NEURONAL NADPH-DIAPHORASE IS A NITRIC OXIDE SYNTHASE

by

BRUCE THOMAS HOPE

B.Sc., The University of British Columbia, 1985

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Division of Neurological Sciences

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1991

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Psychiatry

The University of British Columbia
Vancouver, Canada

Date 3 October 1991

DE-6 (2/88)

ABSTRACT

The enzyme responsible for the neuronal NADPH-diaphorase histochemical reaction was identified in rat brain by employing a variety of histochemical and biochemical techniques. The histochemical reaction catalyzes the NADPH-dependent reduction of tetrazolium dyes to colored insoluble formazans. Although the histochemical reaction has been widely employed in neuroanatomical and neuropathological studies, the identity of the enzyme responsible for the reaction has been unknown. Previous attempts to determine the identity of the enzyme have failed due to the lack of a specific biochemical assay.

Some biochemical characteristics of the histochemical reaction in rat striatum were determined in order to develop a specific biochemical assay for neuronal NADPH-diaphorase. The histochemical reaction used several different analogs of NADPH but did not use β-NADH. All tetrazolium analogs with redox potentials above that for β-NADPH were reduced, although the reduction of some tetrazoliums was oxygen-sensitive. The reaction appeared not to require metal ions, flavins, peroxides, or superoxide anions as all methods to remove these factors did not influence staining. The DT-diaphorase activator, menadione, and the inhibitor, dicumarol, did not affect neuronal NADPH-diaphorase. Electron microscopic results suggested neuronal NADPH-diaphorase was membrane-bound, particularly with the endoplasmic reticulum. These results correlate with no known enzymes, including those previously proposed as neuronal NADPH-diaphorase.

Employing an antiserum which specifically detected neuronal NADPH-diaphorase, we found the enzyme to be nitric oxide (NO) synthase. NO synthase produces the membrane-permeable second messenger NO. Immunoreactive

NADPH-diaphorase activity was copurified to apparent homogeneity with NO synthase activity. The antiserum specifically immunoprecipitated both NO synthase and NADPH-diaphorase activities and specifically labelled a 150 kD band on Western blots, similar to NO synthase from previous reports. The NADPH-diaphorase substrate, NBT, competed with the NO synthase substrate, arginine, for electrons from NADPH. As expected, immunoreactivity for citrulline was found only in NADPH-diaphorase neurons. Citrulline is produced along with NO from the substrate, arginine. NADPH-diaphorase activity was weak to moderate in the cerebellum even though this region contains high levels of NO synthase. The cerebellum also had no citrulline- or NADPH-diaphorase immunoreactivity. We suggest the NO synthase in the cerebellum is a different form from that in the rest of the brain.

We conclude that the neuronal NADPH-diaphorase histochemical reaction is due to a form of NO synthase and therefore NADPH-diaphorase is a histochemical marker of NO synthase in the brain. The product NO is the endogenous activator of soluble guanylate cyclase in the brain. This allows a discussion of the anatomical and functional relationships between NO synthase/NADPH-diaphorase and guanylate cyclase to determine the possible functions of these enzymes and their second messenger products in the brain.

Table of Contents

List of Tables

List of Figures

Acknowledgements

The assistance of many friends in the production of this work was greatly appreciated. The author wishes to thank Bryce Pasqualotto for helping with the work on citrulline immunoreactivity, Esther Leung for photography and electron microscopy, and Paul Cumming for challenging debate. The discussions of neuroscience with Peter Reiner were greatly appreciated. I definitely have to thank Barry Boyes for helping me learn to play with biochemistry toys. Special thanks to my advisor Steve Vincent for his many helpful suggestions which were necessary for me to achieve my goals. I thank Karl Knigge and Greg Michaels for donating the NADPH-diaphorase-immunoreactive antiserum. I wish to thank my parents for their support. I thank my fiancee Cynthia Zonneveld who was perhaps the only one convinced that I would ever finish writing this manuscript. Marcus and Birgitta Von Krosigk deserve a great deal of credit for reviewing the manuscripts.

INTRODUCTION

The NADPH-diaphorase reaction allows particular populations of neurons in tissue sections to be stained with a resolution similar to that seen with the Golgi technique. The simplicity of this histochemical method, plus the demonstration that it can be used to localize specific neurochemically defined neuronal populations throughout the brain, has led numerous investigators to adopt this technique for both experimental and neuroanatomical studies and for the examination of human neuropathology (Beal et al., 1986, Davies and Roberts, 1987, Ellison et al., 1987, Ferrante et al., 1985, Koh et al., 1986, Kowall et al., 1985, 1987b, Sagar, 1986, Sagar and Ferriero, 1987, Sandell, 1985, 1986, Sandell et al., 1986, Wallace, 1986). NADPH-diaphorase may be of clinical importance. NADPH-diaphorase neurons are relatively resistant to a number of chemical insults as well as to anoxia and ischemia. There has been particular emphasis on the relative sparing of NADPH-diaphorase neurons from cell death in Huntington's striatum and in excitotoxic lesions. It is not known if or how the NADPH-diaphorase reaction is involved in this relative sparing.

Although the NADPH-diaphorase histochemical method has now become a popular tool in neuroscience for anatomical studies, very little is known about the reaction and its function in the neurons in which it is found. Until this investigation, only two biochemical characteristics were known about the NADPH-diaphorase reaction: 1) the reaction involved an NADPH-dependent chemical reduction of a tetrazolium dye to an insoluble formazan; and 2) the reaction was assumed to be enzyme-mediated. Numerous suggestions have been made for the identity of this enzyme. Some investigators have even attempted to assay NADPH-diaphorase activity biochemically and to employ the

assay to purify the enzyme. These attempts have been unsuccessful because many enzymes in a brain homogenate have biochemical diaphorase activity. Previous attempts at assaying the enzyme were not specific for the neuronal NADPH-diaphorase seen histochemically.

The purpose of this investigation was to identify the particular enzyme responsible for neuronal NADPH-diaphorase activity. First, the NADPH-diaphorase reaction was shown to be indeed enzyme-mediated. To avoid the pitfalls of previous attempts at biochemical identification, several biochemical characteristics of the histochemical reaction that could lead to a specific biochemical assay were identified. The biochemical characteristics, along with the distribution of NADPH-diaphorase, did not appear to correlate with any known enzymes. This left us with no hypotheses about the identity of NADPH-diaphorase. However, using a novel antibody, an immunoblot assay for the NADPH-diaphorase enzyme was developed which allowed its identification as nitric oxide synthase. This enzyme produces a unique new second messenger called nitric oxide.

I would like to introduce the NADPH-diaphorase histochemical reaction by describing the molecules involved and providing a definition of a "diaphorase". This leads us to a two-part history of neuronal NADPH-diaphorase histochemistry. The first investigations, in the 1960's, noted the general distribution of strong neuronal NADPH-diaphorase activity in unfixed tissue. The second phase, in the 1980's, was a detailed colocalization of NADPH-diaphorase activity with various neurotransmitter-specific populations of neurons in well-fixed tissue. I will then discuss the possible involvement of NADPH-diaphorase activity in neuropathology.

To determine the endogenous biochemical function of NADPH-diaphorase it helps to review the list of known brain diaphorases for

candidates. The last section describes attempts by other investigators to examine and identify neuronal NADPH-diaphorase biochemically. Altogether, this should provide the necessary background for this investigation into the identity of NADPH-diaphorase and its function in the nervous system.

BACKGROUND

Tetrazoliums

Tetrazolium salts, first synthesized in 1894 by von Pechmann and Runge, are soluble compounds that upon reduction form water insoluble precipitates called formazans (for an extensive review see Altman, 1976a). The salts are based on a tetrazole ring of four nitrogens and a carbon (see figure 1). The solubility, redox potential, osmiophilicity, and color of the tetrazoliums and resulting formazans can be varied tremendously by attaching various chemical groups to the basic ring structure (Altman, 1969, 1974). Over 1000 forms of tetrazoliums have been synthesized and identified. Some tetrazoliums have redox potentials such that enzymes are capable of causing their reduction (Altman, 1976a). This led to the use of these salts for the histochemical localization of enzymes, first demonstrated for succinate dehydrogenase by Seligman and Rutenberg (1951) and for the histochemical detection of sulfhydryl groups (Pearse, 1952, Barrnett and Seligman, 1954, Findlay, 1954, Deguchi, 1963). The change in absorbance from tetrazoliums to formazans has also allowed quantitative investigations of enzyme activity in tissue sections by either eluting the formazans from the section (Defendi and Pearson, 1955, Glick and Nayyar, 1956) or by direct measurement employing microspectrophotometry (Wagner et al., 1976, Altman, 1976b,c, 1977, Butcher and Altman, 1973). Tetrazolium salts were first used to assay enzyme activity in homogenates in 1949 (Kun and Abood). Since then tetrazoliums have been used in numerous biochemical studies in a variety of tissues (Levine et al., 1960, Giuditta and Strecker, 1961).

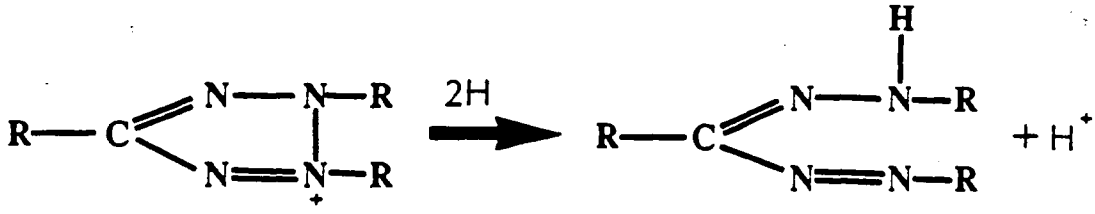
Figure 1

The core of a tetrazolium dye is a heterocyclic ring of four nitrogens and one carbon. Upon reduction the ring opens up to form a formazan which is insoluble and absorbs different wavelengths of light. This results in precipitation of a formazan with a different color than that of the tetrazolium. A large variety of tetrazoliums can be derived by the addition of different chemical groups (R) to the core ring. Tetrazolium rings can even be linked together such as in the commonly employed tetrazolium dye, nitroblue tetrazolium (NBT).

Figure 2

The $NAD(P)^+$-dependent reduction of tetrazoliums during dehydrogenase histochemistry involves two reactions: 1) The dehydrogenase enzyme oxidizes its metabolic substrate and in the process passes electrons from the substrate to the pyridine nucleotide $NAD(P)^+$ to form NAD(P)H. 2) The reduced molecule NAD(P)H can then pass electrons to a tetrazolium dye by a second reaction. The second reaction may involve the small molecule electron mediators, phenazine methane sulphate or menadione. Alternatively, the second reaction may involve an enzyme found in the tissue section. This endogenous enzyme is called a diaphorase.

Figure 1



Tetrazolium Formazan

Figure 2

1) Substrate + NAD(P)+ $\xrightarrow{\text{enzyme}}$ Substrate + NAD(P)H
(reduced) (oxidized)

2) NAD(P)H + Tetrazolium $\longrightarrow$ NAD(P)+ + Formazan
Diaphorase
or PMS
or Menadione

Diaphorases

A variety of metabolic substrates such as glucose-6-phosphate, isocitrate, 6-phosphonogluconate, ethanol, androsterone, and malate have been shown to reduce tetrazoliums in the tissue section in the presence of the oxidized pyridine nucleotides, nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) (when considered together they are abbreviated NAD(P)) (Thomas and Pearse, 1961, Sims et al., 1974, Hess and Pearse, 1963, Van Nooren and Butcher, 1984, Scherer-Singler et al., 1983, Summy-Long et al., 1984, Mellgren, 1973, Kuwabara and Cogan, 1959). The enzymes which oxidize these metabolic substrates cannot reduce tetrazoliums by themselves. NAD(P) is first reduced to NAD(P)H during the first enzymatic step. The NAD(P)H formed then reduces the tetrazoliums independent of the first reaction (see figure 2) (Van Nooren and Butcher, 1984, Farber et al., 1956, Nachlas et al., 1958).

The second reaction can occur in vitro and in situ by either of two methods. The non-enzymatic method uses electron mediators such as menadione or phenazine methane sulphate (PMS) to catalyze the transfer of electrons from reduced NAD(P)H to tetrazoliums (see figure 2) (Altman, 1976a, Van Nooren and Butcher, 1984). Electrons passed non-enzymatically have been called Type II electrons and are believed to be involved in lipid and cholesterol synthesis, glutathione reduction, and reduction of folic acid (Summy-Long et al., 1984, Altman, 1976a).

The second method involves a secondary enzyme already found in the tissue section (see figure 2). These enzymes, called diaphorases are capable of oxidizing NAD(P)H and passing the electrons to dyes such as tetrazoliums and dichlorophenyl indophenol (DCPIP) (Adler et al., 1939). The electrons passed

enzymatically are termed Type I electrons and are believed to be involved in mixed function oxidation of xenobiotics, steroids, and other endogenous substrates as well as oxidizing fatty acids. Many enzymes oxidize NAD(P)H so the term diaphorase is not very informative. More specific functions have been ascribed to most of these enzymes so the term diaphorase is now rarely used.

NADH and NADPH

The electron donors in diaphorase reactions are NADH and NADPH. NADH is primarily involved in catabolic energy producing biochemical pathways. Energy derived from oxidations during glycolysis, fatty acid oxidation, and Kreb's cycle is first trapped as high energy electrons of NADH. These electrons are then passed down the mitochondrial electron transport chain for ATP formation. The electron transport chain is responsible for most of the histochemical NADH-dependent diaphorase activity in the tissue section (Altman, 1976a).

NADPH is more involved in energy utilizing synthetic pathways. Fatty acid synthesis relies on NADPH for reducing equivalents as does much of the nucleotide synthetic pathway. NADPH is also involved in detoxification, primarily through the cytochrome P450 system, where NADPH is oxidized by a reductase which subsequently reduces cytochrome P450 (Naslund et al., 1988, Kapitulnik et al., 1987, Le Goascogne et al., 1987, Walther et al., 1986, Iscsan and Arinc, 1988). Cytochrome P450 can then either hydroxylate foreign molecules, allowing them to be conjugated by sulfotransferase or glucoronotransferase, or it can produce epoxides, which can be conjugated to reduced glutathione or hydrated to diols by epoxide hydrolase. A similar mechanism is involved in steroid metabolism. The cytochrome P450 system is a major source of

histochemical NADPH-dependent diaphorase activity in a number of organs (Altman, 1976a).

Histochemical Detection of NADPH-diaphorase Neurons in Unfixed Tissue

Using histochemical methods on unfixed tissue, Thomas and Pearse (1961, 1964) described the presence of cells in the brain that differed from other nervous elements by their high content of a specific diaphorase activity which reduced tetrazoliums using NADPH, but not NADH. Double staining of these cells with silver had shown these cells to be neurons (Duckett and Pearse, 1964). The neurons were at first called "solitary hyperactive cells" because of their sparse distribution throughout the cortex and basal ganglia, and because of the hypothesis that high diaphorase activity signified high cellular metabolic activity (Thomas and Pearse, 1961). The authors hypothesized that NADPH-diaphorase neurons had "vegetative" functions because other brain nuclei thought to be involved in "vegetative" functions were also relatively high in NADPH-dependent tetrazolium reductase activity, although not as high as that in solitary hyperactive cells.

Employing glucose-6-phosphate dehydrogenase to supply NADPH in unfixed cryostat sections, Shimuzu and Abe found solitary hyperactive cells in the cortex and basal ganglia, as well as in the lamina granulosa of the olfactory bulb, olfactory tubercle, and the islands of Cajella (Abe et al., 1963, Shimuzu and Abe, 1966). In addition, they found a group of solitary hyperactive cells in the lateral dorsal tegmental (LDT) nucleus of the pontine region which extended into the nucleus lateralis profundus of the mesencephalon. These neurons had been noted before by Felgenhauer and Stammler (1962) who incorrectly described them as locus ceruleus neurons. It

should be noted that low levels of NAD(P)H diaphorase activity different from that in solitary hyperactive cells had been found in a variety of other brain regions (Thomas and Pearse, 1961, Duckett and Pearse, 1964, 1967, Abe et al., 1963, Becker, 1961).

NADPH-diaphorase in Well-Fixed Tissue--Colocalization with Neurotransmitter-specific Neuronal Populations

The introduction of a technique to demonstrate NADPH-diaphorase neurons in well-fixed brain tissue led to the discovery that these are not solitary neurons, but rather occur in discrete, well defined cell groups in particular nuclei of the brain (Vincent et al., 1983a,b, Scherer-Singler et al., 1983). The histochemical technique used to detect diaphorase-containing neurons uses material fixed by perfusion with paraformaldehyde or glutaraldehyde solutions. Sections cut on either a vibratome, a freezing microtome, or a cryostat are incubated with the dye nitro blue tetrazolium (NBT) and NADPH. This simple histochemical procedure stains particular populations of neurons with a resolution similar to that seen with the Golgi technique. The histochemical procedure can be combined with immunohistochemistry (Vincent et al., 1982, 1983a,b, 1986, Vincent and Johansson, 1983), other enzyme histochemical techniques (Vincent et al., 1986, Nakamura and Vincent, 1985) and extended to the electron microscopic level (Vincent and Johansson, 1983). The following is a review of these studies on the distribution and morphology of NADPH-diaphorase neurons and their association with several neurotransmitter systems (see Table 1 for a summary). For an extensive review of the distribution of NADPH-diaphorase activity in the brain see Vincent and Kimura (1991).

Table 1

Neuronal NADPH-diaphorase activity is found in a variety of brain regions. NADPH-diaphorase neurons may either be projection neurons sending fibers to other brain regions or interneurons with short processes which stay within the same brain region. NADPH-diaphorase activity colocalizes with a number of neurotransmitters in a given brain region but never in all brain regions.

