

# NADPH-Diaphorase-Positive Neurons in Primate Cerebral Cortex Colocalize with GABA and Calcium-Binding Proteins

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Neurons in the monkey cerebral cortex containing nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) can be divided into two distinct types, both nonpyramidal. Type I neurons have a large soma (diameter 20–50  $\mu\text{m}$ ), a dense NADPH-d histochemical reaction, and are distributed throughout the cortex, but mainly in the subcortical white matter, and are mostly aspiny. Type II cells have a small soma (< 20  $\mu\text{m}$ ) with light NADPH-d reactivity and are distributed primarily in the supragranular layers, particularly layers II and upper III. The numerical density of type II cells is much greater than that of type I. Type I neurons also stain for GABA and a few intracortical type I cells contain calbindin. All type II cells found here are colocalized with both GABA and calbindin. Neither type I nor type II cells are stained for parvalbumin.

Together with previous observations that almost all cortical NADPH-d cells in various subprimates are like type I cells, we suggest that type II cells may form a group of NADPH-d-rich neurons differentiated in higher mammalian cortex from a subpopulation of calbindin-containing GABAergic interneurons, and these nitric oxide-synthesizing cells may play a role in control of intracortical neuronal activity characteristic of higher cerebral functions in advanced mammals.

The identification of neuronal nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) as a nitric oxide synthase (NOS) has enabled NADPH-d histochemistry to be used to localize this enzyme and help elucidate the role of nitric oxide (NO) in the brain (Bredt et al., 1991; Hope et al., 1991; Young et al., 1992). NO is associated with many cellular processes including cerebrovascular activity, neurotransmission, neuronal death, and synaptic plasticity (Garthwaite et al., 1988; Gally et al., 1990; East and Garthwaite, 1991; Moncada et al., 1991; Tanaka et al., 1991, 1993; Adachi et al., 1992; Prado et al., 1992; Snyder and Bredt, 1992; Vincent and Hope, 1992; Iadecola, 1993; Iadecola et al., 1993; Suzuki et al., 1993).

The cerebral cortex in primates is greatly expanded and more structurally and functionally complicated than in lower mammals. Human cortical activity revealed measurement of regional cerebral blood flow (rCBF) or glucose metabolism is focal and highly dynamic (Posner, 1993; Sergent, 1993). Abnormal rCBF has been observed in the brains of patients with various cognitive disorders (Cunningham Owens, 1992; Liddle et al., 1992; Bolwig, 1993; Holcomb et al., 1993; Waddington et al., 1993). Altered patterns of NADPH-d neurons have been observed in certain cortical areas in patients with Alzheimer's disease and schizophrenia (Kowall and Beal, 1988; Unger and Lange, 1992; Akbarian et al., 1993a,b; Gentleman et al., 1995). Thus, the relationship of the NOS system and cortical activity in primates is an important issue.

NADPH-d activity is expressed by certain populations of neurons in the cerebral cortex and subcortical white matter. Their morphology in subprimates is fairly constant with most presenting a Golgi-like appearance in histochemical preparations (Kowall and Beal, 1988; Mizukawa et al., 1988, 1989; Hedlich et al., 1990; Vincent and Kimura, 1992; Valtschanoff et al., 1993; Egberongbe et al., 1994; Yan et al., 1994). However, cortical NADPH-d neurons in primates are subdivisible on the

basis of their soma size and diaphorase activity. In addition to the type described above, there are numerous smaller and more lightly stained neurons (Sandell, 1986; Aoki et al., 1993; DeFelipe, 1993; Hashikawa et al., 1994; Lüth et al., 1994). In the present study, we investigate NADPH-d neurons in various cortical areas in the monkey using NADPH-d histochemistry, and immunocytochemistry for GABA and the calcium-binding proteins calbindin (CB) and parvalbumin (PV). CB and PV are markers of subpopulations of cortical interneurons (Morino-Wannier et al., 1992; Andressen et al., 1993), and colocalization of NADPH-d with GABA or CB has been reported in certain populations of neurons in the cerebral cortex (Valtschanoff et al., 1993).

