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Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues

(endothelium-derived relaxing factor/neurotoxicity)

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ABSTRACT **NADPH diaphorase staining neurons, uniquely resistant to toxic insults and neurodegenerative disorders, have been colocalized with neurons in the brain and peripheral tissue containing nitric oxide synthase (EC 1.14.23.-), which generates nitric oxide (NO), a recently identified neuronal messenger molecule. In the corpus striatum and cerebral cortex, NO synthase immunoreactivity and NADPH diaphorase staining are colocalized in medium to large aspiny neurons. These same neurons colocalize with somatostatin and neuropeptide Y immunoreactivity. NO synthase immunoreactivity and NADPH diaphorase staining are colocalized in the pedunculopontine nucleus with choline acetyltransferase-containing cells and are also colocalized in amacrine cells of the inner nuclear layer and ganglion cells of the retina, myenteric plexus neurons of the intestine, and ganglion cells of the adrenal medulla. Transfection of human kidney cells with NO synthase cDNA elicits NADPH diaphorase staining. The ratio of NO synthase to NADPH diaphorase staining in the transfected cells is the same as in neurons, indicating that NO synthase fully accounts for observed NADPH staining. The identity of neuronal NO synthase and NADPH diaphorase suggests a role for NO in modulating neurotoxicity.**

Nitric oxide, NO, is a prominent vascular and neuronal messenger molecule first identified as the chemical responsible for endothelium-derived relaxing factor activity (1–3). NO is also formed in macrophages and other peripheral blood cells (4, 5), though NO synthase (EC 1.14.23.-) activity of macrophages involves a distinct enzyme protein with different cofactors than the brain/endothelial enzyme (6, 7). NO synthase of brain tissue has been purified to homogeneity and shown to be a monomer of 150 kDa with an absolute requirement for calmodulin, calcium, and NADPH for enzyme activity (8). Utilizing selective antisera, we have localized the brain/endothelial enzyme by immunohistochemistry (9); besides endothelial cells, the only other localization throughout the body is in neurons and nerve processes. In the brain, NO synthase is selectively localized to discrete populations of medium-to-large aspiny neurons of the cerebral cortex and corpus striatum, basket and granule cells of the cerebellum, and other selected sites (9). In the periphery NO synthase is highly concentrated in neurons of the myenteric plexus of the small intestine, ganglion cells in the adrenal medulla, and in nerve fibers of the posterior pituitary derived from NO synthase-containing cells in the supraoptic and paraventricular hypothalamic nuclei (9).

The unique pattern of NO synthase localization throughout the brain does not match precisely with any known neurotransmitters. Identifying some property that is uniquely characteristic of NO synthase-containing neurons might shed light on the biological role of NO. In the present study we show that NO synthase-containing neurons are identical with populations of neurons selectively stained for NADPH diaphorase, an oxidative enzyme localized to unique populations of neurons (10, 11) that resist the toxic effects of excitatory amino acids (12–14) and hypoxia (15) and survive the degenerative processes of Huntington (16) and Alzheimer (17) diseases in select areas. Moreover, we show that NO synthase catalytic activity of these neurons is responsible for their diaphorase staining.

MATERIALS AND METHODS

Colocalization of NO Synthase with NADPH Diaphorase. Adult male Sprague–Dawley rats were perfused with 2% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and postfixed for 2 hr in 2% paraformaldehyde in PB followed by cryoprotection in 20% (vol/vol) glycerol or 10% sucrose. Free-floating thick sections (40 μm) were then cut on a sliding microtome (Reichert-Jung). Thin sections (5 or 10 μm thick) were cut on a cryostat (−18°C) (Microm) and mounted onto gelatin/chrome alum-coated slides. The slide-mounted tissue sections and the free-floating tissue sections were incubated in Tris-buffered saline (TBS; 50 mM Tris/HCl) containing affinity-purified NO synthase antiserum (1:50 dilution) (9) and 2% (vol/vol) normal donkey serum and were incubated overnight at 4°C. Immunofluorescence staining was accomplished with donkey anti-rabbit antibodies conjugated with rhodamine (Jackson ImmunoResearch). Some sections were stained with an avidin–biotin–peroxidase system (Vector Laboratories) with diaminobenzidine as a chromogen. NADPH diaphorase staining was performed by incubating free-floating or slide-mounted tissue sections with 1 mM NADPH/0.2 mM nitroblue tetrazolium/0.1 M Tris·HCl, pH 7.2/0.2% Triton X-100 for 15–30 min at 37°C (18). In all instances colocalization of NO synthase with NADPH diaphorase neurons was confirmed by staining identical sections with both NO synthase and NADPH diaphorase or by examining adjacent thin 5-μm sections.