Table 1

Region	Interneuron or Projection	Colocalizes With	Does Not Colocalize With
Olfactory bulb	Interneurons	SS, NPY	
Striatum	Interneurons	SS, NPY	ACh, GABA
Neocortex	Interneurons	SS, NPY, GABA	
Amygdala	Projection	SS, NPY	
Bas. Forebrain	Projection	ACh, Galanin	SS, NPY
LDT and Pont. Tegmentum	Projection	ACh, SP, CRF, GRP, ANP	SS, NPY, TH
Hypothalamus	Neurosecretory	Oxytocin, Vasopressin	SS, NPY
Retina	Interneurons	GABA	TH

Abbreviations: **Bas. Forebrain**=Basal Forebrain; **LDT**=Lateral Dorsal Tegmentum; **Pont. Tegmentum**=Pontine Tegmentum; **SS**=Somatostatin; **NPY**=Neuropeptide Y; **ACh**=Acetylcholine; **GABA**=γ-amino-butyric acid; **SP**=Substance P; **TH**=Tyrosine Hydroxylase; **CRF**=Corticotropin-releasing Factor; **GRP**=Gastrin-releasing Peptide; **ANP**=Atrial natriuretic peptide.

Olfactory System

The olfactory bulb contains 6-8 types of short axon cells, two of which contain NADPH-diaphorase activity (Scott et al., 1987, Villalba et al., 1989, Gall et al., 1986, Croul-Ottman and Brunjes, 1988, De Quidt and Emson, 1986, Vincent and Kimura, 1991, Vincent et al., 1985). The superficial NADPH-diaphorase neurons are found in the periglomerular region. These neurons receive inputs from periglomerular axons, from tufted cell axons, and centrifugal fibers from the nucleus of the diagonal band of Broca and the locus ceruleus (Gall et al., 1986). These interneurons then send processes amongst the glomeruli with many entering the glomeruli itself (Vincent and Kimura, 1991, Vincent et al., 1985, Scott et al., 1987). They also provide a dense NADPH-diaphorase positive fiber network surrounding granule cells. These NADPH-diaphorase interneurons may thus provide an interaction for the periglomerular region with the internal granule cells (Scott et al., 1987, Gall et al., 1986).

There are also deeper short axon cells containing NADPH-diaphorase activity (Scott et al., 1987, Villalba et al., 1989, Gall et al., 1986, Croul-Ottman and Brunjes, 1988, De Quidt and Emson, 1986, Vincent and Kimura, 1991). These neurons appear to correspond, but not entirely, with the larger Blanes or large Golgi neurons. They lie along the ventricular portion of the granule cell layer. Many of their processes stay in this deep layer and probably innervate the deep granule cell dendrites. These cells may be able to disinhibit granule cells.

Both types of NADPH-diaphorase interneurons contain neuropeptide Y (NPY) (Scott et al., 1987, Gall et al., 1986, De Quidt and Emson, 1986) and its C-terminal flanking peptide (C-PON) from the propeptide of NPY (Villalba et al., 1989), as well as somatostatin (SS) immunoreactivity (Scott et al., 1987, Vincent

et al., 1985). The number of superficial short axon cells containing NADPH-diaphorase activity is similar to the number containing NPY suggesting exclusive colocalization (Scott et al., 1987). However, there appear to be more NADPH-diaphorase neurons in the deeper layer as compared with NPY-immunoreactive neurons suggesting that NADPH-diaphorase function is not necessarily involved with NPY metabolism.

Moving caudally, the periventricular NADPH-diaphorase neurons appear to be continuous with large multipolar neurons in the anterior olfactory nucleus and into the anterior limb of the anterior commisure (Vincent and Kimura, 1991). This layer continues into the piriform cortex, olfactory tubercle and into the rest of the cortex (Vincent et al., 1985). Many of these neurons also contain NPY (De Quidt and Emson, 1986, Gall et al., 1986) and C-PON (Villalba et al., 1989) immunoreactivity, as well as SS immunoreactivity. These neurons appear to contribute centrifugal fibers into the olfactory bulb to innervate exclusively the granule cell layer, similar to the local short axon interneurons, to control granule cell activity (Gall et al., 1986).

Striatum

NADPH-diaphorase neurons make up 1-2% of the striatal neurons (Scherer-Singler et al., 1983, Vincent and Johansson, 1983). They are sparsely distributed throughout the striatum (Scherer-Singler et al., 1983, Kowall et al., 1985, 1987a). These medium-sized interneurons have bipolar and multipolar morphology with long aspiny varicose dendrites that never leave the striatum (Aoki and Pickel, 1990, De Quidt and Emson, 1986, Vincent and Kimura, 1991, Vincent and Johansson, 1983, Vincent et al., 1982). This morphology is similar to the Type II medium-sized aspiny neurons of Dimova (Vincent and

Johansson, 1983, Vincent et al., 1982) or Type I of the Golgi classification (De Quidt and Emson, 1986, Vincent and Johansson, 1983). They are smaller than the large cholinergic striatal interneurons (Vincent and Johansson, 1983, Kowall et al., 1987a). The ventral striatum and nucleus accumbens contain similar NADPH-diaphorase neurons as in the striatum (Vincent, 1986). These neurons appear to be continuous with the polymorphic layer III NADPH-diaphorase neurons of the olfactory tubercle (Vincent and Kimura, 1991). The globus pallidus, by contrast, does not contain NADPH-diaphorase positive neurons or fibers (Vincent, 1986).

NADPH-diaphorase fibers form a dense network in the striatum and nucleus accumbens (Vincent and Kimura, 1991). The fibers of the accumbens appear to cross cell bridges into the olfactory tubercle, olfactory and piriform cortices (Vincent, 1986, Vincent and Kimura, 1991, Vincent and Johansson, 1983). It should be noted that striatal projection areas, such as the substantia nigra and globus pallidus, have few NADPH-diaphorase positive fibers, consistent with the idea that striatal NADPH-diaphorase neurons are local circuit neurons.

Striatal NADPH-diaphorase neurons appear similar to the medium-sized cells studied by Bishop et al. (1982). He found these neurons were depolarized following both cortical and nigral stimulation, suggesting these neurons were the target for convergent inputs from these two sources. As interneurons, striatal NADPH-diaphorase neurons help shape the output of the medium-sized spiny neurons which form the vast majority of striatal neurons as well as being the major striatal output (Sandell et al., 1986).

The striatum can be divided into striosomal "patches" and the surrounding "matrix" (Sandell et al., 1986, Kowall et al., 1987a,b). NADPH-diaphorase neurons and fibers are found most densely in the

acetylcholinesterase (AChE)-rich matrix compartment with less in the AChE-weak striosomes of the cat and human striatum. The matrix and striosomes have differing inputs and outputs. NADPH-diaphorase neurons with fibers entering striosomes may help to connect and integrate these two compartments of the striatum.

Employing double labelling methods, all striatal and nucleus accumbens NADPH-diaphorase neurons were found to contain NPY and SS (Vincent et al., 1982, 1983a, Vincent, 1986, Sandell et al., 1986, Kowall et al., 1987a). C-PON also colocalized with these neurons (Villalba et al., 1989). These neuropeptides always colocalized with each other and with NADPH-diaphorase in the striatum. Morphological and distribution studies of neuropeptide Y (De Quidt and Emson, 1986) and SS (Vincent et al., 1985) immunoreactive neurons agree with the double labelling studies. There is evidence for extrinsic sources of NPY and SS fibers in the striatum since a portion of these fibers do not contain NADPH-diaphorase, suggesting that NADPH-diaphorase is probably not involved in the metabolism of NPY and SS (Sandell et al., 1986, Kowall et al., 1985). Some of this extrinsic NPY input may come from the catecholaminergic neurons of the locus ceruleus (Aoki and Pickel, 1990). NADPH-diaphorase also does not colocalize with SS and NPY in the hypothalamus or brainstem (Vincent, 1986). Few NPY-immunoreactive striatal neurons were double-labelled for GABA immunoreactivity (Aoki and Pickel, 1990). Even these few NPY neurons with GABA immunoreactivity were suspected to contain GABA because of uptake from the extracellular medium and not due to an inherent ability to synthesize GABA.

Cortex

NADPH-diaphorase neurons are found in all cortical fields, primarily in

layers II-III, V-VI, and in the subcortical white matter, including the callosum (Kowall and Beal, 1988, Scherer-Singler et al., 1983, Mizukawa et al., 1988). NADPH-diaphorase neurons in human cortex have varicose aspiny dendrites and beaded axons. The fibers tend to stay in the same layers as the cell bodies but some rise to traverse the superficial layer I. The morphology suggests these neurons to be local circuit interneurons that may inhibit pyramidal neurons in layers II and V.

Most, but not all NADPH-diaphorase neurons in the cortex contain SS and NPY (Vincent, 1986, Ellison et al., 1987, Kowall and Beal, 1988). Some NADPH-diaphorase neurons have only SS or NPY or neither. In human cortex, there is over 90% colocalization of SS and NPY. SS is found in 80% of the NADPH-diaphorase neurons while 10% of the neuropeptide-containing neurons do not contain NADPH-diaphorase. Once more this suggests NADPH-diaphorase is not involved in the metabolism of either neuropeptide. It should be noted that most but not all of the SS and NPY neurons are also GABAergic (Aoki and Pickel, 1990, Kowall and Beal, 1988). This means NADPH-diaphorase is found in a subpopulation of GABAergic neurons in the cortex, similar to NADPH-diaphorase positive amacrine cells in the retina (Vaney and Young, 1988).

NADPH-diaphorase neurons appear similar to cytochrome oxidase-containing neurons in layer IVc of macaque striate cortex (Sandell, 1986). During monocular damage NADPH-diaphorase staining shows ocular dominance columns similar to that with cytochrome oxidase staining. NADPH-diaphorase neurons are found throughout the visual system. This includes the retina and probably the lateral geniculate relay neurons of the thalamus. Rodent somatosensory barrel fields also have a distribution of NADPH-diaphorase neurons similar to cytochrome oxidase (Sandell, 1984, 1986).

Amygdala

NADPH-diaphorase neurons with long varicose processes are found in all subregions of the rat and human amygdala (Scherer-Singler et al., 1983). In human amygdala there is a high level of colocalization of neuropeptide Y and SS while NADPH-diaphorase is found in a large proportion of SS neurons suggesting all three are colocalized, although not necessarily in all these neurons (Unger et al., 1988). NPY and thus probably NADPH-diaphorase neurons in the amygdala appear to innervate the perirhinal and insular cortices through the ventral amygdalofugal pathway (De Quidt and Emson, 1986). The same pathway may also carry NPY and NADPH-diaphorase fibers from the basolateral nucleus of the amygdala to the ventral part of the nucleus accumbens.

Basal Forebrain

There are NADPH-diaphorase projection neurons in the diagonal band of Broca and the medial septum but not in the lateral septum (Vincent and Kimura, 1991, Kinjo et al., 1989). Those in the medial septum project to the ventral hippocampal formation while those from the horizontal limb of the diagonal band of Broca project to the dorsal part of the hippocampal formation (Kinjo et al., 1989). These neurons are cholinergic and contain the neuropeptide galanin (Vincent and Pasqualotto, 1991). The basal forebrain cholinergic neurons which innervate the neocortex, however, do not contain NADPH-diaphorase activity. NPY (De Quidt and Emson, 1986) and SS (Vincent et al., 1985) immunoreactivity are found in the lateral septum but not in the medial septum or diagonal band of Broca. It is, therefore, unlikely that NADPH-diaphorase activity colocalizes with SS and NPY or plays a role in the

metabolism of these neuropeptides.

In humans, NADPH-diaphorase neurons of the substantia innominata have been shown to comingle but never colocalize with the magnocellular cholinergic neurons which provide the major cholinergic innervation of the neocortex (Ellison et al., 1987). NADPH-diaphorase neurons may interact with the cholinergic neurons of the substantia innominata which have been suggested to play a role in learning and memory, and possibly a role in the pathophysiology of Alzheimer's disease.

The Midbrain and Pontine Region

The brainstem contains densely stained NADPH-diaphorase neurons in the lower midbrain and upper pontine tegmentum (Vincent, 1986, Vincent and Kimura, 1991, Scherer-Singler et al., 1983, Vincent et al., 1983b, 1986, Nakamura et al., 1988, Kowall and Mueller, 1988). Rostrally, they are found lateral to the superior cerebellar decussation in the pedunculopontine tegmental nucleus. Caudally, the cells are found more medially in a central gray region called the laterodorsal tegmental nucleus (LDT). In the rat NADPH-diaphorase neurons are found medial to the catecholaminergic neurons of the locus ceruleus.

The morphology and distribution of these NADPH-diaphorase neurons are similar to the cholinergic neurons of the brainstem (Vincent et al., 1983b, Nakamura et al., 1988). Double labelling studies with antibodies to choline acetyltransferase (ChAT) in the rat have shown all NADPH-diaphorase neurons in this group to be cholinergic (Vincent et al., 1983b, Vincent, 1986). However, 10% of the NADPH-diaphorase neurons in the human brainstem are not cholinergic (Mesulam et al., 1989). NADPH-diaphorase neurons in the striatum are not cholinergic so NADPH-diaphorase is unlikely to be involved

in cholinergic metabolism (Vincent et al., 1983c).

Unlike most of the forebrain, the NADPH-diaphorase neurons of the LDT do not contain SS (Vincent et al., 1986) or NPY (Vincent et al., 1982). NPY is found instead in the tyrosine hydroxylase-containing catecholaminergic neurons of the locus ceruleus which lie beside the NADPH-diaphorase neurons (De Quidt and Emson, 1986, Vincent et al., 1982, Vincent et al., 1986). It was previously thought that the locus ceruleus neurons contained NADPH-diaphorase (Felgenhauer and Stammler, 1962, Kauffman et al., 1974). Double labelling studies, however, have found locus ceruleus neurons do not contain NADPH-diaphorase (Vincent et al., 1986). All the cholinergic/NADPH-diaphorase neurons in the midbrain-pontine tegmentum are atriopeptin-immunoreactive (Standaert et al, 1986). Atriopeptin is also known as the atrial natiuretic peptide (ANP). Thirty per cent of the NADPH-diaphorase neurons in this cholinergic column contain substance P (SP) and all SP neurons in this group are NADPH-diaphorase positive (Vincent et al., 1986). Most of these SP neurons also contain gastrin-releasing peptide (GRP) immunoreactivity, which in vertebrates probably signals the presence of neuromedin B. The medial anterior cortex is innervated by fibers from these cholinergic-SP-GRP hindbrain neurons, joining those cholinergic fibers from the basal forebrain (Stivers et al., 1988). This region of cortex can undergo carbachol induced seizures which can be potentiated by SP and decreased by CRF. The caudal cholinergic/NADPH-diaphorase/SP neurons also contain weak CRF immunoreactivity (Vincent et al., 1986).

The major output of the hindbrain cholinergic neurons is to the thalamus where one sees many NADPH-diaphorase positive fibers (Mesulam et al., 1989, Kayama and Ogawa, 1987, Vincent and Kimura, 1991). Correspondingly, these neurons provide the majority (90%) of cholinergic

input (Kayama and Ogawa, 1987) and brainstem afferents to the thalamus (Kayama et al., 1986, Vincent et al., 1986, Mesulam et al., 1989). The neurons have widely branching dendrites that intermingle with passing fiber tracts such as the central tegmental tract and the superior peduncle, allowing them to be diffusely activated by a variety of sensory input (Vincent et al., 1986, Mesulam et al., 1989).

These caudal cholinergic/NADPH-diaphorase neurons are considered to play a major role in attention and arousal. The caudal cholinergic neurons form the major part of the "isodendritic core" of the ascending reticular activating system (ARAS) (Vincent et al., 1983b, Kowall and Mueller, 1988, Mesulam et al., 1989). Stimulation of the ARAS desynchronizes cortical EEG by blockade of rhythmic thalamocortical activity (Semba et al., 1990). Stimulation of the ARAS also facilitates sensory transmission. Two hundred Hertz stimulation of the LDT increased the firing rate of dorsal lateral geniculate relay neurons in a scopolamine-dependent and noradrenergic antagonist-independent manner (Kayama et al., 1986). LDT stimulation also increased responses to photic stimulation in some lateral geniculate neurons.

Descending projections from the caudal cholinergic/NADPH-diaphorase neurons are involved in the initiation of REM sleep (Semba et al., 1990).

The caudal cholinergic/NADPH-diaphorase neurons project to limbic structures such as the medial prefrontal cortex, the cingulate cortex, and the lateral septum (Stivers et al., 1988, Wilcox et al., 1989). The caudal cholinergic neurons project to rostral cholinergic neurons in the basal forebrain which provide the majority of diffuse cholinergic input to the cortex (Kowall and Mueller, 1988, Mesulam et al., 1989, Consolo et al., 1990). It has been suggested these forebrain cholinergic neurons are a rostral extension of the ARAS and thus play a role in attention and arousal. The caudal cholinergic neurons also

provide sparse cholinergic innervation directly to the cortex.

Through connections with the basal ganglia the caudal cholinergic/NADPH-diaphorase neurons may also be involved in extrapyramidal control (Vincent, 1986, Kawamata et al., 1990, Skinner et al., 1989, Mesulam et al., 1989).

Cerebellum

Granule cells of the cerebellar cortex are moderately stained for NADPH-diaphorase (Vincent and Kimura, 1991, Kawamata et al., 1990). There is weak staining in the inner third of the molecular layer, possibly due to stellate neurons or basket cells. Purkinje cells do not have NADPH-diaphorase activity. The weak staining of granule cells may be due to the lack of a significant layer of cytoplasm surrounding the nucleus.

Superior Colliculus

In the upper part of the midbrain NADPH-diaphorase neurons are found in a wedge-shaped area of the central gray substance, in the subependymal layer of the superior colliculus, and in the precommisural nucleus (Vincent and Kimura, 1991, Wallace, 1986, Wallace and Fredens, 1989). These NADPH-diaphorase neurons and those from the caudal cholinergic column contribute a patchy distribution of NADPH-diaphorase positive fibers to the intermediate gray layers of the superior colliculus. Similar to the striatum, these patches of weak NADPH-diaphorase staining neuropil are surrounded by a lattice of strongly staining NADPH-diaphorase positive fibers (Wallace and Fredens, 1989). As expected, this lattice also has strong AChE activity, most likely from the caudal cholinergic column in the hindbrain (Wallace, 1986, Wallace and Fredens, 1989, Mesulam et al., 1989). Similar to the striatum, the lattice has

different afferents than the patches. The weak NADPH-diaphorase staining patches are innervated by the brachium of the inferior colliculus suggesting involvement with the auditory system while the lattice areas are putatively involved in nociception (Wallace and Fredens, 1989). There are also NADPH-diaphorase neurons in the cortical nucleus of the inferior colliculus (Vincent and Kimura, 1991).

