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Resetting the Biological Clock: Mediation of Nocturnal Circadian Shifts by Glutamate and NO

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Circadian rhythms of mammals are timed by an endogenous clock with a period of about 24 hours located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Light synchronizes this clock to the external environment by daily adjustments in the phase of the circadian oscillation. The mechanism has been thought to involve the release of excitatory amino acids from retinal afferents to the SCN. Brief treatment of rat SCN in vitro with glutamate (Glu), *N*-methyl-D-aspartate (NMDA), or nitric oxide (NO) generators produced lightlike phase shifts of circadian rhythms. The SCN exhibited calcium-dependent nitric oxide synthase (NOS) activity. Antagonists of NMDA or NOS pathways blocked Glu effects in vitro, and intracerebroventricular injection of a NOS inhibitor in vivo blocked the light-induced resetting of behavioral rhythms. Together, these data indicate that Glu release, NMDA receptor activation, NOS stimulation, and NO production link light activation of the retina to cellular changes within the SCN mediating the phase resetting of the biological clock.

Diurnal oscillations of endocrine, physiological, and behavioral functions are ubiquitous features of eukaryotes (*1*). In mammals, the mechanisms responsible for the generation and synchronization of these circadian rhythms reside in the hypothalamic SCN (*2*). The dominant signal that coordinates internal time with environmental changes is light. Photic information is conveyed to the SCN by way of a direct retinal projection, the retinohypothalamic tract (*3*). Under conditions of continuous darkness, brief light exposure during the early subjective night causes phase delays, whereas exposure during the late subjective night causes phase advances of circadian rhythms driven by the SCN clock (*4*). Light exposure during the subjective day does not alter the circadian phase. Neither the mechanisms responsible for clock resetting, nor those underlying nocturnal restriction, or "gating," of the response are understood.

Glutamate (Glu) is the putative neurotransmitter that mediates photic entrainment. Glu and its bipeptide precursor are localized in retinal fibers innervating the SCN, and Glu is released after optic nerve stimulation in vitro (*5*). Antagonists of the NMDA subtype of Glu receptor block photic phase shifts of the free-running activity rhythm in rodents (*6*, *7*). Although attempts to affect phase in vivo by Glu with bolus injections near the SCN have failed to elicit lightlike effects on circadian rhythms (*8*), preliminary reports suggest that SCNs in vitro show nocturnal sensitivity to Glu (*9*).

Activation of NMDA Glu receptors in neural systems leads to an influx of Ca^{2+}. This, in turn, can activate nitric oxide synthase (NOS), resulting in the production of nitric oxide (NO) (*10*). NO is a short-lived gaseous neurotransmitter that can readily traverse cell membranes and produce intercellular effects. Thus, NO can activate guanylate cyclase or adenosine diphosphate–ribosyltransferase or facilitate neurotransmitter release in cells neighboring the site of NOS stimulation (*11*).

In order to selectively probe the pathway of phase resetting of the biological clock, we studied a SCN rat brain slice preparation maintained in vitro for 3 days (*12*). The mean firing frequency of SCN neurons in vitro forms a sinusoidal curve, with a 24-hour period and a peak midday, near circadian time 7 (CT 7) (Fig. 1A) (*13*). A microdrop of 10 mM Glu was briefly applied to the SCN and the time of subsequent peaks in neuronal activity assessed (*14*). Glu induced robust phase shifts, depending on the phase of the circadian cycle at which it was administered (Fig. 1, B through D). When applied to the SCN in the early subjective night, Glu caused a 3.0-hour delay in the neuronal circadian rhythm. Peak activity remained delayed by the same interval relative to the normal peak for the 2 days studied after Glu treatment, indicating that a stable phase resetting of the SCN clock had been induced. Later in the subjective night, Glu application advanced

