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Research report

Nitric oxide synthase inhibitor blocks light-induced phase shifts of the circadian activity rhythm, but not c-*fos* expression in the suprachiasmatic nucleus of the Syrian hamster

E. Todd Weber [a], Robert L. Gannon [a], Anna Marie Michel [a], Martha U. Gillette [b], Michael A. Rea [a,*]

[a] *BRAIN Research Institute, Armstrong Laboratory, Brooks AFB, TX 78235, USA*
[b] *Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801, USA*

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Abstract

Circadian rhythms in mammals are entrained to the environmental light cycle by daily adjustments in the phase of the circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus. Brief exposure of hamsters maintained under constant darkness to ambient light during subjective nighttime produces both phase shifts of the circadian activity rhythm and characteristic patterns of c-*fos* protein (Fos) immunoreactivity in the SCN. In this study, we demonstrate that light-induced phase shifts of the circadian activity rhythm are blocked by intracerebroventricular (i.c.v.) injection of the competitive nitric oxide synthase (NOS) inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), but not by the inactive isomer, D-NAME. The effects of L-NAME are reversible and dose-related, and are countered by co-injection of arginine, the natural substrate for NOS. While effects on behavioral rhythms are pronounced, similar treatment does not alter the pattern of light-induced Fos immunoreactivity in the SCN. These results suggest that nitric oxide is a component of the signal transduction pathway that communicates photic information to the SCN circadian pacemaker, and that nitric oxide production is either independent of, or downstream from, pathways involved in induction of c-*fos* expression.

Keywords: Nitric oxide; Circadian rhythm; Suprachiasmatic nucleus; Fos; Photic entrainment

1. Introduction

The light-entrainable circadian pacemaker responsible for the generation of circadian rhythms in mammals has been localized to the hypothalamic suprachiasmatic nuclei (SCN) [22,23,31]. SCN-driven circadian rhythms are entrained to the environmental light–dark (LD) cycle as a consequence of daily, light-induced adjustments in the phase of the circadian pacemaker. Light at dusk causes phase delays, while light exposure at dawn results in phase advances of the circadian pacemaker [9,12]. Light exposure at circadian phases that results in phase shifts of the circadian pacemaker induces expression of a number of immediate early genes [4,14,18,19,25,26,30], including c-*fos*, among discrete populations of SCN cells. Light-induced expression of Fos in the SCN has been postulated to play a role in photic entrainment [4,14,18,19,25,26,29,30].

Previously we demonstrated that NOS activity is necessary for light-induced advances of circadian rhythms [13]. In order to further determine the role of NO in the transduction of photic information in the SCN, we examined the effects of local administration of the competitive NOS inhibitor, *N*-nitro-L-arginine (L-NAME), on light-induced phase advances and delays of the free-running activity rhythm and on light-induced Fos expression in the hamster SCN.

2. Materials and methods

Syrian hamsters (*Mesocricetus auratus*) were obtained from Charles River (Wilmington, MA) and housed under a LD 14:10 lighting cycle for at least 2 weeks prior to surgery. Pentobarbital-anesthetized (90 mg/kg, i.p.) hamsters (130–180 g at the time of surgery) received cannula guides implanted to a depth of 2.9 mm below the dura and

* Corresponding author. Biological Rhythms and Integrative Neuroscience Research Institute, Armstrong Laboratory (CFTO), 2504 Gillingham Drive, Suite 25, Brooks AFB, TX 78235-5104, USA. Fax: (1) (210) 536-3513. e-mail: rea%kirk.decnet@hsdp2.brooks.af.mil

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fixed to the skull with fine machine screws and dental cement. Cannula guides fixed at stereotaxic coordinates of 1.0 mm anterior to bregma at the midline (upper incisor bar at 0) were aimed at the floor of the third ventricle near the SCN [27]. After recovery from surgery (1 week under LD 14:10), animals were transferred to individual cages equipped with 9-inch running wheels and maintained under constant darkness (DD). Wheel-running activity was monitored continuously on a computer running Dataquest III data acquisition software (Minimitter Co., Sunriver, OR). The onset of wheel running activity, designated as circadian time (CT) 12, was used as a phase reference point for the timing of photic stimulation. The period of the free-running rhythm was calculated as the average amount of time between activity onsets over the five days prior to 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. After stable free-running activity rhythms were established (10–14 days), animals received i.c.v. injections of either (1) artificial cerebrospinal fluid (ACSF; 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 $CaCl_2$), (2) 0.1 mM *N*-nitro-L-arginine (L-NAME) in ACSF, (3) 1 mM L-NAME in ACSF, (4) 1 mM L-NAME plus 4 mM arginine in ACSF, or (5) 1 mM *N*-nitro-D-arginine methyl ester (D-NAME) in ACSF. Intracerebroventricular administration was achieved using 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 min prior to 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 10–15 s after the injection. All drugs were prepared fresh the day of injection.