Hypothalamus

The caudal cholinergic/NADPH-diaphorase neurons send fibers to the hypothalamus which itself contains strong NADPH-diaphorase staining in the magnocellular neurons of the supraoptic and paraventricular nuclei (Vincent and Kimura, 1991, Sagar and Ferriero, 1987). The magnocellular neurons send NADPH-diaphorase fibers through the median eminence and terminate on capillary loops in the posterior pituitary. The terminals secrete the neurohormones oxytocin and vasopressin into the bloodstream to control lactation and blood osmolality, respectively (Sagar and Ferriero, 1987).

The lateral hypothalamic nucleus also contains medium-sized NADPH-diaphorase neurons whose processes form a dense meshwork in the nucleus (Vincent and Kimura, 1991). These NADPH-diaphorase neurons are continuous with those in the bed nucleus of the stria terminalis which send fibers caudally in the stria medullaris to the lateral habenula. NADPH-diaphorase neurons of the hypothalamus do not colocalize with NPY or SS (Vincent, 1986). NADPH-diaphorase is thus not involved in the metabolism of SS or NPY.

Medulla

More NADPH-diaphorase neurons can be found scattered in the medulla caudal to the hindbrain cholinergic/NADPH-diaphorase neurons (Vincent and

Kimura, 1991, Kowall and Mueller, 1988, Sakharova et al., 1979). The gracile nucleus contains strongly stained cells and a dense fiber network. The cuneate nucleus has only a dense bundle of positive fibers but no NADPH-diaphorase positive cell bodies. There are NADPH-diaphorase neurons sparsely distributed in the reticular areas near cranial nuclei but never within them. One exception, however, is a plexus of fibers and stained cells in the substantia gelatinosa of the spinal trigeminal tract. None of these neurons contain AChE activity and thus are not a caudal extension of the caudal cholinergic column.

Sensory Ganglia

NADPH-diaphorase neurons can be found in the nodose ganglion and in the dorsal root ganglia from the fifth thoracic to the first lumbar level (Aimi et al., 1991). These neurons may carry autonomic visceral afferent information from the gastric wall and celiac ganglion.

Retina

Subclasses of amacrine neurons in the retina stain for NADPH-diaphorase (Bhattacharjee, 1977, Sandell, 1985, Sagar, 1986, Vaney and Young, 1988, Mitrofanis, 1989, Provis and Mitrofanis, 1990, Sato, 1990b, Cobcroft et al., 1989). The pattern of NADPH-diaphorase staining varies widely between species (Cobcroft et al., 1989). Most vertebrate retina have small, sparsely distributed NADPH-diaphorase amacrine cells at the inner border of the inner nuclear layer (Bhattacharjee, 1977, Sandell, 1985, Sato, 1990b, Cobcroft et al., 1989). Except for the rat (Mitrofanis, 1989), mammalian retinas also have larger NADPH-diaphorase amacrine cells that form the innermost row of the inner nuclear layer (Sagar, 1986, Vaney and Young, 1988, Provis and Mitofanis, 1990, Cobcroft et al., 1989). Both types in the rabbit have been

colocalized with GABA immunoreactivity, but not with tyrosine hydoxylase immunoreactivity (Vaney and Young, 1988). These NADPH-diaphorase staining neurons form only a small proportion of the GABA-immunoreactive amacrine cells, suggesting NADPH-diaphorase is not necessary for GABA metabolism.

Human retinas have an additional class of small amacrine cells as well as cone photoreceptors that contain NADPH-diaphorase activity (Provis and Mitrofanis, 1990). In all species, dendrites of NADPH-diaphorase neurons spread in the middle and outer strata of the inner plexiform layer (Bhattacharjee, 1977, Vaney and Young, 1988, Provis and Mitrofanis, 1990, Cobcroft et al., 1989). This is where the cone bipolar neurons synapse while rod bipolar neurons synapse elsewhere, suggesting that in the retina, NADPH-diaphorase neurons may be involved in cone visual physiology (Provis and Mitrofanis, 1990).

Pineal Gland

Another association of NADPH-diaphorase with visual function is the finding of multipolar NADPH-diaphorase interneurons in the photosensitive frog pineal gland (Sato, 1990a). Rat pineal glands are not photosensitive and contain only a few scattered NADPH-diaphorase positive fibers (Vincent and Kimura, 1991).

NADPH-diaphorase and Pathophysiology

It has been discussed above that NADPH-diaphorase histochemistry is not uniquely associated with any neurotransmitter system yet identified, but rather is found in regions of the central nervous system associated with different neurotransmitter-specific populations of neurons. Thus the

question remains, why do these particular classes of neurons contain this enzyme? What role does it perform in these neurons? Already in the 1960's Thomas and Pearse (1961, 1964) had suggested that neurons containing NADPH-diaphorase were resistant to various forms of damage or chemical insult. Thus, when they examined animals treated with carbon monoxide, sulphanilamide, 3-acetylpyridine, thalidomide, or tetrachloromethane, in no case did they succeed in influencing the high activity of NADPH-diaphorase present in these particular cell groups. No explanation has yet been found for the sparing of these neurons or even if NADPH-diaphorase was involved.

Huntington's Disease and Excitotoxicity

More recently, the striatal neurons containing NADPH-diaphorase have been found to be selectively spared in the striatum of patients with Huntington's disease (Kowall et al., 1987b, Ferrante et al., 1985, 1987). In the late stages of Huntington's disease up to 95% of striatal neurons are lost (Kowall et al., 1987b). The nucleus accumbens which contains a higher density of NADPH-diaphorase neurons is spared except in severe cases. The matrix component, as defined by high AChE activity, undergoes the most severe neuronal loss (Ferrante et al., 1987). NADPH-diaphorase neurons are found most densely in the matrix but are spared from cell death. As expected, SS and NPY increase in relative concentration as Huntington's striatum shrinks (Beal et al., 1986).

Huntington's disease was one of the first suggested to have an etiology involving endogenous excitotoxins (Beal et al., 1988). Excitotoxins are conformationally locked molecules that resemble the active conformation of the excitatory amino acid, glutamate. Excitotoxins are proposed to stimulate glutamate receptors excessively leading to cell death (Koh and Choi, 1988a).

Striatal neurons, including NADPH-diaphorase neurons, receive a strong glutamatergic input and thus have many glutamate receptors (Ferrante et al., 1987). Thus when the excitotoxins AMPA, kainic, ibotenic, quisqualic, and quinolinic acid are injected into the striatum, cell death occurs (Beal et al., 1986, 1988, 1989, 1990, Ferriero et al., 1990, Koh and Choi, 1986, 1988a, Boegman and Parent, 1988, Araki et al., 1985, Davies and Roberts, 1987, 1988, Cohen et al., 1987). MK-801, an NMDA receptor antagonist, blocks the cell death that occurs after injection of some of these excitotoxins (Beal et al., 1989).

Only quinolinic acid injections result in relative sparing of NADPH-diaphorase neurons, similar to that in Huntington's striatum (Beal et al., 1986, 1988, 1989, 1991, Koh and Choi, 1986, 1988a). Higher than normal levels of endogenous quinolinic acid have been proposed to cause striatal cell death during Huntington's disease. Davies and Roberts (1987), unlike the previous authors, found no relative sparing of NADPH-diaphorase neurons after quinolinic acid injections at all distances from the core of the lesion site. For comparison they measured the changes in numbers of NADPH-diaphorase neurons relative to cholinergic neurons. They assumed cholinergic neurons were representative of non-NADPH-diaphorase striatal neurons. Cholinergic neurons, however, are also selectively spared during quinolinic acid injections (Davies and Roberts, 1988, Beal et al., 1988, 1989, 1991, Koh and Choi, 1988a) so it is not surprising Davies and Roberts (1987) found no relative sparing. In contrast, NADPH-diaphorase neurons are relatively more vulnerable to kainic acid and AMPA injections (Koh and Choi, 1988a, Beal et al., 1989, Araki et al., 1985). Similarly, Koh and colleagues have found relative sparing of NADPH-diaphorase neurons in cortical cell cultures after exposure to quinolinic acid and NMDA and relative vulnerability to kainic acid and quisqualate (Koh and Choi, 1986, 1988b).

It has been hypothesized that NADPH-diaphorase neurons are relatively spared during Huntington's disease because the NADPH-diaphorase enzyme metabolizes a toxin, possibly an excitotoxin (Kowall et al., 1987b, Ferrante et al., 1985, 1987). One candidate for the identity of NADPH-diaphorase was DT-diaphorase, an enzyme with NADPH-diaphorase activity and capable of metabolizing quinolinic acid (Cohen et al., 1987, Ferrante et al., 1987). However, studies employing an antibody raised against liver DT-diaphorase found immunoreactivity only in cell bodies in catecholaminergic nuclei, such as the substantia nigra, ventral tegmental area, and A8 region (Schultzberg et al., 1987). There was no DT-diaphorase in the hypothalamic dopaminergic neurons. DT-diaphorase immunoreactive fibers in the striatum disappeared with a 6-hydroxydopamine lesion in the hindbrain, further indicating DT-diaphorase is found only in catecholaminergic neurons. Since NADPH-diaphorase activity is never found in catecholaminergic neurons, DT-diaphorase cannot be a component of neuronal NADPH-diaphorase activity.

Striatal cholinergic neurons, which do not contain NADPH-diaphorase, are also relatively spared in Huntington's striatum and during quinolinic injections (Koh and Choi, 1988a, Beal et al., 1988, 1989, 1991, Davies and Roberts 1988, Kowall et al. 1987b). Although choline acetyltransferase (ChAT) levels decrease in Huntington's striatum and during quinolinic acid injections, ChAT and AChE positive neurons appear to be spared from cell death. This suggests the NADPH-diaphorase enzyme is not necessary for the protection of neurons during quinolinic acid injections and during Huntington's disease. The spared striatal neurons more likely have relatively few of the glutamate receptor subtypes, such as NMDA receptors, that make the neurons susceptible to quinolinic acid (Beal et al., 1989, 1991, Koh and Choi, 1988a,b).

Anoxia-Ischemia

NADPH-diaphorase neurons in gerbil striatum, rat cortex, and fetal tissue grafts were selectively spared after ischemia (Ferriero et al., 1990, Mampalam et al., 1988, Uemura et al., 1990, Ferriero et al., 1988). The mechanism of ischemia-induced cell death may involve excitatory amino acids since glutamate receptor antagonists such as MK-801 can block neuronal death (Beal et al., 1988). Again, selective sparing of NADPH-diaphorase neurons may be due to a relative lack of NMDA receptors rather than having anything to do with NADPH-diaphorase activity. Some investigators suggest selective sparing of NADPH-diaphorase neurons occurs only between post-natal days 2 and 14 (Ferriero et al., 1988, 1990). This might be related to the differential effects of hypoxia-ischemia on adults and neonates, the latter leading to cerebral palsy.

Alzheimer's Disease

Cortical NADPH-diaphorase neurons undergo some degeneration during Alzheimer's disease (Kowall and Beal, 1988). As expected, somatostatin levels are also lower in Alzheimer's cortex relative to that in age-matched controls. When examined histochemically, the processes of NADPH-diaphorase neurons appear to be reduced with no reduction in the number of NADPH-diaphorase neurons. NADPH-diaphorase neurons in Alzheimer's amygdala, however, are relatively spared (Unger et al., 1988). The NADPH-diaphorase neurons of the LDT, which are also cholinergic, shrink with age (Kawamata et al., 1990). They are particularly reduced in Alzheimer's and Parkinson's brains (Zweig et al., 1989). The shrinking with age and disease may contribute to the cholinergic deficits leading to memory loss (Kawamata et al., 1990). Injection of aluminum chloride into the brain has been suggested to model the neuropathology of

Alzheimer's disease (Kowall et al., 1989). NADPH-diaphorase neurons in the rabbit cortex, however, are unaffected by aluminum chloride toxicity which is dissimilar to the pathological findings in Alzheimer's brain and casts further doubt on the aluminum-toxicity model.

Biochemical Studies of Diaphorase Enzymes in the Brain

What enzyme is responsible for the neuronal NADPH-diaphorase reaction? There have been several suggestions but no concerted attempts to answer this question. Many candidates for neuronal NADPH-diaphorase have been detected biochemically in the brain. The following is a list of the possibilities. In the early 1960's, various brain diaphorases were characterized which used NADH only, or NADH and NADPH to reduce various electron acceptors other than tetrazoliums (Giuditta and Strecker, 1960, 1961, Harper and Strecker, 1962, Levine et al., 1960, Briggs et al., 1975, Farber et al., 1956, Nachlas et al., 1958, Roerig et al., 1972).

The brain has an NADH-dependent diaphorase activity dependent on the presence of oxygen and inhibited by antimycin A and amytal (Giuditta and Aloj, 1965, Vesco and Giuditta, 1966). This diaphorase activity is attributed to the mitochondrial electron transport chain.

There are separate NADH and NADPH-dependent cytochrome c and tetrazolium reductase activities associated with microsomes which are not inhibited by antimycin A and amytal (Williams and Kamin, 1962, Vesco and Giuditta, 1966). The NADH-dependent microsomal pathway involves a flavoprotein which passes electrons to cytochrome P450 via cytochrome b_5. The NADPH-dependent microsomal pathway supplies cytochrome P450 with electrons either directly through a flavoprotein called cytochrome P450 reductase (Iscan and Arinc, 1988, Walther et al., 1986, Le Goascogne et al., 1987,

Kapitulnik et al., 1987, Naslund et al., 1988) or through a similar system involving adrenodoxin and adrenodoxin reductase (Tuls et al., 1987).

There is a soluble diaphorase that can use either NADPH or NADH to reduce tetrazoliums (Vesco and Giuditta, 1966, Giuditta and Strecker, 1961, Giuditta and Aloj, 1965). It requires an intermediary redox mediator, such as menadione, for activity and is inhibited by micromolar levels of dicumarol. This activity is called DT-diaphorase, as well as quinone reductase, menadione reductase, and vitamin K reductase (Martius, 1959, Hall et al., 1972). This form was mentioned in the discussion of Huntington's disease. There is also a particulate diaphorase in the brain with characteristics similar to DT-diaphorase.

Glutathione reductase is a soluble, strictly NADPH-dependent diaphorase found in the mammalian brain (Giuditta and Aloj, 1965).

It is notable that the brains of marine animals have relatively little of the above NADPH-dependent diaphorase activities but still have comparable amounts of NADH-dependent diaphorase activity (Giuditta and Aloj, 1965). There are, however, NAD(P)H dependent diaphorase activities, one dicumarol sensitive and two insensitive, found in the optic lobe of cephalopods. Marine animals, similar to mammals, have NADPH and NADH-dependent ferricyanide reductase activity.

Flynn and colleagues (Cromlish and Flynn, 1985, Flynn, 1982, Cromlish et al., 1985) have described four aldehyde reductases capable of diaphorase activity in the cytosol of mammalian brain. One of them is glucuronate reductase which uses NADPH exclusively and is part of the pentose pathway. Two of the other aldehyde reductases use only NADPH. They are succinic semialdehyde reductase, which is involved in the metabolism of the neurotransmitter γ-aminobutyric acid (GABA), and a reductase similar to

prostaglandin-9-ketoreductase and other xenobiotic reductases. Lastly, there is an aldose reductase with tetrazolium reductase activity which uses both NADPH and NADH. This enzyme may be involved in biogenic aldehyde metabolism. It is unknown how these aldehyde reductases are related to the diaphorases described in the 1960's.

Attempts to Identify Neuronal NADPH-diaphorase Biochemically

Sagar and Ferriero (1987) have recently suggested that NADPH-diaphorase activity may correlate with neuronal function and metabolic activity. They confirmed the presence of NADPH-diaphorase neurons in the magnocellular neurosecretory neurons of the paraventricular and supraoptic nuclei of the hypothalamus. Some of these neurons secrete vasopressin which regulates blood osmolality. They hypothesized that stimulation of these neurons by salt loading should increase general metabolism in these cells and therefore increase NADPH-diaphorase activity. However, they found histochemical experiments produced too much variability in the degree of NADPH-diaphorase staining to allow quantitative comparisons between various experimental groups. They, therefore, adapted the histochemical methodology to a biochemical assay and found a marked increase in activity following 8 days of salt loading. This was the second published attempt to assay NADPH-diaphorase biochemically. Previously, Ferrante et al. (1985) had tried a similar biochemical assay for NADPH-diaphorase activity in homogenates of normal human and Huntington's brain. However, when tissue is homogenized there is no way of knowing that the reduction of NBT observed in the presence of NADPH is mediated by the same enzyme activity detected histochemically in fixed tissue. Many enzymes, including non-specific diaphorases which can use both NADH and NADPH, various hemoproteins and cytochrome oxidases,

will be present in such a tissue homogenate and could conceivably reduce tetrazoliums (see above). Thus such studies can only be undertaken once the biochemical characteristics of this particular NADPH-diaphorase have been documented.

Is NADPH-diaphorase Cytochrome P450 reductase?

Roberts and his colleagues have recently made attempts to purify and characterize neuronal NADPH-diaphorase (Kuonen et al., 1988, Kemp et al., 1987a,b, 1988, Davies et al., 1985). Employing non-denaturing polyacrylamide gel electrophoresis (ND-PAGE), one major band of NADPH-dependent tetrazolium reductase activity was detected, mostly in the Triton X-100 solubilized supernatant of microsomes (Kemp et al., 1987a,b, Davies et al., 1985). In solution, the enzyme had a Km of 1.1 μM for NADPH and 7.3 μM for the tetrazolium, NBT (Kemp et al., 1987a). The enzyme could also use cytochrome c with a Km of 1.3 μM (Kemp et al., 1987b). Gel filtration chromatography resulted in a single peak of NADPH-diaphorase activity (Kuonen et al., 1988). This gave them the idea that there was only one NADPH-diaphorase in solution and thus there was only one possible candidate for identity with neuronal NADPH-diaphorase. The native enzyme had a molecular weight of 180 kD, similar to that detected by ND-PAGE. Unlike the NADPH-diaphorase activity found histochemically, the cerebellum and cerebral cortex had the highest levels of activity while the striatum had only moderate levels. They had also found high levels of activity in a number of peripheral tissues, including the kidney and liver.

