

Localization and Characterization of Nitric Oxide Synthase in the Rat Suprachiasmatic Nucleus: Evidence for a Nitrergic Plexus in the Biological Clock

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Abstract: Behavioral and electrophysiological evidence indicates that the biological clock in the hypothalamic suprachiasmatic nuclei (SCN) can be reset at night through release of glutamate from the retinohypothalamic tract and subsequent activation of nitric oxide synthase (NOS). However, previous studies using NADPH-diaphorase staining or immunocytochemistry to localize NOS found either no or only a few positive cells in the SCN. By monitoring conversion of L-[³H]arginine to L-[³H]-citrulline, this study demonstrates that extracts of SCN tissue exhibit NOS specific activity comparable to that of rat cerebellum. The enzymatic reaction requires the presence of NADPH and is Ca²⁺/calmodulin-dependent. To distinguish the neuronal isoform (nNOS; type I) from the endothelial isoform (type III), the enzyme activity was assayed over a range of pH values. The optimal pH for the reaction was 6.7, a characteristic value for nNOS. No difference in nNOS levels was seen between SCN collected in day versus night, either by western blot or by enzyme activity measurement. Confocal microscopy revealed for the first time a dense plexus of cell processes stained for nNOS. These data demonstrate that neuronal fibers within the rat SCN express abundant nNOS and that the level of the enzyme does not vary temporally. The distribution and quantity of nNOS support a prominent regulatory role for this nitrergic component in the SCN.

Key Words: Circadian rhythms—Confocal microscopy—Nitric oxide synthase—Rat—Suprachiasmatic nucleus.

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trolled (Fiallos-Estrada et al., 1993; Matsumoto et al., 1993). Whereas iNOS activity is Ca²⁺-independent, activation of nNOS and eNOS requires a rise in intracellular Ca²⁺ concentration. In the CNS, NO can be released upon glutamate receptor activation and lead to elevation in cyclic GMP levels (Garthwaite et al., 1988). This NO-linked glutamate/cyclic GMP signaling pathway has been best documented in the cerebellum (Bredt and Snyder, 1989; Southam et al., 1991) but operates in other brain regions as well, including the neocortex (Carroll et al., 1996), striatum (Marin et al., 1992), and hypothalamus (Belsham et al., 1996).

Recent studies have demonstrated a role for NO in the glutamatergic regulation of the circadian rhythms (Ding et al., 1994; Watanabe et al., 1994, 1995; Weber et al., 1995). As the master clock programming daily behaviors, the suprachiasmatic nucleus (SCN) in the hypothalamus receives a direct input from the retina through the retinohypothalamic tract (Moore and Lenn, 1972). This projection plays a principal role in synchronizing animal behaviors controlled by the clock to the environmental day–night cycle (Stephan and Nunez, 1976; Johnson et al., 1988). Glutamate is the primary neurotransmitter released from retinohypothalamic tract terminals (Liou et al., 1986; Kim and Dudek, 1991; Rea et al., 1993) and adjusts the phasing of the SCN neuronal firing rhythm in brain slices in a manner very similar to that of the light-induced behav-

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Abbreviations used: CT, circadian time; eNOS, iNOS, and nNOS, endothelial, inducible, and neuronal nitric oxide synthase, respectively; ICC, immunocytochemistry; NADPH-d, NADPH-dependent diaphorase; L-NAME, *N*-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; SCN, suprachiasmatic nucleus.

The inorganic gas, nitric oxide (NO), is produced from arginine (Palmer et al., 1988) by cellular NO synthase (NOS). The NOS family comprises three iso-enzymes (for review, see Griffith and Stuehr, 1995). The inducible form of NOS (iNOS; type II) is rapidly up-regulated following appropriate immunologic stimulation (Hibbs et al., 1987). Neuronal NOS (nNOS; type I) and endothelial NOS (eNOS; type III) are generally constitutive but also can be transcriptionally con-

ioral phase adjustments (Ding et al., 1994; Shibata et al., 1994; Shirakawa and Moore, 1994). Both light and glutamate are effective at phase resetting only at night.

The signaling pathway activated in the SCN by glutamate involves NO. NOS inhibitors or NO donors blocked and mimicked glutamate-induced phase shifts, respectively, in the SCN brain slice (Ding et al., 1994; Watanabe et al., 1994). When the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) was injected into the SCN region of hamsters, light-induced shifts in the phase of wheel-running rhythms were effectively blocked (Watanabe et al., 1995; Weber et al., 1995). L-Arginine, a natural substrate of NOS, outcompeted the inhibitory effect of L-NAME both in vivo and in vitro (Ding et al., 1994; Watanabe et al., 1994; Weber et al., 1995). Also, the SCN is sensitive to NO only during the night phase of the clock (Ding et al., 1994; see, however, Starkey, 1996).

