Circadian Locomotor Analysis of Male Mice Lacking the Gene for Neuronal Nitric Oxide Synthase (nNOS–/–)

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Abstract Nitric oxide (NO) is an endogenous gas that functions as a neurotransmitter. Because NO is very labile with a half-life of less than 5 sec, most functional studies of NO have manipulated its synthetic enzyme, NO synthase (NOS). Three isoforms of NOS have been identified: (1) in the endothelial lining of blood vessels (eNOS), (2) an inducible form found in macrophages (iNOS), and (3) in neurons (nNOS). Most pharmacological studies to date have blocked all three isoforms of NOS. Previous studies using such agents have revealed that NO might be necessary for photic entrainment of circadian rhythms; general NOS inhibitors attenuate phase shifts of free-running behavior, light-induced c-*fos* expression in the suprachiasmatic nucleus (SCN), and phase shifts of neural firing activity in SCN maintained in vitro. To assess the specific role of nNOS in mediating entrainment of circadian rhythms, mice with targeted deletion of the gene encoding the neuronal isoform of NOS (nNOS–/–) were used. Wild-type (WT) and nNOS–/– mice initially were entrained to a 14:10 light:dark (LD) cycle. After 3 weeks, the LD cycle was either phase advanced or phase delayed. After an additional 3 weeks, animals were held in either constant dim light or constant dark. WT and nNOS–/– animals did not differ in their ability to entrain to the LD cycle, phase shift locomotor activity, or free run in constant conditions. Animals held in constant dark were killed after light exposure during either the subjective day or subjective night to assess c-*fos* induction in the SCN. Light exposure during the subjective night increased c-*fos* expression in the SCN of both WT and nNOS–/– mice relative to animals killed after light exposure during the subjective day. Taken together, these findings suggest that NO from neurons might not be necessary for photic entrainment.

Key words circadian, glutamate, NMDA, knockout, retinohypothalamic tract, suprachiasmatic nucleus, temporal cycles

INTRODUCTION

Nitric oxide (NO) is an endogenous gas that was first identified as a regulator of blood vessel tone (Furchgott and Vanhoutte, 1989; Ignarro, 1990). NO is considered to be a neural messenger in both the central and peripheral nervous systems (Dawson and Snyder, 1994; Sanders and Ward, 1992; Toda and Okamura,

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1992). Because NO is extremely labile, several studies have sought to determine the actions of NO by manipulating its synthetic enzyme, NO synthase (NOS). Currently, three isoforms of NOS have been identified: (1) in the endothelial tissue of blood vessels (eNOS), (2) an inducible form in macrophages (iNOS), and (3) in neural tissue (nNOS) (Lowenstein and Snyder, 1992; Marletta, 1993). In the central nervous system, NO synthesis begins when glutamate (Glu) binds to the *N*-methyl-D-aspartate (NMDA) subtype of Glu receptor, causing the opening of cation permeable channels. Calcium (Ca^{2+}) enters through the NMDA channel, binds to calmodulin (a cofactor for NOS), and stimulates NOS activity; NOS converts arginine into NO and citrulline (Marletta et al., 1988; Palmer and Moncada, 1989).

Previous studies have blocked the formation of NO by eliminating arginine or by administering a potent NOS inhibitor such as L-N^G-nitro-Arg-methyl ester (L-NAME). Administration of L-NAME produces a number of anomalies in rats including deficits in male sexual behavior (Hull et al., 1994), abolition of electrophysiologically induced penile erections (Burnett et al., 1992), and blockade of hippocampal long-term potentiation (O'Dell et al., 1994).

