Resetting the Biological Clock: Mediation of Nocturnal CREB Phosphorylation via Light, Glutamate, and Nitric Oxide

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Synchronization between the environmental lighting cycle and the biological clock in the suprachiasmatic nucleus (SCN) is correlated with phosphorylation of the Ca^{2+}/cAMP response element binding protein (CREB) at the transcriptional activating site Ser^{133}. Mechanisms mediating the formation of phospho-CREB (P-CREB) and their relation to clock resetting are unknown. To address these issues, we probed the signaling pathway between light and P-CREB. Nocturnal light rapidly and transiently induced P-CREB-like immunoreactivity (P-CREB-lir) in the rat SCN. Glutamate (Glu) or nitric oxide (NO) donor administration *in vitro* also induced P-CREB-lir in SCN neurons only during subjective night. Clock-controlled sensitivity to phase resetting by light, Glu, and NO is similarly restricted to subjective night. The effects of NMDA and nitric oxide synthase (NOS) antagonists on Glu-mediated induction of P-CREB-lir paralleled their inhibition of phase shifting. Significantly, among neurons in which P-CREB-lir was induced by light were NADPH-diaphorase-positive neurons of the SCN's retinorecipient area. Glu treatment increased the intensity of a 43 kDa band recognized by anti-P-CREB antibodies in subjective night but not day, whereas anti-αCREB-lir of this band remained constant between night and day. Inhibition of NOS during Glu stimulation diminished the anti-P-CREB-lir of this 43 kDa band. Together, these data couple nocturnal light, Glu, NMDA receptor activation and NO signaling to CREB phosphorylation in the transduction of brief environmental light stimulation of the retina into molecular changes in the SCN resulting in phase resetting of the biological clock.

Key words: suprachiasmatic nucleus; circadian rhythm; glutamate; NMDA; nitric oxide; CREB phosphorylation; immunocytochemistry; Western blot; nuclear protein extraction; diaphorase; APV; L-NAME; D-NAME; SNAP

The daily rhythms of life are generated by a biological clock, which in mammals resides within the suprachiasmatic nucleus (SCN) of the hypothalamus (Meijer and Rietveld, 1989; Morin, 1994). Although the time-generating mechanism is endogenous to the SCN, the dominant regulator of circadian clock phasing is environmental light. Photic signals are communicated via the retinohypothalamic tract, a direct neural projection from the retina to the SCN (Moore and Lenn, 1972; Johnson et al., 1988). The clock restricts its own sensitivity to stimulation (Gillette et al., 1995) so that only nocturnal light adjusts its timing, causing phase delays in early night and phase advances in late night (DeCoursey, 1960; Daan and Pittendrigh, 1976; Takahashi and Zatz, 1982; Summers et al., 1984). This ensures that daily behavioral rhythms are synchronized appropriately to phases of the environmental cycle of darkness and light. We sought to discriminate the multiple events linking sensory stimulation with SCN changes that orchestrate these behavioral modulations.

Important features of the photic phase shifting process are (1) the brevity of the light pulse that can permanently reset the near 24 hr cycle of circadian clocks (DeCoursey, 1960; Daan and Pittendrigh, 1976; Takahashi and Zatz, 1982) and (2) alterations of transcriptional elements (Ginty et al., 1993). Within 2 min, a phase-resetting light stimulus induces transcriptional changes in a state variable, the *frequency* gene, of the *Neurospora* clock (Crosthwaite et al., 1995). In the nervous system, too, long-lasting changes induced by a brief stimulus often involve the alteration of gene expression (Goelet et al., 1986; Montarolo et al., 1986; Morgan and Curran, 1989; Sheng and Greenberg, 1990; Alberini et al., 1994). Induction of immediate-early genes, especially members of the *fos* and *jun* families, occurs in the SCN within 1 hr of a photic stimulus that induces phase shifts of circadian rhythms (Rea, 1989; Rusak et al., 1990; Kornhauser et al., 1992; Takeuchi et al., 1993). Neurotransmission is coupled to gene induction in neurons via signaling cascades that activate DNA-binding proteins through transient phosphorylation of transcriptional activating amino acid residues. Brief exposure of hamsters to light at night induces phosphorylation of such a protein, cAMP response element binding protein (CREB), at its transactivation site; Ser^{133}-phosphorylated CREB (P-CREB) appears in the SCN within 5 min on light exposure (Ginty et al., 1993). This duration of light induces robust phase shifts of the circadian rhythm of locomotor activity in the days after stimulation. Thus, P-CREB is the earliest sign in the SCN of transcriptional activation by photic stimulation that leads to adjustments in 24 hr timing.

Although the sequence of events by which light signals P-CREB formation is unknown, essential components of the pathway mediating light-stimulated phase resetting have been identified. Light induces clock resetting through an excitatory signal transduction pathway mediated by glutamate (Glu), NMDA receptor activation, stimulation of nitric oxide synthase (NOS), and intercellular movement of nitric oxide (NO) (Ding et al., 1994b; Shibata et al., 1994; Shirakawa and Moore, 1994; Watanabe et al., 1994). In cultured hippocampal neurons and PC-12 cells, Glu activation of NMDA receptors with subsequent Ca^{2+} influx rapidly induces phosphorylation of CREB (Bading et al., 1993; Gallin and Greenberg, 1995; Ghosh and Greenberg, 1995). Because light triggers P-CREB in the SCN and the Glu/NO pathway mediates light-induced phase shifts, we examined the hypothesis that Glu and NO are components of the signal transduction cascade that activates CREB in the circadian clock.