Colocalization of NO Synthase with Neurotransmitters. Free-floating 40-μm thick sections of rat brain were incubated overnight in TBS (4°C) containing mouse monoclonal somatostatin antibodies (1:250 dilution; Novo Industries, Bagsvaerd, Denmark) and affinity-purified rabbit polyclonal NO synthase antibodies (1:50 dilution). Fluorescent staining was accomplished with donkey anti-mouse antibodies conjugated with rhodamine for somatostatin and donkey anti-rabbit antibodies conjugated with fluorescein for NO synthase. Control sections incubated with the same primary antibodies alone, but with secondary antibodies for the opposite antigen exhibited no staining. In addition, fluores-

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Abbreviations: NPY, neuropeptide Y; PPN, pedunculopontine tegmental nucleus.

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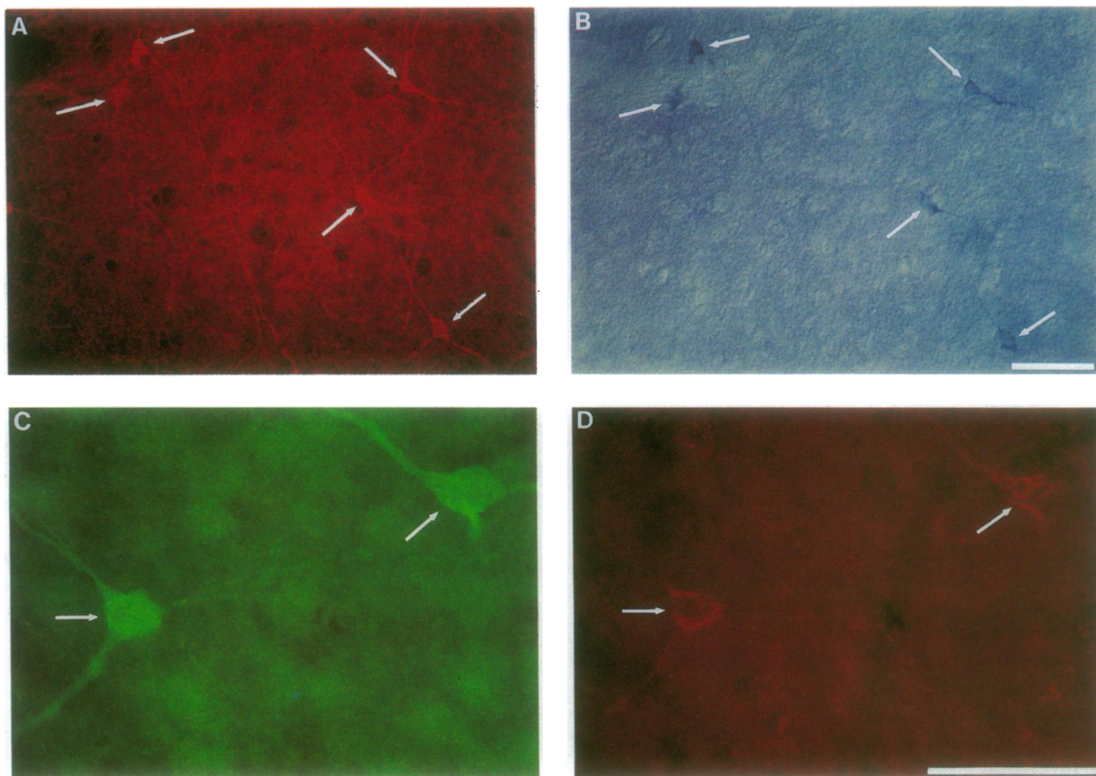

