

# Nitric Oxide Synthase Protein and mRNA Are Discretely Localized in Neuronal Populations of the Mammalian CNS Together with NADPH Diaphorase

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## Summary

**Nitric oxide is a free radical that has been recently recognized as a neural messenger molecule. Nitric oxide synthase has now been purified and molecularly cloned from brain. Using specific antibodies and oligonucleotide probes, we have localized brain nitric oxide synthase to discrete neuronal populations in the rat and primate brain. Nitric oxide synthase is exclusively neuronal, and its localization is absolutely coincident with NADPH diaphorase staining in both rat and primate.**

## Introduction

Nitric oxide (NO) appears to be responsible for endothelium-derived relaxing factor activity in blood vessels (Moncada et al., 1989; Furchgott and Vanhoutte, 1989; Ignarro, 1990). NO is also formed in macrophages and neutrophils in response to endotoxin and appears to mediate the tumoricidal and bactericidal activities of these cells (Hibbs et al., 1987, 1989); NO is also formed in brain tissue (Garthwaite et al., 1988, 1989; Bredt and Snyder, 1989). An understanding of NO disposition in the brain has come in large part from characterization of NO synthase (NOS). NOS forms NO from arginine via oxidation of one of the guanidino groups of arginine with the stoichiometric formation of citrulline (Marletta et al., 1988; Palmer and Moncada, 1989). The NOS protein of brain and endothelium appears distinct from that of macrophages and neutrophils. For instance, the brain/endothelial enzyme activity requires calcium and calmodulin (Bredt and Snyder, 1990), whereas there is no such requirement for the macrophage enzyme, which instead requires tetrahydrobiopterin (Tayeh and Marletta, 1989; Kwon et al., 1989). NOS has been purified from the brain to homogeneity and demonstrated to be a monomer of 150 kd with an absolute requirement for calmodulin and calcium as well as nicotinamide adenine dinucleotide phosphate (NADPH) (Bredt and Snyder, 1990). Molecular cloning of NOS reveals recognition sites for flavin adenine dinucleotide and flavin mononucleotide, as well as NADPH (Bredt et al., 1991). The amino acid sequence of brain NOS has substantial homology with that of cytochrome P-450 reductase.

Our initial immunohistochemical maps of NOS in

the brain reveal discrete localization to limited populations of cells in the cerebral cortex and corpus striatum, to basket and granule cells of the cerebellum, and to other selected sites (Bredt et al., 1990). In these studies we did not observe any extremely close correlation with the localization of known neurotransmitters or other neuronal markers.

NADPH diaphorase was first identified histochemically through the reduction of Nitro Blue Tetrazolium in the presence of NADPH (Thomas and Pearse, 1964). NADPH diaphorase occurs in selected neuronal populations in brain, with colocalization of some NADPH diaphorase neurons with somatostatin, neuropeptide Y, and choline acetyltransferase (Mizukawa et al., 1989). Considerable interest in the function of NADPH diaphorase neurons has followed the findings that these neurons are selectively resistant to destruction in clinical neurodegenerative conditions, such as Huntington's disease (Ferrante et al., 1985), as well as following ischemic destruction of neuronal tissue (Uemura et al., 1990) or neurotoxins (Beal et al., 1986; Koh et al., 1986).

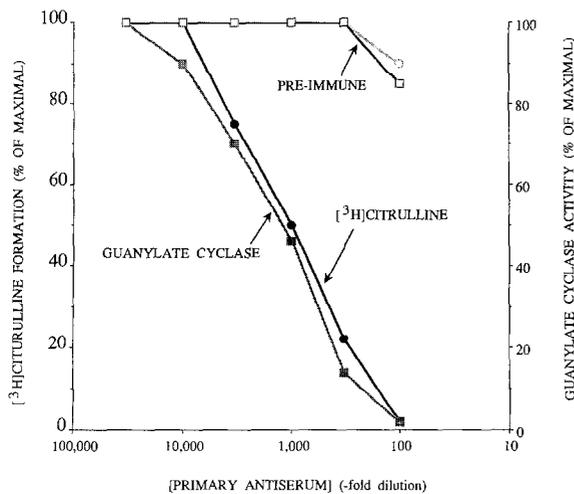
A role for NO in neurotoxicity is evident from our recent observations that N-methyl-D-aspartate destruction of cerebral cortical neurons in primary culture is blocked by inhibitors of NO synthase or deletion of arginine from incubation media (Dawson et al., 1991b). Also, in preliminary studies we detected identical localizations of NOS and NADPH diaphorase (Dawson et al., 1991a).

In the present study we have mapped in detail the localization of NOS protein and mRNA in rat and monkey brain and demonstrate striking colocalization of NOS and NADPH diaphorase in all brain areas.

## Results

### NOS Protein Levels and Enzyme Activity Correspond in Brain Regions

We have developed a rabbit polyclonal antiserum to rat NOS that has permitted immunohistochemical mapping of NOS in the brain and peripheral tissues (Bredt et al., 1990). To characterize the antiserum in greater detail in terms of its affinity for NOS, we have conducted immunoprecipitation studies (Figure 1). In rat cerebellar supernatant preparations, increasing concentrations of the rabbit antiserum deplete the supernatant preparation of NOS activity whether measured by the conversion of arginine to citrulline or the activation of guanylate cyclase by arginine and NADPH. In immunoprecipitation experiments using 1 mg/ml cerebellar soluble extracts, half-maximal effects are apparent at 10 µg/ml antiserum, reflecting a 1:1000 dilution of the crude serum (Figure 1). With preimmune serum only slight precipitation of NOS activity occurs at 1:100 dilutions. Moreover, immuno-