## Materials and Methods

### Animals and Tissue Preparation

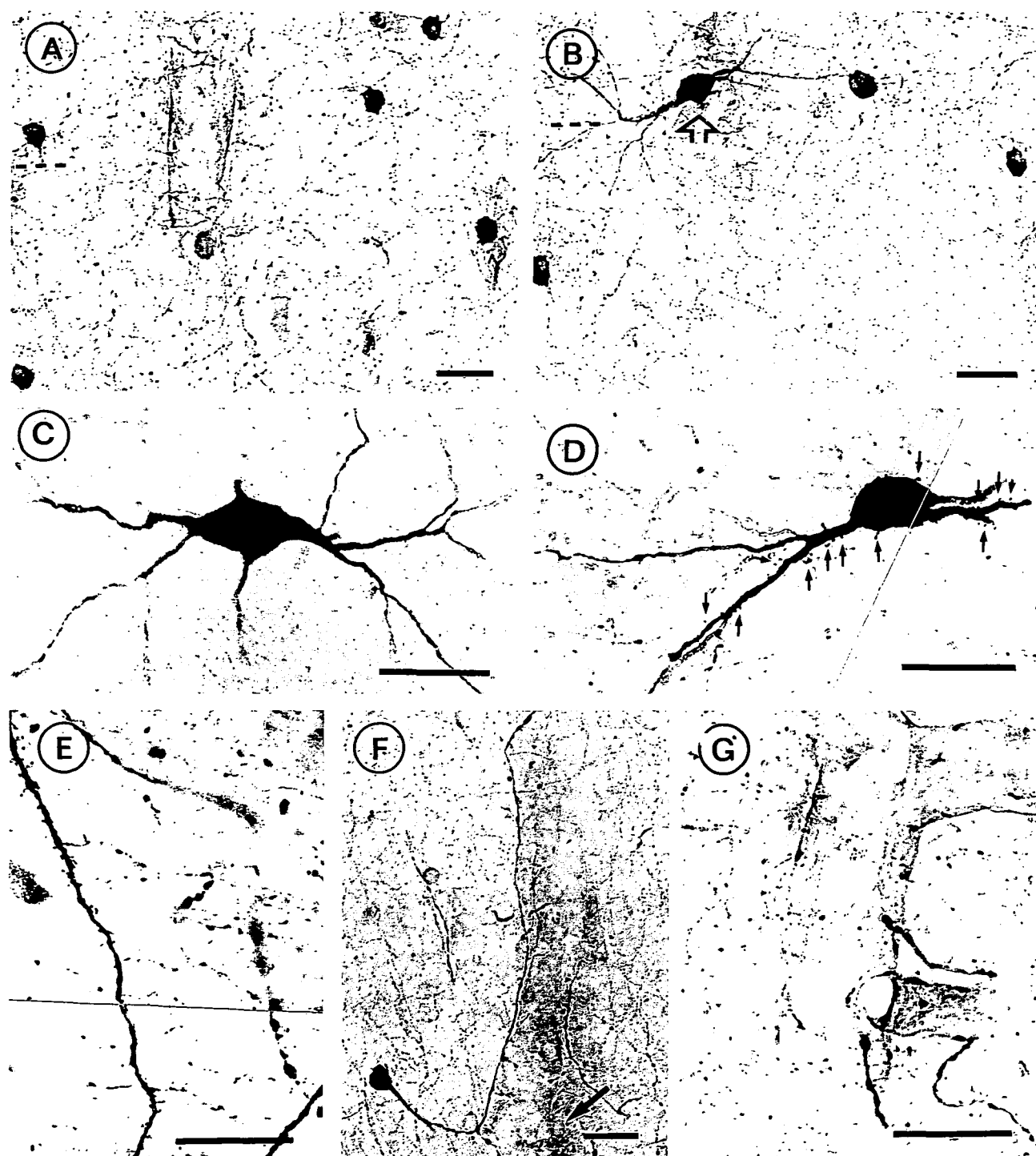
Cerebral cortical tissue was available from three adult male monkeys (*Macaca fascicularis*, 3–5 years old; body weight, 6.5–10.5 kg). They had been deeply anesthetized with ketamine hydrochloride (20 mg/kg, i.m. for induction) and sodium pentobarbital (40 mg/kg, i.p.), before transcardiac perfusion with Ringer solution and 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains had been postfixed in the same fixative. Four blocks were dissected out from both hemispheres. Areas (Brodman, 1909) studied were 4 and 9 from the frontal lobe, 1–3 from the parietal lobe, 20–22 from the temporal lobe, and 17 and 18 from the occipital lobe). Blocks were immersed in 30% sucrose in PBS at 4°C for from overnight to a few days. They were then cut coronally on a cryostat, with alternate sections taken at 40  $\mu\text{m}$  and 20  $\mu\text{m}$  and collected in PBS.