Recently, NO has been implicated in mammalian biological rhythms. Several findings suggest that NO is involved in the transmission of photic information from the retina to the suprachiasmatic nucleus (SCN), the main circadian oscillator (Amir, 1992; Ding et al., 1994; Melo et al., 1997; Watanabe et al., 1994). In mammals, entrainment of circadian rhythms to the environmental light:dark (LD) cycle is mediated by the transmission of photic information from retinal ganglion cells to the SCN via a direct retinohypothalamic tract (RHT) (Moore, 1973; reviewed in Rusak and Bina, 1990). Glu and other excitatory amino acids are involved in the transmission of light information from the retina to the SCN. Glu has been localized in terminals of the RHT in the SCN (de Vries et al., 1993; Castel et al., 1993), and application of Glu to rat SCN slice preparations produces phase shifts predicted by the rat phase response curve (Ding et al., 1994). Glu may act on NMDA receptors to mediate photic entrainment. NMDA applied to SCN slice preparations mimics the effects of Glu application (Ding et al., 1994), and NMDA receptor antagonists prevent light-induced phase shifts in intact animals (Colwell et al., 1990; Colwell and Menaker, 1992).

Glu appears to act on NMDA receptors to mediate photic entrainment, and Glu binding can activate NOS. Several lines of evidence suggest that NO is necessary to transmit photic information from the retina to the SCN. Intracerebroventricular administration of L-NAME (10 min before light exposure) significantly attenuates light-induced phase advances of activity in male Syrian hamsters (*Mesocricetus auratus*) (Ding et al., 1994). Likewise, peripheral injections of L-NAME block light-induced phase delays in wheel-running activity (Watanabe et al., 1995). In addition, NOS inhibitors prevent NMDA- or Glu-induced phase shifts of SCN activity rhythms in vitro (Ding et al., 1994). NOS inhibitors also attenuate light-induced *c-fos* expression and Glu-induced activation of cyclic AMP response element binding protein in the SCN (Amir and Edelstein, 1997; Ding et al., 1997). These findings suggest that NO is involved in transmitting photic information to the SCN. However, treatment with L-NAME has many nonspecific effects; for example, L-NAME affects all three forms of NOS and affects systemic blood pressure and blood flow throughout the brain.

The present study used mutant mice with targeted disruption of the gene encoding the neuronal isoform of NOS to determine whether nNOS is responsible for producing NO to transmit photic information from the retina to the SCN. It is possible that the neuronal isoform of NOS is solely responsible for producing NO to transduce photic information. If this hypothesis is correct, then nNOS–/– animals should exhibit marked abnormalities in entraining to an LD cycle and phase shift behavior after a delay of the LD cycle. Alternatively, another isoform of NOS (e.g., eNOS) might be responsible for generating NO to transmit light information to the SCN. If another isoform of NOS is involved in photic entrainment, then nNOS–/– animals should maintain the ability to entrain to an LD cycle and phase shift behavior. However, it also is possible that more than one isoform of NOS is involved in entrainment. In that case, nNOS–/– animals might maintain or exhibit minor impairments in the ability to entrain to an LD cycle and phase shift behavior. These possibilities were examined in the present experiment.

MATERIALS AND METHODS

Experiment 1

Animals. A total of 9 mutant mice with targeted disruption of the nNOS gene (nNOS–/–) and 9 wild-type (WT) animals (C57BL6J), 4 to 8 months of age at

the time of testing, were housed individually in polycarbonate cages (28 × 17 × 12 cm) and were maintained in LD 14:10 photoperiods (lights on 0700 h [all times Eastern Standard Time]) at 20 ± 2° C temperatures with relative humidity of 50 ± 5%. Food and tap water were available ad libitum for the duration of the study.

Assessment of circadian rhythms. Mice were housed individually in polycarbonate cages equipped with activity wheels. Wheel revolutions were monitored continuously by computer (Dataquest III, Mini-Mitter Inc., Sunriver, OR). Cumulated counts were recorded every 10 min. At the onset of the study, animals were maintained in an LD 14:10 photoperiod (lights on 0600 h) for 3 weeks. Subsequently, behavior was phase delayed by delaying the photoperiod by 4 h (LD 14:10, lights on 1000 h). Animals remained in this photoperiod for 2 weeks. Finally, animals were maintained in constant dim light (60 lux) for a 3-week duration.