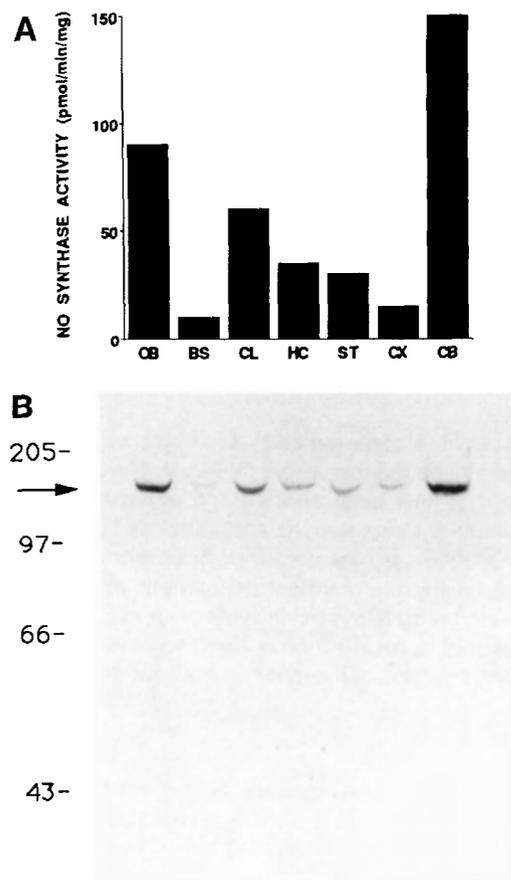
**Figure 1. Immunoprecipitation of NOS Activity**  
NOS activity was assayed in cerebellar homogenates following immunoprecipitation with antiserum. Both assays for NOS activity, the conversion of [<sup>3</sup>H]arginine to [<sup>3</sup>H]citrulline and the activation of guanylate cyclase by newly synthesized NO, were potently and specifically immunoprecipitated with half-maximal precipitation at 1:1000 dilution of serum. Following immunoprecipitation, guanylate cyclase activity could still be activated to maximal levels with sodium nitroprusside, demonstrating that depletion of NOS accounts for the decrease in guanylate cyclase activity. Maximal levels of [<sup>3</sup>H]citrulline formation prior to immunoprecipitation were 11,200 cpm. Basal levels of guanylate cyclase were 5 pmol/min per mg; prior to immunoprecipitation, activity was activated to 200 pmol/min per mg by either 1 mM arginine and 1 mM NADPH, or 300 μM sodium nitroprusside. This is representative of a similar experiment repeated three times with nearly identical results.

precipitation does not reduce the total protein content of the supernatant preparation (data not shown).

To assess the relationship between the amount of immunoreactivity and of NOS catalytic activity, we measured NOS activity in various brain regions and conducted Western blot analysis of NOS protein in the same regions (Figure 2). The highest NOS activity occurs in the cerebellum, with somewhat lesser activity in the colliculi and the olfactory bulb. The lowest levels of activity are apparent in the cerebral cortex and brain stem. Western blot analysis reveals a single immunoreactive protein band at about 160 kd in all brain regions. The relative intensity of NOS staining corresponds well with the relative amount of NOS activity, with highest levels in the cerebellum and lowest levels in the brain stem and cerebral cortex.

**Immunoreactive NOS and NOS mRNA Occur in Discrete Rat Brain Regions**

In sagittal sections of rat brain examined at low magnification, we confirmed the distribution of NOS immunoreactivity reported in a preliminary exploration (Bredt et al., 1990). Highest densities are apparent in the cerebellum and the olfactory bulb (Figure 3A). Staining in the accessory olfactory bulb is even more



**Figure 2. Regional Distribution of NOS Catalytic Activity and Protein in Brain**

NOS activity in brain homogenates (A) displays a regional distribution nearly identical to the density of the immunoreactive NOS band at 160 kd (B). Nitric oxide synthase activity was determined by monitoring the conversion of [<sup>3</sup>H]arginine to [<sup>3</sup>H]citrulline as described (Bredt and Snyder, 1990). Western blot analysis was performed as described in Experimental Procedures. Enzyme activity represents the means of triplicate determinations. This is a representative experiment repeated five times with similar results.

Abbreviations: OB, olfactory bulb; BS, brain stem; CL, colliculi; HC, hippocampus; ST, striatum; CX, cerebral cortex; CB, cerebellum.

prominent than that in the olfactory bulb and cerebellum. Areas of high density staining also are apparent in the pedunculo-pontine tegmental nucleus, the superior and inferior colliculi, the supraoptic nucleus, the islands of Callejae, the caudate-putamen, and the dentate gyrus of the hippocampus. In situ hybridization reveals a similar localization for NOS mRNA, with highest densities in the accessory olfactory bulb, the pedunculo-pontine nucleus, and the cerebellum (Figure 3C). Other regions with increased grain densities include the main olfactory bulb, the dentate gyrus of the hippocampus, the superior and inferior colliculi, and the supraoptic nucleus. Scattered white dots throughout the cerebral cortex, the caudate-putamen, and basal forebrain correspond to NOS protein and mRNA concentrated in isolated cells in these re-

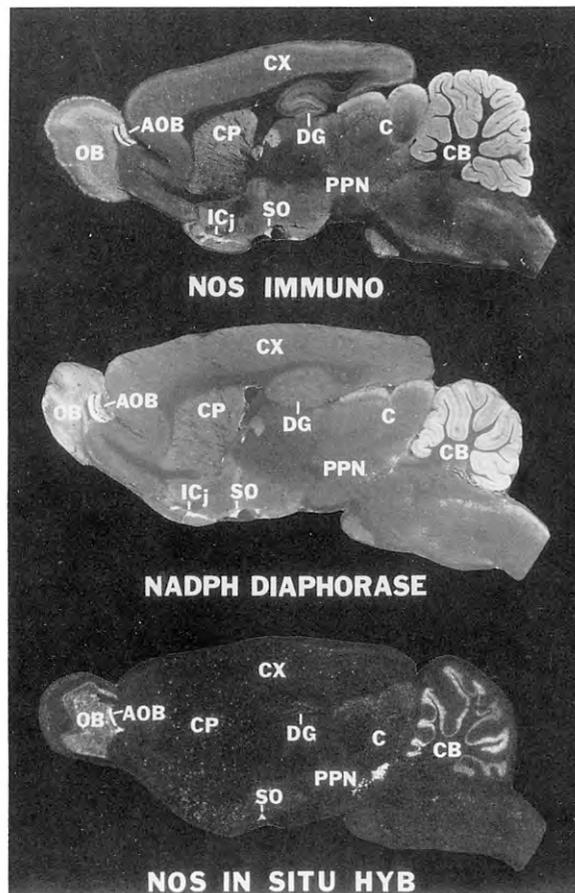


Figure 3. Histologic Localizations of NOS Protein, NADPH Diaphorase Activity, and NOS mRNA in Brain