Most cannulated hamsters received light stimulation (20

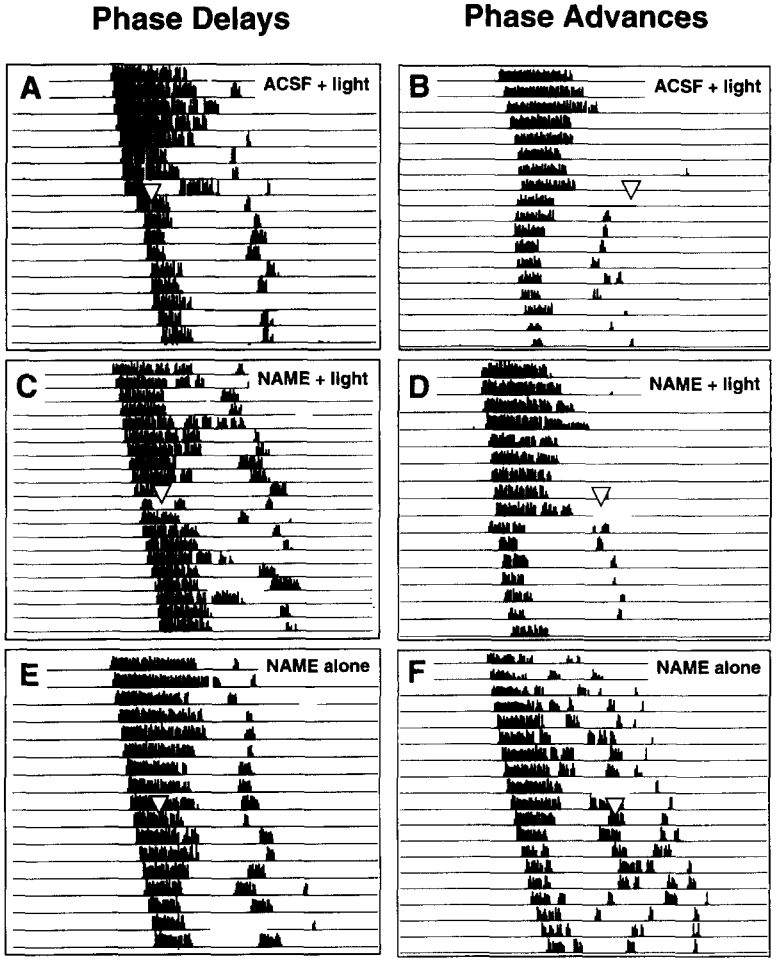

Fig. 1. I.c.v. administration of L-NAME inhibits light-induced phase shifts of the free-running activity rhythm in hamsters. Shown are representative actograms of individual hamsters that received the following treatments at the times indicated by the inverted triangles (▽). A,B: i.c.v. injections of 0.5 μl ACSF followed by exposure to 20 lux of white light for 5 min. C,D: i.c.v. injections of 0.5 μl of 1 mM L-NAME in ACSF followed by exposure to 20 lux of white light for 5 min. E,F: i.c.v. injections of 0.5 μl of 1 mM L-NAME in ACSF without light exposure. Light stimulation was initiated at either CT 14 (left panels) or CT 19 (right panels). Horizontal lines represent successive days while vertical deflections indicate 6-min bins during which wheel running activity occurred. The height of the vertical deflections is proportional to the number of wheel turns that occurred during that 6-min period.

lux white light for 5 min) beginning 10 min after injection. Injections were timed so that light stimulation occurred either 2 circadian hours (1 circadian hour = free-running period/24) after the predicted activity onset (CT 14), or 7 circadian hours after the predicted activity onset (CT 19). After photic stimulation, the hamsters were returned to darkness and wheel-running activity was monitored for an additional 10–14 days [27]. After data collection, the location of the injection site was verified histologically by examining 75-μm-thick vibratome sections cut through the injection site.