The data from the final 10 days of the first and second portions of the experiment (i.e., LD 14:10 and LD 14:10 delayed 4 h) were used to calculate the duration (α) of the active phase. The duration of the active phase was defined as the interval between activity onset and activity offset. Activity onset was operationally defined as the first bout of sustained wheel running after a period of 2 h with less than 20 min of running. Activity bouts were considered sustained if there was less than 20 min of inactivity between successive wheel revolutions. Activity offset was defined as the final bout of sustained wheel running before a period of 2 h with less than 20 min of activity. These data also were used to calculate the phase angle (ψ) of the entrained rhythm. The number of days required to reentrain after the photoperiod was delayed was calculated using the data from the second portion of the experiment. The period (τ) of the free-running rhythm was calculated using the data from the final 10 days of the constant dim light condition.

Statistics. Individual pairwise comparisons were analyzed with independent two-tailed *t* tests. The treatment effects were considered statistically significant if $p < 0.05$.

Experiment 2

Animals. A separate set of 7 nNOS–/– and 8 WT animals (C57BL6J) were used. All animals were 4 to 8 months of age at the time of testing. Animals were housed individually in polycarbonate cages (28 × 17 × 12 cm) and were maintained in LD 14:10 photoperiods (lights on 0700 h) at 20 ± 2° C temperatures with relative humidity of 50 ± 5%. Food and tap water were available ad libitum for the duration of the study.

Assessment of circadian rhythms. Mice were housed individually in polycarbonate cages equipped with activity wheels. Wheels were kept in LD chambers with an internal light source. Wheel revolutions were monitored continuously by computer (Dataquest III; Mini-Mitter Inc., Sunriver, OR). Cumulated counts were recorded every 10 min. At the onset of the study, animals were maintained in an LD 14:10 photoperiod (lights on 0600 h) for 3 weeks. Subsequently, behavior was phase advanced by advancing the photoperiod by 4 h (LD 14:10, lights on 0200 h). Animals remained in this photoperiod for 2 weeks. Finally, animals were maintained in constant darkness for 3 weeks.

The data from the final 10 days of the first and second portions of the experiment (i.e., LD 14:10 and LD 14:10 delayed 4 h) were used to calculate the duration (α) of the active phase. The number of days required to reentrain after the photoperiod was advanced was calculated using the data from the second portion of the experiment. The period (τ) of the free-running rhythm was calculated using the data from the final 10 days of the constant darkness condition.

Statistics. Individual pairwise comparisons were analyzed with independent two-tailed *t* tests. The treatment effects were considered statistically significant if $p < 0.05$.

Experiment 3

Animals. In this experiment, 4 nNOS–/– and 6 WT animals (C57BL6J) from Experiment 2 were used. Animals were housed individually in polycarbonate cages (28 × 17 × 12 cm) and were maintained in LD 14:10 photoperiods (lights on 0700 h) at 20 ± 2° C temperatures with relative humidity of 50 ± 5%. Food and tap water were available ad libitum for the duration of the study.

Treatment. Animals were held in constant darkness for 3 weeks (i.e., conclusion of Experiment 2). WT (*n*'s = 2 and 3 per group, respectively) and nNOS–/– (*n* = 2 per group) were killed after 1 h of light exposure during either the subjective day (circadian time [CT]

6, with CT 12 = onset of activity) or subjective night (CT 19). Mice were killed by lethal injection of sodium pentobarbital and were perfused transcardially with 100 ml 0.9% saline followed by 200 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Brains were removed and placed into fixative for 2 h. Following postfixation, brains were submersed in 30% sucrose overnight. After cryoprotection, brains were frozen on dry ice and stored at –80º C until processed for c-*fos*.

c-fos *immunohistochemistry*. All brains were processed at the same time. Brains were sectioned in the coronal plane at 40 μm using a cryostat. Alternate sections were collected into 0.1 *M* PBS (pH 7.4). Tissue was submersed in 0.5% hydrogen peroxide to reduce endogenous peroxidase activity. Subsequently, samples were incubated in 10% normal goat serum (Vector Laboratories, Burlingame, CA) at room temperature for 1 h. Following preincubation in normal goat serum, the sections were incubated for 48 h at 4º C in the c-*fos* antibody (Oncogene Research Products, Cambridge, MA). Then, sections were sequentially incubated in biotinylated goat anti-rabbit IgG (1:250, Vector Laboratories) and avidin-biotin-HRP complex (Vector Laboratories). HRP label was demonstrated using nickel-intensified 0.04% diaminobenzidine (Polysciences Inc., Warrington, PA) in 0.1 *M* PBS as the chromogen and 0.01% hydrogen peroxide as the substrate. Alternate sections were mounted onto gelatin-coated slides, dehydrated in a graded series of ethanol solutions (30%, 70%, 95%, 100%), and cleared in Hemo-De (Fisher Scientific, Columbia, MD) before the application of coverslips.