Adjacent sagittal brain sections were processed for NOS immunohistochemistry (A), NADPH diaphorase histochemistry (B), and NOS in situ hybridization (C). All three methods show densest staining in the accessory olfactory bulb (AOB), pedunculo-pontine tegmental nucleus (PPN), and cerebellum (CB), with lesser staining in the dentate gyrus of the hippocampus (DG), main olfactory bulb (OB), superior and inferior colliculi (C), and supraoptic nucleus (SO). As depicted, in region CA1 staining for diaphorase exceeds that for NOS; whereas in the superficial cortex staining for NOS exceeds that for diaphorase. However, in other sections these layers have similar staining intensities. Intensely staining isolated cells are apparent scattered throughout the hippocampus, cerebral cortex (CX), caudate-putamen (CP), and basal forebrain. NOS and NADPH diaphorase staining in the hippocampus and cerebral cortex is variable. Some regions enriched in NOS protein and diaphorase staining are devoid of NOS mRNA, suggesting that in these regions the NOS protein has been transported to nerve fibers distant from its site of synthesis. These regions include the molecular layer of the cerebellum, the islands of Callejae (ICj), and the neuropil of the caudate-putamen and cerebral cortex.

gions. In the cerebellum NOS mRNA is apparent only in the granule cell layer, whereas immunoreactive NOS is concentrated in the molecular as well as the granule cell layer. This suggests that NOS in the molecular layer of the cerebellum occurs largely in processes of granule cells (Bredt et al., 1990). NOS mRNA in the caudate-putamen and basal forebrain is pres-

ent only in isolated cells, whereas immunohistochemistry shows diffuse staining of the caudate-putamen and intense staining of the islands of Callejae. This is consistent with NOS protein in the neuropil of the caudate-putamen and the islands of Callejae being present predominantly in nerve fibers.

We employed coronal sections to explore more levels of the neuraxis (Figure 4). In the cerebral cortex light staining for NOS is apparent in superficial areas throughout the cerebral cortex, but with greatest intensity near the rhombic fissure. Somewhat more intense staining appears in the caudate-putamen, whereas in the adjacent globus pallidus, negligible staining is apparent. High concentrations of immunoreactivity are detected in the medial nucleus of the amygdala, with much less NOS in other portions of the amygdala. The islands of Callejae and bed nucleus of the stria terminalis also have high NOS levels. The paraventricular nucleus of the hypothalamus has elevated NOS concentrations, presumably in the cells projecting to the posterior pituitary, which we previously showed to display extremely high concentrations of NOS (Bredt et al., 1990). The cells of the subfornical nucleus possess particularly high concentrations of NOS.

Within the hippocampal formation, highest NOS levels are apparent in the dentate gyrus, with very little in other portions of the hippocampus. The interpeduncular nucleus as well as the central gray and superior colliculus has high densities of NOS. The zona incerta is enriched in NOS immunoreactivity, whereas the underlying substantia nigra is devoid of NOS. Within the hypothalamus, the medial mammillary nucleus has high concentrations of NOS.

In the brain stem NOS levels are low except for discrete portions of the nucleus prepositus hypoglossi and the substantia gelatinosa of the spinal tract for the trigeminal nerve. In the spinal cord NOS is also very highly concentrated in the substantia gelatinosa. The cerebellum displays high NOS levels, with immunoreactivity in the molecular and granule cell layers as previously reported (Bredt et al., 1990).

Examination at higher magnification permits the cellular localization of NOS (Figure 5). In the cerebral cortex immunoreactivity occurs in scattered, isolated cells that are medium to large aspiny neurons (Figure 5A). Staining is apparent within the cell bodies and throughout the several processes of the cells, with some evident varicosities (Figure 5B). Within the hippocampal formation, granule cells of the dentate gyrus are also heavily stained, with the greatest staining apparent in their processes in the molecular layer of the dentate gyrus (Figure 5C). Examination of hippocampal region CA1 under high magnification reveals scattered immunoreactive neurons, with no labeling of pyramidal cells (Figure 5D).

In the islands of Callejae NOS appears largely confined to fibers, with intense staining of a large number of these processes (Figure 5E). By contrast, the nucleus of the diagonal band possesses both NOS cells and