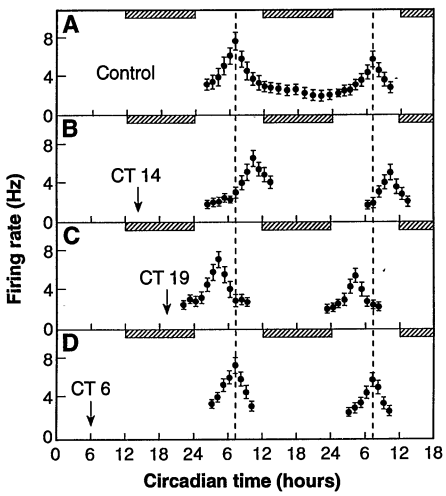

Fig. 1. Sensitivity of the SCN to phase resetting by Glu. We assessed sensitivity by measuring the phasing of endogenous circadian rhythms of neuronal firing rate of the SCN in brain slices (*14*). Circadian rhythms of the ensemble of neurons were derived by random sampling of single units extracellularly at 10-min intervals for two 2-min periods. Units (82 to 124) were sampled per slice; activities were grouped into a 2-hour running average ± SEM to determine the time of peak firing activity (*13*). (**A**) Circadian rhythms of neuronal activity in SCN slices in vitro shown in a continuous record over 38 hours from a single SCN on days 2 to 3 after slice preparation. The horizontal bars indicate the subjective night of the circadian cycle. The dashed vertical lines mark the time of the normal peak activity at CT 7 in unperturbed and EBSS-treated controls (*13*). (**B**) Effect of Glu at CT 14 on the activity rhythm. A 0.2-μl droplet of 10 mM Glu was applied directly to the SCN for 10 min (arrow), followed by EBSS rinse, during the early subjective night at CT 14. (**C**) Effect of Glu at CT 19. (**D**) Effect of Glu at mid-subjective day, CT 6.

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subsequent peaks by 3.5 hours. However, the same Glu treatment in the middle of the subjective day had no effect on the circadian rhythm. This demonstrates a direct effect of Glu on SCN phase and a nocturnally restricted sensitivity of the SCN to Glu, with the specific effect (phase delay or advance) dependent on a specific window of sensitivity (early or late night).

To more fully evaluate the timing of clock sensitivity to Glu, we treated SCN slices at 16 different points across the circadian cycle. The resulting phase-response curve (PRC) (Fig. 2) shows that the SCN is unresponsive to Glu throughout the subjective day; in early subjective night (CT 12 to 16), it responds with phase delays (maximum, CT 14 = −3.0 ± 0.5 hours; n = 6), whereas from CT 18 to 23, it responds with phase advances (maximum, CT 19 to 20 = +3.2 ± 0.5 hours, n = 12).

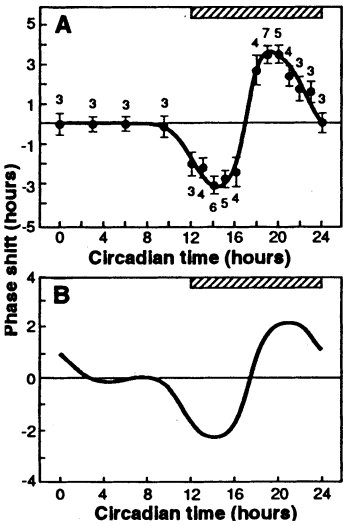

Fig. 2. The PRC for Glu applied to the SCN (A) and the light-induced PRC (B) in rat (*15*). (**A**) The PRC for 10 mM Glu applied to the SCN for 10 min followed by EBSS rinse is derived from 63 experiments at 16 time points across the day-night cycle. Maximum delay is 3.0 ± 0.5 hours at CT 14; maximum advance, 3.2 ± 0.5 hours at CT 19 to 20. Each data point represents the mean ± SD of three to seven experiments, as indicated. (**B**) PRC for 1-hour pulses of light of 150 lux to Sprague-Dawley rats in constant darkness, redrawn from Summers *et al.* (*15*). Symbols are as in Fig. 1.

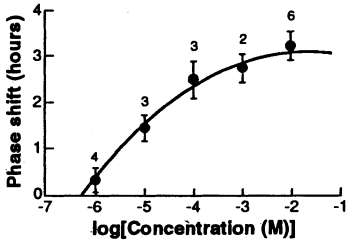

Fig. 3. Dose-response curve for a 10-min pulse of 0.2 μl of Glu applied to the SCN in vitro at CT 19. Each data point represents the mean ± SD of two to six experiments, as indicated, measuring time-of-peak as in Fig. 1.