To selectively probe elements regulating CREB phosphorylation, we compared the response of the SCN *in vivo* to light with that *in vitro* to specific reagents affecting Glu and NO pathways. We used the rat SCN in a hypothalamic brain slice, a preparation in which the circadian clock persists for 3 d (Gillette, 1991). The mean firing frequency of the population of SCN neurons undergoes a 24 hr oscillation *in vitro* (Green and Gillette, 1982) that matches the pattern of SCN neuronal activity *in vivo* (Inouye and Kawamura, 1979, 1982). Likewise, the SCN clock *in vitro* continues to regulate its own sensitivity to stimuli that can adjust its timing over the circadian cycle (Ding et al., 1994b; Gillette et al., 1995; Gillette, 1996).

MATERIALS AND METHODS

Brain slice preparation and electrophysiological recordings of the SCN circadian neuronal activity. The detailed description of this method has been reported previously (Gillette, 1991; Ding et al., 1994b). Briefly, a 500-μm-thick coronal hypothalamic brain slice containing the paired SCN was prepared at least 2 hr before the onset of the dark phase from 7- to 10-week-old, inbred Long–Evans rats housed in a 12:12 hr light/dark cycle. The brain slices survived for up to 3 d *in vitro* with continuous perifusion of Earle's Essential Balanced Salt Solution (EBSS, Life Technologies, Gaithersburg, MD), supplemented with 24.6 mM glucose, 26.2 mM sodium bicarbonate, and 5 mg/ml of gentamicin, and saturated with 95% O_2/5% CO_2 at 37°C, pH 7.4. The single-unit activity of the SCN neurons was recorded extracellularly with a glass microelectrode, and running means were calculated to determine the time-of-peak activity. The unperturbed sinusoidal pattern of neuronal activity is predictably high in the day and low during the night, peaking at mid-day at approximately circadian time 7 (CT 7) (Prosser and Gillette, 1989). The onset of the light phase of the entraining light/dark cycle of the brain slice donor was designated as CT 0. Thus, the time-of-peak of the neuronal firing rate can be used as a reliable assessment of the phase of the circadian rhythm (Gillette et al., 1995). For treatment of the brain slice, the perifusion pump was stopped, and a 0.2 μl microdrop of a test substance dissolved in EBSS was applied bilaterally to the SCN for 10 min before rinsing with EBSS and resuming pumping with normal medium. To evaluate potential blockers of the stimulus, the bathing medium was replaced with antagonists dissolved in EBSS, pH 7.4, for 20 min before the phase-shifting stimulus was applied to the SCN. To determine the phase of the circadian rhythm, the time-of-peak neuronal activity was assessed for the following 1 or 2 d *in vitro*.

Immunocytochemistry and histochemistry. We compared the sensitivity of this biological clock to treatments during the subjective day, when the clock is insensitive to light-induced resetting, with its responsiveness during the subjective night. Reagents were applied directly to the SCN *in vitro*, and their efficacy was assessed with reference to that of light *in vivo*. The appearance of P-CREB in the tissue was assayed using an antibody (anti-P-CREB) that recognizes the peptide sequence containing phosphorylated Ser[133]. This sequence is found within the transcriptional activating domain of the CREB family of transcription factors αCREB, CREM, and ATF-1 (Ginty et al., 1993; Ghosh et al., 1994).

To assess Glu-induced P-CREB-like immunoreactivity (P-CREB-lir) *in vitro*, Glu was applied to the SCN for 10 min, after which the brain slices were placed in 4% paraformaldehyde for 12–18 hr at 4°C. The slices were then transferred to a cryoprotectant (15% sucrose in PBS) for 24 hr before sectioning at 20 μm by cryostat at −15°C; sections were affixed to gelatin-coated microscope slides and stored at −15°C. For light-induced P-CREB-lir, animals were exposed to 150 lux of white light for 10 min at CT 19. After appropriate durations, animals were deeply anesthetized with sodium pentobarbital (75 mg/kg, i.p.) and perfused intracardially with 60 ml of chilled PBS followed by 600 ml of chilled 4% paraformaldehyde. For immunocytochemical detection of P-CREB, the tissue sections were rinsed with PBS and then incubated for 1 hr at room temperature with 0.3% Triton X-100 and 1% heat-inactivated goat serum to permeabilize the lipid membrane and block nonspecific binding sites, respectively. The sections were then incubated for 18–24 hr at 4°C with affinity-purified polyclonal anti-P-CREB antibody diluted 1:1000 in PBS with 0.3% Triton X-100 detergent. The avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) was used with the glucose oxidase reaction as the peroxide generator to form an insoluble brown DAB product localizing the antigen. The sections were then air-dried before alcohol dehydration and xylene defatting, and coverslips were applied with Permount (Ding et al., 1994a).

Because neuronal NOS also mediates the neuronal NADPH-diaphorase histochemical reaction (Hope et al., 1991), NADPH-diaphorase staining is widely used to visualize neuronal NOS. Histochemical detection of neuronal NADPH diaphorase was performed according to Vincent and Kimura (1992) with minor modifications. Briefly, the rat brains were fixed and sectioned as above for immunocytochemistry. The tissue sections were first incubated in PBS with 0.3% Triton X-100 for 30 min at room temperature and then incubated with 0.5 mM nitro blue tetrazolium (NBT, Sigma, St. Louis, MO) and 1.0 mM β-NADPH (Sigma) in 50 mM Tris–HCl buffer, pH 7.8, at 37°C for 30–60 min. The tissue sections were transferred to PBS to stop the color reaction. Histochemical reactions in the absence of NADPH or NBT, respectively, were used as controls.