Despite this compelling functional evidence, demonstration of NOS in the SCN has been elusive. The SCN was not among the positive areas reported by studies mapping NOS throughout the rat brain using either immunocytochemistry (ICC) or NADPH-dependent diaphorase (NADPH-d) histochemistry (Bredt et al., 1991; Vincent and Kimura, 1992). Nevertheless, significant NOS specific activity has been measured in extracts of SCN at nighttime (Ding et al., 1994). To resolve this apparent paradox, we sought to identify and characterize the isoform(s) of the NOS expressed in the SCN and to localize the putative NO-synthesizing components by ICC.

MATERIALS AND METHODS

Brain slice preparation and tissue collection

Inbred Long-Evans rats were born and raised in our colony for 6–9 weeks. The animals were allowed free access to rat chow and water and maintained at the desired 12-h light/12-h dark lighting schedule for at least 2 weeks before they were killed. The procedures for brain slice preparation and tissue collection have been described previously (Prosser and Gillette, 1991). In brief, coronal hypothalamic slices (500 μ M) containing the SCN were either frozen immediately or maintained at the gas-media interface of a brain slice chamber for at least 4 h before tissue collection at the desired circadian time (CT). CT is reckoned from CT 0, "lights on" in the rat colony, and continues for 24 h. Slices were transferred to a dry ice-cooled glass slide, and the SCN was punched out with a flattened 28-gauge syringe needle under a dissecting microscope. Punches were kept at -70°C until use, for up to 1 week. Tissue punches of cerebellar and hippocampal slices were collected in the same way except that they were frozen immediately after removal from the brain.

NOS activity measurement

NOS activity was assayed by monitoring the conversion of radiolabeled arginine to citrulline (Bredt and Snyder, 1990). Tissue was disrupted by five freeze-thaw cycles, placed in 50 mM HEPES buffer (pH 7.1) containing 1 mM dithiothreitol and 1 mM EDTA, and then centrifuged at 10,000 g for

5 min. The supernatant was passed through a Dowex 50 (Na^+ form; Sigma) column to remove endogenous arginine before 0.12 μ M L-[^3H]arginine (36.7 $\mu\text{Ci}/\mu\text{l}$; Du Pont) and 0.3 μ M L-arginine were added. The reaction was initiated by addition of 0.5 mM β -NADPH, 10 $\mu\text{g}/\text{ml}$ calmodulin, and 1.25 mM CaCl_2 in a total volume of 100 μl containing ~ 20 μg of protein. After incubation at 22°C for 10 min, the reaction was stopped by adding ice-cold HEPES buffer (pH 5.5, 4 mM EDTA), and reactants were separated on another Dowex 50 column, eluted, and counted for radioactivity. The identity of [^3H]citrulline was confirmed by paper chromatography. The quantitative recovery of citrulline was determined using L-[^{14}C]citrulline. Protein concentrations were measured by the assay of Bradford (1976).

Western analysis of NOS

Punches of SCN at CT 7 and 14 and cerebellum were prepared as described above. Membrane-bound, soluble, and total protein extracts were prepared immediately before electrophoresis.

Total protein extract. Two punches (~ 10 μg of protein) of each sample were suspended in 40 μl of denaturing buffer, sonicated briefly, and boiled for 3 min.

Membrane-bound and soluble protein extracts. For each sample, two punches were resuspended in 20 μl of fractionation buffer [20 mM HEPES (pH 7.2), 0.2 mM EDTA, 1.0 mM EGTA, 1.0 mM NaF, 5 μM microcystin, 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 0.7 $\mu\text{g}/\text{ml}$ pepstatin, 1.0 $\mu\text{g}/\text{ml}$ aprotinin, 40 $\mu\text{g}/\text{ml}$ bestatin, 10 mM glycerol phosphate, 5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride] and centrifuged at 100,000 g (60 min, 4°C). After centrifugation, both the supernatant (soluble protein) and precipitate (membrane-bound protein) were diluted to final volumes of 40 μl in denaturing buffer and boiled for 3 min. Thirty-five microliters of each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel and transferred to nitrocellulose (pore size, 0.2 μm ; Bio-Rad). The remaining 5 μl of each sample was subjected to silver staining to evaluate protein loading.