FIG. 1. Colocalization of NO synthase immunoreactivity with NADPH diaphorase staining and somatostatin immunoreactivity in the corpus striatum. (*A*) Several medium-to-large aspiny neurons of the corpus striatum exhibit NO synthase immunoreactivity (arrows). (*B*) These exact same neurons also stain positively for NADPH diaphorase activity (arrows). (*C* and *D*) The NO synthase immunoreactive neurons (*C*) also colocalize with somatostatin. (*D*) Choline acetyltransferase immunoreactive neurons do not colocalize with NO synthase or NADPH diaphorase (data not shown). (Bars = 50 μM.)

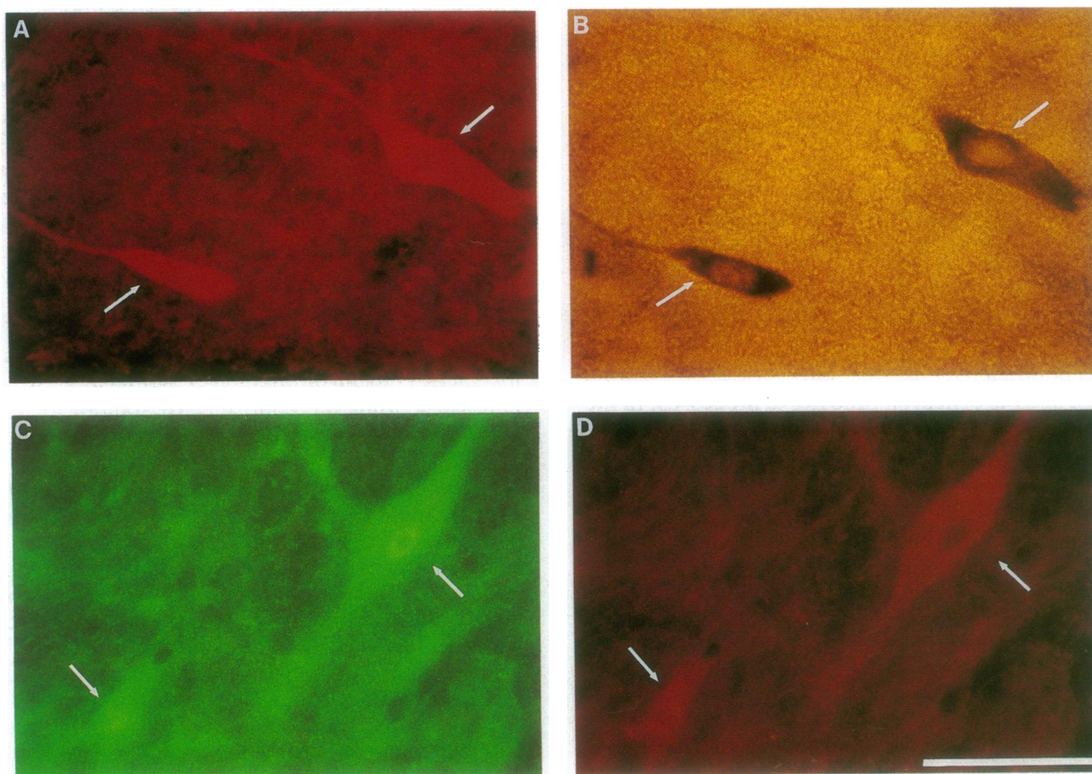

FIG. 2. Identical populations of neurons stain for NO synthase and choline acetyltransferase immunoreactivity and for NADPH diaphorase in the PPN nucleus. All NO synthase-positive cells (*A*) stain for NADPH diaphorase (*B*). The NO synthase-immunoreactive neurons (*C*) also colocalize with all of the choline acetyltransferase-immunoreactive neurons (*D*) in the PPN. No NPY or somatostatin immunoreactivity was observed in the PPN (data not shown). (Bar = 50 μM.)

cence for the appropriate fluorophore was observed only with its appropriate filter. When both primaries were stained with different secondary antibodies, there was no cross-reactivity. These controls ensured no false colocalization (19).

