Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4214–4218, May 1994
Neurobiology

Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: Implications for synaptic plasticity

(long-term potentiation/immunohistochemistry/NADPH diaphorase/carbon monoxide/neuron)

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Contributed by Solomon H. Snyder, January 7, 1994

ABSTRACT **Using antibodies that react selectively with peptide sequences unique to endothelial nitric oxide synthase (eNOS), we demonstrate localizations to neuronal populations in the brain. In some brain regions, such as the cerebellum and olfactory bulb, eNOS and neuronal NOS (nNOS) occur in the same cell populations, though in differing proportions. In the hippocampus, localizations of the two enzymes are strikingly different, with eNOS more concentrated in hippocampal pyramidal cells than in any other brain area, whereas nNOS is restricted to occasional interneurons. In many brain regions NADPH diaphorase staining reflects NOS catalytic activity. Hippocampal pyramidal cells do not stain for diaphorase with conventional paraformaldehyde fixation but stain robustly with glutaraldehyde fixatives, presumably reflecting eNOS catalytic activity. eNOS in hippocampal pyramidal cells may generate the NO that has been postulated as a retrograde messenger of long-term potentiation.**

Nitric oxide (NO) appears to be a biological messenger mediating immune responses of macrophages (1, 2) and endothelium-dependent blood vessel relaxation (3–5) and serving as a neurotransmitter in the peripheral and central nervous systems (6–8). NO is formed from arginine by NO synthase (NOS), which oxidizes a guanidino nitrogen of arginine, releasing NO and citrulline (5). Several distinct NOS enzymes have been cloned and localized (9, 10). Macrophage NOS is localized to macrophages throughout the body but also occurs in the brain in microglial cells, which are modified macrophages. By use of antibodies that recognize both neuronal and endothelial NOS, staining of the endothelium of blood vessels in the periphery and in the brain has been detected (11–15), whereas an antiserum selective for endothelial NOS (eNOS) has detected endothelium of blood vessels in the brain following ischemic injury (16). Extensive mapping with antiserum selective for eNOS has not been reported. Neuronal NOS (nNOS) is localized to neurons throughout the peripheral and central nervous system (11–13, 17). Targeted disruption of nNOS in mice produces a >90% depletion of NOS catalytic activity in the brain (18). However, the residual NOS activity displays discrete regional distributions suggesting that neurons in some areas of the brain express a form of NOS encoded by a different gene (18).

Studies of the role of NO in brain function have implied a participation in long-term potentiation (LTP) in the hippocampus, a form of synaptic plasticity. NOS inhibitors block the induction of LTP in a fashion that is reversed by L-arginine (19–23). Hemoglobin, which binds extracellular NO, blocks LTP (21–23). NO produces an increase in the frequency of spontaneous miniature excitatory postsynaptic currents in cultured hippocampal neurons (21), and NO application may itself elicit LTP (20, 21). The notion that NO is a retrograde messenger that migrates from pyramidal cells to stimulate release of excitatory transmitter from the Schaffer collaterals is supported by the ability of NOS inhibitors injected into pyramidal cells to block LTP (23). Though there has been some difficulty in replicating effects of NOS inhibitors on LTP (24, 25), consistent effects are obtainable under appropriate experimental conditions (19, 26, 27).

Central to the notion that NO is the retrograde messenger of LTP is the assumption that hippocampal pyramidal cells possess abundant NOS. However, while immunohistochemical (13, 17) and *in situ* hybridization (13) studies demonstrate substantial NOS in small interneurons in the hippocampus, NOS is not readily evident in CA1 pyramidal cells (11, 13). Some studies have shown faint, inconsistent staining of CA1 pyramidal neurons for NADPH diaphorase activity (13, 17, 28), a histochemical stain which colocalizes with nNOS (12, 29). However, NADPH diaphorase staining can be elicited by any NADPH-dependent oxidative enzyme that survives fixation. In a preliminary report, acetone fixation revealed low levels of nNOS staining in CA1 pyramidal cells, but only with antiserum more concentrated than that required to detect nNOS in most brain regions (30).