Nitrocellulose blots were probed with the following monoclonal anti-peptide antibodies (Transduction Labs): rabbit anti-nNOS, rabbit anti-eNOS, rabbit anti-iNOS, mouse anti-nNOS, mouse anti-eNOS, and mouse anti-iNOS. A rabbit polyclonal antibody against the entire nNOS protein (1:1,000) was also tested (Kummer et al., 1992). Immunoreactivity was visualized using horseradish peroxidase-linked goat anti-rabbit (Chemicon) or goat anti-mouse (Zymed) secondary antibody and an ECL fluorescence detection system (Amersham). Specificity of immunoreactive bands was determined by preabsorbing the polyclonal nNOS antibody with 25 $\mu\text{g}/\text{ml}$ nNOS purified from baculovirus-infected insect cells (Harteneck et al., 1994) for 60 min at room temperature before probing nitrocellulose blots.

ICC and confocal microscopy

Four animals were anesthetized with sodium pentobarbital (75 mg/kg i.p.) and perfused intracardially with chilled 0.1 M phosphate-buffered saline (pH 7.4) followed by chilled 4% paraformaldehyde in phosphate-buffered saline. The brains were removed, postfixed, and sectioned at 50–80 μm by a Vibratome. Tissue sections were incubated with 0.3% Triton X-100 detergent and 5% heat-inactivated goat serum to permeabilize the membrane and to block nonspecific binding sites, respectively. The sections were next incubated with

the rabbit polyclonal anti-nNOS antibody (Kummer et al., 1992). Alternate sections were tested with preabsorbed antibody as described earlier and processed for the fluorescein isothiocyanate-conjugated avidin–biotin (Vector Laboratories) amplification reaction.

The fluorescent specimens were imaged at 488 nm using helium–neon and argon ion lasers on a Zeiss LSM 10 confocal laser scanning microscope at the Optical Visualization Laboratory of the Beckman Institute for Advanced Science. Images were collected as optical sections through the entire thickness of each tissue section. The confocal laser scanning microscope was programmed to collect a set of images by optical scanning of planes at consecutive 1–2- μm depths through the 50–80- μm section. In doing so, we were able to collect only the in-focus information resulting in a set of clear, high-resolution images. The individual images were then added together with perfect alignment to obtain a composite image. The composite image of the whole section was enhanced by Kalman filtration during data acquisition to improve depth of field. Confocal data sets were imported to an IBM 6000 workstation to perform adaptive histogram equalization and to render them as peak intensity projections.

RESULTS

Characterization of NOS activity in the SCN

The conversion of L-[^3H]arginine to L-[^3H]citrulline by SCN extract was linear within the first 10 min (data not shown). Previous measurements in SCN reduced slices indicated that 2 mM L-NAME inhibited NOS activity completely (Ding et al., 1994). Here we use punches confined to SCN tissue to examine the NADPH and Ca^{2+} /calmodulin dependence of the reaction. Omitting Ca^{2+} or NADPH from the reaction mixture abolished NOS activity. At 32 μM , trifluoperazine, a calmodulin antagonist, reduced the enzyme activity by half (Fig. 1A). The isoform was further characterized by measuring pH dependence of this activity. NOS activity in the SCN exhibited a pH optimum of 6.7 (Fig. 1B).

We compared NOS activity among different brain regions. The specific activity of NOS in the SCN was ~80% of that in the cerebellum and about threefold higher than in the hippocampus (Fig. 1C). No detectable difference in activity was measured among SCN samples collected in daytime (CT 7) and early (CT 14), and late night (CT 19) (Fig. 1D).

Western blot analysis of NOS in the SCN

Western blots were performed to determine antigenic type, molecular weight, and relative levels of NOS protein expression in the SCN as compared with cerebellum (Fig. 2). Immunoreactivity was observed in blots using anti-nNOS antibodies, which identified a band of 155 kDa. Antibodies against eNOS and iNOS did not reveal either isoform. Like the results from enzyme activity assays (Fig. 1C), the amount of nNOS protein in the SCN was comparable to that in the cerebellum, and nNOS protein amounts were similar in SCN collected at CTs 7 and 14 (Fig. 2A; $n = 4$). To