The NADPH-diaphorase enzyme of Roberts and colleagues was purified to homogeneity (Kemp et al., 1988). SDS-PAGE of the purified protein had a molecular weight of 73 kD, similar to that for cytochrome P450 reductase.

Other similarities included equimolar amounts of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) per subunit, competitive inhibition by 2'-AMP, NADP, and noncompetitive inhibition by the sulfhydryl reagents, p-mercuribenzoate (PCB) and 5,5'-dithio-(2-nitrobenzoic acid) (DTNB). Dicumarol, the potent inhibitor of DT-diaphorase had no effect on the purified enzyme. The results strongly indicated that the purified enzyme was indeed cytochrome P450 reductase. This led Kemp et al. (1988) to suggest that the neuronal NADPH-diaphorase activity seen histochemically was cytochrome P450 reductase.

Five years earlier, however, Haglund et al. (1984) had localized cytochrome P450 reductase immunoreactivity in only catecholaminergic nuclei such as the subtantia nigra, ventral tegmental area, the rostral part of the dorsal raphe, the locus ceruleus, and the ventrolateral medulla. Immunoreactivity was also detected in the periventricular and arcuate nucleus of the hypothalamus and the medial aspect of the zona incerta. In contrast, there were no immunoreactive cell bodies in the striatum or cortex where many NADPH-diaphorase neurons are found. Employing double-labelling techniques and consecutive sections, cytochrome P450 reductase immunoreactivity was found in cells which also had tyrosine hydroxylase immunoreactivity. NADPH-diaphorase neurons never contain tyrosine hydroxylase. Therefore, although cytochrome P450 reductase has NADPH-dependent tetrazolium reductase activity it is unlikely to be responsible for the activity seen in NADPH-diaphorase positive neurons.

Roberts and colleagues failure to measure specifically neuronal NADPH-diaphorase activity in homogenates was due to the large number of possible diaphorase enzymes in the brain and, most importantly, due to the lack of a specific biochemical assay. This is why it was necessary to determine specific

biochemical characteristics of the histochemical reaction. It is only with a specific biochemical assay that neuronal NADPH-diaphorase could be purified and identified. These steps allowed determination of the function of neuronal NADPH-diaphorase in the brain.

METHODS

NADPH-Diaphorase Histochemistry

Adult male Wistar rats were anesthetized with 0.5 g/kg chloral hydrate intraperitoneally. The rats were perfused through the ascending aorta with 50 ml of 0.9% saline followed by 250 ml of 4% paraformaldehyde, buffered with 0.1 M sodium phosphate, pH=7.4. The brains were then removed, post-fixed for two hours and placed in 15% sucrose for 1-2 days. Thirty micrometer coronal sections were cut on a freezing microtome and collected in 50 mM Tris-buffered saline (TBS), pH=7.4. The normal histochemical reaction for NADPH-diaphorase consisted of incubating the free-floating sections for 45 minutes at 37°C in 1 mM NADPH, 0.5 mM nitro blue tetrazolium (NBT), and 0.2% Triton X-100 in 50 mM Tris-HCl, pH=8 (Scherer-Singler et al., 1983). Peripheral tissue was processed in the same way.

Protease digestion. Because the tissue was very difficult to handle after protease digestion, sections were first mounted onto subbed slides. The sections were covered with 0.1% trypsin or 0.01% proteinase K at room temperature (21°C) for 15 minutes, followed by repeated rinses with TBS. As a control, trypsin previously inhibited with 0.05% phenylmethanesulfonyl fluoride (PMSF) was applied to some sections.

Heat Stability. Sections were placed in TBS in a microfuge tube and placed in a water bath heated to a selected temperature for 5 minutes. The sections were removed to ice cold TBS and reacted for NADPH-diaphorase activity.

Acid and Alkaline Stability. Sections were left overnight at 4°C in 0.1 M buffers of various pH: glycine-HCl (pH=3); sodium acetate (pH=4.5); sodium phosphate (pH=6.7); Tris-HCl (pH=8); glycine-NaOH (pH=9 and 10); and

sodium phosphate (pH=11). The next day the sections were rinsed repeatedly with TBS and then reacted for NADPH-diaphorase activity at pH=8.

pH of Reaction Solution vs Activity. Using the buffers described above, the pH of the reaction solution was varied between 6-10.

NADPH Analogs. The following analogs were substituted at 1 mM final concentration for the usual β-NADPH in the reaction for NADPH-diaphorase activity: α-NADPH; deamino-NADPH; 3'-phosphate-NADPH (3'-PO4-NADPH); β-NADH, and the reduced form of nicotinamide mononucleotide (NMNH).

Tetrazolium Analogs. The following tetrazolium analogs were substituted for the usual nitro blue tetrazolium (NBT): triphenyl tetrazolium (TT), tetrazolium blue (TB), tetranitro blue tetrazolium (TNBT), iodonitrotetrazolium (INT), neotetrazolium (NT), thiocarbamyl nitro blue tetrazolium (TC-NBT), benzothiazolyl styryl pthalhydrazidyl tetrazolium (BSPT), and monotetrazolium (MTT). Both MTT and TT were tested with and without 100 mM cobalt chloride. Some of the tetrazoliums were examined in reaction solutions that were vacuum degassed, and sparged with nitrogen before and during the reaction with the tissue section for NADPH-diaphorase activity.

Cyanide and Chelators. Sections were pre-treated by placing them overnight in 0.1 M ethylenediaminetetraacetic acid (EDTA), trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), or potassium cyanide, all at pH=7.5. The sections were then reacted for NADPH-diaphorase activity in the presence of the same chelator.

Menadione and Dicumarol. Sections were pretreated with 0.1 mM dicumarol or 5 mM menadione for 1 hour at room temperature. The sections were then reacted for NADPH-diaphorase activity with the same concentration of menadione or dicumarol.

Free Radical Scavengers. Either 500 U/ml of superoxide dismutase or 5000 U/ml of catalase, or both were included in the usual histochemical reaction solution.

Flavin Removal. Attempts to remove a possible flavin from NADPH-diaphorase were performed employing the four different methods described by Husain and Massey (1978).

(a) Acid-ammonium sulfate treatment:

Sections were placed in one ml of 0.1 M TrisCl, pH=8.0. One ml of 3 M KBr was added. Thirty seconds later one ml of saturated ammonium sulfate was added slowly over twenty seconds with gentle swirling. The ammonium sulfate had been acidified to pH=1.5, 2.0, 3.0, 4.0. Swirling of the reaction continued for forty seconds before four more ml of ammonium sulfate were added. The solution was kept on ice for five min before the sections were removed and quickly rinsed in 0.1 M TrisCl, pH=8.0, 1 mM EDTA. The sections were placed in ice cold 0.1 M TrisCl, pH=8.0, 1 mM EDTA, 1.5 M KBr for forty min. The whole procedure was repeated two more times before rinsing three times in TBS and reacting for NADPH-diaphorase activity.

Attempts to reconstitute NADPH-diaphorase activity with exogenous flavins involved incubating the treated sections in 0.1 M TrisCl, pH=8.0, 1 mM EDTA, 200 μM FAD or FMN or both for 24 hours at 4°C. The sections were rinsed three times in TBS and reacted for NADPH-diaphorase activity.

b) Dialysis Against Potassium Bromide:

Sections were placed in the dialysis buffer: 2 M KBr, 1 mM EDTA, 0.1 M phosphate buffer, pH=4.0, 5.5, 7.0, 8.5 at 4°C. The sections were transferred to fresh buffer 6 times over two days. The sections were rinsed 3 times in TBS and reacted for NADPH-diaphorase activity. Attempts to reconstitute NADPH-diaphorase activity with exogenous flavins were performed as above.

c) Guanidine Hydrochloride:

Sections were placed in ice cold 2 or 5 M guanidine hydrochloride, 0.1 M phosphate buffer, pH=7.6, 1 mM EDTA for 5 min. The sections were rinsed 3 times in TBS and reacted for NADPH-diaphorase activity. Attempts to reconstitute NADPH-diaphorase activity with exogenous flavins were performed as above.

d) Calcium Cloride Treatment:

Sections were incubated in 0.1 M TrisCl, pH=8.0, 1 mM salicylate, 0.3 mM EDTA, and 2 M calcium chloride for 90 min at room temperature (21°C). The sections were then placed in 0.1 M TrisCl, pH=8.0, 1 mM salicylate, 0.3 mM EDTA, and 10 mM cysteine at 4°C with four changes over 24 hours. The sections were rinsed 3 times in TBS and reacted for NADPH-diaphorase activity. Attempts to reconstitute NADPH-diaphorase activity with exogenous flavins were performed as above.

Sulfhydryl Inhibitors. Sections were pre-treated for 90 min at room temperature with the following sulfhydryl inhibitors: 1 mM 5,5'-dithio-(2-nitrobenzoic acid) (DTNB); 5 mM p-mercuribenzoate (PMB); or 5 mM N-ethyl maleimide (NEM). They were then rinsed repeatedly in TBS and reacted for NADPH-diaphorase activity.

Electron Microscopy. To examine the ultrastructural localization of the NADPH-diaphorase reaction, animals were perfused through the ascending aorta with 300 ml of 0.1 M phosphate buffer, pH=7.4, containing 4% paraformaldehyde and 1.0% glutaraldehyde. Striatal sections were cut on a vibratome at 100 μm thickness and collected in TBS. The sections were incubated for NADPH-diaphorase activity with either NBT or BSPT and no Triton X-100 detergent (Kalina et al., 1972). After the reaction the sections were rinsed in TBS, post-fixed in osmium tetroxide, dehydrated, and embedded

in Epon. Ultra-thin sections were cut and both unstained and lead citrate-stained sections were examined with a Phillips 201 electron microscope.

Histochemistry with Species other than Rat

Painted turtles, rainbow trout, leopard frogs, and hagfish were guillotined without anesthetic. These brains, along with cow brain, were immersion fixed in 4% paraformaldehyde, buffered with 0.1 M phosphate buffer, pH=7.4, for 24 hours followed by 1-2 days in 15% sucrose. The brains of cats and guinea pigs were perfusion fixed similar to the rat. Thirty micrometer sections were made and reacted for NADPH-diaphorase activity as before.

Conjugation of Citrulline to Bovine Serum Albumin

One ml of citrulline (10 mg/ml) was mixed with 1 ml of bovine serum albumin (BSA) (30 mg/ml in 3 M sodium acetate buffer, pH=7.8). One ml of 5% glutaraldehyde was added and allowed to react for 3 minutes. The solution turned a strong yellow color. One ml of 10 mM sodium borohydride was added to reduce the double bond. The solution was dialyzed overnight into one liter of 0.9% saline at 4°C. The next day the solution was centrifuged for 30 min at 20,000xg to remove any insoluble material.

Immunization

Two rabbits were immunized with the citrulline-BSA conjugate. The immunogen was made 1 mg/ml in sterile saline and sterile filtered through a 0.22 µm syringe filter. 500 µl of this solution was emulsified with 1 ml of Freund's complete adjuvant before injecting 1.5 ml subcutaneously into each rabbit. Booster injections in incomplete adjuvant were given every 2-3 weeks for 3 months. The rabbits were bled and the blood centrifuged for 30 minutes at 20,000xg. 0.02% sodium azide was added to the supernatent. Aliquots of the antiserum were frozen at -70°C.

Specificity of Citrulline Antisera

The antibody specificity was tested using conjugates of various amino acids with whole brain macromolecules (Otterson and Storm-Mathieson, 1984). Briefly, whole rat brain was homogenized and dialyzed, and the resultant macromolecular suspension (10 mg protein/ml) was mixed with an amino acid (2.5 nmoles amino acid/mg protein) in 0.1M sodium phosphate buffer together with 25 nmoles/ml glutaraldehyde, at 22°C for 1 hour. After dialyzing overnight, the conjugates were blotted onto nitrocellulose membranes. The membranes were then incubated in the primary antibody and stained with the same avidin-biotin complex immunoperoxidase technique used for immunohistochemistry (Hsu et al., 1981).

NADPH-diaphorase Immunoreactivity

The antiserum for NADPH-diaphorase immunoreactivity, called HAN antiserum, was kindly donated by Greg Michaels and Karl Knigge of Rochester University. HAN antiserum was developed as an anti-idiotypic antibody to an anti-CRF (corticotropin-releasing-factor) antibody. It was the hope of Michaels and Knigge (1989) that HAN antiserum would recognize the CRF receptor. However, the distribution of immunoreactivity in no way correlated with CRF autoradiographic or membrane binding studies of CRF receptor distribution (De Souza, 1987, De Souza et al., 1985). For example, CRF receptors have been found at high levels in the cerebellum and at very high levels in the anterior pituitary whereas the HAN antiserum showed no intense staining in these regions. Instead, the staining followed the pattern revealed by NADPH-diaphorase histochemistry. The reason for cross-reactivity of HAN antiserum with neuronal NADPH-diaphorase is unknown.

Immunohistochemistry

Three hundred gram male Wistar rats were perfused through the heart

with 50 ml of 0.1M phosphate-buffered saline, pH=7.4 (PBS) followed by 200 ml of 0.1M phosphate buffered 4% paraformaldehyde. The brains were post-fixed for 2 hr and placed in cryoprotectant solution (15% sucrose, 10% glycerol, 0.02% sodium azide) for two days at 4°C. Twenty µm sections were cut on a cryostat and placed in PBS.

For single labelling of citrulline immunoreactivity, free floating sections were incubated with rabbit anti-citrulline antibody diluted 1:20,000 in PBS with 0.3% Triton X-100, 2% normal goat serum, and 0.01% sodium azide (PBST) for 2 days at 4°C. After rinsing 3x20 min in PBS, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector ABC Kit) for 1 hour at room temperature, washed again and incubated in the avidin-biotinylated horseradish peroxidase complex for 1 hour. The sections were then washed, and reacted for peroxidase using the glucose oxidase-diaminobenzidine-nickel technique (Shu et al., 1988).

For double labelling of citrulline and NADPH-diaphorase enzyme activity, sections were incubated in anti-citrulline serum diluted 1:2000 in PBST for 2 days at 4°C. For double labelling of NADPH-diaphorase immunoreactivity and NADPH-diaphorase enzyme activity, sections were incubated in HAN antiserum diluted 1:200 in PBST for 2 days at 4°C. After washing, sections were incubated in Texas Red conjugated goat anti-rabbit IgG (Jackson Labs) diluted 1:40 in PBST for 1 hour at room temperature. Sections were then examined and photographed with a Leitz fluorescence microscope. After photography the sections were rinsed for 15 minutes in 50 mM TrisCl buffer, pH=8.0 with 0.3% Triton X-100, and then reacted for NADPH-diaphorase histochemistry as described above for 20 minutes. They were then re-examined and photographed under bright field illumination.

Immunoprecipitation

Samples to be immunoprecipitated were first centrifuged for 10 min at 10000xg to remove insoluble precipitates. 200 µl of the supernatant were mixed with 2 µl of HAN antiserum or non-immune normal rabbit serum (NRS). The mixture was placed on a rotating platform at 4°C for 1 hour. 100 µl of a 10% solution of Protein-G Sepharose beads in Tris-Cl buffered saline, pH=7.4 (TBS) was added to the sample and agitation continued for another hour. The solution was centrifuged at 10000xg for 2 min. The supernatant was assayed for NO synthase or NADPH-diaphorase activity.

The pellet was washed twice with 750 µl of TBS and assayed for NADPH-diaphorase activity by adding 270 µl of 50 mM Tris-Cl, pH=8.0, 0.2% Triton X-100, 0.5 mM NBT, 1 mM NADPH and incubating at 37°C for 16 min. The reaction was stopped with 300 µl of 100 mM sulfuric acid. The beads were allowed to settle by gravity and the supernatant pipetted off. Activity could be seen as the precipitated blue formazan on the beads. The formazan could not be quantitatively precipitated from the beads so a positive reaction was registered if the beads were a dark blue color with the HAN antisera and not colored with NRS.

The pellet could also be assayed for NO synthase activity by the addition of 100 µl of NO synthase assay solution (see below) and 25 µl of 90 µM [^{3}H]-arginine (3 µCi/ml). The solution was incubated for 10 min at room temperature and stopped by washing the pellet twice with 1 ml washes of 20 mM HEPES, pH=5.5, 2mM EDTA. The washes were pooled and assayed for [^{3}H]-citrulline.

Biochemical NADPH-diaphorase Assay

In a total volume of 300 µl the assay mixture contained 30 µl of sample, 50 mM Tris-Cl, pH=8.0, 0.2% Triton X-100, 1 mM NADPH, and 0.5 mM NBT. The

assay was started with the addition of NBT and incubated for 16 min at 37°C. The assay was stopped with 300 µl of 100 mM sulfuric acid and the absorbance measured at the isobestic wavelength of 585 nm. This is the wavelength at which the absorbance of the NBT diformazan is twice that of the NBT monoformazan (Butcher and Altman, 1973, Eadie et al., 1970). The absorbance is thus proportional to the number of reducing equivalents accepted by the tetrazolium.

NO Synthase Assay

Twenty-five µl of sample were mixed with 100 µl of 50 mM HEPES, pH=7.4, 1 mM EDTA, 1 mM DTT, 1.25 mM CaCl , 10 µg/ml calmodulin, and 1 mM NADPH. The assay was started by adding 25 µl of 90 µM [^{3}H]-arginine (3 µCi/ml) and incubating at room temperature (20°C) for 10 min. The assay was stopped with the addition of 2 ml of 20 mM HEPES, pH=5.5, 2 mM EDTA. The [^{3}H]-citrulline product was detected by passage through a 1 ml column of AG-50W-X8 Dowex cation exchanger, followed by 2 ml of water. The pooled eluents were collected in scintillation vials, mixed with 12 ml of aqueous counting scintillant and counted for 10 min in a liquid scintillation counter. The Dowex columns were washed before each assay with 2 ml of 1 M NaOH, followed by 10 ml of water.