Analysis of c-fos *immunreactivity*. Immunopositive cells were counted in alternate sections through the entire SCN by two experimenters unaware of the experimental treatment conditions. Total bilateral counts were performed under bright field microscopy using a Nikon Optiphot microscope. All cell counts were performed at 20× magnification.

RESULTS

Experiment 1

Both WT and nNOS–/– animals entrained to the 14:10 LD cycle during the first portion of the experiment. During the final 10 days of this photoperiod, animals ran for approximately the same duration as during the active phase ($p > 0.05$) (Fig. 1 and Table 1). The phase relationship between photoperiod and activity onset was essentially equivalent for both WT and nNOS–/– mice ($p > 0.05$) (Fig. 1 and Table 1). Similar results were obtained during the final 10 days of the second portion of the experiment (i.e., after the photoperiod was delayed). WT and nNOS–/– animals did not differ in their ability to reentrain to the LD cycle after the photoperiod was delayed by 4 h ($p > 0.05$) (Fig. 1 and Table 1). In addition, during the final 10 days of this portion of the experiment, WT and nNOS–/– mice were entrained to the new photoperiod and

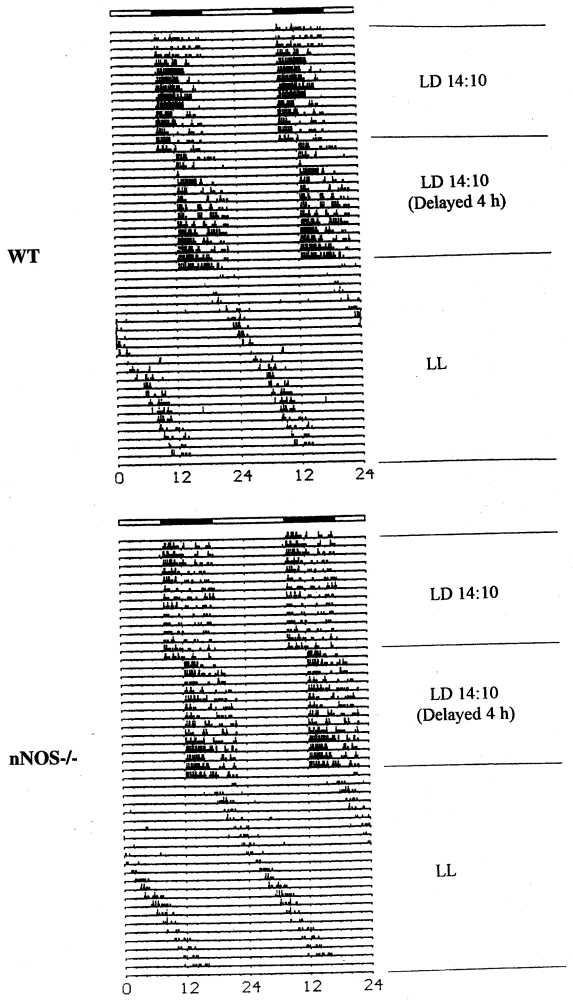

Figure 1. Double-plotted activity record of a wild-type (WT) mouse and a mouse lacking the gene for neuronal nitric oxide synthase (nNOS–/–) entrained to an LD cycle, after a 4-h phase delay of the LD cycle, and in constant dim light.

Table 1. Mean (± *SEM*) phase angles (ψ), durations of the active phase (α), and periods of the free-running rhythm (τ) of wild-type (WT) mice and mice lacking the gene for neuronal nitric oxide synthase (nNOS–/–) from Experiment 1 (hours).