Because P-CREB is a nuclear protein and NOS/diaphorase is a cytoplasmic enzyme, it is possible to discriminate the two proteins in the same neurons to evaluate their colocalization. For double-label immunocytochemistry and histochemistry, the animals were exposed to a light pulse for 10 min at CT 19. The animals were perfused, and the brains were removed and sectioned as described above. The tissue sections were first incubated with the primary and secondary antibodies recognizing P-CREB to generate a brown DAB–peroxidase staining in the nucleus. After PBS washes, the sections were then incubated with NADPH and NBT. A diaphorase histochemical reaction will generate blue NBT staining in the cytoplasm. The sections were then dehydrated and mounted as described above.

Quantitative analysis of P-CREB-lir. Because the tissue fixation and immunocytochemical reaction may vary from time to time, all experimental and control slices were processed simultaneously with the same batch of reagents. Cell counting was performed manually to include all visible P-CREB-lir-positive cells within one focal plane of each 50 μm section of the entire SCN. For the supraoptic nucleus (SON), only the sections that were on the same level of the nucleus circularis were counted. For computerized imaging analysis of P-CREB-lir, brain sections were placed under a Zeiss microscope, and the images were captured by a CCD video camera (NEC). The digitalized image was displayed on a Macintosh computer equipped with National Institutes of Health Image software. By moving a cursor masking the SCN, the image intensity in nonresponsive brain regions was determined. A frequency histogram of the image intensity was used to select a threshold cutoff for signal from background. By calculating the histogram for image intensity above the threshold, the average intensity of the signal and the number of pixels exceeding the threshold were determined (data not shown). Statistical analyses were performed using the software SAS.

Nuclear protein extraction and Western blot. Brain slices were maintained *in vitro* for >5 hr until the appropriate CT, when they were quick-frozen on a glass slide cooled on dry ice. A stainless steel needle (400 μm inner diameter) was used to punch both SCN from each 500-μm-thick brain slice. For each experimental condition, the SCN from five animals were collected (~50 μg protein) and stored at −80°C until use. Samples enriched for nuclear protein were prepared according to Dash et al. (1995). All steps were carried out at 4°C with the following reagents present in all solutions: 1 mM EGTA, 5 mM EDTA, 2 mM NaF, 1 mM sodium orthovanadate, 10 mM glycerol phosphate, 200 μM sodium
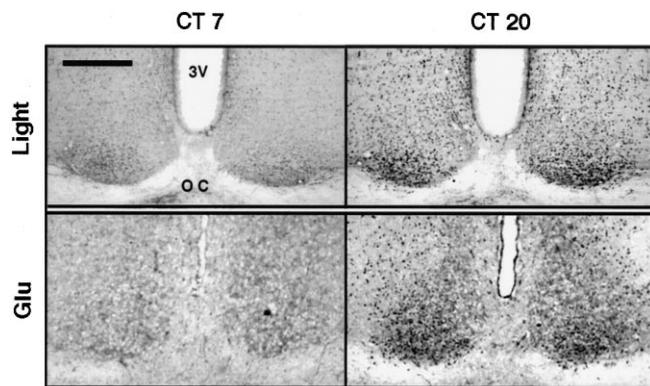
Figure 1. Sensitivity of SCN to CREB phosphorylation by light or Glu is restricted to the night. Robust nuclear staining of P-CREB-lir was induced in the SCN under free-running conditions after stimulation with light *in vivo* (150 lux, 10 min) or Glu *in vitro* (10 mM in 0.2 μl drop, 10 min) during subjective night (CT 19–20). In contrast, only a weak basal level of P-CREB-lir was detected at CT 7 after the same light or Glu treatment. When the anti-P-CREB antibody was preabsorbed with equal amount of phospho-CREBtide, no positive immunoreactivity was detected. Scale bar, 200 μm.

Figure 2. Time course of P-CREB-lir in the SCN after light exposure. After a light pulse at CT 19, P-CREB-lir in the SCN reached maximum level within 10 min and remained at this level at 30 min. It declined to approximately half-maximum by 1 hr and returned to nearly basal level in ~2 hr. In contrast, the P-CREB-lir in the SON of the same animals remained unchanged throughout the entire duration after the light exposure. General linear regression (GLM) for unbalanced ANOVA and *post hoc* test (Duncan) revealed that the levels of P-CREB-lir in the SCN at 10, 30, and 60 min groups are significantly different from basal level and from each other except for the values at 10 and 30 min. Each data point represents at least three animals; $**p < 0.01$.

pyrophosphate, 5 μM microcystin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1.0 μg/ml aprotinin, 40 μg/ml bestatin, and 1 mM PMSF. The SCN tissue was washed with sucrose buffer (0.25 M sucrose, 15 mM HEPES, 60 mM KCl, 10 mM NaCl, pH 7.2), and centrifuged at $2000 \times g$ for 10 min. Pellets were resuspended in cell lysis buffer (1.5 mM $MgCl_2$, 10 mM KCl, 15 mM HEPES, pH 7.2), agitated gently, and centrifuged at $4000 \times g$ for 10 min. Pellets were resuspended in 1 μl/SCN of nuclear lysis buffer (1.5 mM $MgCl_2$, 0.8 mM KCl, 100 mM HEPES, pH 7.2), tapped 3–4 times during a 30 min agitation, and centrifuged at $14,000 \times g$ for 30 min. Supernatants containing the enriched nuclear extract were then stored at −80°C.