### Histochemistry and Immunocytochemistry

Histochemical and immunocytochemical agents, except the ABC kit, were obtained from Sigma. Sections 40  $\mu\text{m}$  thick were used for NADPH-d histochemistry and staining with cresyl violet. Briefly, free-floating sections for NADPH-d histochemistry were washed several times in PBS, incubated in a solution containing 1 mM  $\beta$ -NADPHG, 0.4 mM nitro blue tetrazolium (NBT), 10% dimethylsulfoxide, and 0.3% Triton X-100 in PBS for 0.5–2 hr at 37°C. After histochemical staining, the sections were washed in PBS, mounted on gelatinized slides, air dried, and coverslipped.

As the tissue penetrability of antibodies is weaker than that of the histochemical agents (Valtschanoff et al., 1993), 20  $\mu\text{m}$  thick sections were used for double staining for NADPH-d and GABA, CB, and PV. The sections were processed first for NADPH-d histochemistry as described above, followed by immunocytochemistry for GABA, CB, and PV, respectively. After histochemical staining, the sections were washed in several changes of PBS, and incubated in PBS with 0.3% Triton X-100, 5% normal goat or horse serum, and rabbit anti-GABA (1:10,000), mouse anti-PV (1:3000) or CB-D-28K (1:5000), respectively, for 24 hr at room temperature. The sections were rinsed again and incubated in PBS containing normal goat or horse serum, goat anti-rabbit, or horse anti-mouse IgG (5%) for 1 hr, and finally in avidin-biotin solution (ABC, Vector, 1%) for another hour. The immunoreactive product was visualized with diaminobenzidine tetrahydrochloride (0.05%) and hydrogen peroxide (0.01%), and the sections were mounted and coverslipped.

Controls for histochemistry were carried out with incubation in NADPH or NBT omitted, and for immunostaining followed the same



**Figure 1.** NADPH-d reactive cells and processes in area 9 of the monkey cerebral cortex. *A*, Type II cells in layers II and III (*broken line* indicates the border between these two layers). *B*, Type I (*arrow*) and type II cells in layers III and IV (*broken line* indicates laminar border). *C*, Aspiny type I cell at high magnification. *D*, High-power view of a spiny type I cell (spines are indicated by *arrows*). *E*, High-power view of spiny (*left*) and aspiny (*right*) processes. *F*, Type I cell with a dendrite running along a blood vessel from a branching point (*arrow*). *G*, Thick NADPH-d processes terminate on a vessel near a branch. Scale bars, 50  $\mu$ m.

protocol with the primary antibodies replaced by PBS, or normal rabbit (for GABA) and mouse (for CB and PV) sera. No positive staining was found in the control sections.

### Results

The overall distribution pattern of NADPH-d neurons in the monkey cerebral cortex was similar in different areas, and no difference was detected between the two hemispheres. Mor-

phology and distribution of NADPH-d neurons. Cortical NADPH-d neurons were all classifiable as nonpyramidal, but could be divided into two types, which we defined as type I and type II cells.