	WT	*nNOS–/–*
ψ_{LD1}	0.20 ± 0.01	0.28 ± 0.11
ψ_{LD2}	0.22 ± 0.03	0.27 ± 0.05
α_{LD1}	9.51 ± 0.31	8.15 ± 0.59
α_{LD2}	7.39 ± 0.99	8.72 ± 0.69
α_{LL}	4.77 ± 0.61	4.28 ± 0.43
τ_{LL}	24.9 ± 0.001	25.0 ± 0.001

NOTE: ψ_{LD1} = phase angle during LD 14:10; ψ_{LD2} = phase angle during LD 14:10 (delayed 4 h); α_{LD1} = activity duration during LD 14:10; α_{LD2} = activity duration during LD 14:10 (delayed 4 h); α_{LL} = activity duration during constant dim light; τ_{LL} = period of the free-running rhythm in constant dim light.

exhibited a similar phase relationship between the onset of the dark cycle and the onset of activity ($p > 0.05$) (Fig. 1 and Table 1). During the final portion of the experiment (i.e., constant dim light), both WT and nNOS–/– animals exhibited a similar period of free-running activity ($p > 0.05$). The duration of the active phase did not differ between WT and nNOS–/– mice during this free-running condition.

Experiment 2

Both WT and nNOS–/– animals entrained to the initial 14:10 LD cycle. nNOS–/– mice tended to run for a slightly shorter duration during the active phase of the initial LD cycle ($p < 0.05$) (Fig. 2 and Table 2). The phase relationship between photoperiod and activity onset was essentially equivalent for both WT and nNOS–/– mice ($p > 0.05$) (Fig. 2 and Table 2). Similar results were obtained for the second portion of the experiment (i.e., following entrainment after the photoperiod was advanced). WT and nNOS–/– animals did not differ in their ability to reentrain to the LD cycle after the photoperiod was advanced by 4 h ($p > 0.05$) (Fig. 2 and Table 2). In addition, during the final 10 days of this portion of the experiment, WT and nNOS–/– mice were entrained to the new photoperiod and exhibited a similar phase relationship between the onset of the dark cycle and the onset of activity ($p > 0.05$) (Fig. 2 and Table 2). During the final portion of the experiment (i.e., constant darkness), both WT and nNOS–/– animals exhibited a similar period of free-running activity ($p > 0.05$). The duration of the active phase did not differ between WT and nNOS–/– mice during this free-running condition.