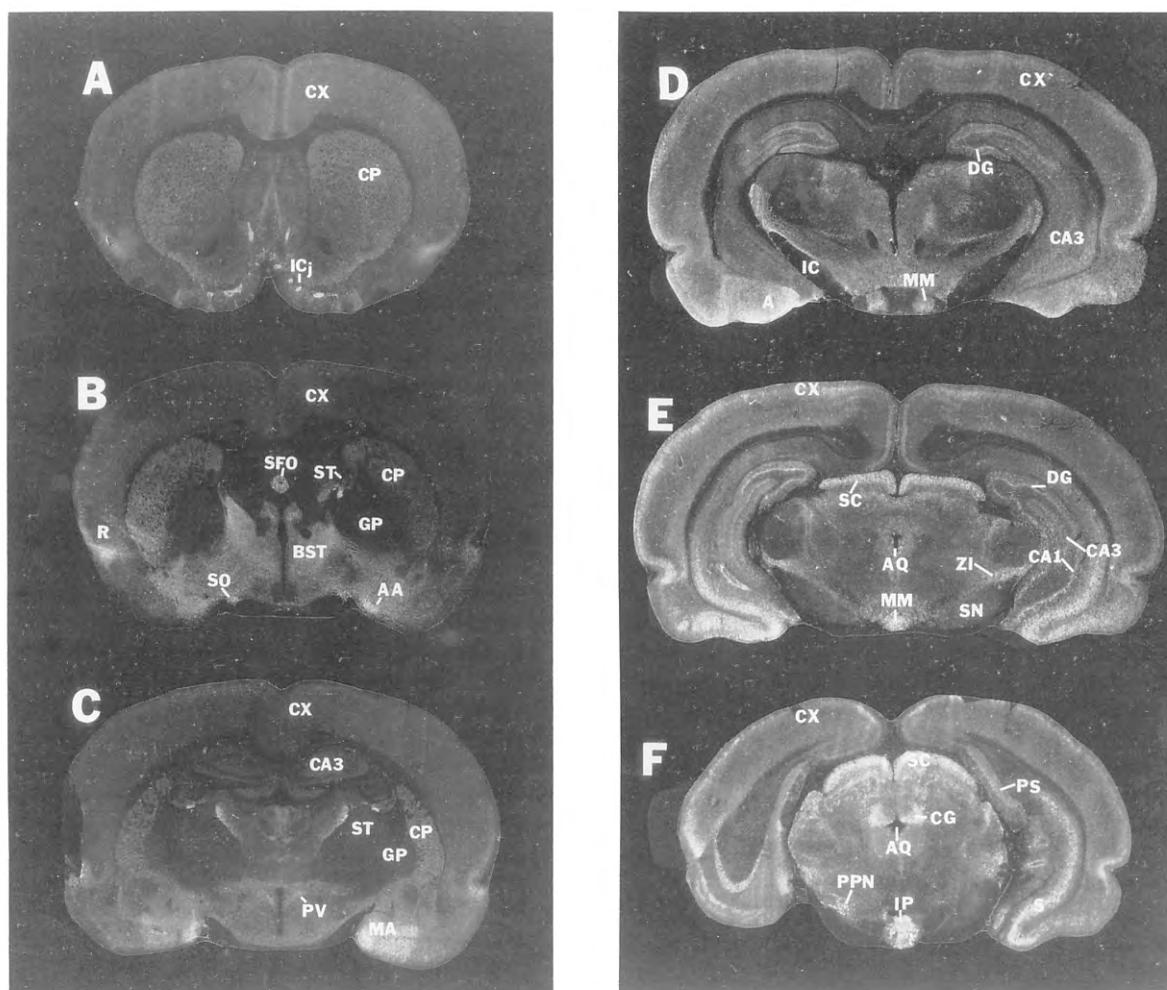


Figure 4. Localization of NOS in Coronal Brain Sections

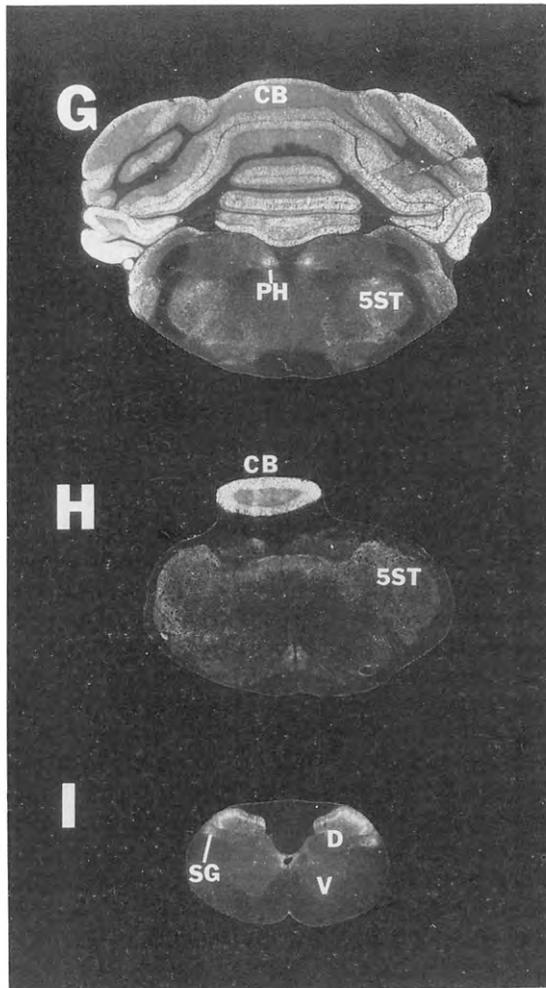
Thin sections (16  $\mu$ m) of rat brain were processed for NOS immunohistochemistry. Immunoreactive structures appear white in these dark-field images. Labeled structures are described in the text. Abbreviations: A, amygdala; AA, anterior amygdala; AQ, cerebral aqueduct; BST, bed nucleus of the stria terminalis; CB, cerebellum; CG, central gray; CP, caudate-putamen; CX, cerebral cortex; D, dorsal horn of the spinal cord; DG, dentate gyrus of the hippocampus; GP, globus pallidus; IC, internal capsule; ICj, islands of Callejae; IP, interpeduncular nucleus; MA, medial amygdala; MM, medial mammillary nucleus; PH, nucleus prepositus hypoglossi; PPN, pedunculopontine tegmental nucleus; PS, presubiculum; PV, paraventricular nucleus; R, rhombic fissure; SC, superior colliculus; SFO, subfornical organ; SG, substantia gelatinosa; SN, substantia nigra; SO, supraoptic nucleus; ST, stria terminalis; V, ventral horn of spinal cord; ZI, zona incerta; 5ST, spinal tract of the trigeminal nerve.

processes. Within the corpus striatum, NOS occurs in scattered, medium to large aspiny neurons, with staining evident in cell bodies and extensive staining seen in the neuropil (Figure 5F).

#### NOS and NADPH Diaphorase Are Colocalized in Rat and Monkey Brain

In preliminary studies (Snyder and Brecht, 1991; unpublished data) we noted colocalization of NOS immunoreactivity with NADPH diaphorase in a few areas of the brain and in peripheral tissues, including the posterior pituitary, the retina, the adrenal medulla, and the myenteric plexus in the intestine. We now have explored a wide range of brain regions and ob-

served colocalization for NOS and NADPH diaphorase in virtually every brain area examined (Figure 3B). At low magnification of sagittal sections, the highest densities of NADPH diaphorase are apparent in the accessory olfactory bulb, with slightly lower levels in the main olfactory bulb, the pedunculopontine nucleus, and the molecular and granule cell layers of the cerebellum, essentially the same as the distribution of NOS (Figure 3C). Lower levels of NADPH diaphorase are evident in the superior and inferior colliculi, the dentate gyrus of the hippocampal formation, the supraoptic nucleus, and the corpus striatum, again quite similar to the overall distribution of NOS. Higher magnifications emphasize the colocalization of these



two enzymes. For instance, in the cerebellum Purkinje cells display absolutely no NOS or NADPH diaphorase. On the other hand, all basket cells stain for NOS and NADPH diaphorase, as do glomeruli and granule cells (Figure 6). Within the pedunculopontine tegmental nucleus, we conducted *in situ* hybridization for NOS mRNA on sections first stained for NADPH diaphorase (Figure 7). Every NADPH diaphorase-positive cell displays NOS mRNA. We have conducted similar studies on the scattered NADPH diaphorase-positive cells throughout the cerebral cortex, corpus striatum, and hippocampal formation. In all instances, every NADPH diaphorase-positive cell displays prominent NOS mRNA autoradiographic grains (data not shown). Moreover, in no instance is NOS mRNA present in the absence of NADPH diaphorase staining.

We conducted a detailed cellular analysis of the colocalization of NOS and NADPH diaphorase in the corpus striatum (Figure 8). In adjacent sections of the corpus striatum, virtually identical numbers of cells stain for NADPH diaphorase and NOS.

To ascertain whether the unique localization of NOS in rat brain is species specific or might be generalized, we have mapped NOS immunoreactivity throughout monkey brain. In all regions examined, the cell and fiber groups stained are the same as in rat (data not shown). In certain areas of monkey brain we have compared NOS and NADPH diaphorase localization. In the monkey, large cells in the basal forebrain, corresponding to the nucleus basalis of Meynert of humans, project cholinergic neurons to the cerebral cortex. This group of cells does not exist as a discrete nucleus in the rat. In the monkey, NOS staining is evident in the medium to large cells of the basal forebrain, but not in the smaller cells (Figure 9). All NOS-staining cells also stain for NADPH diaphorase in the basal forebrain.