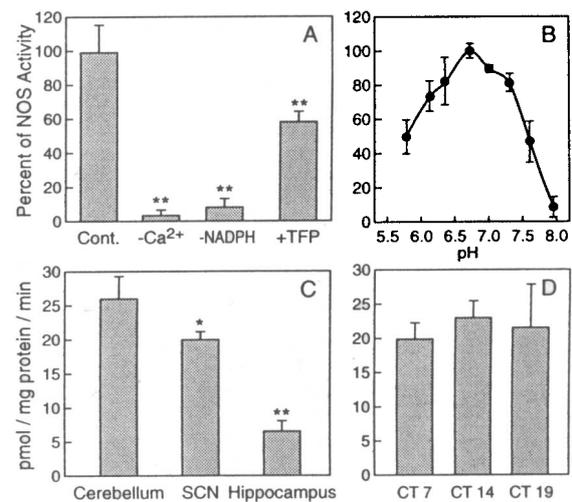


FIG. 1. Tissue extracts of SCN exhibited significant NOS activity. **A:** NOS activity in the rat SCN is Ca^{2+} /calmodulin- and NADPH-dependent. TFP, trifluoperazine. **B:** NOS activity peaked at pH 6.7 in SCN. Tris-bis-propane buffer (50 mM) was used to adjust the pH. **C:** NOS specific activities are similar in cerebellum and SCN and lower in hippocampus. **D:** NOS activities measured from the rat SCN punches collected at CT 7 (midday), CT 14 (early night), and CT 19 (late night) showed no significant variation. Data are mean \pm SEM (bars) values of three or more experiments performed in triplicate. Asterisks indicate significant differences from far-left column values: * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA and Duncan post hoc test.

localize NOS within the cell, the western blot was performed on SCN tissue separated into membrane-bound and soluble fractions (Fig. 2B). About half of NOS protein was associated with the membrane fraction in the SCN. Immunoreactivity at lower molecular weights increased in direct correlation with sample storage time (authors' unpublished data; Fig. 2A). Furthermore, there is a larger relative amount of this lower-molecular-weight species in fractionated samples that have spent 1–2 h at 4°C before denaturing as compared with the whole samples that were immediately denatured on thawing (Fig. 2B). This suggests that the lower-molecular-weight species may be proteolytic degradation products of nNOS. Blots of SCN and cerebellum probed with rabbit polyclonal antibody against the entire nNOS protein revealed one major band at 155 kDa, which specifically disappeared when primary antibody was preabsorbed with purified nNOS.

Immunocytochemical localization of nNOS in the SCN

The composite confocal micrographs revealed both neuronal somata and processes immunoreactive to nNOS in the SCN and surrounding regions of the hypothalamus (Fig. 3). nNOS-positive neuronal somata in the SCN were sparse and mostly located in the ventrolateral region. Whereas few cell bodies were stained,

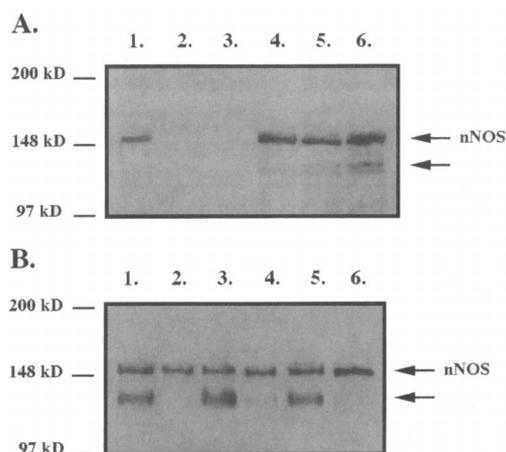


FIG. 2. Western blots of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels probed with a mouse anti-nNOS antibody. **A:** Immunoreactive band at 155 kDa from extracts of pituitary tissue (lane 1; control for nNOS), mouse macrophages (lane 2; control for iNOS), human endothelial cells (lane 3; control for eNOS), rat cerebellum (lane 4), rat SCN at CT 7 (lane 5), and rat SCN at CT 14 (lane 6). **B:** Subcellular localization of NOS. Tissues from cerebellum and SCN were separated into membrane-bound (lane 1, cerebellum; lane 3, SCN at CT 7; lane 5, SCN at CT 14) and soluble (lane 2, cerebellum; lane 4, SCN at CT 7; lane 6, SCN at CT 14) fractions. The upper arrows correspond to the expected molecular mass (155 kDa) of nNOS as seen in rat pituitary extract. Bands indicated by the lower arrows (140 kDa) are not eNOS or iNOS (see control lanes in A) and may correspond to degradation products. Results are representative of four similar blots.

nNOS-positive processes were intense and extensive, pervading the entire nucleus. Thus, they form a nitrergic plexus throughout the SCN. The nNOS-positive neurons within the SCN varied greatly in size, arborization, and intensity. nNOS-positive processes were more intense inside the SCN than in the surrounding hypothalamus, whereas the intensely stained neuronal somata appeared outside the SCN (Fig. 3A). Preabsorption of the antibody with nNOS, the antigen used to raise the antibody, completely blocked nNOS immunoreactivity (Fig. 3B). High-magnification confocal imaging of single optical sections in the ventrolateral SCN demonstrated both nNOS-positive somata and fibers with dense puncta (Fig. 3C). In contrast to the SCN, confocal scans of the striatum exhibited much larger nNOS-positive somata without densely packed puncta (Fig. 3D).