The radioactive arginine from the supplier contained a radioactive contaminant that appeared in the flow-through from the cation exchange chromatography step. The cpms from this contaminant were high enough to interfere significantly with the [^{3}H[-citrulline signal formed from NO synthase activity. The contaminant was removed by mixing the [^{3}H]-arginine with a small amount of Dowex cation exchange beads in a microcentrifuge tube. The beads were washed several times with water to remove the non-adsorbed contaminant. The purified [^{3}H]-arginine was then eluted from the beads with a small volume of 4 M NaCl. The NaCl was greatly diluted in the NO

synthase assay mixture and thus did not interfere with the assay.

Inhibition of NO synthase by NBT

Twenty µl of the supernatant of a brain homogenate which was centrifuged at 30000xg for 30 minutes (see below) were mixed with 115 µl of NO synthase buffer. The assay was started with the addition of 15 µl of 80 mCi/mmole [^{3}H]-arginine, final concentration in the reaction was 0.833, 1.071, 1.5, 2.5, and 7.5 µM. The assay continued for 10 min at room temperature (21^{o}C). The reaction was stopped and assayed for [^{3}H]-citrulline formation as before. The reaction was then performed in the presence of 50 µM NBT. The cpms of the blank reactions, no supernatant added, were subtracted from those of the test samples. All reactions were performed in triplicate.

Copurification of NO synthase and NADPH-diaphorase

Thirteen whole rat brains were homogenized in 5 volumes of 50 mM Tris-Cl, pH=7.4, 1 mM EDTA, 10 mg/l each of soybean trypsin inhibitor, bacitracin, and aprotinin, and 100 mg/l phenylmethanesulfonyl fluoride and centrifuged at 30000xg for 30 min. The supernatant was mixed with 2',5'-ADP agarose for 30 min, poured into a 1 cm diameter column and washed with 40 ml of 0.5 M NaCl in 10 mM Tris-Cl, pH=7.4, 1mM EDTA followed by 8 ml of buffer without the salt. The column was eluted with 10 mM β-NADPH in buffer. The fractions containing NO synthase and NADPH-diaphorase activities were then applied to a Protein Pak DEAE-5P HPLC anion exchange column (Waters Assoc.) and eluted with a 0 to 0.4 M NaCl gradient. NO synthase activity of the fractions was assayed for 10 min at room temperature. NADPH-diaphorase activity was assayed for 8 min at 37^{o}C.

SDS-PAGE

SDS-PAGE electrophoresis was performed according to the method of Laemlii et al. (1970). The resolving gel was 9% acrylamide, 0.25% bis-

acrylamide in 375 mM Tris-HCl, pH=8.8, 0.1% SDS. The stacking gel was 3.75% acrylamide, 0.1% bis-acrylamide in 125 mM Tris-HCl, pH=6.8, 0.1% SDS. The initiator and catalyst for the polymerization reaction were TEMED and ammonium persulfate, respectively. The samples were mixed 1:1 with 125 mM Tris-HCl, pH=6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.05% bromphenol blue and placed in a boiling water bath for 3 min. The samples were allowed to cool to room temperature before centrifuging 5 min at 9000xg to remove any insoluble precipitate. The samples were electrophoresed at 10 mA (constant current) till the bromphenol blue band was near the bottom of the gel. The running buffer in both top and bottom chambers was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH=8.2-8.3.

Western Blot

The gel from SDS-PAGE electrophoresis was equilibrated in the transfer buffer (50 mM Tris, 192 mM glycine, pH=8.2-8.3, 15% methanol) for 30 minutes. After immersing the Immobilon membrane (Waters Assoc.) in methanol for several seconds to wet it, the membrane was rinsed with water and allowed to equilibrate along with blotting papers in the transfer buffer for 15 min. The gel was placed on one of the blotting papers, then the membrane and another blotting paper. This was placed in the sandwich apparatus of a Hoeffer Transphor unit, put in the tranfer chamber with 3 liters of transfer buffer and tranferred at 25 V (constant voltage) for 4-5 hours (approx. 125 V-hours).

The membrane was rinsed in transfer buffer and immersed in 200 ml of blocking buffer (10% non-fat dried milk powder in TBS) on a rotating platform at room temperature for four hours. The membrane was rinsed 2x5 min with 0.3% milk powder in TBS and placed in a Seal-a-meal bag with either the HAN antiserum or normal rabbit serum (NRS) (1:1000) in 2% normal goat serum (NGS), 0.3% Tween-20, 0.02% sodium azide, and 10% milk powder in TBS. The

bag was sealed with a Decasonic bag sealer and shaken vigorously on a rotating platform for two days at 4°C. The membrane was rinsed 3x30 min with 0.3% milk powder in TBS and incubated with the secondary antibody (goat anti-rabbit antibody conjugated to alkaline phosphatase), diluted 1:3000, in 10% milk powder, 0.3% Tween-20, 0.5% NGS in TBS for 2 hours at room temperature. The membrane was rinsed twice with 0.3% milk powder in TBS and reacted for alkaline phosphatase activity with the substrates 5-bromo-4 chloro-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) according to the method described in the Biorad technical sheet for 5-30 min. The bands appeared purple.

RESULTS

Light Microscopy and Biochemical Characteristics

We examined the biochemical characteristics of NADPH-diaphorase activity in rat striatal tissue sections. Except for the nucleus, NADPH-diaphorase neurons stained in their entirety. During the reaction, staining was first apparent in the cell body and then in the major processes and neuropil. Thus any relative decrease in activity resulted in less staining first of the neuropil, then of the major processes, and eventually the soma became clear. During the 60 min reaction, the cell cytosol quickly became saturated, and, therefore, any decreases in the cytosol were evident only after considerable inhibition of the reaction. Sections pre-treated and reacted under the same conditions always gave reproducible results.

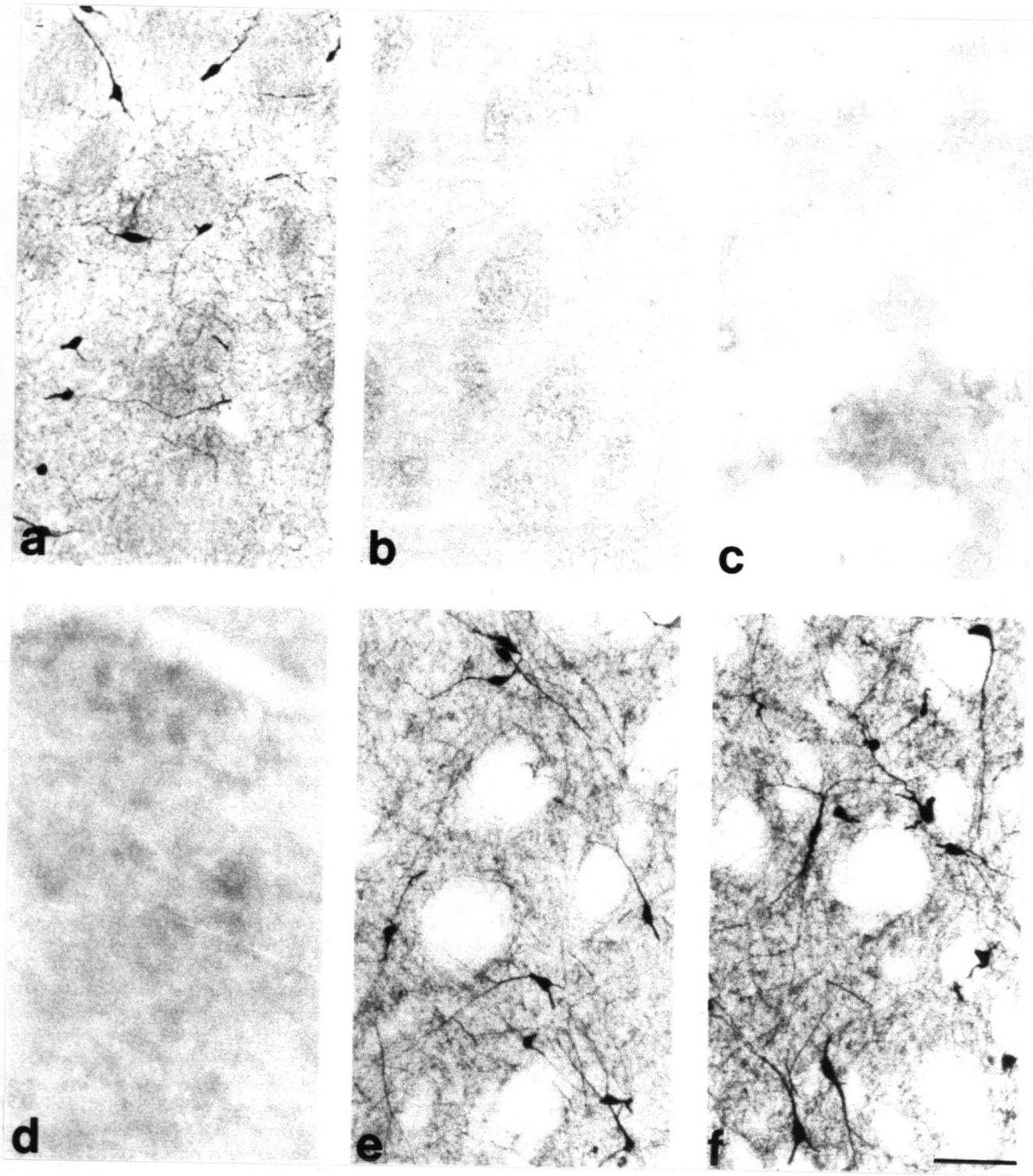
Pre-heating the sections to 55°C for 5 min had no apparent effect, whereas pre-heating to 65°C produced a slight reduction in NADPH-diaphorase activity (Figure 3a). Activity could be completely abolished by pre-heating the sections to 77°C for 5 min (Figure 3b). After treatment with trypsin (Figure 3c) or proteinase K, NADPH-diaphorase activity was drastically decreased. One could still observe very light staining in some neurons. To demonstrate that activity was abolished due to proteolytic activity, trypsin was pretreated with PMSF, a serine protease inhibitor. Subsequently, NADPH-diaphorase activity was not decreased in the section (not shown).

Pre-treatment of sections with the sulfhydryl inhibitors DTNB, NEM, and PMB resulted in strong inhibition of NADPH-diaphorase activity (Figure 3d). Treatment of sections with the metal chelators EDTA, CDTA, and with cyanide did not inhibit the reaction (Figures 3e and 3f). Attempts to inhibit NADPH-diaphorase activity by selective removal of flavin groups were unsuccessful.

Figure 3

NADPH-diaphorase histochemistry of rat striatal sections after pre-incubation at 65°C (**a**) or 77°C (**b**) for 5 min, or with 0.1% trypsin for 15 min at room temperature (**c**), or for 90 min at room temperature with 5 mM p-mercuribenzoate (PMB) (**d**), or overnight at 4°C with 0.1 M potassium cyanide (**e**) or CDTA (**f**), before incubation. Bar=100μm.

Figure 3



Those deflavination treatments that did inhibit activity in the section involved non-specific protein denaturing conditions such as acidic pH or chaotropic salts. Addition of exogenous flavins to these sections to reconstitute NADPH-diaphorase activity were without effect.

Addition of excess superoxide dismutase and/or catalase to the histochemical reaction had no effect on NADPH-diaphorase staining.

Incubating tissue sections overnight in buffers of various pH had little effect from pH=5-10. Activity was reduced at pH=3 and was abolished at pH=11, whereas pH=4 resulted in only small decrease (Figures 4a-4c). It should be noted that the normal NADPH-diaphorase histochemical reaction also stains blood vessels in the brain. In comparison with the reaction within neurons, the staining of the blood vessels was very weak and required long incubation times. This staining of blood vessels decreased when the pH of the histochemical reaction solution was lowered from pH=10 to pH=6 (Figure 4d). Staining of the neurons, however, did not decrease noticeably within this pH range. Below pH=6, NADPH degraded non-enzymatically too rapidly to give satisfactory staining. Above pH=10, tetrazoliums were reduced non-enzymatically by sulfhydryls in the tissue section.

Addition of 0.1 mM dicumarol in the reaction solution was without effect (Figure 4e), whereas menadione enhanced the staining of structures, including blood vessels, that were completely different from those stained in the normal reaction (Figure 4f).

The following tetrazolium analogs were reduced by the diaphorase in the presence of β-NADPH: TNBT, NBT, MTT (with and without cobalt), INT, BPST, and TC-NBT. If BT, NT, or TT (with or without cobalt) were employed under the usual aerobic conditions, there was no staining (Figure 5b). When the tissue sections were allowed to react under anaerobic conditions, all the

Figure 4

NADPH-diaphorase histochemistry of rat striatal sections. Sections were pre-incubated at pH=3 (**a**), pH=7 (**b**), or pH=11 (**c**) overnight at 4°C and then reacted at the usual pH=8. Section (**d**) was incubated directly in the reaction mixture buffered to pH=6. The effects of 1 hr pre-treatment and reaction in the presence of 0.1 mM dicumarol (**e**) or direct incubation in the presence of 5 mM menadione (**f**) are shown. Bar=100 μm.

Figure 4

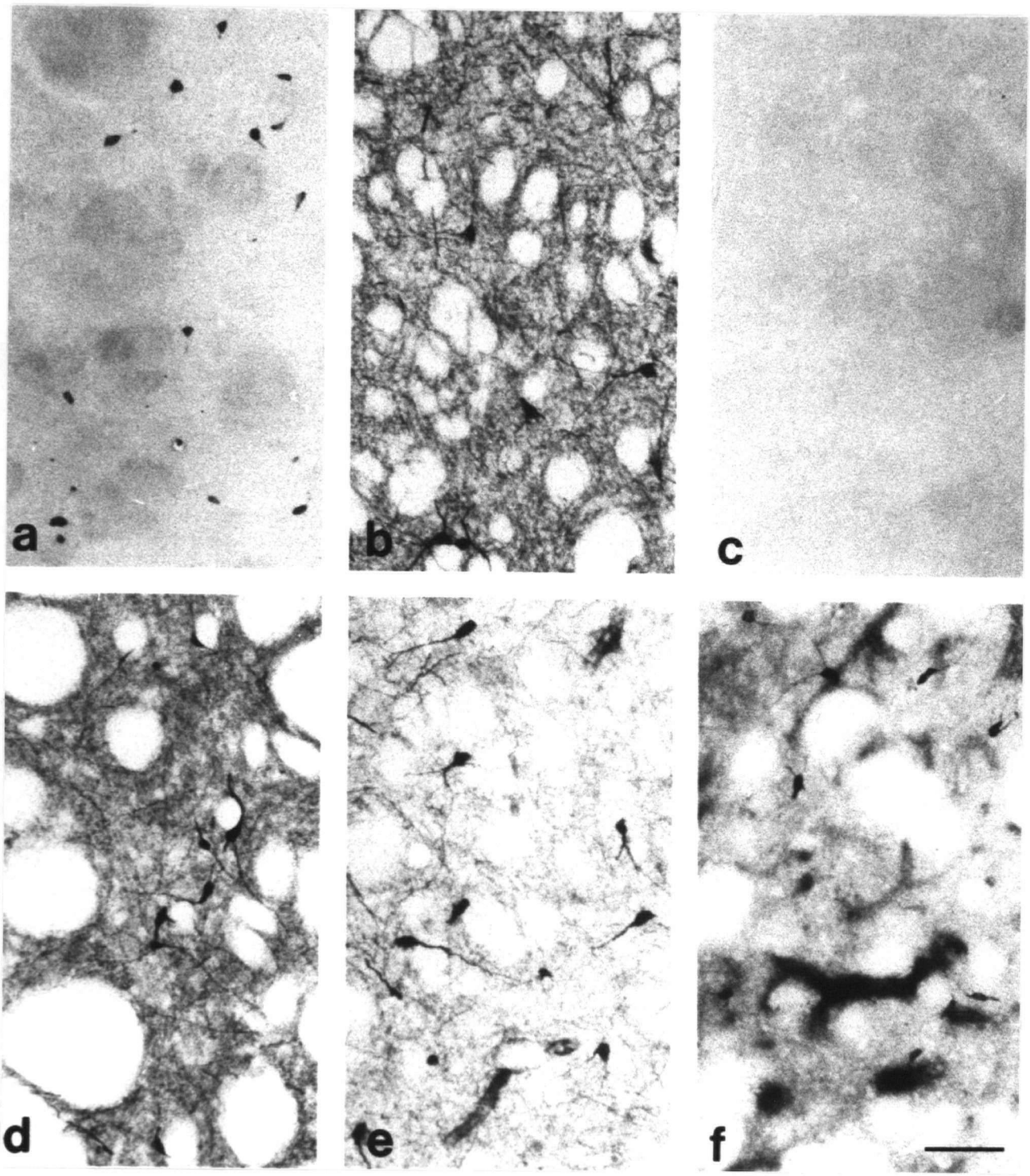
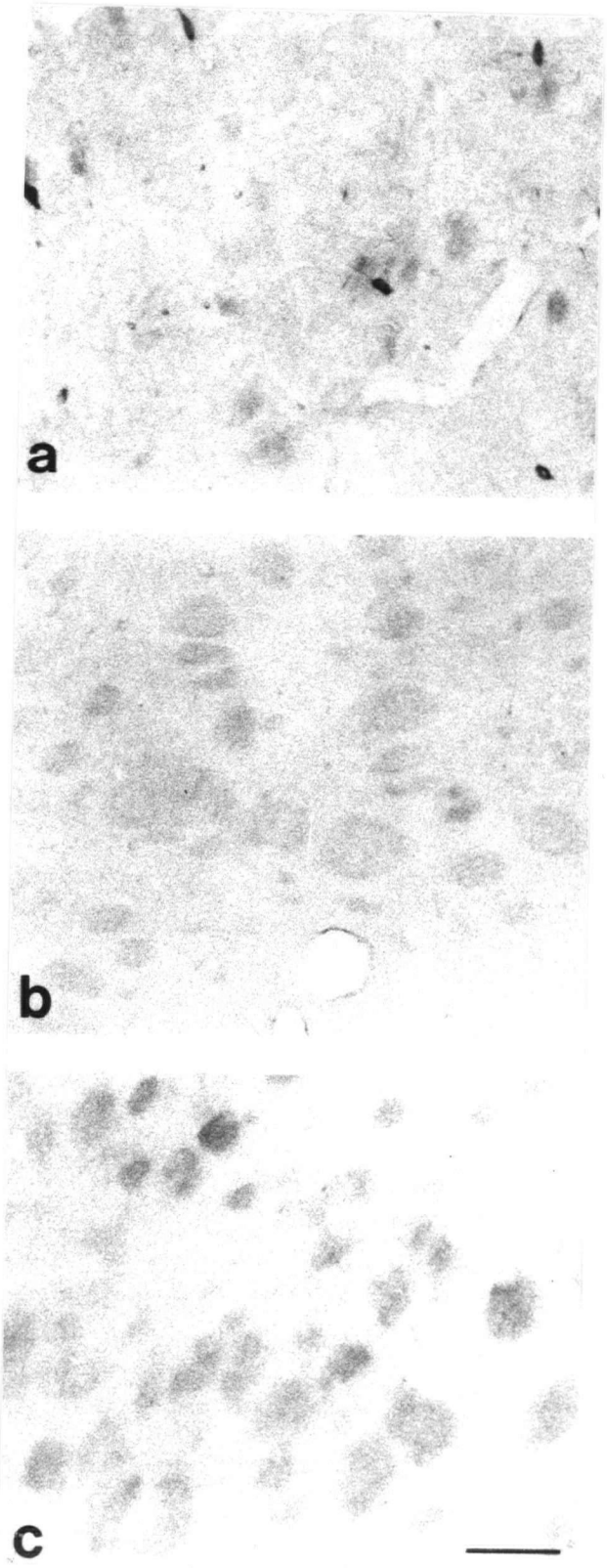


Figure 5

NADPH-diaphorase histochemistry demonstrated with β-NADPH and neotetrazolium under anaerobic (**a**) and aerobic (**b**) conditions, or with β-NADPH and triphenyl tetrazolium under anaerobic conditions (**c**). Bar=100 μm.