Type I NADPH-d neurons were darkly stained from the soma to the fine processes (Fig. 1*B-D*) and were of medium to large size (soma diameter from 20 to 50  $\mu$ m). Most were located in the white matter up to 200  $\mu$ m deep to layer VI,

where they formed loose clusters. The remaining type I cells were distributed throughout the cortex, with their numerical density greatest in layers II/III. NADPH-d-positive processes from type I cell bodies could be traced for several hundred micrometers and were highly varicose. The main branches of intracortical type I cells were usually radially orientated, but those of the subcortical type I cells were mainly tangential. Most were aspiny, though a few had spines on both soma and dendrites (Fig. 1C-E).

Type II cells had small, round, or oval somata (diameter < 20  $\mu\text{m}$ ; Fig. 1A,B). They were lightly stained, though in some cases a few thin processes were observed to run from the soma to join a fine NADPH-d fiber plexus. Most type II cells were in supragranular layers, particularly in layers II and upper III. Their number was up to 20 times that of type I neurons in the same region. Unlike the irregular tangential distribution pattern of type I cells, type II NADPH-d neurons in the supragranular layers were distributed more regularly across the cortex, either between adjacent gyri or adjacent regions in the same gyrus.

Distinct plexuses formed by numerous thin and fewer thick highly varicose processes were observed throughout the cortex and the white matter, and fibers often appeared to innervate cerebral blood vessels. In layer I and the supragranular layers, numerous fine NADPH-d fibers contacted small vessels that penetrated the cortex from the pial surface (Fig. 1A). In some cases, processes seemed to contact the vessels preferentially at their branching points and then run along the vessels (Fig. 1F,G).

#### **Colocalization of NADPH-d with GABA and Calcium-Binding Proteins**

In 20  $\mu\text{m}$  thick sections double-labeled cells contained both the bright blue histochemical product and brown immunocytochemical product in the same perikaryon (Fig. 2). The histochemical reactivity was granular and generally localized in the peripheral cytoplasm and sometimes the processes, while the immunoreactive product was evenly distributed and also present in the nuclear region. Colocalization was found between NADPH-d and GABA or CB, but not between NADPH-d and PV in both types of cortical NADPH-d neurons. All type II neurons in at least two double-stained sections from each animal were checked for double labeling; this amounted to a minimum of 500 cells per section, and all were found to be doubly reactive for NADPH-d and GABA or CB (Fig. 2C-F). Their overlapping staining patterns indicated a triple colocalization of all three molecules in type II cells. For type I cells, 100 from of the four blocks from each animal were counted, and more than half (58%) of the intracortical type I NADPH-d neurons, and about a tenth (9%) of the subcortical ones, were also concurrently stained for GABA (Fig. 2A-C). A few (4%) type I NADPH-d cells in the cortex were also clearly stained for CB (Fig. 2D,E).

#### **Discussion**

Using NADPH-d histochemistry and immunocytochemistry for GABA and calcium-binding proteins, we have confirmed a morphological and chemical diversity of NADPH-d neurons in the monkey cerebral cortex.

Darkly stained NADPH-d neurons are found in the mammalian cortex in various species including rat, cat, monkey, and man, and their distribution pattern is fairly constant across species, characterized by a predominant localization in the subcortical white matter and an apparently random tangential distribution (Sandell, 1986; Mizukawa et al., 1988, 1989; Meyer et al., 1992; Vincent and Kimura, 1992; Akbarian et al., 1993a,b; Hashikawa et al., 1994; Lüth et al., 1994; Yan et al., 1994). Such cells correspond to type I neurons of the

present study. They are interneurons and are partially colocalized with GABA and various neuropeptides (Mizukawa et al., 1989; Unger and Lange, 1992; Valtchanoff et al., 1993). The numerical density of type I NADPH-d cells in the cortex is lower in the monkey than in the rat (Yan et al., 1994).