## Discussion

The main findings of this paper are the highly selective localizations of NOS neurons throughout the brain and their colocalization with NADPH diaphorase. In some areas of the brain, such as the corpus striatum, NADPH diaphorase is colocalized with somatostatin and neuropeptide Y. However, several areas of the brain possess NADPH but are devoid of somatostatin and neuropeptide Y. Similarly, in the pedunculopontine nucleus, NADPH diaphorase occurs together with choline acetyltransferase (Mizukawa et al., 1989), while in other parts of the brain, like the corpus striatum, they are not colocalized. By contrast, the coincidence of NADPH diaphorase and NOS is absolute in all cells and in all brain areas examined.

The colocalization of NOS and NADPH diaphorase is not restricted to the brain. In other studies (Snyder and Bredt, 1991; Dawson et al., 1991a) NOS and NADPH diaphorase are colocalized in neuronal fibers in terminals of the posterior pituitary derived from the supraoptic and paraventricular nuclei of the hypothalamus. In the eye NOS and NADPH diaphorase are colocalized in a plexus of nerve fibers in the choroid as well as in the retina in a discrete population of amacrine cells in the inner nuclear layer, together with occasional cells in the ganglion cell layer. In the myenteric plexus of the small intestine neurons and processes possess both NOS and NADPH diaphorase. In the adrenal medulla NADPH diaphorase and NOS occur together in ganglion cells and fibers that contact the chromaffin cells. NADPH diaphorase in peripheral tissues can occur in the absence of NOS in limited instances. For instance, the adrenal cortex stains for NADPH diaphorase but not NOS. Presumably high levels of oxidative enzyme involved in steroid synthesis account for NADPH diaphorase activity in the adrenal cortex. Additionally, the liver stains for NADPH diaphorase but not NOS.

The striking colocalization of NADPH diaphorase and NOS and the fact that both enzymes have oxidative activity dependent upon NADPH suggest that in

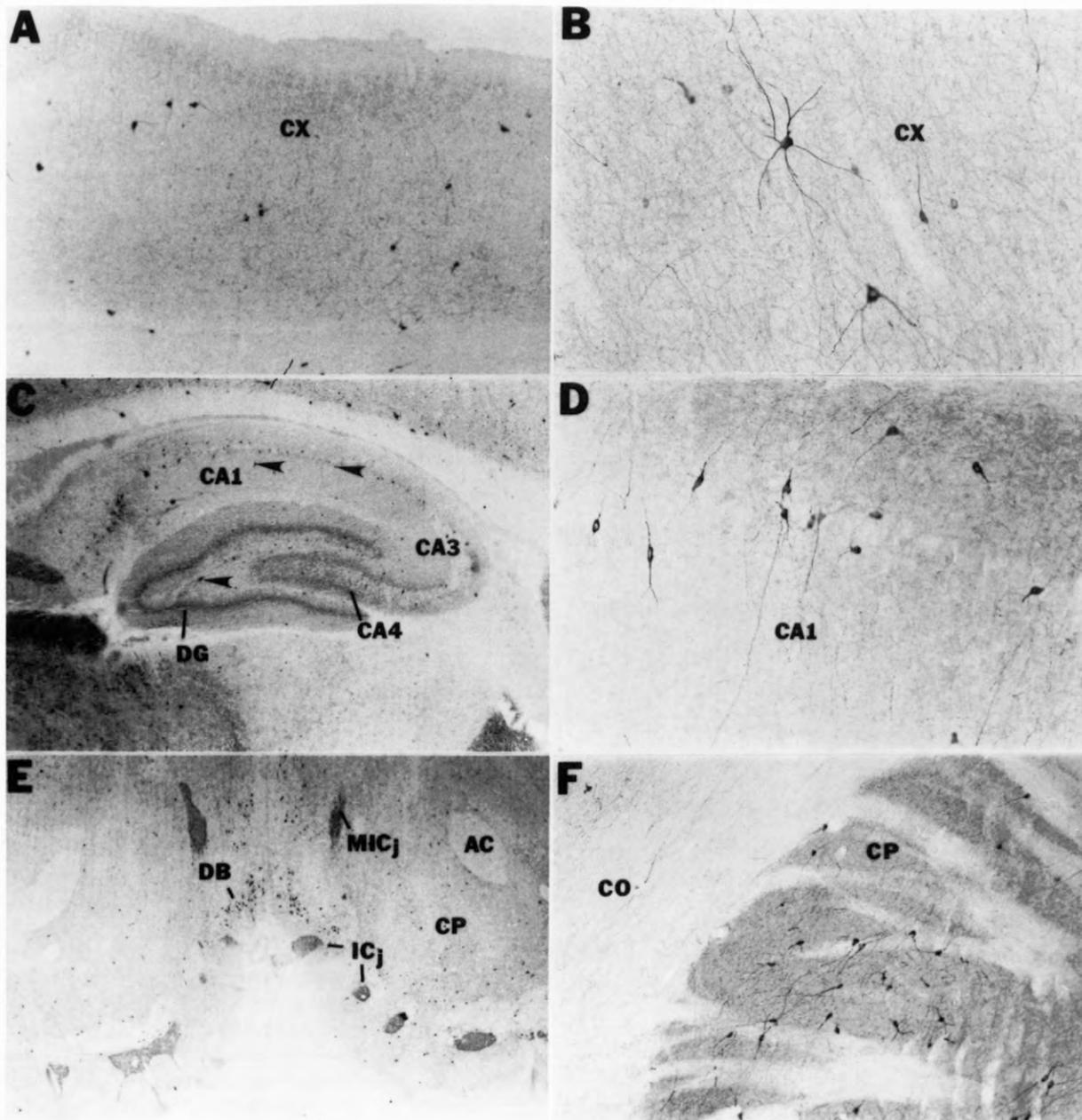
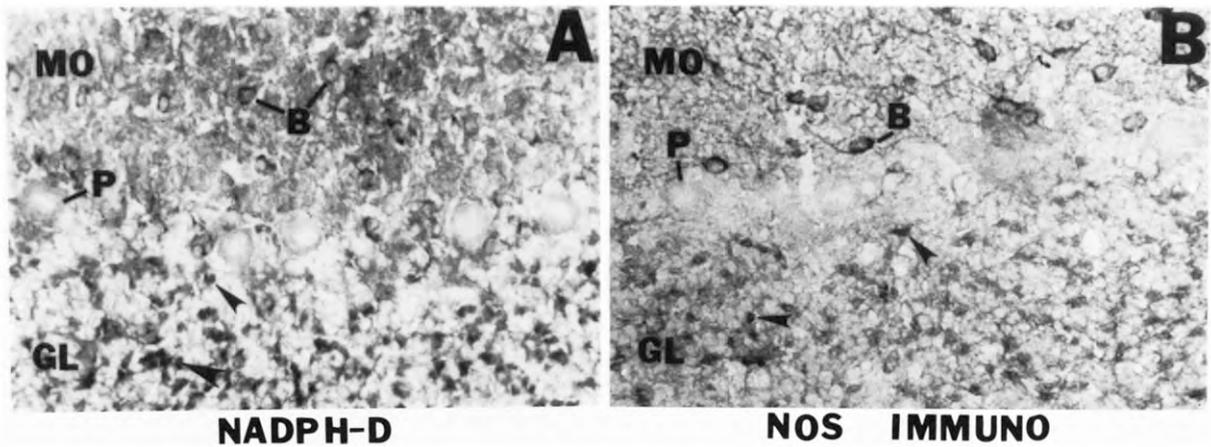