Immunostaining for choline acetyltransferase and NO synthase was accomplished as described above for somatostatin and NO synthase. Choline acetyltransferase antibody (Boehringer Mannheim) was used at a dilution of 1:250.

Transfections. We recently cloned NO synthase cDNA from a rat brain library (20, 21), transfected the cDNA into human kidney 293 cells, and demonstrated encoded protein and catalytic activity in the transfected cells. Human 293 kidney cells were transfected with 1 or 10 μg of cDNA spanning the full open-reading frame for NO synthase. A control expression vector was used for nontransfected cells. After transfection the cells were plated on tissue culture chamber slides and stained for NO synthase immunoreactivity and NADPH diaphorase. The density of the staining was evaluated and graded from low (1+) to very high (8+). The amount of NO synthase immunoreactivity and NADPH diaphorase staining was also determined in individual neurons of several regions by examining adjacent sagittal or coronal sections. The density of staining was evaluated by two independent observers and graded on the same scale as the transfected cells.

RESULTS

In the corpus striatum a discrete population of medium-to-large aspiny neurons stained for NO synthase, and in all instances we also observed costaining for NADPH diaphorase (Fig. 1 *A* and *B*). Somatostatin- and neuropeptide Y (NPY)-containing neurons in the corpus striatum have been reported to stain for NADPH diaphorase (22). Similarly, all NO synthase-staining neurons in the striatum also exhibited immunoreactivity for somatostatin (Fig. 1 *C* and *D*) and NPY (data not shown). In the corpus striatum, choline acetyltransferase did not occur in the same neurons as NADPH diaphorase, and we also did not detect choline acetyltransferase staining of NO synthase-positive cells (data not shown). In the cerebral cortex NO synthase staining was evident in a discrete population of medium-to-large aspiny cells in apparent random distribution in all layers of the cerebral cortex, including fiber areas such as the corpus callosum. These cells had a variety of shapes, including pyramidal, stellate and fusiform, and had a similar morphology to the NO synthase-positive cells of the corpus striatum. All of the NO synthase-positive cells in the cerebral cortex stained for NADPH diaphorase, NPY, and somatostatin (data not shown). Others have shown that only 1–2% of neurons in the cerebral cortex and corpus striatum are positive for NADPH diaphorase (18), which we confirmed for NO synthase and NADPH diaphorase, making the colocalization of these two proteins striking.

In the PPN, the pattern of NO synthase staining was markedly different from that in the cerebral cortex and corpus striatum. Virtually all neuronal cells in the PPN stained for both NO synthase and NADPH diaphorase (Fig. 2 *A* and *B*). Also in contrast to corpus striatum and cerebral cortex, in the PPN choline acetyltransferase and NO synthase occurred in the same cells (Fig. 2 *C* and *D*). In studies of others (22) as well as our own examination, no neuronal cells in the PPN stained for somatostatin or NPY (data not shown).

We previously reported that in the cerebellum NO synthase is in virtually all basket cells, granule cells, and mossy fiber terminals in glomeruli (9). We confirmed these localizations and observed NADPH diaphorase staining with an identical pattern (data not shown), though others have not found the characteristic intense neuronal staining for this enzyme in the cerebellum (23, 24). In our experiments, NADPH diaphorase staining of the cerebellum and NO synthase immunoreactivity were consistent and present only with mild fixation with paraformaldehyde (2% or less) (unpublished observations).

We previously reported NO synthase immunoreactivity in neuronal fibers of the posterior pituitary and in cells of the supraoptic and paraventricular hypothalamic nuclei that project to the posterior pituitary (9). We now show a virtually identical pattern for NADPH diaphorase staining, with the