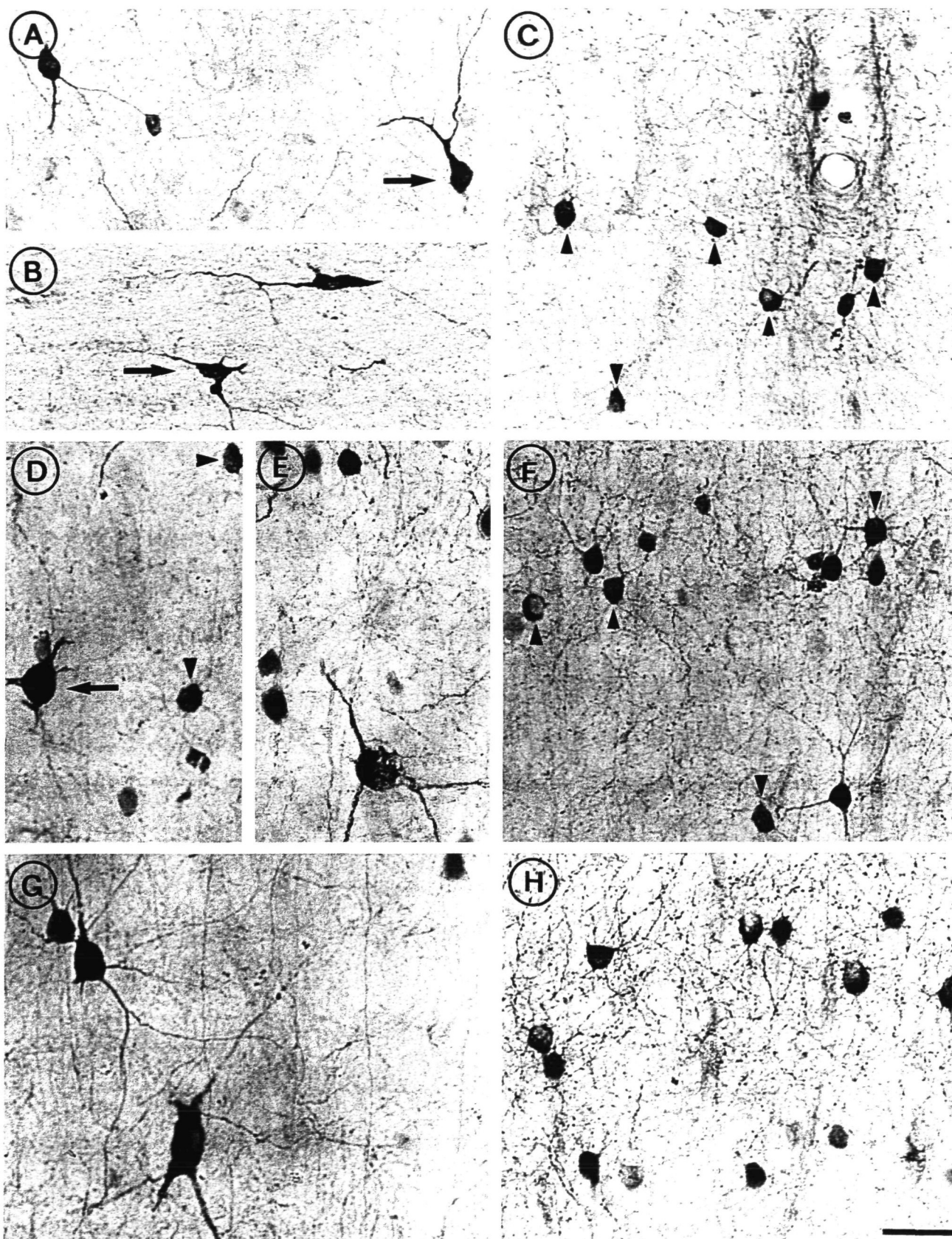
In addition to this population of darkly stained cells, similar to those that comprise the entire population of cortical NADPH-d neurons in the rat, another distinct group, our type II cells, is found in the monkey cortex, mainly in the supragranular layers. Type II cells have a smaller soma and lower NADPH-d activity, and are much more numerous than type I cells.