The PRC for a brief pulse of Glu directly applied to the SCN (Fig. 2A) is similar to the light pulse–induced PRC in rat wheel running (Fig. 2B) (*15*). However, phase shifts, both advances and delays, were larger in the isolated SCN than in intact animals and occurred more rapidly than most phase shifts of behavior. This difference could be due to the lack of feedback inhibition to SCN in isolated slices. Alternatively, the maximum dosage of Glu used in these experiments may be considerably higher than the normal amount of neurotransmitter released upon light stimulation. At CT 19, the dose dependency to Glu applied in microdrops ranged from 10^{-6} to 10^{-2} M, with a half-maximal response near 1.25×10^{-5} M (Fig. 3). In subsequent experiments, 10 mM Glu, the lowest concentration that fell within the plateau range, was used. However, even at this dosage the SCN tissue did not show signs of Glu toxicity; SCN neurons continued to fire vigorously for up to 3 days and to generate normal circadian rhythms. This corroborates previous observations that SCN tissue shows a greater tolerance for the effect of Glu than other brain regions (*16*).

Long-lasting changes due to Glu neurotransmission are often mediated by the NMDA subtype of postsynaptic Glu receptors. Therefore, the effect of NMDA on SCN circadian rhythms was examined. NMDA, applied by microdrop at CT 6, 14, or 20, elicited effects indistinguishable from those of Glu at these CTs (Fig. 4). The specific NMDA antagonist 2-amino-5-phosphonovaleric acid (APV) abolished the phase-shifting effects of Glu at CT 14 and CT 20, but had no effect on the rhythm when applied alone (Fig. 5A). This observation suggests that whereas other ionotropic Glu receptors may play a role in the Glu-induced depolarization of SCN neurons (*17*), activation of NMDA receptors, and attendant Ca^{2+} entry, must be critical to phase-shifting and would lead to activation of Ca^{2+}-sensitive processes, possibly including the NOS-NO pathway (*10*).

To test the effect of NO on circadian rhythms, we applied directly to the SCN three exogenous NO generators with different mechanisms of action. Sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) release NO in tissue; hydroxylamine is converted to NO by intracellular catalase (*18*). Treatments with SNP, SNAP, or hydroxylamine at CT 6 produced no change in phase (Fig. 4). However, nocturnal treatments at CT 14 or CT 20 induced phase delays or advances, respectively. The responses were similar to those elicited by Glu and NMDA both in direction and magnitude (Fig. 4).

To further evaluate the signal transduction sequence downstream from Glu, we examined the SCN for evidence of NOS activity and function. NOS converts L-arginine (Arg) to L-citrulline (Cit) and releases NO in the process. Significant NOS

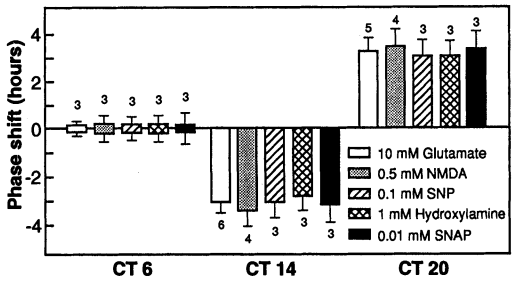

Fig. 4. Phase shifts induced by NMDA and NO donors. NMDA, SNP, hydroxylamine, or SNAP was applied for 10 min in 0.2-μl microdrops to the SCN in brain slices. Each data point represents the mean ± SD of three to six experiments, as indicated. To compare groups of unequal sizes, a general linear regression model was used for unbalanced analysis of variance; despite small sample sizes, the results did not vary significantly among the treatments at each time point.

Table 1. NOS specific activity in the SCN. We determined NOS enzymatic activity by monitoring the conversion of [^{3}H]Arg to [^{3}H]Cit (*19*). Each value represents the mean ± SD of three SCNs. Analysis of variance with the new Duncan post-hoc test confirms the significant difference ($P < 0.01$) between the conversion of Arg to Cit in SCN tissue compared with reactions containing competitors of [^{3}H]Arg: L-NAME, a competitive nonhydrolyzable analog, excess unlabeled Arg substrate, and SCN tissue incubated in buffer without added Ca^{2+}

SCN tissue treatment	NOS activity expressed as conversion to	
	[^{3}H]Cit (cpm/μg protein/30 min)	[^{3}H]Cit (pmol/mg protein/30 min)
[^{3}H]Arg	909 ± 155	218 ± 48
2 mM L-NAME + [^{3}H]Arg	19.8 ± 6.8	3.8 ± 1.9
2 mM Arg + [^{3}H]Arg	18.0 ± 5.4	3.7 ± 1.3
Ca^{2+} free + [^{3}H]Arg	14.5 ± 6.7	3.5 ± 1.6