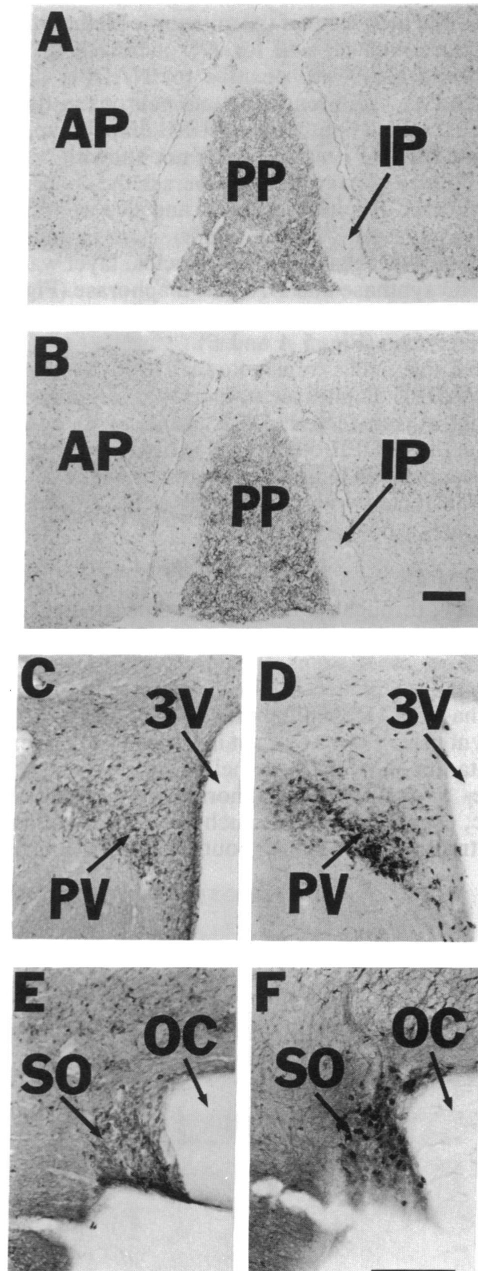

FIG. 3. Distribution of NO synthase (*A*, *C*, and *E*)- and NADPH diaphorase (*B*, *D*, and *F*)-containing neurons and processes in the hypothalamic–pituitary axis. Bright-field photomicrographs of NO synthase and NADPH diaphorase shown in *A* and *B* illustrate a similar pattern of distribution in the posterior pituitary (PP) and occasional staining of cells in the anterior pituitary (AP) and intermediate pituitary (IP). Bright-field photomicrographs demonstrate a similar pattern of distribution of NO synthase and NADPH diaphorase in the supraoptic (SO) (*C* and *D*) and paraventricular (PV) hypothalamic nuclei (*E* and *F*), which project to the posterior pituitary. 3V, third ventricle; OC, optic chiasm. (Bar = 250 μM.)

same cells in the supraoptic and paraventricular nuclei being positive for the two proteins (Fig. 3).

We confirmed our earlier observations of NO synthase staining in ganglion cells of the adrenal medulla (9) and observed NADPH diaphorase staining in the same population of cells (Fig. 4 *A* and *B*). Additionally, NADPH diaphorase stained heavily in the adrenal cortex, where we observed no NO synthase staining. The adrenal cortex possesses high levels of several oxidative enzymes associated with steroid synthesis, which may account for the NADPH diaphorase staining there (25).

Neurons and processes of the myenteric plexus of the small intestine were well stained for NO synthase as observed earlier (9) and also were positive for NADPH diaphorase (Fig. 4 *c* and *d*). Additionally, some neurons in the submucosal plexus stained lightly for NADPH diaphorase, but were not positive for NO synthase (data not shown).

In the retina, a plexus of nerve fibers in the choroid stained for NO synthase as noted earlier (9) and also was positive for NADPH diaphorase (Fig. 5 *A* and *B*). Also, a limited population of amacrine cells in the inner nuclear layer was positive for both NO synthase and NADPH diaphorase (Fig. 5 *C* and *D*). Occasional cells in the ganglion cell layer stained lightly for both enzymes (Fig. 5 *A* and *B*).

Human kidney cells transfected with NO synthase cDNA showed NADPH diaphorase staining that was proportional to the amount of transfected cDNA (Table 1). In addition, the proportion of NADPH diaphorase staining and NO synthase immunoreactivity in individual neurons was the same as that in cells transfected with NO synthase cDNA (Table 1).