The colocalization of GABA in NADPH-d interneurons suggests that these cells may also serve as inhibitory neurons. The difference between type I and type II cells in terms of colocalization with GABA and particularly CB, points to a distinct difference between them. NO synthesis is a response to a rise in intracellular calcium ions; the consistent colocalization of NOS and CB in type II cells implies that this calcium-binding protein may play a role in the regulation of NO generation by changing the concentration of calcium in the cytoplasm, which might be a mechanism related to the changeable NOS activity in these cells. Type I cells may mainly use other calcium binding proteins, such as calmodulin, for this purpose (Garthwaite et al., 1988; East and Garthwaite, 1991; Snyder and Brecht, 1992; Andressen et al., 1993).

Type II cells may represent a group of cortical NADPH-d neurons newly differentiated in higher mammals, or even only in primates, during evolution from a subpopulation of calbindin-containing GABAergic interneurons, and this group of cells may serve as a new cortical NO source in these advanced mammals. The evolution of the cerebral cortex in mammals is largely marked by increases in volume, in differentiation of cortical neurons, particularly interneurons, and in complexity of functional areas and pathways. The morphological basis of higher cerebral function in advanced mammals, particularly in man, may include a rich set of inter- and intracortical connections mainly originating and terminating in the supragranular layers to provide pathways for processing and integrating cortical activity (Fisken et al., 1975; Burkhalter and Bernardo, 1989; Burkhalter et al., 1993; Lund, 1987; Hirsch and Gilbert, 1991; McGuire et al., 1991; Lachica et al., 1992, 1993). Thus, NADPH-d neurons, particularly type II cells, in these cortical layers may play a significant role in intracortical neuronal activation.

Cortical NADPH-d neurons are selectively resistant to excitotoxicity, which is linked to excessive intracellular concentration of calcium upon stimulation of glutamate receptors. Calbindin might act to protect these neurons from excitotoxicity by binding excess intracellular calcium (Mattson et al., 1991).

The NO transmitter/modulator system may play a significant role in human cerebral activity, either in health or in neuropathological conditions. Although certain alterations of type I NADPH-d neurons have been observed in conditions such as schizophrenia and Alzheimer's disease (Kowall and Beal, 1988; Meyer et al., 1992; Unger and Lange, 1992; Akbarian et al., 1993a,b), we must now consider what changes might affect type II NADPH-d neurons under various circumstances in the normal or diseased human cortex. It is possible that type II cells are more sensitive to the influence of post-mortem delay and tissue processing (Young et al., 1992), which is supported by a recent report that many lightly stained small perikarya were present in supragranular layers in human cerebral cortex obtained at neurosurgery and immediately processed (DeFelipe, 1993). Whether type II cells are involved in neuropathological states obviously deserves further investigation. Equally, it remains to determine how the



**Figure 2.** Colocalization of NADPH-d with GABA and calcium-binding proteins in neurons in monkey area 17. The sections were first stained for NADPH-d, followed by GABA (A–C), CB (D–F), and PV (G and H) immunocytochemistry. Note the partial colocalization of NADPH-d with GABA or CB in type I cells in A and B and D and E. There is complete colocalization in type II cells in C and F, and no colocalization of NADPH-d and PV in type I and type II cells in G and H. Colocalized type I neurons are indicated by *arrows*, and type II cells by *arrowheads*. Scale bar, 50  $\mu$ m.

NOS system is regulated by subcortical and inter- and intracortical inputs in different normal and pathological conditions. On the other hand, how will cortical neurons and cerebral function be affected if the NOS system is structurally or functionally disturbed?

## Notes

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