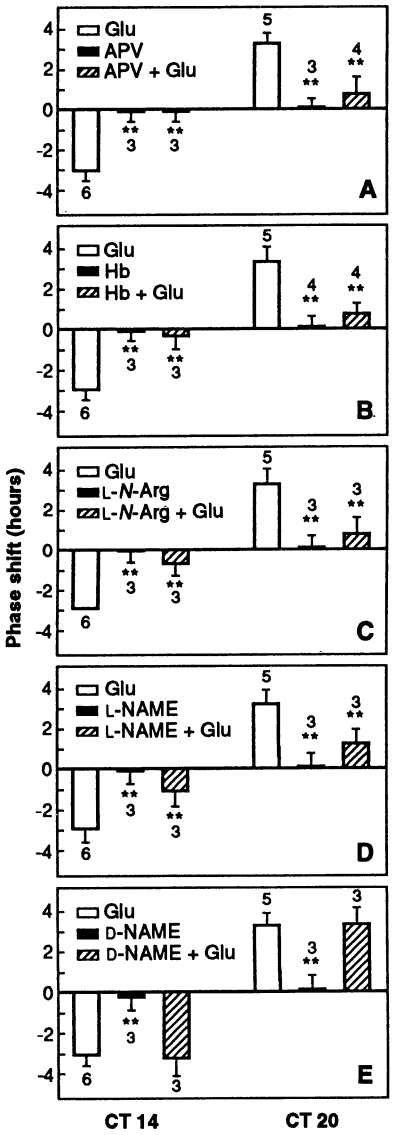

Fig. 5. Block of Glu-induced phase shift by inhibitors of the NMDA-Glu receptor and the NO pathway. Brain slices were incubated for 30 min with potential blockers alone or for 20 min with 0.1 mM APV, Hb, L-*N*-Arg, L-NAME, or D-NAME in the bath before Glu application to the SCN for 10 min. Phase delays or advances normally induced by Glu at CT 14 or CT 20, respectively, were evaluated. Each data point represents the mean ± SD of three to six experiments, as indicated. General linear regression for unbalanced analysis of variance followed by the new Duncan post-hoc test showed significant differences between Glu and four other treatments that inhibit NO pathways. (**A**) Effect of APV, a specific blocker of NMDA receptors, on Glu-induced phase shifts. (**B**) Effect of Hb, which binds NO released extracellularly, on Glu-induced phase shifts. (**C**) Effect of L-*N*-Arg, a competitive inhibitor of NOS, on Glu-induced phase shifts. (**D**) Effect of L-NAME, also a competitive inhibitor of NOS, on Glu-induced phase shifts. (**E**) Effect of D-NAME, an inactive stereoisomer of L-NAME, on Glu-induced phase shifts. With the exception of D-NAME, no significant difference was detected between the effect of each inhibitor alone and its effect in combination with Glu. The double asterisks indicate that $P < 0.01$.

activity, measured as conversion of [^{3}H]Arg to [^{3}H]Cit (*18*), was present within the SCN (Table 1). This conversion was blocked by an excess of alternate substrates: Both L-N^G-nitro-Arg-methyl ester (L-NAME) and unlabeled Arg reduced the rate of [^{3}H]Cit formation to one-fiftieth of previous levels. Furthermore, this NOS activity was abolished in buffer containing no added Ca^{2+}. Ca^{2+} dependency links NOS activation to NMDA receptor stimulation and is characteristic of constitutively expressed isoforms of this enzyme (*10*).

Antagonists of this pathway include inactive competitive substrates for NOS as well as extracellular molecules containing heme groups. Because NO has high binding affinity to heme moieties, hemoglobin (Hb) serves as an NO scavenger, competing with other heme-containing targets of NO, such as guanylate cyclase (*20*). Twenty-minute bath incubation with Hb or with competitive inhibitors of NOS (*21*), L-N^G-nitro-Arg (L-*N*-Arg) or L-NAME, before Glu application to the SCN significantly attenuated phase shifts by Glu at CT 14 and CT 20 (Fig. 5). The inactive stereoisomer of L-NAME, D-NAME, was unable to antagonize the effect of Glu on phase, which supports the specificity of L-NAME as an NOS antagonist.