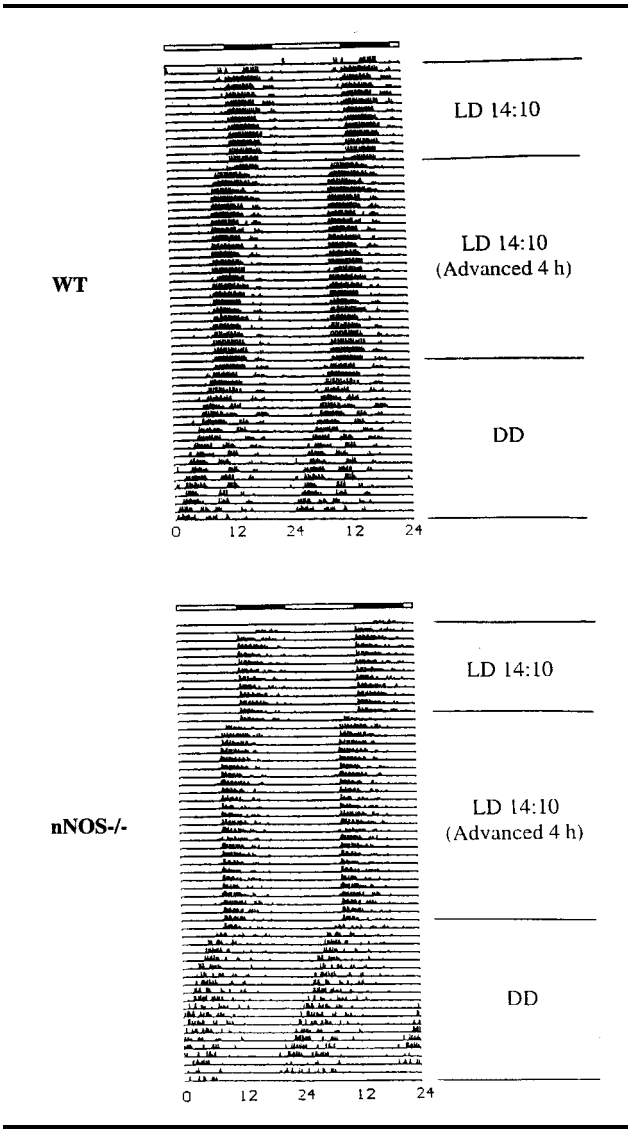

Figure 2. Double-plotted activity record of a wild-type (WT) mouse and a mouse lacking the gene for neuronal nitric oxide synthase (nNOS–/–) entrained to an LD cycle, after a 4-h phase advance of the LD cycle, and in constant darkness.

Table 2. Mean (± *SEM*) phase angles (ψ), durations of the active phase (α), and periods of the free-running rhythm (τ) of wild-type (WT) mice and mice lacking the gene for neuronal nitric oxide synthase (nNOS–/–) from Experiment 2 (hours).

	WT	*nNOS–/–*
ψ_{LD1}	0.34 ± 0.03	0.15 ± 0.08
ψ_{LD2}	0.15 ± 0.23	–0.99 ± 1.18
α_{LD1}	11.14 ± 0.38	9.16 ± 0.68*
α_{LD2}	11.13 ± 0.31	10.64 ± 0.87
α_{DD}	12.53 ± 0.24	9.74 ± 3.42
τ_{DD}	23.39 ± 0.02	23.41 ± 0.04

NOTE: ψ_{LD1} = phase angle during LD 14:10; ψ_{LD2} = phase angle during LD 14:10 (advanced 4 h); α_{LD1} = activity duration during LD 14:10; α_{LD2} = activity duration during LD 14:10 (advanced 4 h); α_{DD} = activity duration during constant darkness; τ_{DD} = period of the free-running rhythm in darkness.
*Significantly less than WT mice.

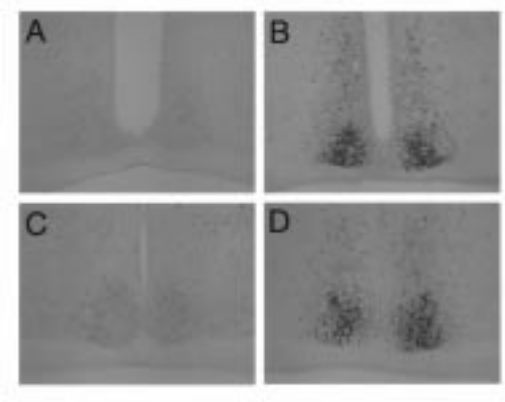

Figure 3. c-*fos* expression in the SCN of a representative wild-type (WT) mouse and a representative mouse lacking the gene for neuronal nitric oxide synthase (nNOS–/–). (A) WT mouse after 1 h of light exposure during the subjective day (CT 6). (B) WT mouse after 1 h of light exposure during the subjective night (CT 19). (C) nNOS–/– mouse after 1 h of light exposure during the subjective day. (D) nNOS–/– mouse after 1 h of light exposure during the subjective night.

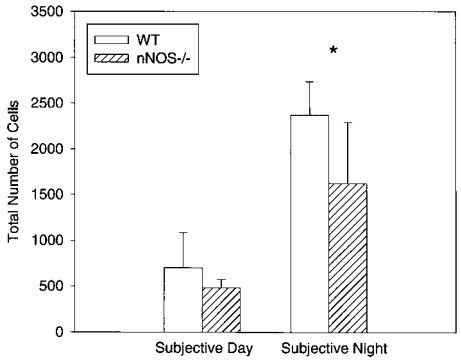

Figure 4. Mean (± *SEM*) number of c-*fos* immunoreactive cells in the SCN of wild-type (WT) mice and mice lacking the gene for neuronal nitric oxide synthase (nNOS–/–) killed after 1 h of light exposure during either the subjective day or the subjective night. Asterisk (*) indicates significantly different from WT and nNOS–/– mice killed after light exposure during the subjective day ($p < .05$).