DISCUSSION

Functional studies have demonstrated a requirement for NO in resetting the biological clock (Ding et al., 1994; Watanabe et al., 1994; Weber et al., 1995), but the source of this diffusible messenger has been unclear. We report here that the rat SCN possesses substantial NOS that is attributable to nNOS based on

molecular weight, Ca^{2+} /calmodulin requirement, and pH dependence. Both nNOS activity and levels remain constant between day and night. Consistent with the high specific NOS activity, nNOS was found to be localized both ventrolaterally in sparse, weakly positive neuronal somata and in an intense plexus of processes that permeates the entire SCN.

Previously, NOS had been detected consistently in two hypothalamic areas: The supraoptic and paraventricular nuclei stain intensely both by histochemistry using NADPH-d activity of NOS and by ICC (Bredt et al., 1991; Vincent and Kimura, 1992). The SCN, however, had been refractive to staining by either method. Owing to the paucity of morphological evidence for NOS in the SCN, we measured and compared NOS specific activity in SCN tissue punches and other brain regions. Our results agree with previous observations that NOS activity measured from hippocampal tissue is ~25% of the cerebellar levels (Lin et al., 1994). It was, however, surprising that the SCN exhibited a high level of NOS activity only 20% lower than that of the cerebellum, the brain region reported to have the highest NOS expression (Bredt et al., 1991).

Our characterization of SCN NOS by examining Ca^{2+} /calmodulin dependence suggests constitutive NOS expression in the SCN. To distinguish nNOS from eNOS, the enzyme activity was measured over a range of pH values. The highest activity was measured at pH 6.7, a value characteristic for nNOS (Hecker et al., 1994b) and distinct from the pH 7.6 optimum of eNOS (Hecker et al., 1994a). Western blot analysis of SCN samples with anti-nNOS antibody recognized a 155-kDa band present in nearly equivalent levels in both day and night. The quantitative similarity between nNOS levels in the SCN and cerebellum observed in specific activity measurements was confirmed by western blot. Thus, the aggregate data fully support nNOS as the prominent and abundant isoform expressed in the SCN in both day and night.

Several studies examining NOS expression in SCN from hamster and different rat strains report weak ICC or NADPH-d staining in ventrolateral perikarya (Decker and Reuss, 1994; Amir et al., 1995; Reuss et al., 1995). In contrast, the present study demonstrates nitrergic processes spread throughout the nucleus. In the Long-Evans rat, a relatively small number of cell bodies stained by anti-nNOS antibody were found in the retinorecipient ventrolateral region as well as borders of the core area of the SCN. Under confocal imaging analysis, however, an intensely staining profusion of fibers was evident, revealing a nitrergic plexus of collaterals and terminals. Staining of these fine structures was not evident under conventional microscopy; indeed, processes within the SCN have been reported to be of small caliber (van den Pol, 1980). Localization of NOS in this plexus of fine fibers very likely accounts for the high NOS activity we have measured in the SCN. It is also possible that a portion