To assess whether the NO pathway mediates photic phase shifts, we determined the effects of intracerebroventricular (icv) injection of L-NAME near the SCN on the light-induced phase advances of the free-running activity rhythm of Syrian hamsters maintained under constant darkness (*22*). Brief light exposure (20 lux for 5 min) 10 min after icv administration of vehicle (0.5 μl) at CT 19 resulted in typical phase advances of the activity rhythm (mean ± SEM = 89 ± 7 min; n = 7) (Fig. 6). Injection of 1 mM L-NAME before light exposure significantly (67%; $P < 0.05$) attenuated light-induced phase advances (30 ± 8 min; n = 11). This effect was completely blocked by co-administration of 4 mM L-Arg, the substrate for NOS (Fig. 6). These results suggest that the transduction of photic signals in the SCN requires the formation of NO.

Together, our results indicate that Glu is the primary messenger mediating photic signals from retinal afferents to the SCN. They suggest that activation of NMDA receptors by Glu is a critical event in this signaling cascade, although non-NMDA receptor agonists can also induce shifts (*23*). It is likely that the rise in the intracellular Ca^{2+} concentration through activated NMDA receptors results in NOS stimulation and production of NO. NOS activity can be measured in the SCN at this time (Table 1), and NOS inhibitors effectively block Glu-induced phase shifts. Furthermore, NOS inhibitors can block both acute (*24*) and circadian behavioral responses to photic stimuli. Thus, NO must be a further critical element in the light signal transduction pathway, linking Glu neurotransmission to phase shifting of the circadian clock.

The mechanism that gates nocturnal sensitivity to this activation sequence is most likely downstream from NO, as NOS activity is present in the SCN in both day and night (*25*). Although the components downstream in this cascade are unknown in the SCN, they may involve cyclic guanosine monophosphate (cGMP). NO can directly activate guanylate cyclase (*11*), and analogs of cGMP induce phase advances in the SCN only at night (*26*). However, the bidirectional response to

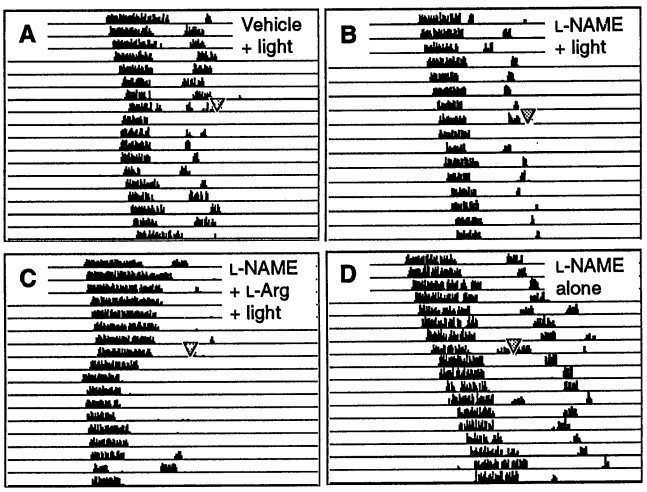

Fig. 6. Block of photic phase shifts in vivo by an inhibitor of NOS. Intracerebroventricular (icv) administration of L-NAME inhibits the light-induced phase advances of the free-running activity rhythm in hamsters. Shown are representative records of activity of individual hamsters that received the following treatments (*22*) at the times indicated by the inverted triangles (▽). (**A**) Injection (icv) of 0.5 μl of vehicle followed by exposure to 20 lux of white light for 5 min at CT 19. (**B**) Injection (icv) of 0.5 μl of 1 mM L-NAME followed by exposure to 20 lux of white light for 5 min at CT 19. (**C**) Injection (icv) of 1 mM L-NAME and 4 mM Arg followed by exposure to 20 lux of white light for 5 min at CT 19. (**D**) Injection (icv) of 0.5 μl of 1 mM L-NAME without light exposure at CT 19. Horizontal lines represent successive days, whereas vertical bars indicate 6-min bins during which wheel-running activity occurred. The height of the vertical bars is proportional to the number of wheel turns that occurred during that 6-min period.

NO agonists indicates that events downstream are complex.

In view of these robust physiological and biochemical data supporting a role for NOS in this Glu activation cascade, it may be surprising that studies using either immunocytochemistry for neuronal NOS (nNOS), or the reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase reaction in paraformaldehyde-fixed tissue, found little conventional NOS staining in SCN neurons (*27*). This raises the possibility that alternative isoforms of the enzyme may mediate this effect. These NOS isoforms could exist in neurons, glia, or endothelia of this brain region. Residual NOS catalytic activity can be detected in brain tissue from a homozygous mutant mouse lacking the gene for nNOS (*28*), and a Ca^{2+}-sensitive endothelial NOS isoform in CA1 hippocampal neurons mediates long-term potentiation in these $nNOS^{-}$ mice (*29*).