For Western blots, denaturing buffer was added to a 2 μl aliquot of SCN nuclear extract from each treatment. Samples were subjected to SDS-PAGE on a 12% gel, transferred to nitrocellulose, and probed as in Ginty et al. (1993), with the exception that a horseradish peroxidase linked to goat anti-rabbit secondary (1:1000) and an ECL fluorescence system (Amersham, Arlington Heights, IL) were used for detection. To compare intersample protein loading, gels were silver-stained (Morrissey, 1981), and each lane was scanned densitometrically.

RESULTS

Circadian sensitivities of the SCN to Glu- and light-induced CREB phosphorylation are coincident

To evaluate the temporal sensitivities of the SCN to Glu- and light-induced P-CREB formation, we compared the effects of these stimuli in subjective day and night. Induction of P-CREB-lir in the SCN was dependent on the CT of treatment. When Glu (0.2 μl drop at 10 mM) was applied to the surface of the SCN for 10 min during the subjective day (CT 6–7), no increase in P-CREB-lir above basal level was observed. In contrast, during the subjective night (CT 19–20), this treatment induced robust nuclear P-CREB-lir within the SCN ($n = 8$) (Fig. 1). Application of a microdrop of the medium only at CT 20 for 10 min did not change P-CREB-lir from the basal level ($n = 6$). Staining was fully blocked by preincubating the antibody with P-CREBtide.

When the efficacy of Glu applied *in vitro* was assessed with reference to that of light *in vivo*, we observed that the patterns of P-CREB-lir were similar (Fig. 1), despite differences in the spatial character of stimulation. After a 10 min exposure of rats

to light, distinctive nuclear staining of neurons was localized primarily in the ventrolateral region of the SCN, where projections from the retina terminate. However, P-CREB-lir induced by Glu in the brain slice was not always restricted to the ventral SCN, possibly because the microdrop can exceed the retinorecipient region. Furthermore, despite the less localized P-CREB staining, the phase shifts induced by Glu microdrops are similar in timing, direction, and amplitude to those induced by light.

Induction of P-CREB-lir in the SCN after light exposure is rapid and transient

To establish a time course for the level of light-induced P-CREB-lir in the SCN, animals were killed at 10, 30, 60, and 120 min after the 10 min light exposure at CT 19. Quantitative analysis of P-CREB-lir was performed by cell counting as well as by computerized imaging analysis. Although immunocytochemical methods cannot determine the exact amount of antigen, they do permit the measurement of relative changes between control and treated groups, while preserving neuroanatomical localization (Ding et al., 1994a; Mize, 1994). Basal P-CREB-lir was low in the rat SCN, similar to basal levels in the hamster (Ginty et al., 1993). However, a high level of P-CREB-lir appeared rapidly in response to a light stimulus at night (Fig. 2). Quantitative analysis of P-CREB-lir in the SCN showed that after a light pulse at CT 19, P-CREB-lir reached maximum level within 10 min and remained at this level at 30 min. It declined to approximately half-maximum by 1 hr, and returned to nearly basal level in ~2 hr ($n = 18$) (Fig. 2). In contrast to the SCN, the level of P-CREB-lir in the supraoptic nucleus SON of the same animals was unaffected by light exposure.

CT 20
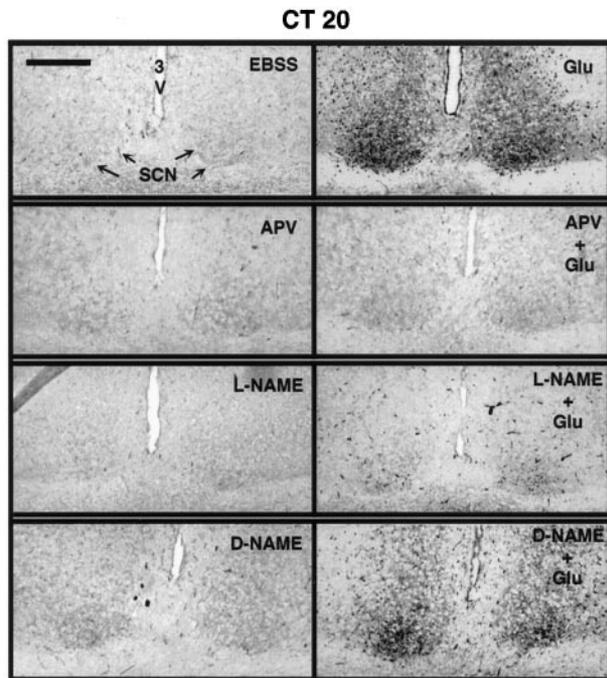
Figure 3. Blockade of Glu-induced P-CREB-lir in the SCN brain slices by inhibitors of the NMDA receptor and NOS. A 20 min preincubation in the NMDA receptor blocker APV (0.1 mM) or the NOS inhibitor, L-NAME (0.1 mM) diminished Glu-induced P-CREB-lir in the SCN at CT 20, whereas D-NAME (0.1 mM), the inactive stereoisomer of L-NAME, failed to block Glu-induced P-CREB-lir. These levels of staining are representative of four to six replications of each condition. Scale bar, 200 μm.

Figure 4. CT-dependent P-CREB induction by exogenous NO donor in the SCN. A microdrop (0.2 μl) of NO generator SNAP (0.01 mM) applied directly to the SCN induced P-CREB-lir at CT 19 (*A*), but not at CT 7 (*B*). Nissl stain demonstrates the histological boundary of the rat SCN (*C*). Scale bar, 200 μm.