Since mice with targeted disruption of nNOS possess residual NOS activity with moderate levels in the hippocampus, we sought evidence for other forms of NOS localized to neurons in the brain. We now report the selective enrichment of eNOS in hippocampal pyramidal cells.

MATERIALS AND METHODS

Generation of Polyclonal Antisera to eNOS and nNOS. A peptide based on amino acids 1185–1205 of bovine eNOS (31) was synthesized, conjugated to bovine serum albumin, and injected into rabbits to raise antiserum (32). Antibodies were affinity purified on a column consisting of ovalbumin-eNOS peptide conjugate immobilized on cyanogen bromide-activated Sepharose (32).

A fusion protein corresponding to amino acids 1–181 of rat nNOS (33) was produced (T.M.D., J. A. Mong, and S.H.S., unpublished data) and purified by using the pGEX expression vector (34) and was injected into rabbits to raise antiserum (32). Antibodies were affinity purified on a column of expressed protein that was immobilized on cyanogen bromide-activated Sepharose (32).

Localization of eNOS and nNOS in Brain and Peripheral Tissues by Western Blotting and Immunohistochemistry. Tissues were homogenized in ice-cold buffer [50 mM Tris·HCl, pH 7.4/1 mM EDTA containing antipain (10 μg/ml), leupep-

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Abbreviations: NOS, nitric oxide synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; LTP, long-term potentiation.

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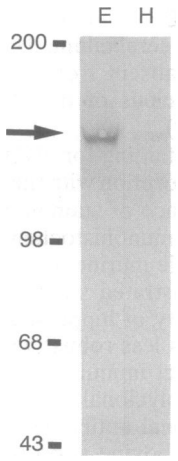

FIG. 1. Western blot analysis of bovine aortic endothelial cells (lane E, 50 μg of protein) and rat hippocampus (lane H, 200 μg) demonstrates much greater eNOS immunoreactivity in endothelial cells than in homogenates of whole hippocampus. Molecular sizes are indicated in kilodaltons.

tin (10 μg/ml), and phenylmethylsulfonyl fluoride (100 μg/ml)] and centrifuged at 100,000 × g for 1 hr. Supernatant and pellets (resuspended in buffer) were analyzed for total protein (Coomassie protein assay reagent, Pierce) and subjected to SDS/PAGE, transferred to Immobilon-P membranes (Millipore), and probed overnight with affinity-purified antibodies (1:500 dilution) for eNOS and nNOS. Blots were washed, developed by enhanced chemiluminescence (Amersham), and exposed to Kodak XAR film. For preabsorption experiments the antibodies were preincubated with 20-fold excess eNOS peptide or nNOS fusion protein at 4°C for 24 hr.

Adult male Sprague–Dawley rats were perfused with 4% freshly depolymerized paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed and postfixed for 2 hr in 4% paraformaldehyde in PB before cryoprotection in 20% (vol/vol) glycerol in PB. Tissues were harvested and processed as described (12). Slide-mounted sections or free-floating tissue was incubated with affinity-purified eNOS antibodies (1:50 dilution) or affinity-purified nNOS antibodies (1:1000 dilution) and 2% (vol/vol) normal goat serum or with a commercially available murine monoclonal antibody against amino acids 1030–1209 of human eNOS (Transduction Laboratories) and 2% normal goat serum overnight at 4°C. Staining was visualized with an avidin–biotin-peroxidase system (Vector Laboratories) using diaminobenzidine as a chromogen.