The efficacy of extracellular Hb in blocking the effects of Glu on the phase resetting of the SCN demonstrates that a signaling step mediated by this pathway requires nonsynaptic intercellular communication. Nonsynaptic intercellular communication would rapidly amplify signals from the retinohypothalamic tract so that they spread beyond primary synapses at the ventral SCN. NO permeates the membrane of all cells, and glia can function as NO targets (*30*), which raises the possibility that both neurons and glia may participate in the photic regulation of circadian rhythmicity. Intercellular diffusion of the short-lived messenger NO might serve to synchronize activities within this multicellular oscillator (31). Elevated concentrations of NO may also lead to the release of additional neurotransmitters at SCN synaptic sites (*11*). Indeed, substance P, which is localized in retinal fibers, and somatostatin, which is endogenous to SCN neurons, elicit phase shifts that are similar, though not identical, to those mediated by Glu (*32*). Regardless of the process, the requirement for intercellular movement of NO points to an intersection between this signal transduction cascade and the clock mechanism in cells beyond those cells initially activated by Glu.

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12. A 500-μm coronal hypothalamic slice containing the paired SCN was prepared at least 2 hours before the onset of the dark phase from 7- to 10-week-old inbred Long-Evans rats housed according to a 12 hours of light and 12 hours of dark lighting schedule. Brain slices survived for up to 3 days with continuous perfusion (34 ml/hour) by Earle's balanced salt solution (EBSS), supplemented wth 24.6 mM glucose, 26.2 mM sodium bicarbonate, and 5 mg/l of gentamicin and saturated with 95% O_2:5% CO_2 at 37°C (pH 7.4). The single-unit activity of SCN neurons was recorded extracellularly with a glass microelectrode, and running means were calculated to determine the time of peak activity (Fig. 1) [for details, see M. Medanic and M. U. Gillette, *J. Physiol. (London)* **450**, 629 (1992)]. The onset of the light phase of the entraining light-dark cycle of the brain slice donor is CT 0.
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14. For treatments, perfusion was stopped and a 0.2-μl microdrop of a test substance dissolved in EBSS was applied directly to the SCN. After 10 min, the SCN surface was washed with EBSS, perfusion was resumed, and the time of peak neuronal activity was assessed for 2 days. To evaluate potential blockers of the stimulus, we replaced the bathing medium with the test substance in EBSS (pH 7.4) 20 min before the phase-shifting stimulus was applied to the SCN. A number of experiments of each condition were performed "blind," where the individual recording the neuronal activity had no knowledge of the application time or the contents of the microdrop. When Glu was applied at CT 17 (the point where maximal phase delay changes rapidly to maximal phase advance), multiple peaks of firing rate occurred, which suggests that within this particular time frame the SCN clock mechanisms are unstable.
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19. NOS activity was determined by modification of the method of D. S. Bredt and S. H. Snyder [*Proc. Natl. Acad. Sci. U.S.A.* **86**, 9030 (1989)]. Slices were prepared at CT 9, surgically reduced to only the SCN and contiguous optic chiasm, incubated in the brain slice chamber for 5 hours, and quick-frozen in dry ice at CT 14. The tissue was sonicated for 10 s, mechanically disrupted with a glass rod in 50 mM Hepes (pH 7.4) containing 0.5 mM EDTA, and then incubated for 30 min at 22°C with 0.2 μCi of 36.1 Ci/mmol [^{3}H]Arg, 5 mM NADPH, 1.25 mM calcium chloride, and calmodulin (10 μg/ml). Control samples contained (i) the reactants plus 2 mM unlabeled Arg, (ii) the reactants plus 2 mM L-NAME, (iii) the reactants without calcium chloride, or (iv) the reactants without SCN tissue. When the reaction mixture was chromatographed, no radioactive species other than Arg and Cit were detected. The amino acids in the supernatants were adsorbed to Dowex-50W (H form; Sigma; we changed it to the Na form by equilibrating with 0.5 N NaOH and washing with water and Hepes buffer). Cit was eluted with water. We verified [^{3}H]Cit in the eluant by thin-layer chromatography by using as solvent methyl ethyl ketone:1 M HCl:isopropyl alcohol (15:25:60). Protein concentrations were measured by the Bradford assay in tissue pellets after sonicating [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)]. [^{3}H]Cit was quantitated by liquid scintillation. Specific activity is based on published endogenous amounts of Arg in rat brain [G. Siegel *et al.*, *Basic Neurochemistry* (Raven, New York, 1991), pp. 558–559].
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22. Male Syrian hamsters (*Mesocricetus auratus*) weighed 130 to 180 g at the time of surgery. Under pentobarbital anesthesia (90 mg per kilogram of body weight, administered intraperitoneally), 26 gauge cannula guides were stereotaxically implanted to a depth of 2.9 mm below the dura and fixed to the skull with fine machine screws and dental cement. Cannula guides were fixed at stereotaxic coordinates of 1.0 mm anterior to the bregma at the midline (upper incisor bar at 0). After 1 week in 14 hours of light and 10 hours of dark per day, cannulated hamsters were transferred to individual cages equipped with 9-inch running wheels and maintained in constant darkness (DD). Wheel-running activity was monitored continuously on a computer running Dataquest III data acquisition software (Minimitter, Sunriver, OR). The onset of wheel-running activity, designated as CT 12, was used as a phase reference point for the timing of photic stimulation. The onset of activity on the day of stimulation was predicted by extrapolation of the regression line fitted to activity onsets from the 5 days preceding the day of stimulation. Intracerebroventricular administration was achieved with use of a 33-gauge infusion cannula attached to a 1-μl Hamilton syringe. The infusion cannula extended 4.4 mm beyond the tip of the cannula guide to a position near the floor of the third ventricle. Ten minutes before light exposure, animals received 0.5-μl injections under dim (<1 lux) red illumination. Animals were gently restrained for approximately 30 s during the injection, and the infusion cannula remained in place for 10 to 15 s after the injection. Animals were returned to their respective cages and maintained for an additional 10 to 14 days under DD. Phase shifts of the free-running activity rhythm were calculated as described previously (*7*). Pharmacological solutions were prepared fresh the