Experiment 3

Light exposure during the subjective night increased c-*fos* expression in the SCN of both WT and nNOS–/– mice compared to light exposure during the subjective day ($p < 0.05$) (Figs. 3 and 4). There were no differences between genotypes after exposure to light during either the subjective day or the subjective night ($p > .05$). Consistent with other studies in mice (e.g., Colwell and Foster, 1992), c-*fos* was expressed throughout the entire dorsoventral extent of the SCN in both WT and nNOS–/– mice killed after light exposure during the subjective night.

DISCUSSION

In contrast to what most pharmacological studies to date suggest, mice with targeted disruption of the nNOS gene do not exhibit any abnormalities in the period of the free-running rhythm, the ability to entrain to an LD cycle, or the ability to phase shift locomotor activity in response to a change in the LD cycle. These data appear to rule out an important role for nNOS in mediating photic entrainment of circadian rhythms. However, these data do not rule out the possibility that another isoform of NOS might be responsible for generating NO to mediate photic entrainment of circadian rhythms.

Several alternative possibilities may explain the apparently normal circadian entrainment of nNOS–/– mice. First, nNOS and eNOS might work in conjunction in genetically unmanipulated mice to transmit photic information to the SCN. In that case, nNOS–/– mice might maintain the ability to entrain to an LD cycle and phase shift behavior because eNOS is necessary and sufficient to maintain circadian entrainment. Alternatively, only nNOS might be involved in photic entrainment of circadian rhythms in genetically unaltered mice, yet nNOS–/– animals have a developmental compensatory increase in eNOS to maintain the ability to entrain to an LD cycle. eNOS is found primarily in the endothelial lining of blood vessels in the central nervous system and periphery. However, there is some evidence that eNOS is expressed in neuronal populations (Dinerman et al., 1994). Finally, nNOS–/– mice might have a deficit in their ability to entrain to an LD cycle, but the light stimulus used in the present study might have been too intense to detect a change in the sensitivity to light. Future studies of nNOS–/– mice using lighting schedules of different intensities are necessary to examine this possibility.

NOS protein and messenger RNA have been localized to discrete neuronal populations within the brain (Bredt et al., 1991; Vincent and Kimura, 1992). Recently, immunohistochemical staining for nNOS and NADPH-diaphorase have revealed staining for nNOS in and surrounding the SCN (Lupi et al., 1996; Decker and Reuss, 1994; Reuss et al., 1995; Wang and Morris, 1996). This finding suggests that nNOS might be important for photic entrainment in genetically unaltered mice. Conceivably, because the gene for nNOS is missing throughout ontogeny, nNOS–/– mice might

have developed a compensatory mechanism(s) that allows entrainment to the environmental LD cycle (reviewed in Nelson and Young, 1998). For example, mice missing only the gene for nNOS or eNOS exhibit no deficit in long-term potentiation, whereas double-mutant mice (missing eNOS and nNOS) have profound deficits in long-term potentiation (Son et al., 1996). If nNOS–/– mice entrain to the LD cycle due to a developmental compensatory mechanism, then this mechanism is unlikely to involve increased eNOS; nNOS–/– mice do not show any evidence of staining for NOS or citrulline in or directly surrounding the SCN (L. J. Kriegsfeld, unpublished observations).

Light exposure during a circadian time capable of phase shifting behavior induced c-*fos* in the SCN of both WT and nNOS–/– mice, suggesting that light information is being transmitted to the SCN of nNOS–/– mice. Importantly, one study found that intracerebroventricular injections of L-NAME are capable of blocking light-induced phase shifts of circadian activity without blocking light-induced c-*fos* expression in the SCN (Weber et al., 1995). Therefore, NO mediation of circadian entrainment might be independent of, or downstream from, c-*fos* induction pathways.

Taken together, the data from the present three experiments suggest that NO from neurons might not be necessary for photic entrainment. Future studies in which specific neuronal NOS inhibitors are used in vivo and in vitro are necessary to clarify the discrepancy between the data obtained from pharmacological and genetic manipulations.

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