For NADPH diaphorase staining, fixation was performed two different ways. For paraformaldehyde fixation, rats were perfused with 2% freshly depolymerized paraformaldehyde in PB, as described (12). For glutaraldehyde fixation, rats were perfused with 2% glutaraldehyde and 0.5% paraformaldehyde in PB. Postfixation for both procedures was for 3 hr at 4°C. After equilibration overnight in 20% glycerol/PB, sections of 40 μm were cut on a sliding microtome, collected in phosphate buffer, and incubated in 0.1 M Tris·HCl, pH 7.2/0.2% Triton X-100/0.2 mM nitroblue tetrazolium/0.01% NaN_3/1 mM NADPH for 15–45 min at 37°C.

RESULTS

Western blots probed with affinity-purified antibodies to eNOS revealed a discrete band at 135 kDa in the rat hippocampus and in cultures of bovine aortic endothelial cells (BAECs) (Fig. 1). A band with the same molecular mass was evident in most other brain regions of rat and cow. In both BAECs and brain tissue the immunoreactivity was predominantly in the particulate fraction, with negligible staining in supernatant fractions, consistent with the largely particulate localization of eNOS. In contrast, nNOS immunoreactivity was characterized by a single band at 150 kDa in both supernatant and particulate fractions (data not shown).

At low magnification, immunohistochemical visualization revealed contrasting localizations of eNOS and nNOS (Fig.

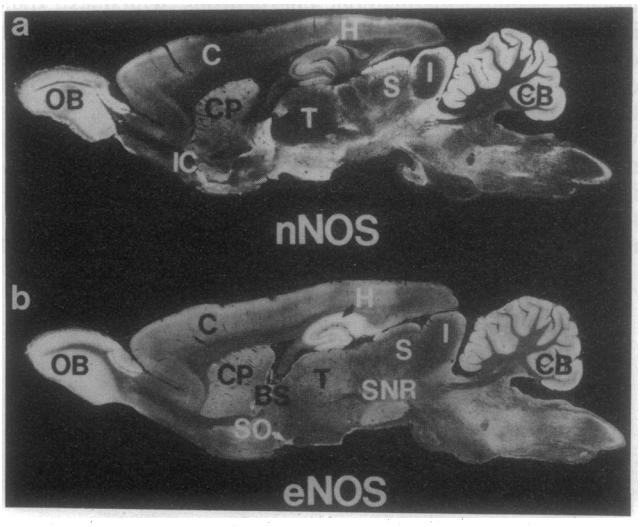

FIG. 2. Contrasting localizations of eNOS and nNOS immunoreactivity in the brain. (a) nNOS immunoreactivity. (b) eNOS immunoreactivity. (Preadsorption of the eNOS antibodies with 20-fold excess peptide antigen eliminates eNOS immunoreactivity; data not shown). OB, olfactory bulb; CP, caudate–putamen; IC, islands of Callaja; S and I, superior and inferior colliculi; C, cerebral cortex; H, hippocampus; SO, supraoptic nucleus; BS, bed nucleus of the stria terminalis; T, thalamus. (Bar = 2.50 mm.)