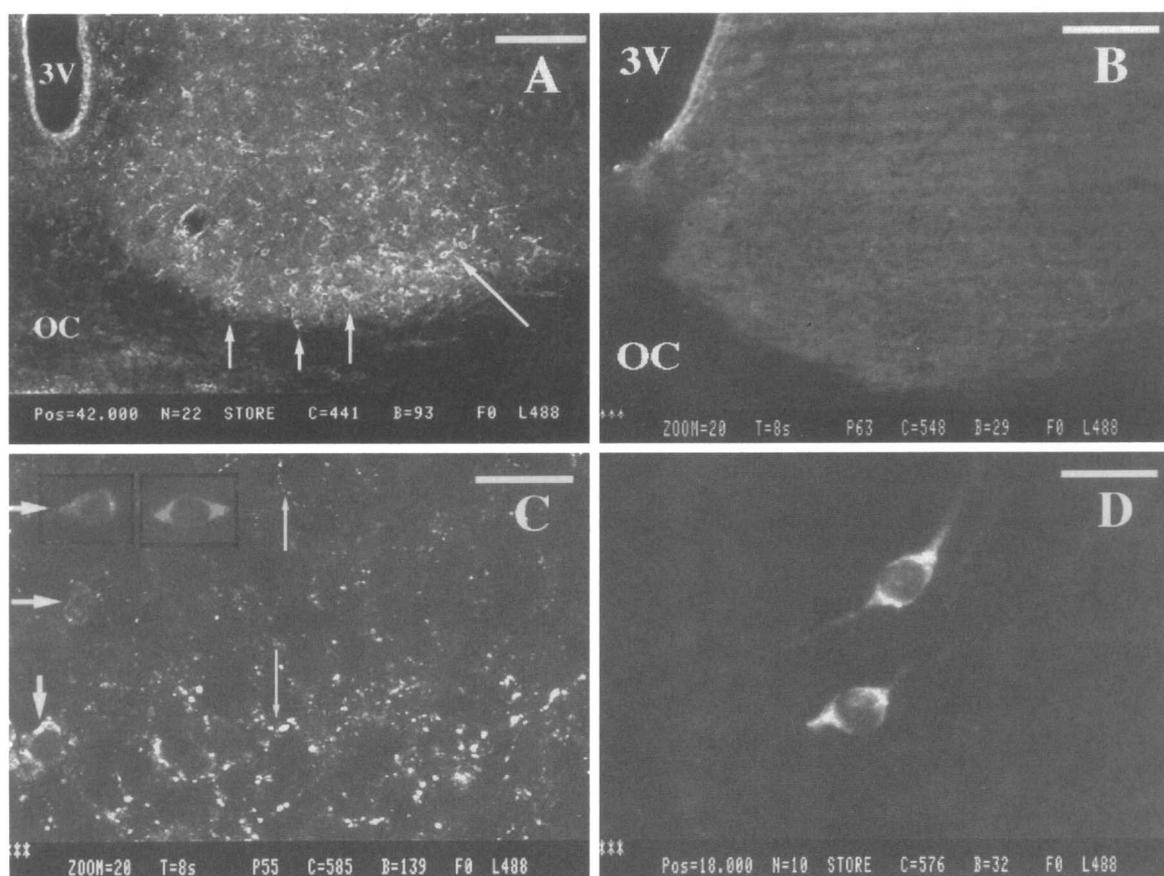


FIG. 3. Confocal imaging of nNOS immunoreactivity. **A:** A composite image of 22 confocal sections at 2- μ m increments shows the nNOS-positive elements within one SCN. The SCN is visible as an ovoid structure in this coronal section of the basal hypothalamus. The third ventricle (3V) is on the left; the optic chiasm (OC) borders the ventral SCN. Somata containing nNOS immunoreactivity (highlighted by arrows) are in the ventrolateral region, whereas nNOS-positive fibers permeate the entire SCN. Bar = 120 μ m. **B:** Preabsorption of the anti-nNOS antibody with nNOS completely blocked SCN immunoreactivity. Bar = 100 μ m. **C:** A high-magnification confocal image of a single 2- μ m optical section in the ventrolateral SCN shows nNOS-positive somata (highlighted by thick arrows) and puncta (highlighted by thin arrows). **Insets:** Composite image of 10 optical scans at 2- μ m increments shows two neurons in the ventrolateral SCN. Bar = 12 μ m. **D:** A composite image of 10 confocal scans at 2- μ m increments in the striatum shows larger nNOS-positive somata without densely packed puncta. Bar = 20 μ m.

of NOS in the SCN remains unstained owing to some unusual aspect of physical disposition such as interaction with anchoring molecules.

NOS-positive fibers and terminals have also been reported in other brain regions (Reuss et al., 1995). Electron microscopic evaluation of monkey visual cortex identified 78% of nNOS immunoreactivity in axonal or dendritic profiles (Aoki et al., 1993). Moreover, a recent study demonstrated that nNOS forms a complex through PDZ-containing protein domains with postsynaptic density-95 protein and NMDA receptors (Brenman et al., 1996). This pattern suggests localization of functionally related elements near synaptic subcellular compartments. Also, we found ~50% of nNOS in the SCN and cerebellum was associated with the membrane portion. This is consistent with a recent detailed study in cerebellum (Hecker et al., 1994b).

Because nNOS in the SCN partitions with the membrane fraction, NO likely contributes to glutamatergic signal amplification and synchronization among the multiple cellular oscillators of the SCN.