Glu-induced CREB phosphorylation in the SCN is channeled via the NMDA receptor and NO signaling pathway

To determine whether Glu effects on P-CREB were mediated via an NMDA receptor, SCN slices were bathed in a specific NMDA receptor antagonist, 2-amino-5-phosphonovaleric acid (APV, 0.1 mM), for 20 min before Glu treatment. Although APV alone had no effect on the basal level of P-CREB-lir ($n = 6$) (Fig. 3), it prevented Glu-induced P-CREB-lir at CT 20 ($n = 6$). Thus, as in Glu-stimulated phase resetting (Ding et al., 1994b), P-CREB formation requires NMDA receptor activation.

To test whether NOS activation is in the pathway leading to P-CREB induction, a competitive NOS inhibitor, L-N^G-nitro-Arg-methyl ester (L-NAME) was used. L-NAME has been shown to block both GLU-induced phase shifts of SCN neuronal circadian rhythms *in vitro* and light-induced phase changes of locomotory rhythms *in vivo* (Ding et al., 1994b; Weber et al., 1995a). Preincubation in L-NAME (0.1 mM) for 20 min significantly diminished the P-CREB-lir normally induced by Glu at CT 20 ($n = 8$), whereas the inactive stereoisomer D-N^G-nitro-Arg-methyl ester (D-NAME, 0.1 mM) failed to prevent the effect of Glu on P-CREB-lir ($n = 6$) (Fig. 3). Neither L-NAME nor D-NAME, when applied alone, had apparent effects ($n = 6$ each).

Microdrops of an exogenous NO generator, *S*-nitroso-*N*-acetyl-penicillamine (SNAP; 0.01 mM), induced P-CREB-lir in the SCN during the subjective night but not the subjective day ($n = 4$) (Fig. 4). This coincides with the circadian sensitivity of the SCN to NO-induced phase resetting. The pattern of P-CREB-lir induced
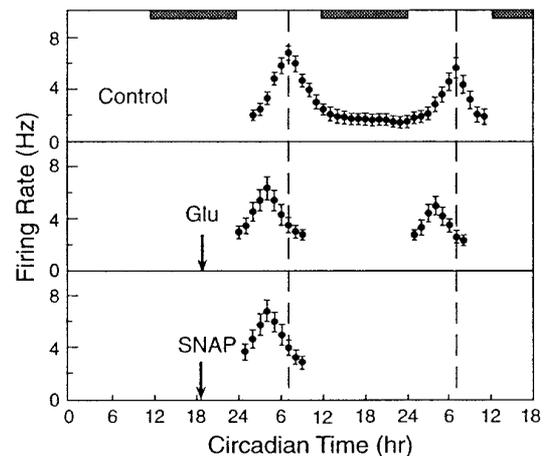
Figure 5. Circadian sensitivity of SCN to phase resetting. Phase resetting was assessed by measuring the time-of-peak of the endogenous circadian rhythms of the neuronal activity of the SCN in brain slice. *Top panel*, Continuous single-unit extracellular recording of the unperturbed SCN neuronal activity from 112 units over 38 hr. The firing rate of this circadian rhythm peaked in midsubjective day at CT 7, on both days 2 and 3 *in vitro*. *Middle panel*, At CT 19, Glu advanced the peak of the SCN activity rhythm by 3 hr. A 0.2 μl droplet of 10 mM Glu was directly applied to the SCN for 10 min (*arrow*), followed by EBSS wash. Neuronal activity was recorded over 10–12 hr on each of the next two cycles to define the time-of-peak activity. *Bottom panel*, At CT 19, a 0.2 μl droplet of 0.01 mM SNAP advanced the peak of the SCN firing rate by 3.5 hr. The *horizontal bars* indicate the subjective night of the circadian cycle. The *dashed vertical lines* mark the time (CT 7) of the normal peak of the circadian rhythm of the neuronal activity in unperturbed and EBSS-treated controls.
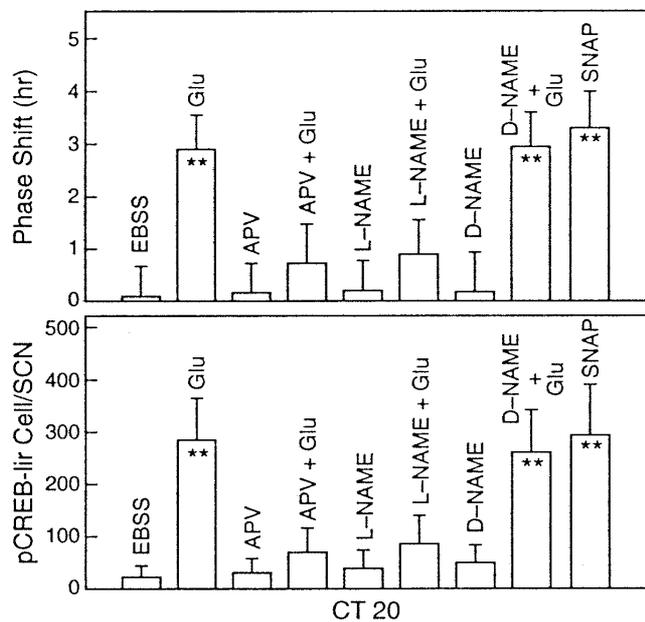
Figure 6. Quantitative comparison of phase shifts and P-CREB-lir induced by various reagents affecting the Glu/NMDA receptor/NOS/NO pathway at CT 19–20. All measurements were made on SCN studied *in vitro*. Phase shifting data are replotted from Ding et al. (1994b) to facilitate direct comparison. P-CREB-lir was determined after treatment, fixation, and sectioning so that *n* = each SCN brain slice per treatment (1 SCN slice was obtained from each animal). GLM for unbalanced ANOVA and *post hoc* test (Duncan) revealed that only Glu, SNAP, and Glu + D-NAME significantly induced phase shifts as well as increased P-CREB-lir from basal levels in the SCN *in vitro*. Each data point represents the mean ± SD of four to eight animals; **$p < 0.01$.

by SNAP was similar to that induced by Glu, except that the P-CREB-like nuclear staining was sometimes also found outside the SCN, possibly because NO can diffuse to a greater radius than the microdrops. However, the P-CREB-lir inside the SCN was always stronger than the surrounding hypothalamus (Fig. 4).