Figure 5



tetrazoliums except TT resulted in specific staining of the neurons (Figures 5a and 5c).

Substitution of β-NADH for β-NADPH resulted in a completely different staining pattern. The entire section, except for the white matter, was strongly stained. No particular structure stained more than another, and none stained as strongly as the neurons stained with β-NADPH (Figures 6a and 6b). There were clearly NADPH-diaphorase-containing neurons lying within the corpus callosum (Figure 6a). When β-NADH was employed there was absolutely no staining of such neurons in this region (Figure 6b).

Both α-NADPH and deamino-NADPH stained the same population of striatal neurons as β-NADPH. Replacement with the isomer α-NADPH resulted in a slight decrease in staining intensity compared with β-NADPH, but staining of the blood vessels was almost absent (Figure 6c). Deamino-NADPH stained neurons as well as β-NADPH with very weak staining of blood vessels (Figure 6d). Replacing β-NADPH with the analogue 3'-PO_4-NADPH resulted in very poor staining of the neurons (Figure 6e). Indeed, the blood vessels were now stained darker than the neurons.

Electron Microscopy

Ultrastructural localization of the NADPH-diaphorase reaction product varied depending on the tetrazolium employed. The formazan product of NBT formed small granules dispersed throughout the cytoplasm but not associated with any organelles (Figure 7a). The reaction product of BSPT was found associated with various organelles but not in the cytoplasm (Figures 7b-7d). The endoplasmic reticulum and the nuclear envelope were stained, but not the Golgi apparatus or the plasma membrane. Organelles in some nerve terminals, which may be secretory vessels or lysosomes, also had reaction

Figure 6

Diaphorase histochemistry in sections of rat striatum demonstrated with β-NADPH (**a**), β-NADH (**b**), α-NADPH (**c**), deamino-NADPH (**d**), and 3'-phosphate-NADPH (**e**). Note the presence of positive neurons in the corpus callosum in **a** and the absence of such cells in **b**. Bar=100 μm.

Figure 6

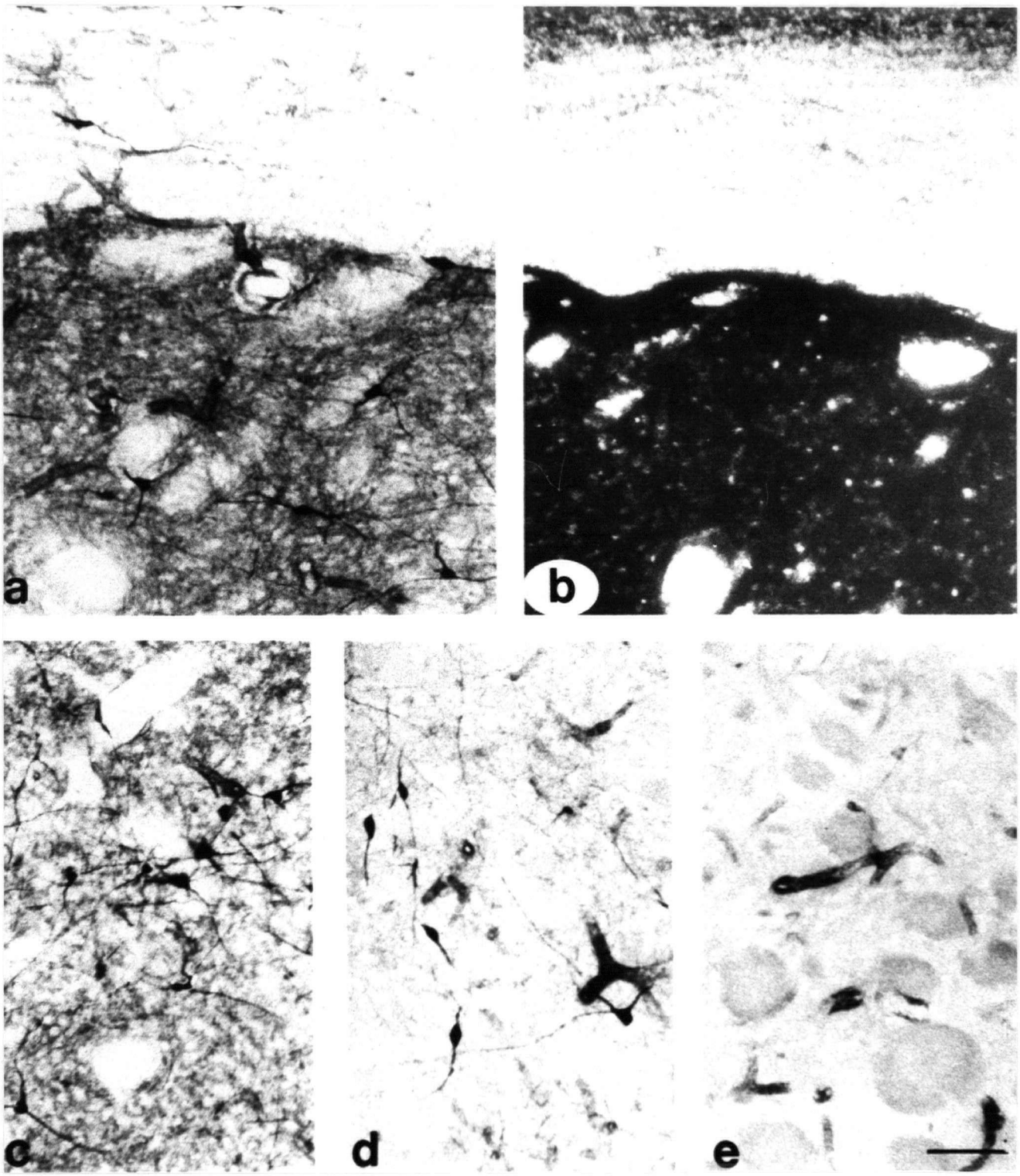
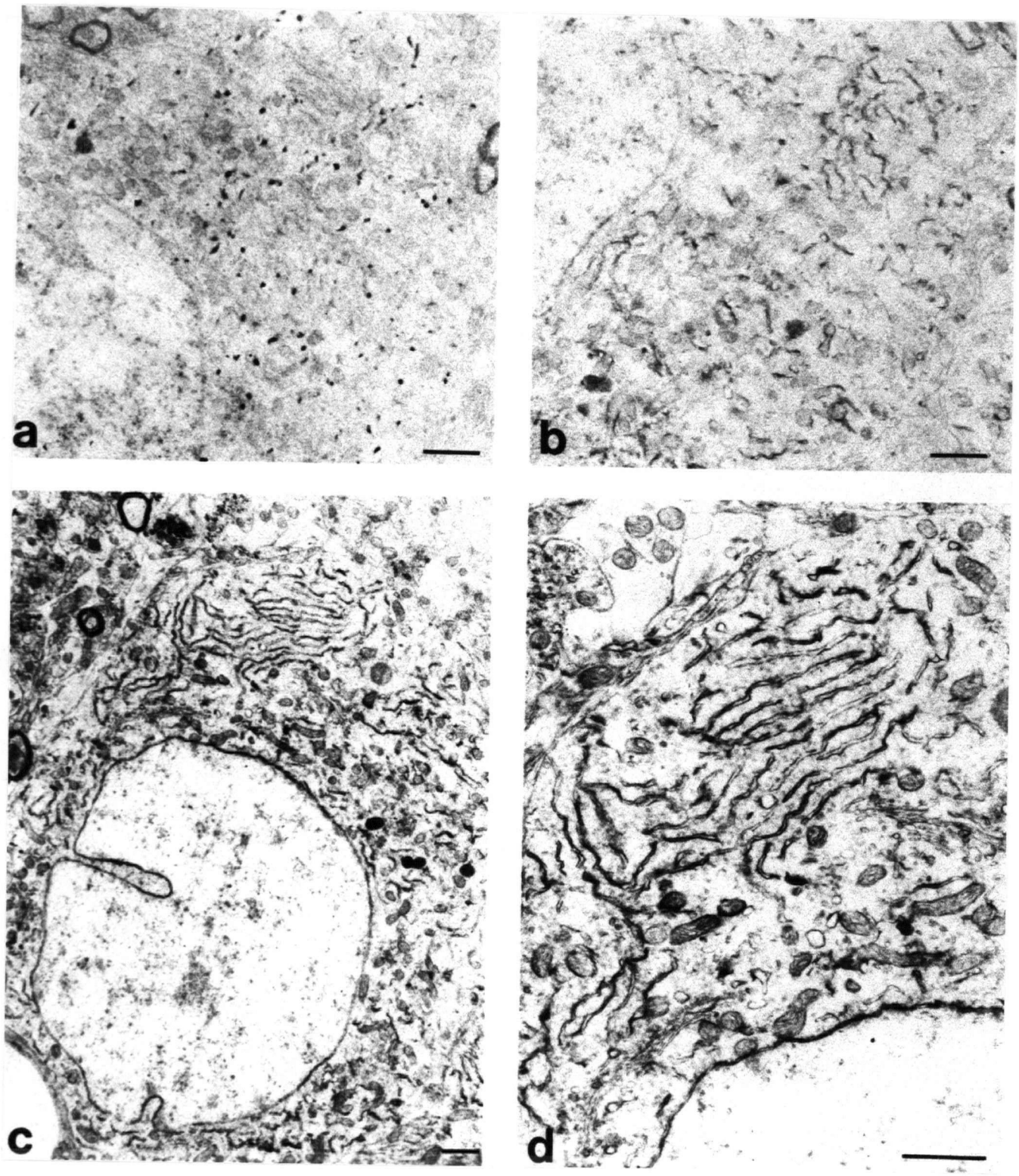


Figure 7

Electron micrographs of NADPH-diaphorase activity demonstrated with nitroblue tetrazolium (**a**) or benzothiazolyl styryl pthalhydrazidyl tetrazolium (BSPT) (**b**,**c**,**d**). **d** is an enlargement of a portion of **c**. Sections **c** and **d** are counterstained with lead citrate. Bars=1 μm.

Figure 7



product on their membranes. This appeared to be associated with only a minority of these organelles in a given terminal. Some mitochondria also had reaction product on their outer membranes.

NADPH-diaphorase Activity in Non-rat Species

We investigated the NADPH-diaphorase histochemical reaction in a variety of animals. We detected NADPH-diaphorase activity in the basal ganglia and other brain regions of the leopard frog and the Northeastern snapping turtle. Activity could not be detected in trout brain or the nervous system of the hagfish. Various brain regions of cats, guinea pigs, and cows strongly stained for NADPH-diaphorase activity.

HAN Antiserum Specifically Binds NADPH-diaphorase

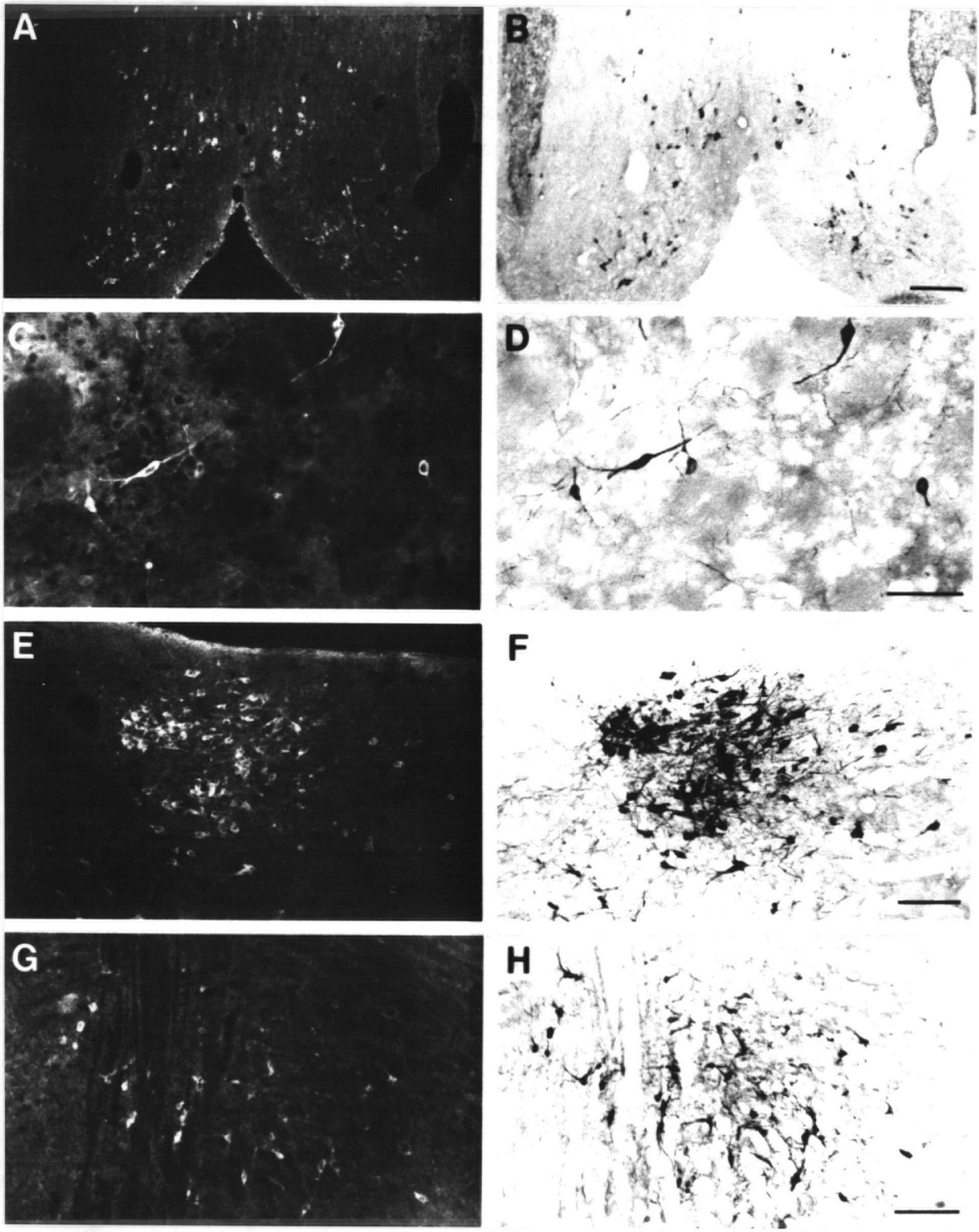
The HAN antiserum immunostains neurons with a distribution similar to that observed with NADPH-diaphorase histochemistry (Michaels and Knigge, 1989). We extended this finding by performing double-labelling experiments. All neurons labelled with the HAN antiserum were also NADPH-diaphorase positive (Figure 8).

There are a number of NADPH-diaphorases in a brain homogenate (see introduction). Therefore, we employed the HAN antiserum with immunoprecipitation to identify the HAN-immunoreactive NADPH-diaphorase activity responsible for the histochemically defined neuronal NADPH-diaphorase. The protein G-Sepharose beads used to pellet the antibody-antigen complex during immunoprecipitation became dark blue from the formation of formazan from the diaphorase reaction. There was little staining of the beads when normal rabbit serum was used instead of HAN antiserum. Since the HAN antiserum could also selectively immunoprecipitate NADPH-diaphorase

Figure 8

Double staining of the rat brain using the indirect immunofluorescence procedure with the HAN antiserum (**A**, **C**, **E**, and **G**) followed by the NADPH-diaphorase histochemical method on the same sections (**B**, **D**, **F**, and **H**). The two techniques label identical neuronal populations throughout the brain, including the magnocellular basal forebrain (**A** and **B**), the striatum (**C** and **D**), the laterodorsal tegmental nucleus (**E** and **F**), and the pedunculopontine tegmental nucleus (**G** and **H**).

Figure 8



activity, this demonstrated that the antigen bound by HAN antiserum was not only found in NADPH-diaphorase neurons but also had NADPH-diaphorase activity. Therefore, we assumed HAN antiserum was specific for the neuronal NADPH-diaphorase seen histochemically.

Copurification

Since NADPH-diaphorase is an NADPH-dependent enzyme, we attempted a purification protocol similar to that used for other NADPH-dependent enzymes, such as NO synthase (Bredt and Snyder 1990). NADPH-diaphorase activity could be detected and immunoprecipitated from the soluble supernatant after centrifugation of a rat brain homogenate. The supernatent was applied to a 2',5'-ADP agarose column and washed with 10 mM Tris-Cl, pH=7.4, 1mM EDTA. Although some NADPH-diaphorase activity was present in the flow-through and wash fractions, no activity could be immunoprecipitated with the HAN antiserum. Immunoreactive NADPH-diaphorase activity was then eluted with 1 M NaCl. When the eluted fractions were transferred from an SDS-PAGE gel to a Western blot and incubated with HAN antiserum, one band with a molecular weight of 150 kD was stained. This molecular weight was similar to NO synthase, another NADPH-dependent enzyme that was previously shown to adsorb to 2',5'-ADP agarose.