Our findings that nNOS is largely localized to fibers and that the level and activity do not vary significantly between day and night contribute to an integrated assessment of nNOS function in the SCN. NOS is a necessary component of the signaling pathway by which light, glutamate, and NMDA receptor activation lead to resetting the clock phase (Ding et al., 1994). NO acts as a paracrine messenger in the SCN, as the extracellular NO scavenger, hemoglobin, blocks the effect of glutamate (Ding et al., 1994). The activation of the few, weakly stained nNOS-positive cells in the ventrolateral SCN is unlikely to mediate such a global effect. The prevalence of nNOS in a fiber plexus

throughout the SCN is compatible with the major signaling and synchronizing role of NO during nocturnal phase resetting by light/glutamate stimulation. It will be important to determine the sources of these NOS-containing fibers so that the role of NO in regulating circadian clock function can be further understood.

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REFERENCES

- Amir S., Robinson B., and Edelman K. (1995) Distribution of NADPH-diaphorase staining and light-induced FOS expression in the rat suprachiasmatic nucleus region supports a role for nitric oxide in the circadian system. *Neuroscience* **69**, 545–555.
- Aoki C., Fenstemaker S., Lubin M., and Go C. G. (1993) Nitric oxide synthase in the visual cortex of monocular monkeys as revealed by light and electron microscopic immunocytochemistry. *Brain Res.* **620**, 97–113.
- Belsham D. D., Wetsel W. C., and Mellon P. L. (1996) NMDA and nitric oxide act through the cGMP signal transduction pathway to repress hypothalamic gonadotropin-releasing hormone gene expression. *EMBO J.* **15**, 538–547.
- Bradford M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.
- Bredt D. S. and Snyder S. H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* **86**, 9030–9033.
- Bredt D. S. and Snyder S. H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* **87**, 682–685.
- Bredt D. S., Glatt C. E., Hwang P. M., Fotuhi M., Dawson T. M., and Snyder S. H. (1991) Nitric oxide synthase protein and messenger RNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* **7**, 615–624.
- Brenman J. E., Chao D. S., Gee S. H., McGee A. W., Craven S. E., Santillano D. R., Wu Z., Huang F., Xia H., Peters M. F., Froehner S. C., and Bredt D. S. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha 1-syntrophin mediated by PDZ domains. *Cell* **84**, 757–767.
- Carroll F. Y., Beart P. M., and Cheung N. S. (1996) NMDA-mediated activation of the NO/cGMP pathway: characteristics and regulation in cultured neocortical neurones. *J. Neurosci. Res.* **43**, 623–631.
- Decker K. and Reuss S. (1994) Nitric oxide-synthesizing neurons in the hamster suprachiasmatic nucleus: a combined NOS- and NADPH-staining and retinohypothalamic tract tracing study. *Brain Res.* **666**, 284–288.
- Ding J. M., Chen D., Weber E. T., Faiman L. E., Rea M. A., and Gillette M. U. (1994) Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. *Science* **266**, 1713–1717.
- Fiallos-Estrada C. E., Kummer W., Mayer B., Bravo R., Zimmermann M., and Herdegen T. (1993) Long-lasting increase of nitric oxide synthase immunoreactivity, NADPH-diaphorase reaction and c-JUN co-expression in rat dorsal root ganglion neurons following sciatic nerve transection. *Neurosci. Lett.* **150**, 169–173.
- Garthwaite J., Charles S. L., and Chess-Williams R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336**, 385–388.
- Griffith O. W. and Stuehr D. J. (1995) Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol.* **57**, 707–736.
- Harteneck C., Klatt P., Schmidt K., and Mayer B. (1994) Expression of rat brain nitric oxide synthase in baculovirus-infected insect cells and characterization of the purified enzyme. *Biochem. J.* **304**, 683–686.
- Hecker M., Mulsch A., Bassenge E., Forstermann U., and Busse R. (1994a) Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiological implications. *Biochem. J.* **299**, 247–252.
- Hecker M., Mulsch A., and Busse R. (1994b) Subcellular localization and characterization of neuronal nitric oxide synthase. *J. Neurochem.* **62**, 1524–1529.
- Hibbs J. B. Jr., Vavrin Z., and Taintor R. R. (1987) L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* **138**, 550–565.
- Johnson R. F., Moore R. Y., and Morin L. P. (1988) Loss of entrainment and anatomical plasticity after lesions of the hamster retinohypothalamic tract. *Brain Res.* **460**, 297–313.
- Kim Y. I. and Dudek F. E. (1991) Intracellular electrophysiological study of suprachiasmatic nucleus neurons in rodents: excitatory synaptic mechanisms. *J. Physiol. (Lond.)* **444**, 269–287.
- Kummer W., Fischer A., Mundel P., Mayer B., Hoba B., Philippin B., and Preissler U. (1992) Nitric oxide synthase in VIP-containing cholinergic vasodilator nerves. *Neuroreport* **3**, 653–655.
- Lin A., Schaad N. C., Schulz P. E., Coon S. L., and Klein D. C. (1994) Pineal nitric oxide synthase—characteristics, adrenergic regulation and function. *Brain Res.* **651**, 160–168.
- Liou S. Y., Shibata S., Iwasaki K., and Ueki S. (1986) Optic nerve stimulation-induced increase of release of ³H-glutamate and ³H-aspartate but not ³H-GABA from the suprachiasmatic nucleus in slices of rat hypothalamus. *Brain Res. Bull.* **16**, 527–531.
- Marin P., Lafon-Cazal M., and Bockaert J. (1992) A nitric oxide synthase activity selectively stimulated by NMDA receptors depends on protein kinase C activation in mouse striatal neurons. *Eur. J. Neurosci.* **4**, 425–432.
- Matsumoto T., Pollock J. S., Nakane M., and Forstermann U. (1993) Developmental changes of cytosolic and particulate nitric oxide synthase in rat brain. *Dev. Brain Res.* **73**, 199–203.
- Moore R. Y. and Lenn N. J. (1972) A retinohypothalamic projection in the rat. *J. Comp. Neurol.* **146**, 1–14.
- Palmer R. M. J., Ashton D. S., and Moncada S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**, 664–666.
- Prosser R. A. and Gillette M. U. (1991) Cyclic changes in cAMP concentration and phosphodiesterase activity in a mammalian circadian clock: studies in vitro. *Brain Res.* **568**, 185–192.
- Rea M. A., Buckley B., and Lutton L. M. (1993) Local administration of EAA antagonists blocks light-induced phase shifts and c-fos expression in hamster SCN. *Am. J. Physiol.* **265**, R1191–R1198.
- Reuss S., Decker K., RoBeler L., Layes E., Schollmayer A., and Spessert R. (1995) Nitric oxide synthase in the hypothalamic suprachiasmatic nucleus of rat: evidence from histochemistry, immunohistochemistry and western blot; and colocalization with VIP. *Brain Res.* **695**, 257–262.
- Shibata S., Watanabe A., Hamada T., Ono M., and Watanabe S. (1994) N-Methyl-D-aspartate induces phase shifts in circadian rhythm of neuronal activity of rat SCN in vitro. *Am. J. Physiol.* **267**, R360–R364.
- Shirakawa T. and Moore R. Y. (1994) Glutamate shifts the phase of the circadian neuronal firing rhythm in the rat suprachiasmatic nucleus in vitro. *Neurosci. Lett.* **178**, 47–50.
- Southam E., East S. J., and Garthwaite J. (1991) Excitatory amino