To compare the effect of Glu and SNAP on phase resetting, single-unit recording was performed to determine the time-of-peak of the SCN neuronal activity. At CT 19, the phase advance (3.5 ± 0.79 hr, $n = 4$) induced by a microdrop of SNAP (0.01 mM) applied for 10 min was overlapping with the response to Glu (3.3 ± 0.6 hr, $n = 6$) (Fig. 5).

To evaluate the effects of stimulating or inhibiting elements of the Glu, NMDA, and NO signaling pathway on both production of P-CREB-lir and phase shifts, the results of each treatment were compared quantitatively (Fig. 6). Only in SCN exposed to Glu, SNAP, and Glu + D-NAME were there significant phase shifts and P-CREB-induction ($p < 0.01$) (Fig. 6). This demonstrates that both the stimulatory effects of Glu, NMDA, and NO donors and the inhibitory effects of NMDA and NOS antagonists on Glu induction of P-CREB-lir matched their effects on SCN clock phasing ($n = 4–6$ per condition) (Fig. 6). Together, these data reveal a quantitative correlation between phase shifts of the circadian rhythm and putative P-CREB formation in the SCN during the nocturnal period of sensitivity of the clock to light-, Glu-, and NO- stimulated resetting.
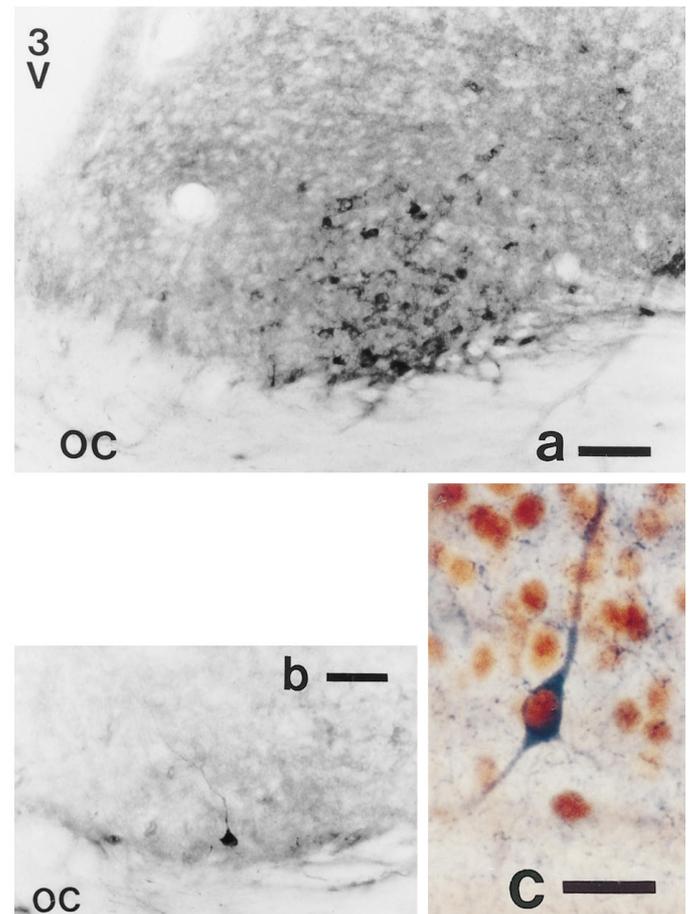
Figure 7. Colocalization of NADPH diaphorase and light-induced P-CREB-lir in the SCN. NADPH-diaphorase-positive neurons of varying size, arborization, and intensity are localized in the ventrolateral region of the SCN (*a*). Some diaphorase-containing neurons in the ventrolateral SCN have long processes projecting to the core of the SCN (*b*). The double-label immunocytochemistry and histochemistry revealed that the brown nuclear staining of P-CERB-lir was colocalized with the blue cytoplasmic staining of neuronal diaphorase within SCN neurons (*c*). Scale bars: *a*, 50 μm; *b*, 40 μm; *c*, 20 μm.