day of injection. The vehicle was a bicarbonate-buffered physiological saline of 122 mM NaCl, 3.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 1.2 mM $CaCl_2$. After data collection, we verified the location of the injection site histologically by examining 75-μm-thick vibratome sections cut through the injection site.
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33. We thank H. Li for statistical counseling; L. Kuriashkina for technical assistance; and C. Q. Doe, M. G. Erlander, J. G. Malpeli, G. E. Robinson, and members of the Gillette lab for critical evaluation of this manuscript. M.U.G. is supported by grants from the National Institute of Neurological Diseases and Stroke (NS22155) and the Air Force Office of Scientific Research (AFOSR) (90-0205, F-49620-93-1-0413). M.A.R. is supported by AFOSR grant 2312W6. E.T.W. is an Air Force Graduate Research Fellow.

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Light-Regulated Translation of Chloroplast Messenger RNAs Through Redox Potential

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Translation of key proteins in the chloroplast is regulated by light. Genetic and biochemical studies in the unicellular alga *Chlamydomonas reinhardtii* suggest that light may regulate translation by modulating the binding of activator proteins to the 5′ untranslated region of chloroplast messenger RNAs. In vitro binding of the activator proteins to *psbA* messenger RNA and in vivo translation of *psbA* messenger RNA is regulated by the redox state of these proteins, suggesting that the light stimulus is transduced by the photosynthesis-generated redox potential.

Synthesis of specific proteins in the chloroplast is enhanced about 50- to 100-fold after illumination of plant and algae cells (*1–4*). This marked increase in translation occurs in the absence of a corresponding change in the amount of the mRNA (*1, 3, 5, 6*), indicating that translation of chloroplast mRNAs is light-regulated. Translation of these chloroplast mRNAs requires nuclear-encoded translational activators that interact with the 5′ untranslated region (5′-UTR) of the mRNAs (*7–10*). We have identified mRNA-binding proteins that bind to a stem-loop RNA structure, which contains the ribosome-binding site, in the 5′-UTR of *psbA* mRNA (*11, 12*) (encoding the D1 protein of photosystem II). The amount of *psbA* mRNA–protein complex (RNP) correlates with the amount of translation of *psbA* mRNA in light- and dark-grown *Chlamydomonas reinhardtii* cells, suggesting that modulation of RNP complex formation may regulate the translation of this mRNA (*11*). We therefore set out to identify the mechanism by which the binding of these proteins to the mRNA—and consequently the translation of the mRNA—is regulated in response to light. Adenosine diphosphate (ADP)–dependent phosphorylation inactivates *psbA* mRNA binding after transfer of cells into the dark (*13*). Here, we show that binding of the protein complex to *psbA* mRNA and translation of *psbA* mRNA are modulated by redox potential, perhaps through ferredoxin and thioredoxin. Utilizing the reducing power generated by photosynthesis to regulate translation provides a dynamic and direct link between the amount of light absorbed by photosynthesis and the synthesis of the photosystem II (PS II) reaction center proteins.