In order to further elucidate the mechanism by which L-NAME-sensitive photic information is transduced in the SCN, the effect of L-NAME on photically-stimulated Fos production was examined. Animals received i.c.v. injections of either ACSF or 1 mM L-NAME 10 min prior to light stimulation (10 min of 20 lux white light) at CT 19, a time in the circadian cycle at which light induces both Fos expression and phase advances of the activity rhythm [25,26], and returned to darkness. Hamsters were anesthetized 90 min after the onset of light stimulation and perfused transcardially [25]. Brains were removed and post-fixed in 4% paraformaldehyde overnight at 4°C, followed by 24 h at 4°C in 0.1 M sodium phosphate buffer (pH 7.4). Seventy micron-thick frontal sections were cut on a vibratome and incubated overnight at 4°C in Fos antiserum (c-*fos* Ab-2 @ 1:2000; Oncogene Science, Manhasset, NY). This antiserum was raised against a synthetic peptide (SGFNADYEASSSRC) corresponding to residues 4–17 of human Fos. Fos-like immunoreactivity (Fos-lir) was detected using a Vectastain ABC kit (Vector Labs, Burlingame, CA). Total immunoreactive cell counts for each SCN were acquired by two raters without knowledge of the treatment for each animal. Cells demonstrating specific nuclear staining above background levels were

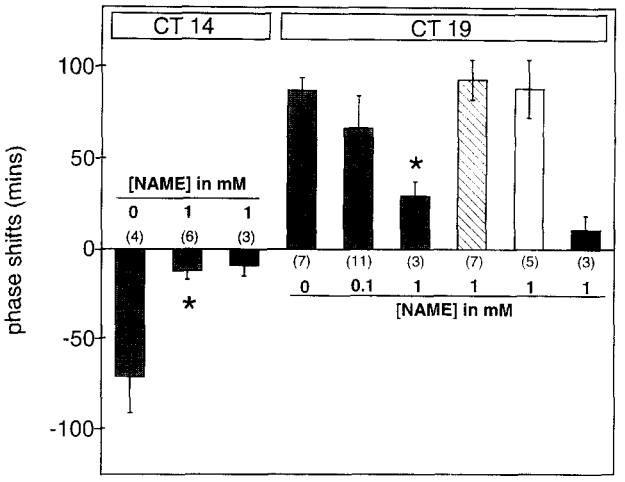

Fig. 3. Effect of i.c.v. L-NAME (gray bars) on light-induced phase shifts is statistically significant. Co-administration of 4 mM arginine with 1 mM L-NAME (crosshatched bar) blocks the inhibition. The inactive isomer, D-NAME (open bar) does not inhibit light-induced phase advances at CT 19. L-NAME alone (solid bars) has no effect on phase. Data represent the mean ± S.E.M. of the number of determinations indicated in parentheses under each bar. Asterisks (*) indicate statistically significant differences ($P < 0.01$) relative to the vehicle + light group as determined by Student's two-tailed t-test.

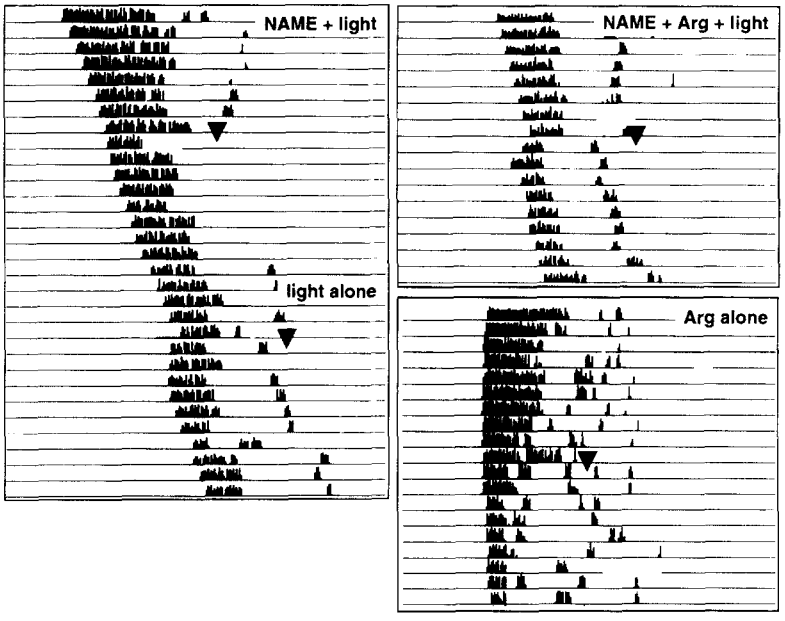

Fig. 2. The effect of L-NAME injection is reversible and blocked by co-administration of arginine. Shown are representative actograms of individual hamsters that received the following treatments at the times indicated by the inverted triangles (▽). Left panel: i.c.v. injection of 1 mM L-NAME followed by exposure to 20 lux of white light for 5 min at CT 19. Thirteen days later, the same hamster received an additional light exposure at CT 19 without injection of L-NAME. Right top panel: i.c.v. injection of 1 mM L-NAME + 4 mM arginine in ACSF followed by exposure to 20 lux of white light for 5 min at CT 19. Right bottom panel: i.c.v. injection of 4 mM arginine in ACSF at CT 19 without light exposure.

counted using a reticle, on a predetermined area corresponding to the Nissl-stained borders of the SCN.