- acid receptors coupled to the nitric oxide/cyclic GMP pathway in rat cerebellum development. *J. Neurochem.* **56**, 2072–2081.
- Starkey S. J. (1996) Melatonin and 5-hydroxytryptamine phase-advance the rat circadian clock by activation of nitric oxide synthesis. *Neurosci. Lett.* **211**, 199–202.
- Stephan F. K. and Nunez A. A. (1976) Role of retino-hypothalamic pathways in the entrainment of drinking rhythms. *Brain Res. Bull.* **1**, 495–497.
- van den Pol A. N. (1980) The hypothalamic suprachiasmatic nucleus of the rat: intrinsic anatomy. *J. Comp. Neurol.* **191**, 661–702.
- Vincent S. R. and Kimura H. (1992) Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* **46**, 755–784.
- Watanabe A., Hamada T., Shibata S., and Watanabe S. (1994) Effects of nitric oxide synthase inhibitors on *N*-methyl-D-aspartate-induced phase delay of circadian rhythm of neuronal activity in the rat suprachiasmatic nucleus in vitro. *Brain Res.* **646**, 161–164.
- Watanabe A., Ono M., Shibata S., and Watanabe S. (1995) Effect of a nitric oxide synthase inhibitor, *N*-nitro-L-arginine methyl ester, on light-induced phase delay of circadian rhythm of wheel-running activity in golden hamsters. *Neurosci. Lett.* **192**, 25–28.
- Weber E. T., Gannon R. L., Michel A. M., Gillette M. U., and Rea M. A. (1995) Nitric oxide inhibitor blocks light-induced phase shifts of the circadian activity rhythm, but not *c-fos* expression in the suprachiasmatic nucleus of the Syrian hamster. *Brain Res.* **692**, 137–142.