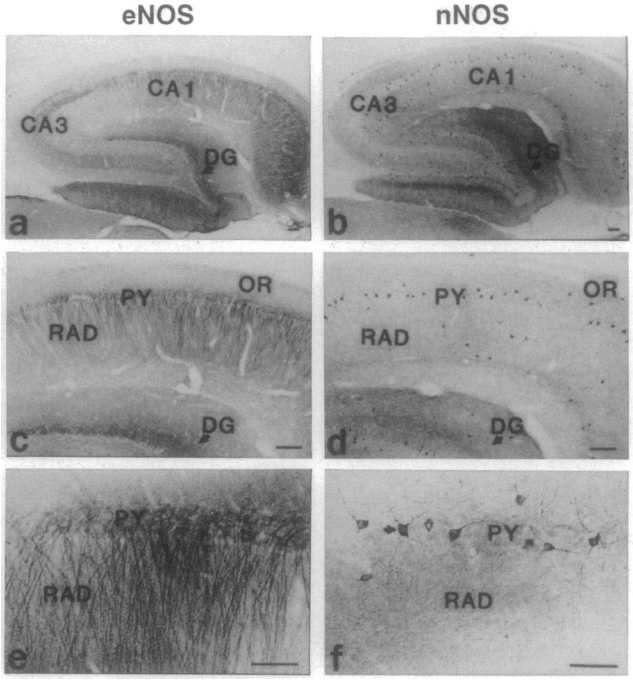

FIG. 3. Prominent immunoreactivity of CA1 hippocampal pyramidal neurons for eNOS (a, c, and e) but not nNOS (b, d, and f). eNOS immunoreactivity is noted in the cell bodies and dendrites of CA1 pyramidal neurons. nNOS immunoreactivity is limited to interneurons (f) (filled arrow) with only faint staining of pyramidal neuron cell bodies (open arrow). DG, dentate gyrus; OR, striatum oriens; PY, pyramidal-cell layer of CA1; RAD, stratum radiatum. (Bar = 100 μm in a–d and 50 μm in e and f.)

2). Whereas both proteins demonstrated immunoreactivity in the olfactory bulb, caudate–putamen, supraoptic nucleus, and cerebellum, eNOS was selectively concentrated in the hippocampus. This staining was evident in pyramidal cells of the CA1 region and in granule cells of the dentate gyrus (Fig. 3*a*). Higher-power magnification confirmed these localizations (Fig. 3 *c* and *e*). Hippocampal pyramidal cells demonstrated eNOS immunoreactivity throughout their perikarya, with staining at the cell body surface, consistent with a localization to the plasma membrane. Staining was also continuous and extensive along the dendritic processes of pyramidal cells. Limited immunostaining was evident in the superior axonal processes. Staining for eNOS contrasted with the localization of nNOS (Fig. 3 *b* and *d*), which was concentrated in γ-aminoglutamatergic interneurons (Fig. 3*f*) in the hippocampus, as observed previously, but not in pyramidal cells of CA1. However, nNOS was abundant in pyramidal cells of the subiculum (data not shown).

eNOS also occurs in other brain regions. In the olfactory bulb, immunoreactivity was concentrated within neurons and neuropil of the internal granule-cell layer and neuropil of the glomerular and external plexiform layers (Fig. 4*a*). By contrast, nNOS displayed intense staining of individual neurons in the glomerular layer with processes that formed a dense plexus (Fig. 4*b*). nNOS was less prominent than eNOS in the neuropil of the external plexiform layer, whereas nNOS localizations in the internal granule-cell layer were similar to those of eNOS. Both nNOS and eNOS were absent from mitral cells. In the caudate–putamen moderate eNOS immunoreactivity occurred in cell bodies of small–medium spiny neurons (Fig. 4*c*), contrasting with intense nNOS immunoreactivity in the cell bodies and processes of medium–large aspiny neurons (Fig. 4*d*). In the cerebellum eNOS immunoreactivity of granule cells and their processes in the molecular layer was less intense than nNOS immunoreactivity (Fig. 4*e*).