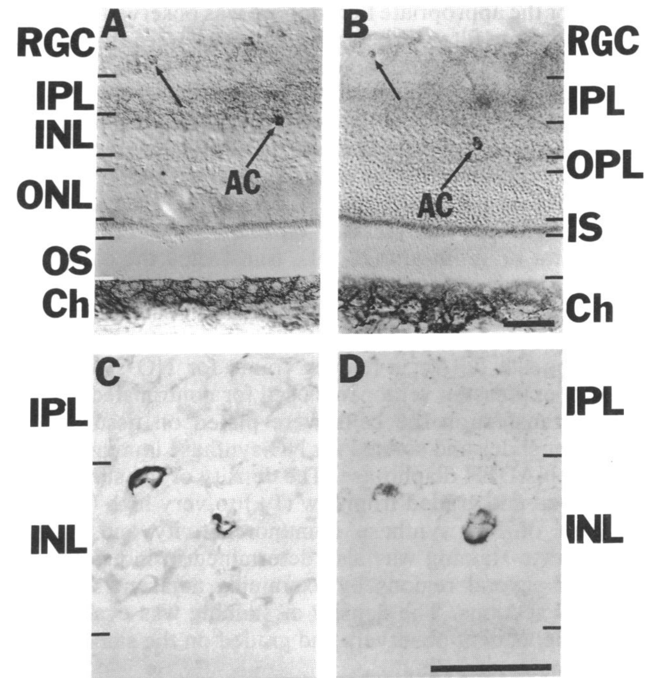

FIG. 5. Distribution of NO synthase immunoreactivity (*A* and *C*) and NADPH diaphorase (*B* and *D*) staining in the retina. In the retina NO synthase immunoreactivity (*A*) and NADPH diaphorase staining (*B*) occur in a plexus of nerve fibers in the choroid (Ch), amacrine cells (AC) of the inner nuclear layer (INL), and an occasional retinal ganglion cell (RGC) (arrows). Identical amacrine cells of the INL are shown for NO synthase immunoreactivity and NADPH diaphorase staining in *C* and *D*, respectively. IPL, inner plexiform layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment. (Bar = 50 μM.)

DISCUSSION

In summary, throughout the brain and peripheral tissues, all NO synthase-staining cells also stain for NADPH diaphorase, and in most areas the great majority of NADPH diaphorase-containing cells also exhibit immunoreactivity for NO synthase. NADPH diaphorase does occur in the absence of NO synthase, as was evident in the adrenal cortex and the liver (data not shown). Nonetheless, the coincidence of NO synthase- and NADPH diaphorase-containing neurons is dramatic, especially in areas such as the cerebral cortex and corpus striatum, where only about 1% of the cells are positive for NO synthase and NADPH diaphorase, which in all instances are colocalized. While somatostatin and NPY occur together with NO synthase and NADPH diaphorase in certain parts of the brain, such as the corpus striatum, in other areas NO synthase-containing cells are not positive for somatostatin and NPY. Similarly, choline acetyltransferase-immunoreactive cells in the PPN possess NO synthase, while