Figure 5. Cellular Localizations of NOS in Rat Brain

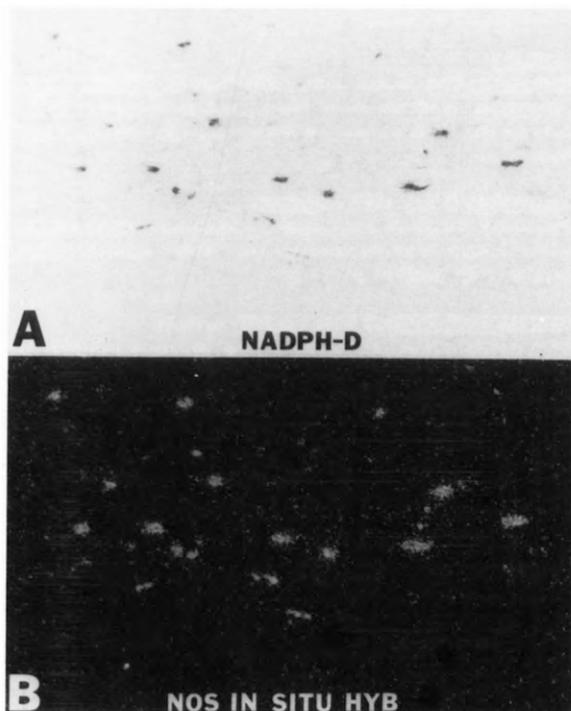
(A) Throughout the cerebral cortex (CX), NOS is concentrated in only 1%-2% of all neurons (90 $\times$ ). (B) Viewed at high magnification, these scattered positive neurons are labeled throughout their cell bodies and elaborate processes (360 $\times$ ). (C) Within the hippocampus, there is intense staining of the outer granule cell layer and inner molecular layer of the dentate gyrus (DG), as well as isolated immunoreactive cells (arrowheads) throughout the hippocampus (90 $\times$ ). (D) High magnification of the hippocampus shows densely labeled cells in the CA1 region (360 $\times$ ). (E) In the basal forebrain at low magnification, staining is apparent in nerve fibers of the islands of Callejae (ICj) and in cell bodies of the caudate-putamen (CP) and diagonal band of Broca (DB) (90 $\times$ ). AC, anterior commissure; MICj, major island of Callejae. (F) Staining of medium, aspiny neurons and their processes is apparent in the caudate-putamen viewed at higher magnification, with staining absent from the corpus callosum (CO) (360 $\times$ ).

cells where they are colocalized, NOS accounts for NADPH diaphorase activity. In principle, one might simply purify NADPH diaphorase protein and determine whether it is identical to NOS protein. However, NADPH diaphorase activity measured in tissue homogenates may well not reflect the enzyme moni-

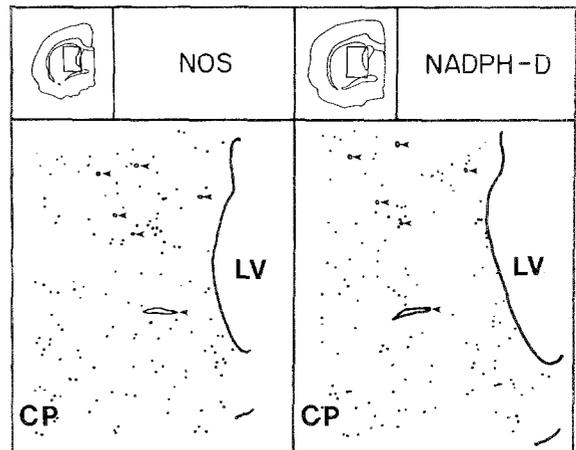
tored by histochemical staining. Histochemistry reveals enzyme activity that is highly concentrated in discrete localizations, whereas catalytic activity in homogenates often reflects the contribution of multiple sources of enzyme diffusely distributed and thus not evident in histochemistry. Very recently, Hope et al.



**Figure 6. Colocalization of NADPH Diaphorase and NOS Protein in Rat Cerebellum**  
Thin sagittal sections of cerebellum stained for NADPH diaphorase (A) or NOS immunohistochemistry (B) reveal that both stain the same cell types (400×). Within the granule cell layer (GL), both granule cells and large glomerular synapses (arrowheads) are labeled, whereas in the molecular layer (MO), basket cells are densely stained, with diffuse labeling of the neuropil likely due to NOS within parallel fibers. Purkinje cells (P) are notable for their lack of staining. Sections were processed for NADPH diaphorase and immunohistochemical staining as described in Experimental Procedures, except that sections were fixed with only 0.5% paraformaldehyde, as this more gentle fixation was specifically required for robust staining in the cerebellum.



**Figure 7. Colocalization of NADPH Diaphorase and NOS mRNA in the Pedunclopontine Tegmental Nucleus**  
A thin section of rat brain stained for NADPH diaphorase was then processed for NOS in situ hybridization. (A) Intense diaphorase staining of large neurons in the pedunclopontine tegmental nucleus is apparent in bright-field. (B) These same neurons are selectively enriched in NOS mRNA silver grains visualized in dark-field (280×).