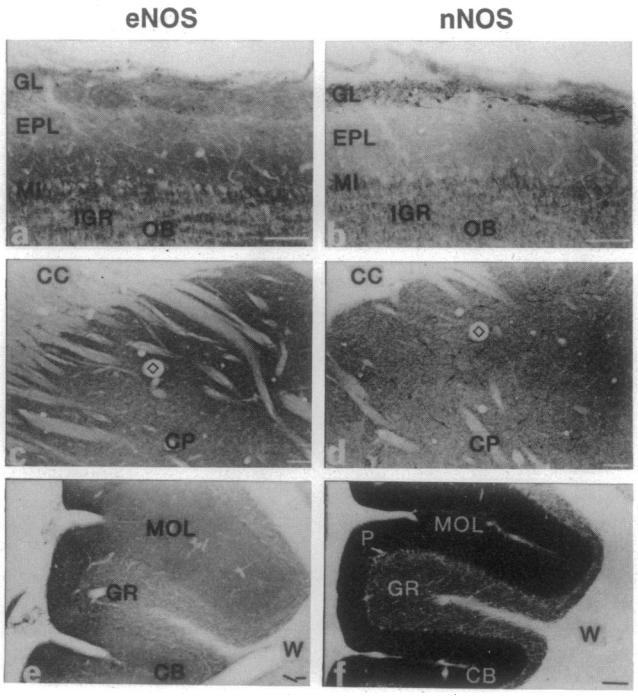

FIG. 4. Contrasting localizations of eNOS (*a*, *c*, and *e*) and nNOS (*b*, *d*, and *f*) immunoreactivity. OB, olfactory bulb; IGR, internal granule-cell layer; GL, glomerular cell layer; MI, mitral cell layer; EPL, external plexiform layer; CP, caudate–putamen; CC, corpus callosum; CB, cerebellum; GR, granule cells of the cerebellum; MOL, molecular cell layer of cerebellum; P, Purkinje cells; W, white matter. Open diamonds in *c* and *d* indicate identical blood vessels. (Bar = 100 μm.)

Both eNOS and nNOS immunostaining were absent from Purkinje cells of the cerebellum. Unlike nNOS, eNOS immunoreactivity was absent from the superior and inferior colliculi, the bed nucleus of the stria terminalis, and the hypothalamus (Fig. 2).

Specificity of the staining for eNOS was evident from its elimination by preabsorption with the eNOS peptide (data not shown) and the absence of staining with preimmune serum (data not shown). Immunohistochemistry performed with a commercially available murine monoclonal antibody against human eNOS demonstrated similar neuronal localizations. However, the intensity of hippocampal CA1 pyramidal cell dendritic staining was less robust with the monoclonal antibody. This may reflect immunohistochemical limitations of monoclonal versus polyclonal antibody preparations.

The eNOS polyclonal antibodies immunostained the endothelium of rat and bovine aorta (data not shown). Blood vessels in the brain were also stained (Fig. 5*a*), further supporting the specificity of the antiserum for eNOS. While the endothelium of blood vessels has been stained in several previous studies with antiserum raised against purified nNOS, the present results represent definitive evidence that the unique eNOS sequence is selectively localized to the endothelium of blood vessels from normal animals. Antibodies raised against a fusion protein specific for nNOS stained adventitial neuronal fibers but not vascular endothelium (Fig. 5*b*). If the staining reflects an eNOS sequence which is not contained in nNOS, then mice with targeted disruption of nNOS should retain eNOS staining. Indeed, eNOS staining was essentially the same in control and in nNOS knock-out mice (T.M.D., J.L.D., P. L. Huang, M. C. Fishman, and S.H.S., unpublished observations).

In NADPH diaphorase histochemical staining, the oxidative activity of NOS in fixed brain sections reduces tetrazolium dyes, the electron acceptor in the presence of NADPH but not NADH, forming an insoluble blue precipitate (35, 36). NADPH diaphorase has been extensively characterized in the brain, where it colocalizes with NOS (12, 13, 29). All forms of NOS possess NADPH diaphorase activity (37). Other flavoproteins possess such activity as well, but their activity is destroyed by fixation (38). In most previous studies in the hippocampus, NADPH diaphorase staining was confined to isolated γ-aminoglutamatergic interneurons, which also stain intensely with antibodies to nNOS. We wondered why the eNOS we have detected in the pyramidal cells did not produce diaphorase staining. We have previously noted an occasional but inconsistent NADPH diaphorase activity in these cells with the standard fixation protocol (13), and some groups have reported diaphorase staining in this region (39, 40). Accordingly, we systematically varied fixation conditions in an attempt to make the diaphorase staining in this region more robust and consistent. Valtschanoff *et al.* (41) have reported