Colocalization of NADPH diaphorase and light-induced P-CREB-lir in the SCN

To study the spatial relationship between P-CREB- and NOS-containing neurons, a double-label immunocytochemistry and histochemistry procedure was used to evaluate potential colocalization of light-induced P-CREB-lir and NADPH-diaphorase staining in the SCN ($n = 12$). The neuronal NADPH-diaphorase histochemical reaction has been shown to visualize neuronal NOS (Hope et al., 1991). Consistent with previous reports (Decker and Reuss, 1994; Amir et al., 1995; Reuss et al., 1995; Chen et al., 1997), NADPH-diaphorase- and neuronal NOS-containing neurons are sparse in the SCN. They are concentrated in the ventrolateral region (Fig. 7A). SCN neurons of varying size, arborization, and intensity were observed with diaphorase staining (Fig. 7A,B). The double-label histochemistry and immunocytochemistry procedure revealed that the blue cytoplasmic staining of neuronal diaphorase was colocalized in a subset of neurons with the brown nuclear staining of P-CERB-lir after a 10 min light pulse (Fig. 7C). However, not every diaphorase-containing neuron in
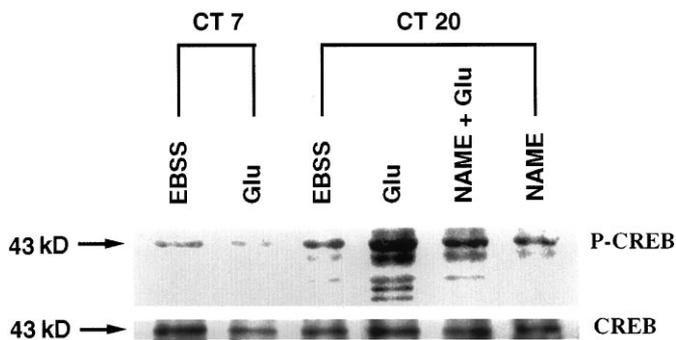
Figure 8. Glu induces a 43 kDa phosphoprotein in the SCN at night. *Top panel,* Affinity-purified anti-P-CREB antibody was used in Western blot analysis of the SCN nuclear extracts. A robust increase above basal level of a 43 kDa band was detected after Glu treatment at CT 20, but not at CT 7. A less intense reaction was also seen in bands of 33–36 kDa. The NOS inhibitor L-NAME diminished the amount of the 43 kDa band recognized by the anti-P-CREB antibody. *Bottom panel,* When the same gel was reprobed with the anti-αCREB antibody, equivalent amounts of the 43 kDa protein were present under all conditions. All experiments were repeated at least three times.

the SCN was colocalized with P-CREB-immunoreactive neuron and visa versa. Both P-CREB-lir neurons and diaphorase-positive neurons were localized in the ventrolateral region of the SCN. However, there were more P-CREB immunoreactive neurons than diaphorase-containing neurons in the SCN. Cell counts of both P-CREB-lir and diaphorase-positive neurons revealed that the ratio between them was ~50:1.

Glu induces the appearance of a 43 kDa CREB-positive phosphoprotein in SCN nuclear extracts at night

To further characterize the protein species representative of P-CREB-lir induced by Glu, SCN nuclear extracts (Dash et al., 1995) were denatured (Laemmli, 1970) and subjected to Western blot analysis with the anti-P-CREB antibody. An increase above basal P-CREB-lir was observed in a 43 kDa nuclear protein after Glu treatment in the subjective night, but not subjective day ($n = 6$) (Fig. 8). In addition to this major band, increased immunoreactivity in minor bands of 33–36 kDa was also observed after night-time treatment. Consistent with the results of quantitative immunocytochemistry, the NOS inhibitor L-NAME greatly diminished the amount of the 43 kDa band recognized by the anti-P-CREB antibody. When the same gel was reprobed with the anti-αCREB antibody, which recognizes αCREB regardless of the phosphorylation state, the quantity of this 43 kDa protein was constant across samples and between day and night.

DISCUSSION

Phosphorylation of the transcription factor CREB has been identified as a key step coupling extracellular stimuli to long-lasting intracellular responses (Sheng and Greenberg, 1990; Brindle and Montminy, 1992). When CREB is phosphorylated on Ser[133], by cAMP or Ca^{2+}/calmodulin-dependent protein kinases (Gonzalez and Montminy, 1989; Dash et al., 1991; Sheng et al., 1991), it becomes active in promoting transcription at the cAMP-response element (CRE) (Hunter and Karin, 1992; Chrivia et al., 1993).

P-CREB can activate the CREs of a number of genes, including the immediate-early gene c-*fos* (Sassone-Corsi et al., 1988; Sheng et al., 1990) and *zif/268* (Sakamoto et al., 1991), and genes encoding synaptic vesicular protein synapsin I (Sauerwald et al., 1990). CREB phosphorylation has been implicated in the learning and memory processes in a wide range of organisms, including *Aplysia* (Dash et al., 1990; Alberini et al., 1994), *Drosophila* (Tully et al., 1994; Yin et al., 1994, 1995), and mammals. CREB-deficient mutant mice exhibit impaired long-term memory, whereas short-term memory remains intact (Bourtchuladze et al., 1994). Phase shifting of the circadian clock is a neuronal phenomenon consistent with this coupling pattern between short-term extracellular stimuli, P-CREB induction, and long-term response with behavioral consequences; a brief light pulse to free-running animals during their subjective night can rapidly induce P-CREB in the SCN and reset the phase of circadian behavioral cycles (Ginty et al., 1993).

The present study demonstrates that direct application of Glu or an NO-releasing agent to the SCN, treatments that induce phase resetting, can induce phosphorylation of the transcription factor CREB at the transcriptional regulatory site Ser[133]. P-CREB was induced only at CTs when the clock is sensitive to phase resetting by light, Glu, and NO. Further, we demonstrate that production of the gaseous neurotransmitter NO, a critical element in light- and Glu-induced phase shifting of circadian rhythms (Ding et al., 1994b; Weber et al., 1995a), is required for the phosphorylation of CREB through the Glu pathway. The P-CREB-lir induced *in vitro* by Glu possesses the same molecular weight as P-CREB-lir induced by light *in vivo*.

