. (1997) J. Neurosci. 17, 3920–3931

50. Yang, J., Tominaga, K., Otori, Y., Fukuhara, C., Tokumasu, A., and Inouye, S. (1998) Neuron 20, 659–670

51. Ban, Y., Shigeyoshi, Y., and Okamura, H. (1997) J. Neurosci. 17, 3920–3931

52. Deutsch, P., Hoeffer, J. P., Jameson, J. L., Lin, J. C., and Habener, J. F. (1988) J. Biol. Chem. 263, 18466–18472

53. Montminy, M. R., and Bilezikjian, L. M. (1987) Nature 328, 175–178

54. Parry, K. Adan, R. A., Carter, D. A., Seab, V., Burbach, J. P., and Murphy, D. (1992) J. Biol. Chem. 267, 21746–21752

55. Beluscak, J., and Schwaninger, M. (1995) J. Biol. Chem. 270, 4224–4235

56. Shinohara, K., Honma, S., Tominaga, K., Otori, Y., Fukuhara, C., Tokumasu, A., and Inouye, S. (1994) Mol. Cell. Neurosci. 5, 97–102

57. Deutsch, P., Hoeffer, J. P., Jameson, J. L., Lin, J. C., and Habener, J. F. (1988) J. Biol. Chem. 263, 18466–18472

58. Montminy, M. R., and Bilezikjian, L. M. (1987) Nature 328, 175–178

59. Parry, K. Adan, R. A., Carter, D. A., Seab, V., Burbach, J. P., and Murphy, D. (1992) J. Biol. Chem. 267, 21746–21752

60. Beluscak, J., and Schwaninger, M. (1995) J. Biol. Chem. 270, 4224–4235

61. Beluscak, J., and Schwaninger, M. (1995) J. Biol. Chem. 270, 4224–4235