**Figure 8. Camera Lucida Illustration of NOS Immunohistochemistry and NADPH Diaphorase Staining in the Caudate-Putamen**  
Adjacent thick sagittal sections of rat brain were stained for NOS immunoreactivity and NADPH diaphorase. Using blood vessels as landmarks (arrowheads), virtually identical numbers of neurons show positive staining for NOS and NADPH diaphorase in the caudate-putamen (CP). LV, lateral ventricle. In the boxed fields, 140 cells stained for NOS and 142 cells were NADPH diaphorase-positive.

(1991) found that a portion of the NADPH diaphorase activity in rat cerebellar homogenates copurifies with NOS (Hope et al., 1991). Taking advantage of our recent molecular cloning of the gene for NOS, we have transfected the cDNA for NOS into kidney cells in culture and found that this transfection produces

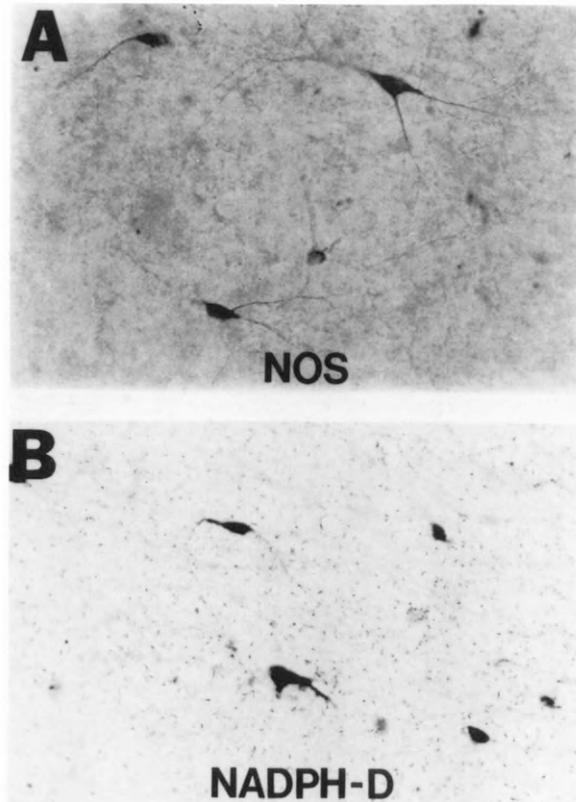


Figure 9. Localization of NOS and NADPH Diaphorase in the Nucleus Basalis of the Rhesus Monkey

Consecutive thick (40  $\mu$ m) sections were stained for either NOS immunohistochemistry or NADPH diaphorase. Both NOS immunohistochemistry (A) and NADPH diaphorase (B) are selectively enriched in the medium-sized neurons in this nucleus, with no staining of other cell bodies in this region (280 $\times$ ).

both substantial NOS activity and diaphorase staining (Dawson et al., 1991a). Moreover, the relative amounts of staining for NOS immunohistochemistry and NADPH diaphorase in the transfected cells are the same as those in brain neurons, establishing that NOS accounts for NADPH diaphorase staining in brain.

In preliminary experiments we have examined the effects on NADPH diaphorase staining in brain sections of nitroarginine (1 mM), an inhibitor of NOS activity, and EGTA (1 mM), which chelates the calcium required for NOS activity. In these experiments we have not observed changes in NADPH diaphorase staining (data not shown). The diaphorase activity of NOS likely derives from the reduction by NADPH of a flavin associated with NOS that in turn directly reduces the Nitro Blue Tetrazolium dye. This portion of NOS enzyme activity must then be independent of arginine, calcium, and calmodulin. Consistent with this model for the way in which NOS provides NADPH diaphorase staining, we have shown that NOS activity is potently inhibited by Nitro Blue Tetrazolium, with 50% inhibition apparent at a 10  $\mu$ M concentration of the dye (data not shown). This is quite similar to the

potency of Nitro Blue Tetrazolium in providing NADPH diaphorase staining.

One of the most dramatic features of NADPH diaphorase neurons is their resistance to various neurotoxic insults. It seems possible that the NOS activity of these neurons may be responsible for this resistance. Neurotoxicity has been ascribed to an overload of calcium within neurons (Choi, 1988). NO can reduce intracellular levels of calcium by mechanisms unrelated to cGMP (Garg and Hassid, 1991), which therefore might afford protection from high levels of calcium. The diaphorase activity of NOS may provide neuroprotection, as induction of a similar enzyme, DT diaphorase, in neuronal cultures can protect neurons from oxidative toxicity (Murphy et al., 1991). Garthwaite and Garthwaite (1988) provided evidence in cerebellar slices that cGMP has neuroprotective effects mediated via unspecified mechanisms. Conceivably, cGMP formation stimulated by NO may provide enhanced neuronal survival. However, while glutamate enhancement of cGMP levels in the cerebellum is mediated via NO (Bredt and Snyder, 1989; Garthwaite et al., 1989), NOS occurs in many parts of the brain in which guanylate cyclase levels have not been detected in abundance by histochemical analysis (Nakane et al., 1983), suggesting that NO has roles in brain function other than activation of guanylyl cyclase.

#### Experimental Procedures

##### Materials

[ $^3$ H]arginine (53 Ci/mmol) and [ $\alpha$ - $^{32}$ P]GTP and (3000 Ci/mmol) were obtained from NEN/DuPont. The Vectastain immunocytochemistry kit was from Vector. All other materials were purchased from Sigma.

##### Preparation of Anti-NOS Antiserum

Polyclonal antiserum was raised in rabbits following a series of immunizations with the homogeneous NOS enzyme (Bredt and Snyder, 1990; Bredt et al., 1990).

##### Immunoprecipitation of NOS Catalytic Activity

NOS activity was assayed by measuring the conversion of [ $^3$ H]arginine to [ $^3$ H]citrulline exactly as described (Bredt and Snyder, 1990) and by monitoring the cyclization of [ $\alpha$ - $^{32}$ P]GTP to [ $^{32}$ P]cGMP as described (Bredt et al., 1991). For immunoprecipitation, crude soluble extracts of rat cerebellum (1 mg/ml) were incubated overnight at 4 $^{\circ}$  C with varying amounts of primary antiserum or preimmune serum. Protein A-Sepharose was then added for 1 hr, and the antibody complexes were sedimented by centrifugation. Enzyme activity and total protein concentration in the supernatant were quantified.

##### Western Blot Analysis

Soluble homogenates from various brain regions were prepared in buffer containing 25 mM Tris-HCl (pH 7.4) with 1 mM EDTA. Proteins (200  $\mu$ g per lane) were separated on a 7.5% SDS polyacrylamide gel, transferred to nitrocellulose, and probed with crude antiserum (1:5000) overnight. Bands were visualized with an alkaline phosphatase-linked secondary antibody.