3. Results

Syrian hamsters that received photic stimulation after injection of ACSF showed stable phase advances of the free-running activity rhythm of 89 ± 7 min (mean $\pm$ S.E.M.; $n = 7$), while similar treatment at CT 14 resulted in stable phase delays of -71 ± 20 min ($n = 4$; Figs. 1 and 3). Administration of 1 mM L-NAME prior to light exposure markedly attenuated light-induced phase advances (by 84%) and delays (by 66%) ($P < 0.05$; Figs. 1 and 3). This effect was reversible (Fig. 2) and dose-related (Fig. 3). Furthermore, the effect of 1 mM L-NAME was blocked by co-injection of 4 mM arginine (Fig. 2, right top panel), which overcomes the competitive blockade of nitric oxide synthase by the inhibitor [5]. Neither 1 mM L-NAME nor 4 mM arginine administered alone altered the phase of the circadian activity rhythm (Fig. 1E,F, Fig. 2, right bottom panel), and 4 mM arginine had no effect on light-induced phase advances (data not shown). The inactive isomer, D-NAME, did not attenuate photic phase shifts (90 ± 16 min, $n = 5$).

Light stimulation of animals receiving ACSF resulted in characteristic patterns of Fos expression in the SCN (Fig. 4A). In three independent experiments, local administration of L-NAME did not appear to alter the pattern of light-induced Fos immunoreactivity in the SCN (660 ± 160