The possibility that neuronal NADPH-diaphorase and NO synthase were the same protein was investigated by attempting to copurify both enzyme activities. We found purification to be more efficient by reversing the order of chromatographic steps used by Bredt and Snyder (1990), ie. 2',5'-ADP agarose affinity chromatography followed by anion exchange chromatography. NO synthase and NADPH-diaphorase activities copurified during both affinity and anion exchange chromatography (Figure 9). The

Figure 9

Copurification of nitric oxide (NO) synthase and NADPH-diaphorase activities from rat brain. Fractions containing NO synthase and NADPH-diaphorase activities eluted from an adenosine 2',5'-diphosphate-agarose affinity column with 10 mM β-NADPH (*) (**a**) were subsequently run on DEAE anion-exchange HPLC and eluted with a 0-0.4 M NaCl gradient (**b**).

Figure 9a

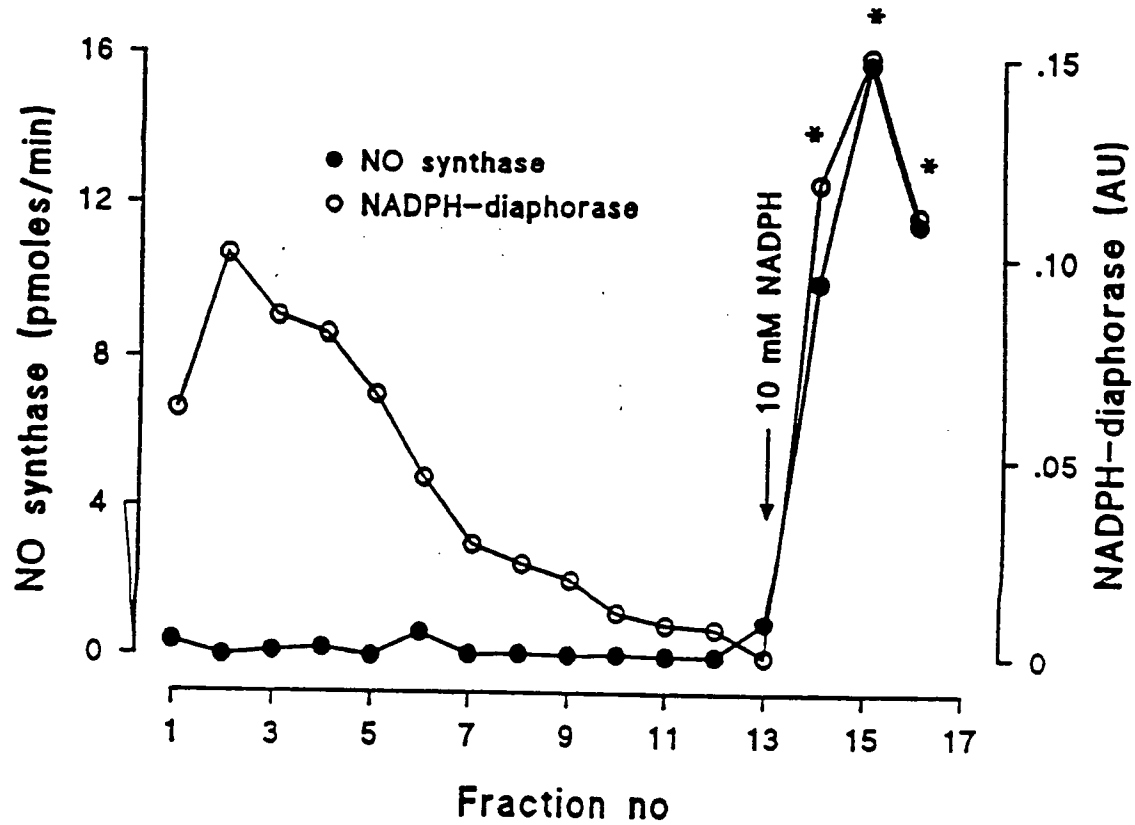


Figure 9b

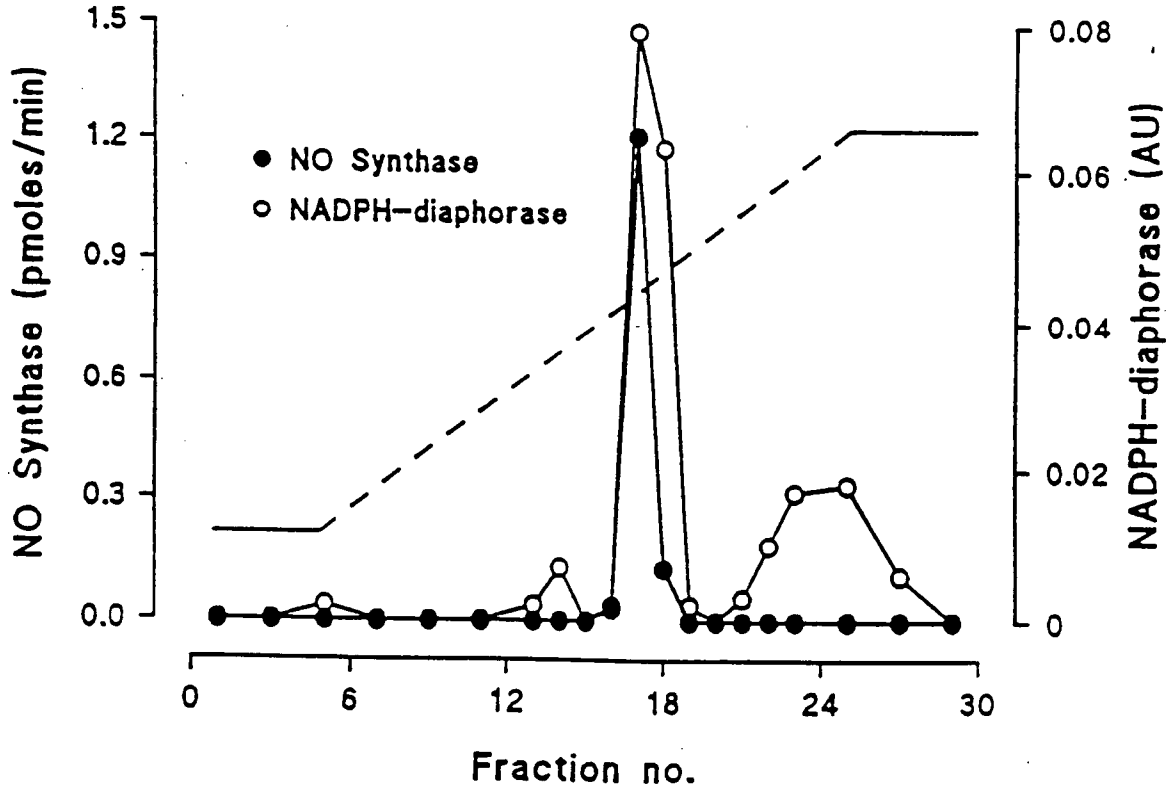
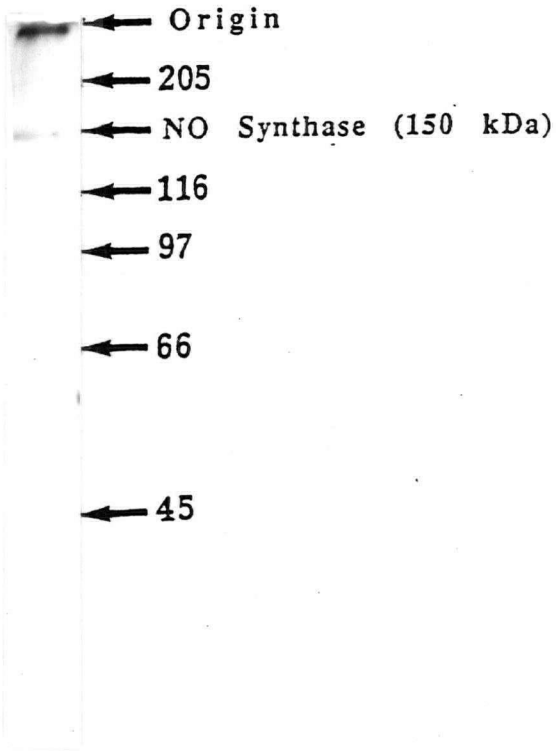


Figure 10

SDS-PAGE electrophoresis of the fraction containing NO synthase and NADPH-diaphorase activity after affinity chromatography and anion-exchange HPLC. There is only one major silver-stained band (arrow) with an apparent molecular weight of 150 kD.

Figure 10



peak fraction of NADPH-diaphorase and NO synthase activity eluted from anion-exchange chromatography resulted in a single silver-stained band with an apparent molecular weight of 150 kD on SDS-PAGE (Figure 10).

Western Blot

Immunostaining Western blots of the unpurified whole homogenate or the 20000xg supernatant with HAN antiserum resulted in faint, almost undetectable staining of a 150 kD band. When immunoblot staining was performed on the partially purified fractions which contained both NO synthase and NADPH-diaphorase activity from either the anion-exchange or affinity chromatography steps, a strongly stained band appeared which corresponded to a molecular weight of 150 kD (Figure 11).

Immunoprecipitation

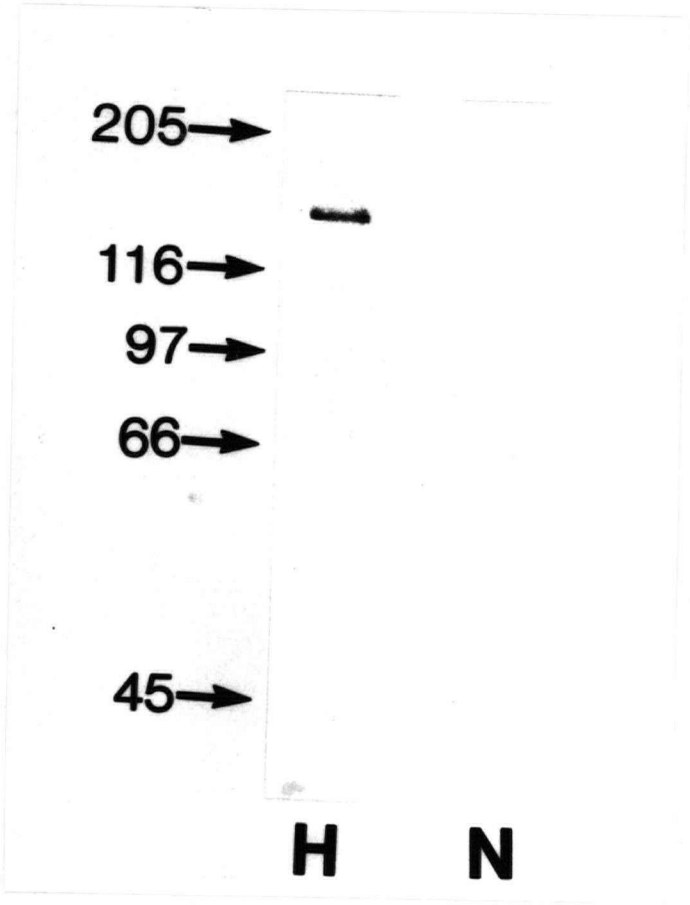
Both NO synthase and NADPH-diaphorase activities could be immunoprecipitated from crude supernatants and purified fractions with the HAN antiserum but not with non-immune normal rabbit serum. As mentioned above, when the pellet immunoprecipitated with HAN antiserum was tested for NADPH-diaphorase activity, the beads turned a dark blue color due to the formazan product. The formazan could not be completely solubilized so the reaction product was not measured spectrophotometrically as was done with the homogenate samples.

NO synthase activity was assayed from the supernatants and pellets of immunoprecipitation experiments. To assay the amount of citrulline formed by NO synthase activity, the product, [^{3}H]-citrulline had to be separated from the substrate, [^{3}H]-arginine, by ion-exchange chromatography. Arginine

Figure 11

Immunoblot of the fractions containing NO synthase and NADPH-diaphorase activities after affinity chromatography. A single protein with an apparent molecular weight of 150 kDa is recognized by the HAN antiserum (**H**), but not by normal rabbit serum (**N**).

Figure 11



adsorbs to the column while citrulline appears in the flow-through. Unfortunately, we found significant levels of background cpms in the flow-through from NO synthase assays where no test sample was added. These background cpms appeared to be due to the remaining radioactive contaminant in the [^{3}H]-arginine stock that was left after initial purification of the radioisotope. To determine the cpms due to [^{3}H]-citrulline formed by NO synthase, background cpms were subtracted from the total cpms of the flow-through of test sample assays.

The HAN antiserum immunoprecipitated NO synthase activity from the crude supernatant. The total number of cpms that resulted from 30 min assays of HAN-immunoprecipitated pellets were 170±32 cpms (n=3) above the background of 1154±14 (n=3). Counting efficiency was 11-15%. This increase over background was significant to $p<0.005$ employing the two-tailed Student t-test (Figure 12). 170 cpms corresponded to 2.2 pmoles of [^{3}H]-citrulline formed during the 30 minute assay. Immunoprecipitation with normal rabbit serum (NRS) showed no significant increase in total cpms (7±13) over background ($p>0.5$). Therefore, immunoprecipitation with HAN antiserum was specific for NO synthase activity, as well as for NADPH-diaphorase activity.

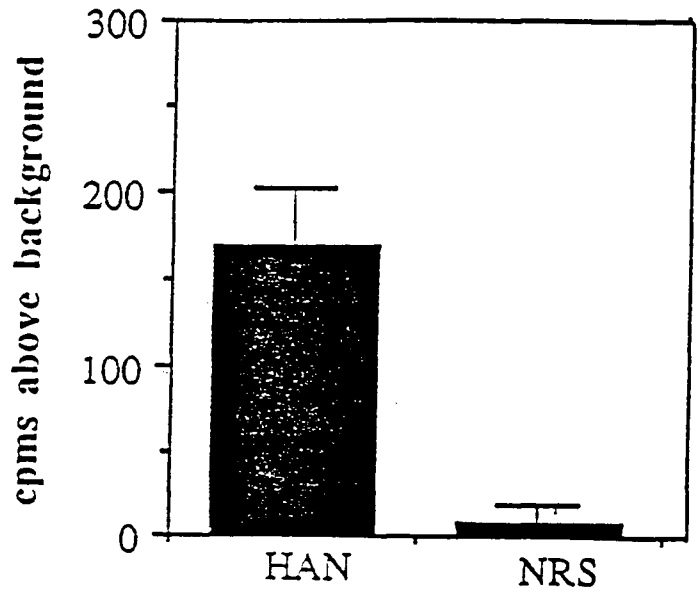
Immunoprecipitation of NO synthase activity with HAN antiserum was performed three times and all gave similar results. The decreases in NO synthase and NADPH-diaphorase activities in the supernatant after immunoprecipitation were small, approximately 2%.

Figure 12

Specific immunoprecipitation of NO synthase activity with **HAN** antiserum but not with normal rabbit serum (**NRS**). Immunoprecipitated activity was assayed by measuring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline. Results are expressed as the increase in cpms from the test assay over those from background (see results section). Significance was calculated by comparing the increase in cpms due to [^{3}H]-citrulline in the test assay with the cpms from background. The increase in cpms due to immunopreciptation with **HAN** antiserum was significant to $p<0.005$ using the two-tailed Student t-test. The increase due to **NRS** was not significant ($p>0.5$).

Figure 12

Immunoprecipitation of NO Synthase Activity: HAN vs. NRS



NBT Inhibition

If NADPH-diaphorase is identical to NO synthase then nitroblue tetrazolium (NBT), the substrate for the NADPH-diaphorase reaction should beable to compete with arginine, the substrate of NO synthase, for electrons from NADPH. We found the Km for arginine to be 2.9 μM for NO synthase from whole rat brain, similar to previous reports for NO synthase from the forebrain and cerebellum (Bredt and Snyder 1989, 1990, Knowles et al 1990). NBT inhibited NO synthase activity competitively with respect to arginine, with a Ki of 11 μM (Figure 13).