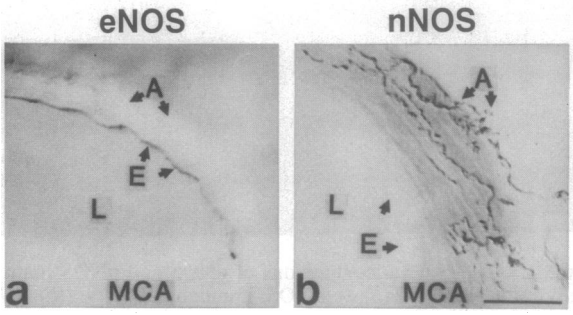

FIG. 5. eNOS and nNOS immunostaining of cerebral arteries. eNOS immunoreactivity (*a*) is prominent in the endothelial cell layer (E) of the middle cerebral artery (MCA), whereas nNOS immunoreactivity (*b*) is confined to nerve fibers of the adventitia (A). L, lumen. (Bar = 10 μm.)

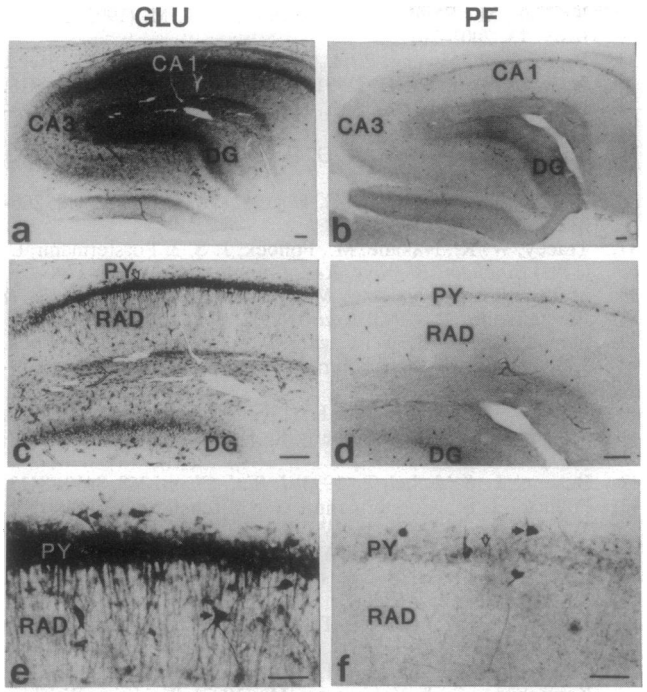

FIG. 6. NADPH diaphorase reactivity in the hippocampus. (*a*, *c*, and *e*) Fixation with 2% glutaraldehyde (GLU) allows demonstration of reactivity in the cell bodies and dendrites of CA1 pyramidal neurons in addition to interneurons (filled arrow in *e*). (*b*, *d*, and *f*) Conventional diaphorase staining employing fixation with 2% paraformaldehyde (PF). Only reactivity of interneurons is demonstrated (open arrow in *f*). DG, dentate gyrus; PY, pyramidal-cell layer of CA1; RAD, stratum radiatum. (Bar = 100 μm in *a–d* and 50 μm in *e* and *f*.)

enhanced diaphorase staining of the rat spinal cord, using fixatives containing high concentrations of glutaraldehyde, and our studies have confirmed this. The background staining with glutaraldehyde was lower, and fine varicose neuronal processes with NADPH diaphorase activity were better preserved throughout the brain. With glutaraldehyde-containing fixatives, the cell bodies and dendritic processes of the pyramidal cells of the CA1 region were stained prominently (Fig. 6 *a*, *c*, and *e*), supporting our immunocytochemical evidence that NOS is localized in these cells. Except for the hippocampus, only slight differences in the diaphorase staining pattern occurred with the two fixation techniques. In the cerebellum, for example, glutaraldehyde fixation better preserved staining of the processes in the molecular layer relative to staining in the granule-cell bodies (data not shown).

DISCUSSION

The major finding of this study is that eNOS occurs in neuronal populations in the brain. Both eNOS and nNOS occur throughout the brain in a wide range of neuronal cell types, but we detected only eNOS in hippocampal pyramidal cells.