##### Immunohistochemistry and NADPH Diaphorase Staining

Adult male Sprague-Dawley rats and rhesus monkey were perfused with 2% freshly depolymerized paraformaldehyde in 0.1 M PBS. Thin sections (12  $\mu$ m) were prepared as described (Bredt et al., 1990). For thick sections (40  $\mu$ m), the brains were removed and postfixed for 2 hr in 2% paraformaldehyde in PBS

followed by cryoprotection in 20% glycerol. Sections (40  $\mu$ m) were cut on a sliding microtome and immediately transferred to 50 mM Tris-HCl buffered saline. Immunostaining was done essentially as described (Bredt et al., 1990). Sections were blocked for 1 hr in PBS containing 1% normal goat serum and then incubated overnight at 4°C in the same buffer containing crude (1:2000) or affinity-purified (1:300) antiserum (Bredt et al., 1990). Horseradish peroxidase staining was done using an avidin-biotin kit (Vectastain). NADPH diaphorase staining was performed by incubating sections with 1 mM NADPH, 0.2 mM Nitro Blue Tetrazolium in 0.1 M Tris-HCl buffer (pH 7.4) containing 10% dimethyl sulfoxide for 30–90 min at room temperature (Mizukawa et al., 1989).

#### In Situ Hybridization

A pool of three 45-mer antisense oligonucleotides, complementary to nucleotides 211–266, 795–840, and 4712–4757 of the cloned cDNA (Bredt et al., 1991), were end-labeled with [<sup>35</sup>S]dATP. In situ hybridization was done exactly as described (Ross et al., 1989) on slide-mounted sections. Some sections were stained with NADPH diaphorase immediately prior to preparing the sections for in situ hybridization. The sequences of the nucleotides used are as follows: oligo #1, GGCCTTGGGCATGCTGAGGGC-CATTACCCAGACCTGTGACTCTGT; oligo #2, AGAGTCTGT-CGCCTCTGTCTGACGTCAACTTGGCGTCATCTGC; oligo #3, AGGCAGACAGGGCCACTCGGTTAGCCGTGTGTGCCG-ATTTA.

#### Acknowledgments

This work was supported by United States Public Health Service grants DA-00266, Research Scientist Award DA-00074 to S. H. S., training grant GM-07309 to D. S. B. and P. M. H., NIMH predoctoral fellowship MH-10017 to C. E. G., a Pfizer fellowship; by grants from the Dana Foundation, the French Foundation for Alzheimer's Research, and the American Academy of Neurology to T. M. D., and by a gift from Bristol-Myers Squibb.

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Received May 17, 1991; revised July 29, 1991.

#### References

Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J., and Martin, J. B. (1986). Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* 321, 168–171.

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., Hwang, P. M., and Snyder, S. H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347, 768–770.

Bredt, D. S., Hwang, P. H., Glatt, C., Lowenstein, C., and Snyder, S. H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351, 714–718.

Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1, 623–634.

Dawson, T. M., Bredt, D. S., Fotuhi, M., Hwang, P. M., and Snyder, S. H. (1991a). Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissue. *Proc. Natl. Acad. Sci. USA*, in press.

Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S., and Snyder, S. H. (1991b). Nitric oxide mediates glutamate neurotox-

icity in primary cortical culture. *Proc. Natl. Acad. Sci. USA* 88, 6368–6371.

Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D., and Martin, J. B. (1985). Selective sparing of a class of striatal neurons in Huntington's disease. *Science* 230, 561–563.

Furchgott, R. F., and Vanhoutte, P. M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.* 3, 2007–2018.

Garg, U. C., and Hassid, A. (1991). Nitric oxide decreases cytosolic free calcium in BALB/c 3T3 fibroblasts by a cyclic GMP-independent mechanism. *J. Biol. Chem.* 266, 9–12.

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.

Garthwaite, J., Garthwaite, G., Palmer, R. M. J., and Moncada, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur. J. Pharmacol.* 172, 413–416.

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.

Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J.-C., Amber, I. J., and Lancaster, J. R., Jr. (1989). Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In *Nitric Oxide from L-Arginine. A Bioregulatory System*, S. Moncada and E. A. Higgs, eds. (Amsterdam: Elsevier Science Publishers B. V.), pp. 189–223.

Hope, B. T., Michael, G. J., Knigge, K. M., and Vincent, S. R. (1991). Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* 88, 2811–2814.

Ignarro, L. J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* 30, 535–560.

Koh, J.-Y., Peters, S., and Choi, D. W. (1986). Neurons containing NADPH-diaphorase are selectively resistant to quinolate toxicity. *Science* 234, 73–76.

Kwon, N. S., Nathan, C. F., and Stuehr, D. J. (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.* 264, 20496–20501.

Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D., and Wishnok, J. S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* 27, 8706–8711.

Mizukawa, K., Vincent, S. R., McGeer, P. L., and McGeer, E. G. (1989). Distribution of reduced nicotinamide-adenine-dinucleotide-phosphate diaphorase-positive cells and fibers in the cat central nervous system. *J. Comp. Neurol.* 279, 281–311.

Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem. Pharmacol.* 38, 1709–1715.

Murphy, T. H., DeLong, M. J., and Coyle, J. T. (1991). Enhanced NAD(P)H: quinone reductase activity prevents glutamate toxicity produced by oxidative stress. *J. Neurochem.* 56, 990–995.

Nakane, M., Ichikawa, M., and Deguchi, T. (1983). Light and electron microscopic demonstration of guanylate cyclase in rat brain. *Brain Res.* 273, 9–15.

Palmer, R. M. J., and Moncada, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 158, 348–352.

Ross, C. A., MacCumber, M. W., Glatt, C. E., and Snyder, S. H. (1989). Brain phospholipase C isozymes: differential mRNA localizations by in situ hybridization. *Proc. Natl. Acad. Sci. USA* 86, 2923–2927.

Snyder, S. H., and Bredt, D. S. (1991). Nitric oxide as a neuronal messenger. *Trends Pharmacol. Sci.* 12, 125–128.

Tayeh, M. A., and Marletta, M. A. (1989). Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. *J. Biol. Chem.* 264, 19654-19658.

Thomas, E., and Pearse, A. G. E. (1964). The solitary active cells. Histochemical demonstration of damage-resistant nerve cells with a TPN-diaphorase reaction. *Acta Neuropathol.* 3, 238-249.

Uemura, Y., Kowall, N. W., and Beal, M. F. (1990). Selective sparing of NADPH-diaphorase-somatostatin-neuropeptide Y neurons in ischemic gerbil striatum. *Ann. Neurol.* 27, 620-625.