The time course of P-CREB induction by light in the SCN is rapid and transient; peak P-CREB-lir appears 10–30 min after the onset of the light pulse. It remains significantly elevated at 60 min but returns to basal level by 120 min. The timing of this response is consistent with findings of P-CREB induction in the magnocellular hypothalamic SON by salt loading (Shiromani et al., 1995) and the parvocellular neurons in the PVN by stress (Kovacs and Sawchenko, 1996), respectively. In addition, a quantitative correlation exists both between phase shifts of circadian rhythms and the level of P-CREB-lir stimulated by light and Glu at CT 20. Because NO is a freely diffusible intercellular messenger and intercellular diffusion is required for the alteration in phase (Ding et al., 1994b), it is likely that a relatively smaller population of NOS-containing neurons may be able to activate a larger population of target cells. We found that Glu-induced P-CREB-lir in ~50 times more neurons than the number of diaphorase-containing neurons in the SCN. Furthermore, the extensive spatial overlap and colocalization between light-induced P-CREB-lir and NADPH diaphorase in the ventrolateral retinorecipient region of the SCN support the idea that NOS-containing neurons may be targets of photic entrainment signals from the retina (Decker and Reuss, 1994; Ding et al., 1994b).

Although it remains to be proven whether P-CREB formation is essential for phase shifting of circadian rhythms, it is clear that P-CREB is useful as a marker to study the signal transduction elements linking nocturnal light and Glu/NO elevation to P-CREB formation and its molecular consequences. The signaling steps between Glu/NO and P-CREB are presently unknown. However, Ca^{2+} and/or cGMP are likely to play important roles. NO can potentiate Ca^{2+}-induced gene expression involving the activation of CREB in neuronal cells (Peunova and Enlkolopov, 1993), and NO can stimulate transcription via a cGMP pathway, including expression of immediate-early genes c-*fos* and *jun*B in

PC-12 cells (Haby et al., 1994). A study of hippocampal neurons demonstrated a functional correlation between CREB phosphorylation and the induction of both long-term potentiation (LTP) and long-term depression (LTD). P-CREB formation triggered by LTP- or LTD-inducing stimuli required calmodulin and Ca^{2+} calmodulin-dependent protein kinase (CaM kinase), but not cAMP-dependent kinase (PKA) activities (Deisseroth et al., 1996). Both CaM kinase and cGMP-dependent protein kinase (PKG) have been implicated as mediators of light-induced phase shifts in hamster behavioral rhythms (Golombek and Ralph, 1994; Weber et al., 1995b; Mathur et al., 1996), and a CaM kinase inhibitor has recently been reported to attenuate light-induced P-CREB in hamster SCN (Glolombek and Ralph, 1995).

This study has identified the initial signal transduction elements linking photic stimulation to CREB phosphorylation in the SCN. These signaling elements are the same as those that are critical to light-induced phase resetting. The aggregate data support a signaling pathway in which nocturnal light impinging on the retina stimulates glutamate release from terminals of the retinal ganglion cells projecting to the ventrolateral SCN. Necessary elements at SCN neurons include NMDA receptor activation, NOS stimulation, and intercellular movement of NO (Ding et al., 1994). Because both the NMDA and non-NMDA Glu receptors are present in the SCN (Mikkelsen et al., 1993; Gannon and Rea, 1994) and non-NMDA receptor agonists can induce phase shifts (Shibata et al., 1994), non-NMDA glutamatergic neurotransmission may also contribute to stimulating CREB phosphorylation in the SCN. However, it is likely that the influx of Ca^{+2} attending NMDA receptor activation leads to activation of kinases such as CaM kinase as well as of NOS. The resulting rise in NO, in turn, could activate PKG. The relative contributions of these potential signaling pathways to CREB phosphorylation requires careful evaluation. Nevertheless, the data thus far place P-CREB at the earliest point of the critical transactivational sequence that intersects with the clock mechanism.

Although the target gene(s) of P-CREB in the SCN have yet to be identified, c-*fos* has been suggested as a candidate (Ginty et al., 1993). Induction of c-*fos* is regulated by P-CREB in response to Ca^{2+}, cAMP, and growth factors in hippocampal and PC-12 cells (Sheng et al., 1988; Berkowitz et al., 1989; Sheng et al., 1990). In the SCN, P-CREB formation precedes the appearance of c-*fos* mRNA in response to light (Kornhauser et al., 1992), and c-*fos* mRNA is induced in proportion to the intensity of the light stimulus (Kornhauser et al., 1990). In addition, intracerebroventricular injection of antisense oligonucleotides for c-*fos* and *jun*B attenuates c-*fos* transcription and light-induced phase shifts (Wollnik et al., 1995). However, a recent study reported an uncoupling between NO-mediated photic stimulation of hamster behavioral phase shifts and Fos expression in the SCN (Weber et al., 1995a). Furthermore, systemic saline injection and handling can induce behavioral phase shifting and Fos expression (Edelstein and Amir, 1995) but not P-CREB-lir (Sumova et al., 1994) in the SCN. Moreover, it was reported recently that light-induced Fos expression in the rat SCN was codistributed but not colocalized with NADPH diaphorase (Amir et al., 1995). Our present study demonstrates that a number of NADPH-diaphorase-positive neurons do colocalize with the light-induced P-CREB-lir in the rat SCN. Therefore, it is likely that multiple cellular components, signal transduction pathways, and transcriptional activations contribute to the SCN response to light.

Finally, the free-running biological clock shows circadian changes *in vivo* and *in vitro* in its response to light, Glu, and NO (Ding et al., 1994b). Therefore, the clock controls its own temporal sensitivity to these stimuli (Gillette, 1996). The consequence is that P-CREB formation and phase resetting can only be stimulated through this signaling pathway in the nocturnal domain of the clock's cycle. Yet, NMDA receptors (Gannon and Rea, 1994), NOS (Chen et al., 1997), and CREB (Ginty et al., 1993) each are present in both night and day. Therefore, among the signaling elements between NO and CREB must lie the clock-controlled molecular gate(s) through which the clock regulates its own temporal sensitivity to these resetting stimuli.

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