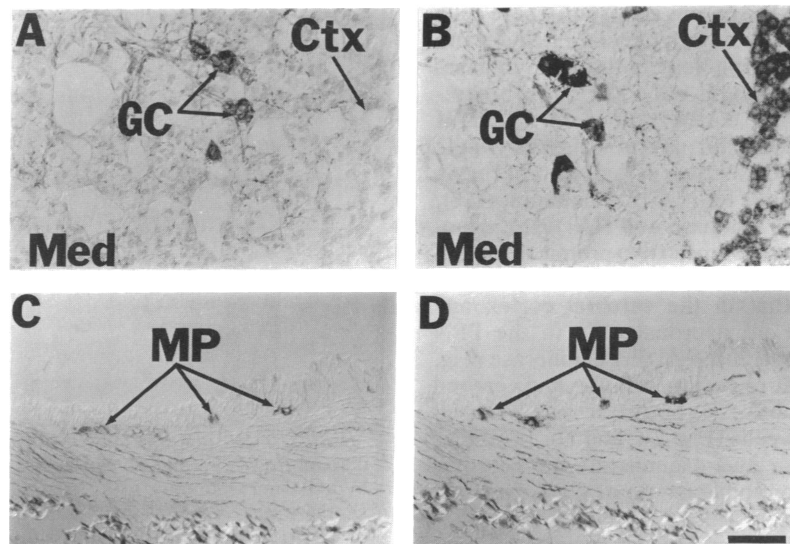

FIG. 4. Colocalization of NO synthase immunoreactivity (*A* and *C*) with NADPH diaphorase (*B* and *D*) in peripheral tissues. (*A* and *B*) Bright-field photomicrographs of NO synthase immunoreactivity (*A*) and NADPH diaphorase staining (*B*) in the adrenal gland. In all instances ganglion cells (GC) of adrenal medulla (Med) stain positively for both NO synthase and NADPH diaphorase. Note the intense NADPH diaphorase staining in the adrenal cortex (Ctx) and the lack of NO synthase staining. (*C* and *D*) NO synthase immunoreactivity in the myenteric plexus (MP) of the duodenum (*C*) and a serial section illustrating a similar pattern of distribution of NADPH diaphorase staining (*D*). (Bar = 50 μM.)

Table 1. Comparison of NO synthase immunoreactivity and NADPH diaphorase staining

Tissue	NO synthase	NADPH diaphorase
Recombinant NO synthase protein		
Non-transfected cells	0	0
NO synthase-transfected cells		
1 μg of cDNA	4+	4+
10 μg of cDNA	8+	8+
Cerebellum		
Basket cells	2+	2+
Granule cells	2+	2+
Purkinje cells	0	0
Cortex; medium-large aspiny neurons	4+	4+
Hypothalamus		
Paraventricular nucleus	3+	3+
Supraoptic nucleus	3+	3+
PPN	5+	5+
Retina		
Amacrine cells	2+	2+
Ganglion cells	1+	1+
Striatum; medium-large aspiny neurons	4+	4+

In four to six separate experiments in which 20–40 neurons were examined per region, the density of NO synthase immunoreactivity is identical to that of NADPH diaphorase staining (1+ = low; 8+ = high). In kidney cells transfected with NO synthase cDNA, the ratio of immunoreactive NO synthase and NADPH diaphorase staining is identical to that seen in positive neurons.

in the corpus striatum the two proteins are in divergent populations of neurons.

The extraordinary concurrence of NO synthase and NADPH diaphorase implies that NO synthase accounts for the diaphorase staining. By applying the NADPH diaphorase stain to human kidney cells transfected with cDNA for NO synthase (20, 21), we show that diaphorase staining reflects and is proportional to the amount of transfected cDNA. Relative levels of staining for NO synthase immunoreactivity and NADPH diaphorase are closely similar in transfected cells. Moreover, the proportion of NO synthase and diaphorase staining in individual neurons is the same as observed for cells transfected with NO synthase cDNA. Thus, the NO synthase content of each neuron can fully account for its diaphorase activity. These findings establish that NO synthase is responsible for NADPH diaphorase staining of neurons.

Another approach to link NO synthase and NADPH diaphorase would be to purify the diaphorase enzyme and monitor NO synthase activity. However, in our efforts to purify NADPH diaphorase by monitoring nitroblue tetrazolium reduction, we observe multiple protein bands with this catalytic activity that are distinct from NO synthase fractions (data not shown), as noted also by other workers (26). Recently Hope *et al.* (27) have also observed substantial NADPH diaphorase catalytic activity that could be separated by column chromatography from NO synthase activity. Hope *et al.* (27) did show that purified NO synthase possesses NADPH diaphorase activity, similar to our observations.

The selective resistance of NADPH diaphorase-positive neurons to neurotoxic insults has long been a puzzle. We have recently shown that NO mediates *N*-methyl-D-aspartate neurotoxicity in primary cortical cultures (28) and that these NO synthase/NADPH diaphorase neurons are the source of neurotoxic NO (unpublished observations). Conceivably the NO synthase catalytic activity of these neurons accounts for their survival. Alternatively, NO synthase-containing neurons might possess uniquely high levels of NADPH serving as a NO synthase cofactor, which could reduce oxidative neurotoxins. In addition, these cells may possess other unique protective mechanisms against NO. Whatever the exact mechanisms, the identity of NO synthase and NADPH diaphorase may clarify the physiologic role of NO and mechanisms of neurotoxicity.

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