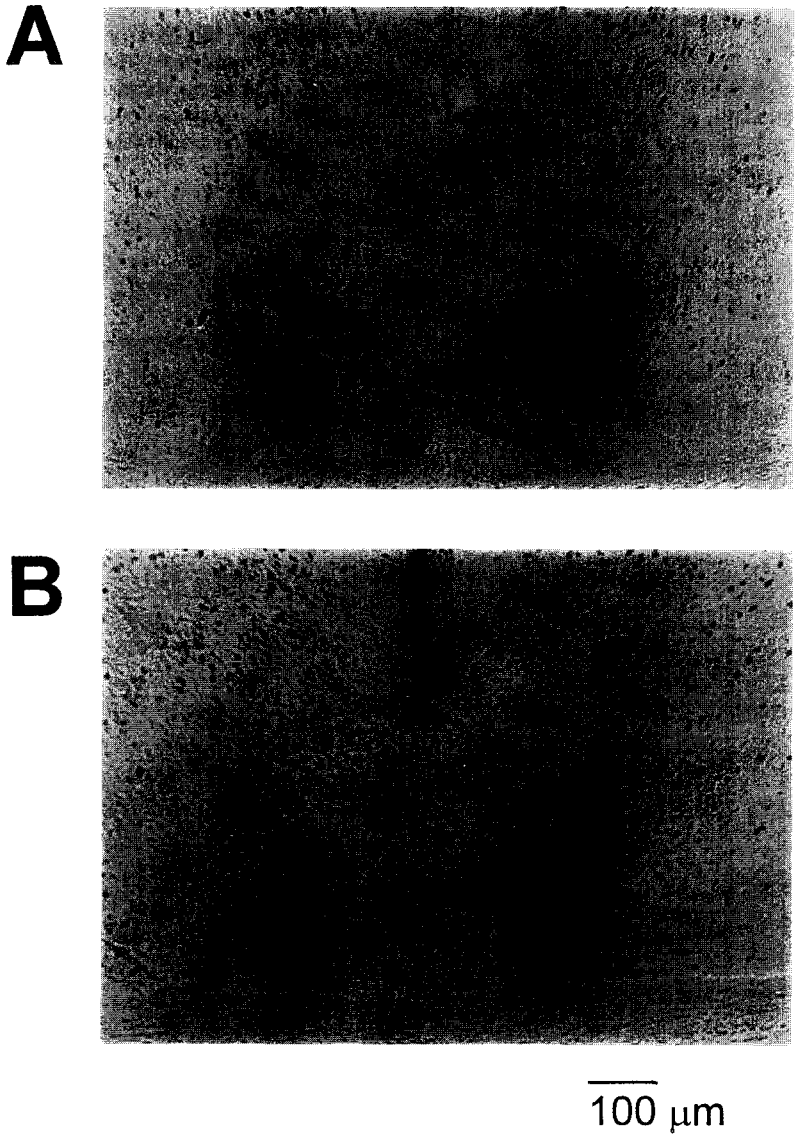

Fig. 4. Intracerebroventricular administration of L-NAME does not inhibit light-induced Fos expression. Shown are representative photomicrographs of individual hamsters that received icv injections of either ACSF (A) or 1 mM L-NAME (B) 10 min prior to exposure to 20 lux of white light for 10 min at CT 19. The number of Fos-immunoreactive cell nuclei observed in the SCN after photic stimulation did not differ significantly (Student's two-tailed t-test, $P > 0.05$).

cells/SCN), compared to vehicle-injected controls (730 ± 140 cells/SCN; Fig. 4). Injection of L-NAME alone did not induce Fos expression (not shown).

4. Discussion

The SCN serve as the primary mammalian circadian clock. Light is capable of resetting the phase of SCN-generated circadian rhythms, as well as inducing production of the immediate-early gene product, Fos, at temporally restricted portions of the circadian cycle. In a previous study, we showed that light-induced phase advances require activation of nitric oxide synthase [13]. The results of the present study demonstrate that both light-induced advances and delays in phase of the circadian system are attenuated by the NOS inhibitor, L-NAME, while the inactive isomer, D-NAME, has no effect. The effects of L-NAME are dose-related, and are reversed by co-administration of arginine, the natural substrate for NOS [5,24]. Furthermore, L-NAME does not inhibit Fos expression, suggesting that nitric oxide production is either downstream of Fos production in the light-entrainment pathway, or that light-induced Fos expression occurs via a parallel, NO-independent mechanism within the SCN.

Initial observations implicated Fos expression in the process of light-induced resetting of the circadian pacemaker. Light causes phase shifts in activity rhythms as well as Fos expression at similar portions of the circadian cycle, indicating that these processes are gated by the circadian clock [19,25,30]. Both pathways are blocked by excitatory amino acid receptor antagonists [1,2,35]. In addition, attenuating c-*fos* transcription by local injection of antisense oligonucleotides for c-*fos* and Jun-B blocks phase shifts induced by light [36]. However, Fos expression is clearly not required for phase shifting in non-photic paradigms [6–8,21,37]. While our findings do not preclude an involvement for Fos in photic phase shifting, they do suggest either that Fos production is upstream of NO synthesis, or that transcription of c-*fos* is regulated by a parallel pathway also activated by photic stimulation.

Evidence from several laboratories suggests that NO appears to act primarily, if not exclusively, as an extrasynaptic transcellular messenger [13,15,32]. Thus, the current results raise the possibility that cellular elements in the vicinity of retinorecipient neurons in the SCN may be involved in the processing of photic information relevant to phase regulation of the circadian clock. One interesting possibility is that SCN astrocytes may serve as targets for NO produced in response to retinohypothalamic tract neurotransmission. SCN astrocytes display circadian rhythms in morphology and GFAP immunoreactivity [20], and have been postulated to regulate the excitability of SCN neurons [16,28,34]. Both guanylate cyclase and nitric oxide synthase have been identified in glial cultures [3,11], and reports from several laboratories indicate that astrocytes accumulate cGMP in response to nitric oxide [3,10,17,33]. The role of SCN astrocytes in the processing of information conveyed by NO, as well as in the regulation of the circadian clock, remains to be elucidated.

Since our drug injections were delivered intracerebroventricularly, we cannot exclude the possibility that phase-shifting of circadian rhythms requires nitric oxide at sites afferent to the SCN, and does not directly regulate transduction of photic information within the SCN. However, those projections would most likely be restricted to areas immediately adjacent to the SCN included in the hypothalamic brain slice, in which L-NAME also inhibits phase shifts of circadian electrical activity rhythms in the SCN [13].

In summary, local administration of the NOS inhibitor, L-NAME, in the region of the SCN blocks light-induced phase advances and delays of the circadian activity rhythm. The inhibition by L-NAME is reversible, stereospecific, dose-related and blocked by co-administration of arginine, the natural substrate for the enzyme. Similar treatment with L-NAME does not attenuate light-induced Fos expression in the SCN. The results suggest that Fos expression occurs either upstream of the nitric oxide-sensitive step of the signal transduction pathway or via a parallel biochemical pathway.

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