The specificity of this staining is evident from its elimination by absorption with eNOS peptides, but not peptides selective for nNOS, and from the absence of staining with preimmune serum. Moreover, identical staining was observed with two eNOS antibodies directed toward distinct peptide sequences that do not occur in other forms of NOS. The NADPH diaphorase staining in hippocampal pyramidal cells observed here and in other studies further supports the existence of NOS in these neurons.

NADPH diaphorase staining in hippocampal pyramidal cells is much more robust when the brain is perfused with fixatives containing glutaraldehyde. Glutaraldehyde fixation is irreversible and produces many more intermolecular crosslinks than formaldehyde. Since eNOS is myristoylated (42) and largely membrane associated (43), glutaraldehyde-containing fixatives should better preserve the active form of eNOS by more efficiently crosslinking it to other components of the plasma membrane. When eNOS is not membrane associated, it is much less active (44). NADPH oxidase of phagocytes, another plasma membrane flavoprotein with diaphorase activity, can be crosslinked to the membrane with glutaraldehyde in a way that preserves its active conformation (45). Furthermore, phospholipid components of the plasma membrane enhance the activity of eNOS (46).

The localization of eNOS to hippocampal pyramidal cells is consistent with a role for NO as a retrograde messenger in this system. Heme oxygenase, which generates carbon monoxide (CO), is localized to discrete neuronal populations throughout the brain (47, 48) and may be a neurotransmitter (47). Heme oxygenase is heavily concentrated in hippocampal pyramidal cells, suggesting that CO could also be a retrograde messenger. Zinc-protoporphyrin IX, a potent inhibitor of heme oxygenase (49), blocks the induction of LTP (50, 51), while application of CO gas enhances LTP (50). Conceivably CO and NO function in a coordinate fashion in hippocampal LTP and perhaps in other parts of the body as well. For instance, in the carotid body, NOS is concentrated in dendrites of the carotid sinus nerve (52), and NOS inhibitors accelerate firing of these neurons (53). The glomus cells of the carotid, which synapse upon the carotid nerve dendrites, are enriched in heme oxygenase, and zinc-protoporphyrin IX markedly accelerates firing of the carotid sinus nerves (54). NO may act as a retrograde messenger released by carotid body neuronal dendrites to influence release by the glomus cells of its transmitter (52, 53).

The localization of eNOS to particulate fractions, presumably plasma membrane, fits well with a function for NO as a retrograde messenger. NO formed at the plasma membrane is more likely to be released to the extracellular environment than NO formed in cytosol. When eNOS is phosphorylated in endothelial cells, it translocates from membranes to cytosol and displays reduced catalytic activity (44). While the cytoplasmic proportion of NOS is greater in the brain than in endothelial cells, substantial levels of particulate NOS occur in the brain (55). Thus, in neurons as well as blood vessels, catalytically active NOS may form NO at the plasma membrane.

The localization of eNOS to selected neuronal populations raises questions about the appropriate designation for the various forms of NOS. Our findings reveal eNOS associated with neurons as well as endothelium. eNOS and nNOS had been regarded as noninducible and were often referred to as "constitutive NOS." However, following neuronal damage, nNOS levels increase markedly in discrete cellular populations (56, 57). In contrast to eNOS and nNOS, macrophage NOS has been regarded as the only form which is inducible and which, by definition, is localized to macrophages. However, following treatment with endotoxin, inducible NOS activity is evident in a wide variety of organs that lack macrophages (58). Moreover, an inducible form of NOS has been cloned from hepatocytes (59) and chondrocytes (60), which are not related to macrophages.

We thank Dawn Dodson and Nancy Bruce for secretarial assistance. This work was supported by U.S. Public Health Service Grants MH18501 and DA00266 and Research Scientist Award DA00074 (S.H.S.). T.M.D. is supported by grants from the American Academy of Neurology and by Public Health Service Clinical Investigator Development Award NSO1578.

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