Light may affect chloroplast metabolism by its effect on a photoreceptor, such as phytochrome, which in turn affects nuclear gene transcription (*14*). Light also generates the products of photosynthesis, such as redox potential, adenosine triphosphate, or carbohydrates, which can subsequently affect activity of chloroplast enzymes (*15*). For example, chloroplast enzymes can be activated by the ferredoxin-thioredoxin system (*16*). Reducing power, generated by the light-reactions of photosynthesis, is used to reduce thioredoxin, which in turn activates enzymes of the reductive pentose phosphate cycle to regulate carbon assimilation.

To investigate whether redox potential affects the assembly of the *psbA* mRNA–binding protein complex, we subjected purified *psbA* RNP to either reducing or oxidizing reagents and analyzed binding activity by T1-ribonuclease (RNase) gel mobility–shift assay (T1-GMS) (*11*). Incubation of the protein complex with the reductants β-mercaptoethanol (β-Me) or dithiothreitol (DTT), prior to T1-GMS, did not alter the RNA-binding capacity of the RNP (Fig. 1, lanes 2 to 4). However, addition of the oxidant dithionitrobenzoic acid (DTNB) (*17*) completely abolished the RNA-binding capacity of the protein complex (Fig. 1, lane 5). This inhibition of RNP formation persisted after removal of the oxidant by gel permeation chromatography (*18*). To assess whether inactivation of RNA binding by DTNB could be reversed by reduction of the protein complex, we incubated the DTNB-oxidized proteins with either β-Me or DTT and subjected the proteins to T1-GMS. Addition of up to 50 mM β-Me did not restore the RNA-binding activity (Fig. 1, lanes 6 and 7), in-

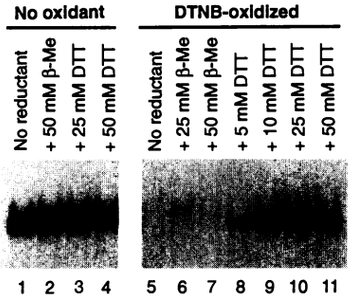

Fig. 1. Redox control of *psbA* mRNA binding in vitro. Autoradiograph of gel mobility–shift assay, displaying the labeled 5′-UTR of *psbA* mRNA complexed with *psbA* mRNA–affinity chromatography–purified proteins (*psbA*-RACPs) (*11*). RNA-binding assays contained *psbA*-RACPs that were treated for 10 min with the reductants β-mercaptoethanol (β-Me, lane 2) or dithiothreitol (DTT, lanes 3 and 4) at concentrations indicated above each lane, or the oxidant dithionitrobenzoic acid (DTNB, lane 5) before the addition of labeled RNA. To examine whether the RNA-binding capacity of the oxidized *psbA*-RACPs could be restored by reduction, we treated DTNB-oxidized *psbA*-RACPs (lane 5) with either β-Me (lanes 6 to 7) or DTT (lanes 8 to11) for 10 min before incubation with labeled RNA and compared RNA binding of these proteins to that of untreated proteins (lane 1). Proteins were then incubated with 0.25 U of the RNase inhibitor Inhibit-Ace (5 Prime→3 Prime) in the presence of 3 mM $MgCl_2$ for 15 min before the addition of 0.065 pmol of in vitro–synthesized ^{32}P-labeled 5′-UTR of *psbA* mRNA and 20 μg of wheat-germ tRNA. The binding reactions were then digested with 0.25 U of RNase T1 and fractionated with a nondenaturing polyacrylamide gel electrophoresis. The presence of bound RNA-protein complex was detected by autoradiography. Only the portions of the gel containing the RNA-protein complexes are shown.

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