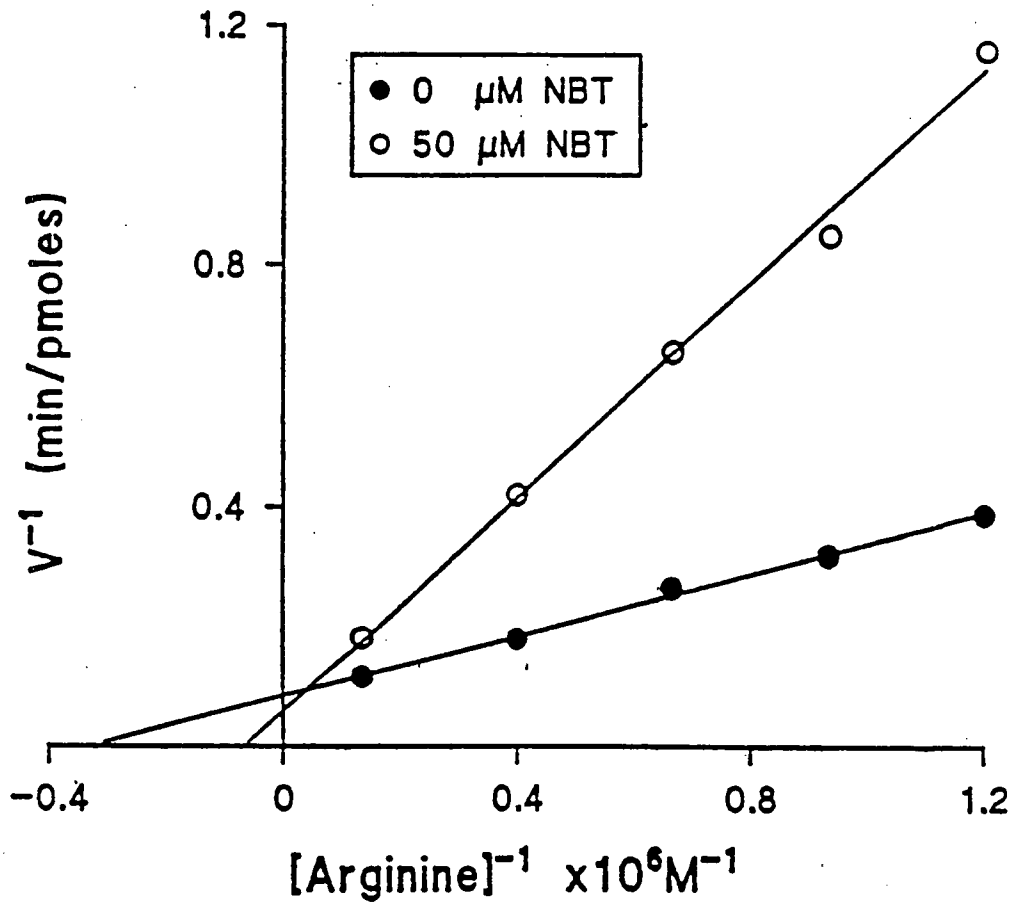
Citrulline Immunohistochemistry

Citrulline is the by-product of the NO synthase reaction from arginine (Moncada et al. 1989). The urea cycle is the only other known chemical pathway that involves citrulline. The complete urea cycle is not found in the brain so we reasoned that citrulline should be found only in those cells that contain NO synthase activity. We developed an antibody to citrulline. Since citrulline alone is far too small to be immunogenic we conjugated the amino acid to bovine serum albumin (BSA). After repeated injections of rabbits with the citrulline-BSA conjugate the antiserum was tested for antigenic specificity. Immunoperoxidase staining of nitrocellulose membranes on which various amino acid-brain protein conjugates had been blotted resulted in staining of only the citrulline conjugate (Figure 14). The antibody did not cross-react with lysine, glutamine, arginine, or argininosuccinate conjugated to whole brain homogenate, nor with brain proteins alone. Preabsorption of the diluted antibody overnight with 10 nmoles/ml of citrulline-BSA conjugate abolished cell staining. Preabsorption with 100 nmoles/ml free citrulline, or with BSA alone, did not block staining. Thus, the antiserum specifically

Figure 13

Inhibition by NBT of NO synthase activity in a crude supernatant (see results) from whole rat brain. The activity (velocity, V) was measured at various substrate concentrations with or without added inhibitor.

Figure 13


1.2
0.8
0.4
0 μM NBT
50 μM NBT
V⁻¹ (min/pmoles)
−0.4
0
0.4
0.8
1.2
[Arginine]⁻¹ x10⁶M⁻¹

recognizes aldehyde conjugates of citrulline and protein.

Immunoperoxidase staining with the highly diluted citrulline antibody (1:20,000) revealed numerous citrulline-immunoreactive cell groups, particularly in the forebrain. Strong citrulline immunoreactivity was displayed by numerous cells scattered throughout the striatum (Figure 15a,b), nucleus accumbens and olfactory tubercle. Occasionally, isolated cells embedded within the corpus callosum displayed citrulline-immunoreactivity. A small scattered population of positive neurons was also present in the piriform cortex and a few positive cells were found in the endopiriform nucleus. Within the hypothalamus, well defined clusters of positive neurons were observed in the paraventricular (Figure 15c,d) and supraoptic (Figure 15e) nuclei. All of these citrulline-immunoreactive forebrain neurons displayed intensely stained soma as well as staining of larger processes.

Several citrulline-immunoreactive cell groups were also observed in the hindbrain. A few small, strongly stained cells were present in the interpeduncular nucleus (Figure 15f). A small number of isolated cells were observed scattered in the external layers of the inferior (Figure 15g) and superior colliculi. A moderately dense cluster was observed within the dorsal raphe nucleus (Figure 15h), and additional groups of cells were present in the laterodorsal (Figure 15h,i) and pedunculopontine tegmental nuclei. Most of the positive cells in the hindbrain were noticeably less intensely stained than the forebrain neurons. There were no citrulline-immunoreactive cells in the cerebellum.

Since all the above cell groups have previously been shown to be selectively stained by NADPH-diaphorase histochemistry (Vincent 1986), double staining experiments were undertaken in which citrulline was detected using indirect immunofluorescence, the sections photographed and then

Figure 14

Specificity of whole brain-amino acid conjugates after ABC-immunoperoxidase staining. The various amino acids fixed to whole brain homogenate by glutaraldehyde are: lysine (**LYS**), citrulline (**CIT**), arginine (**ARG**), glutamine (**GLN**), and argininosuccinate (**ASA**). The positive staining of the citrulline conjugates and the absence of cross reaction with the other conjugates indicates the specific affinity of the antibody for aldehyde conjugates of citrulline and protein.

Figure 14

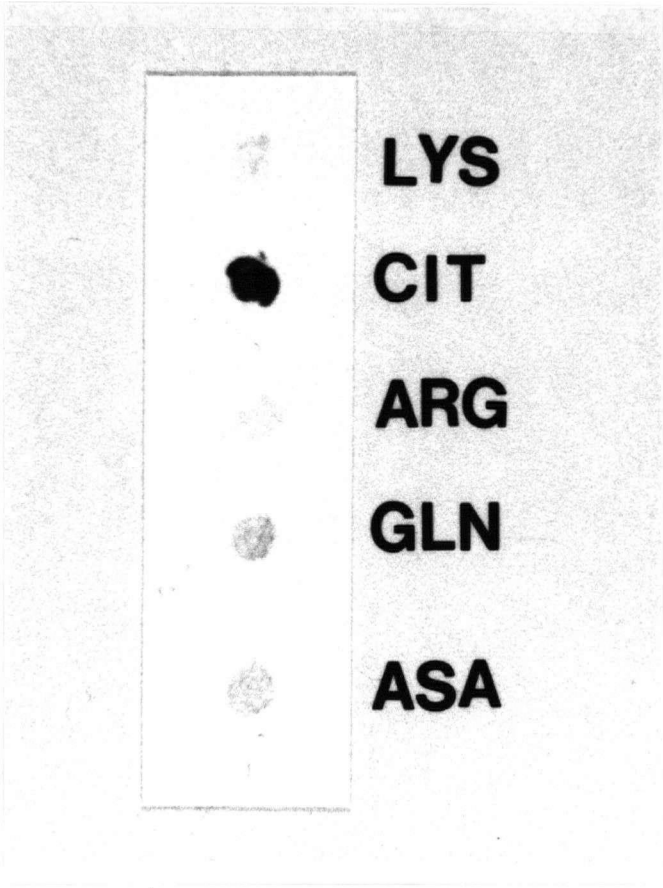
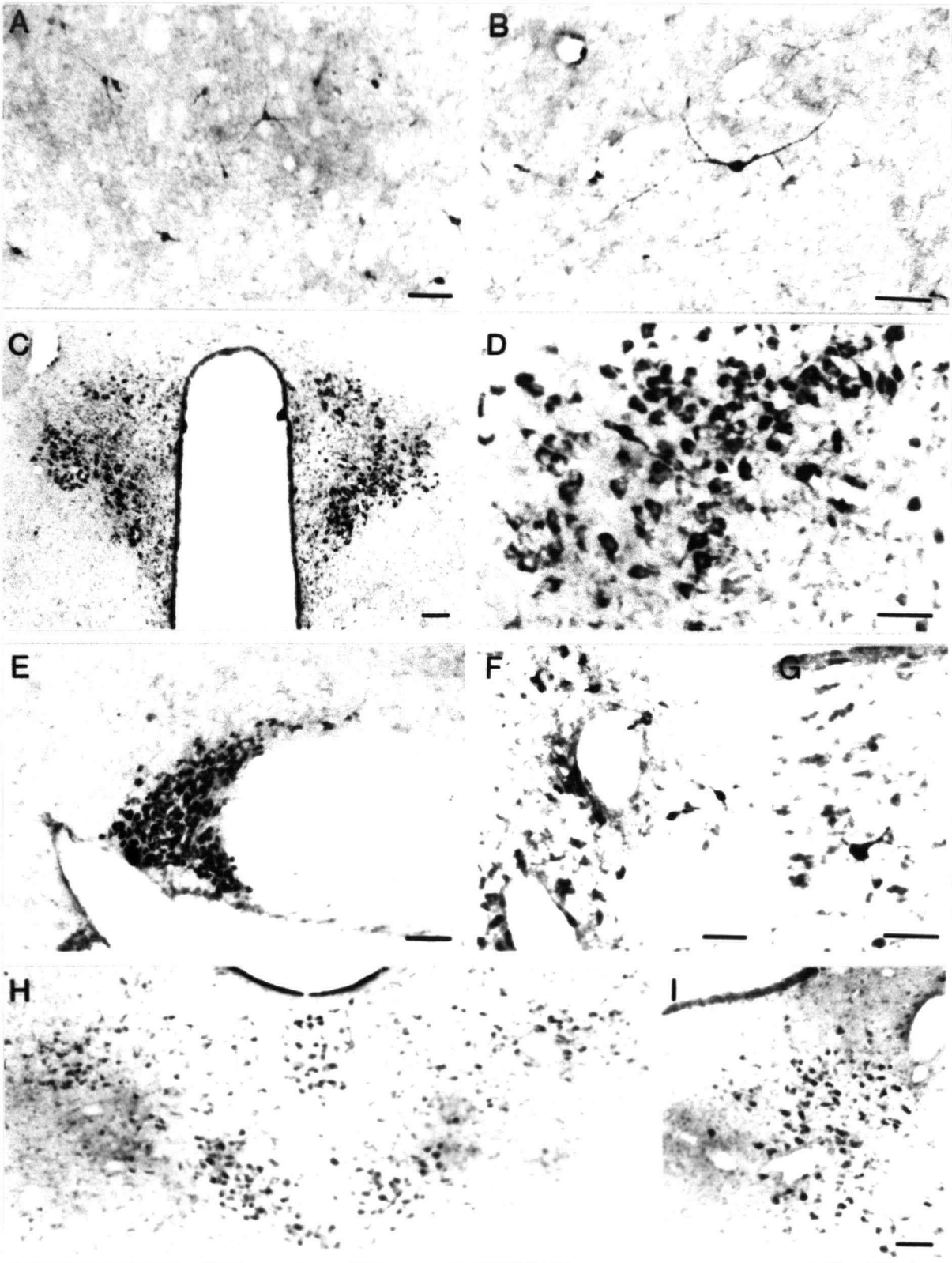


Figure 15

Sections stained for citrulline immunoreactivity by the ABC-immunoperoxidase method. Citrulline positive cells exist in the striatum (**A**, **B**), the paraventricular nucleus of the hypothalamus (**C**, **D**), the supraoptic nucleus (**E**), the interpeduncular nucleus (**F**), inferior colliculus (**G**), dorsal raphe nucleus (DR) (**H**), and the laterodorsal tegmental nucleus (TLD) (**H**, **I**). Scale bars indicate 100 (**A**, **E**, **H**, **I**) and 50 (**B**, **C**, **D**, **F**, **G**) microns.

Figure 15

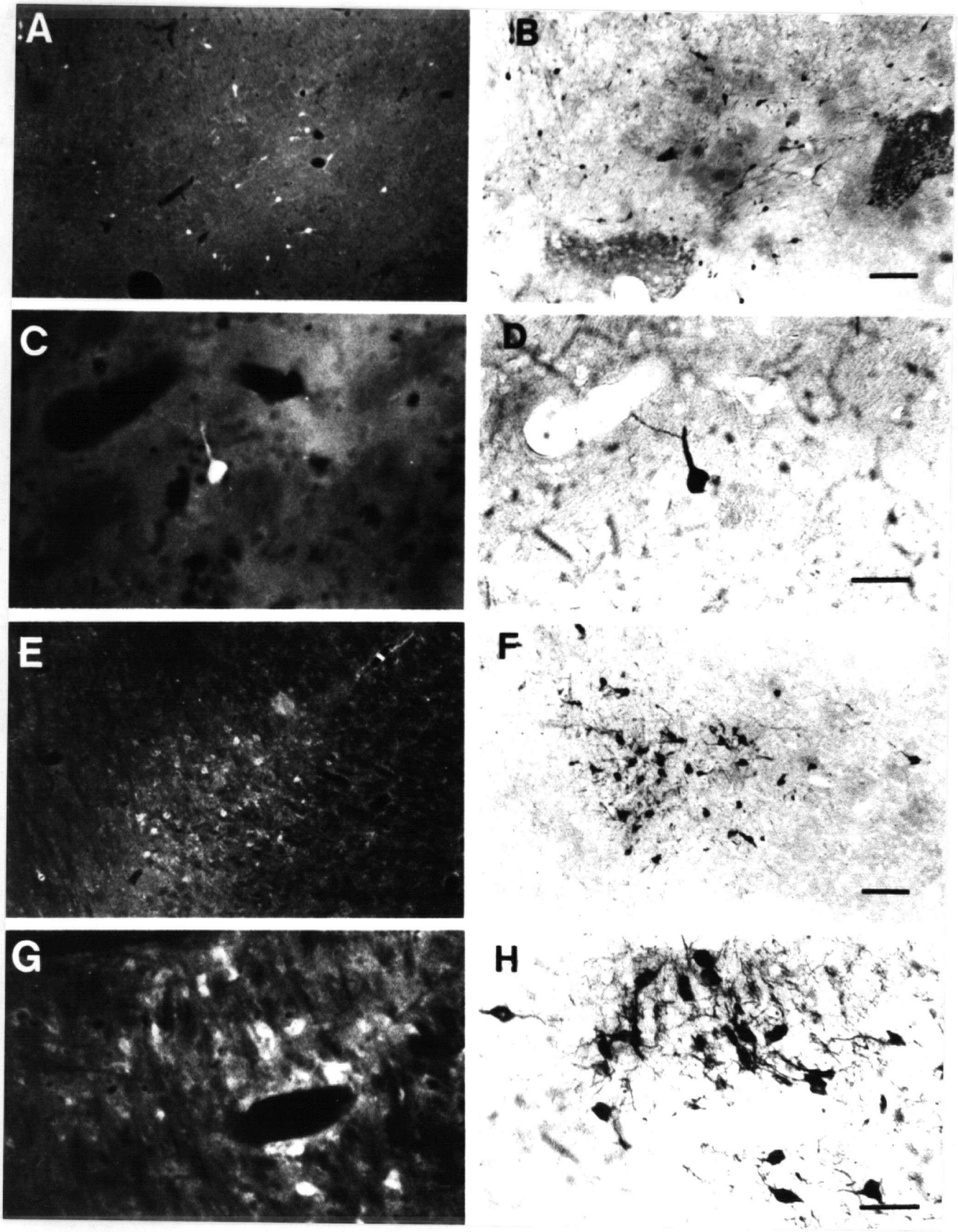


restained for NADPH-diaphorase activity. All citrulline positive cells detected were also NADPH-diaphorase positive (Figure 16). However, many more cells were labelled by NADPH-diaphorase histochemistry than by citrulline immunostaining. Approximately 60% of striatal NADPH-diaphorase neurons contained citrulline immunoreactivity. No cases of citrulline positive and NADPH-diaphorase negative cells were observed.

Figure 16

Citrulline immunofluorescence in the striatum (**A**, **C**) and the pedunculopontine tegmental nucleus (**E**, **G**) followed by NADPH-diaphorase histochemistry (**B**, **D**, **F**, **H**). Note that all of the citrulline-immunoreactive neurons also contain NADPH-diaphorase activity but many NADPH-diaphorase positive neurons do not display citrulline immunoreactivity. Scale bars represent 100 (**A**, **B**, **E**, **F**), 50 (**G**, **H**) or 30 (**C**, **D**) microns.

Figure 16



DISCUSSION

There are four parts to the discussion: 1) A comparison of the biochemical characteristics of the histochemical reaction with that of known brain diaphorase enzymes. 2) A comparison of the biochemistry and anatomy of neuronal NADPH-diaphorase with that of nitric oxide synthase. 3) A review of nitric oxide synthase and other enzymes necessary for its function in the brain. 4) Speculations on the function of neuronal NADPH-diaphorase and nitric oxide synthase in the brain.

I have left the review of nitric oxide synthase for the discussion because identity of this enzyme with neuronal NADPH-diaphorase activity was an unexpected result of our findings and not an initial hypothesis.

Biochemical Characteristics of Neuronal NADPH-diaphorase Derived from Histochemical Investigation

Although it had not been clearly demonstrated previously, the neuronal NADPH-diaphorase reaction had been assumed to be enzyme mediated. The abolishment of the reaction with proteases, heat denaturation, and acid or alkaline pre-treatment shown in this study indicates the NADPH-diaphorase reaction is indeed enzyme catalyzed. Examination of various co-factor analogs demonstrated evidence of structural specificity, again indicative of an enzyme-mediated reaction.

When various tetrazolium dyes were examined histochemically the reaction showed little specificity. In the presence of oxygen, all tetrazoles with a redox potential equal to or more positive than that estimated for MTT (-0.110 V) were reduced (Altman, 1976a) (see Figure 17a). Under anaerobic conditions,

Figure 17

a) Redox potentials for tetrazoliums and other electron acceptors are shown. Tetrazoliums and other molecules have redox potentials which correspond to their relative abilities to attract electrons. Electrons will travel from molecules with lower redox potentials to molecules with higher redox potentials. For example, triphenyl tetrazolium (-0.490) has a lower redox potential than that for NADPH (-0.320) so electrons will not transfer from NADPH to triphenyl tetrazolium under normal circumstances.

b) Electrons can be passed to tetrazoliums one at a time. One electron reduced tetrazoliums may then pass these single electrons to oxygen if the redox potential of oxygen is higher than that of the singly reduced tetrazolium. This may explain the oxygen sensitivity of the reduction of neotetrazolium and blue tetrazolium by the NADPH-diaphorase histochemical reaction.

Figure 17a

Electron Acceptor	Redox Potential
Triphenyl Tetrazolium	-0.490
Neotetrazolium	-0.170
Blue Tetrazolium	-0.160
Monotetrazolium	-0.110
Iodonitrotetrazolium	-0.090
Nitroblue Tetrazolium	-0.050
Tetranitroblue Tetrazolium	-0.050
Dichlorophenolindophenol	+0.175
Cytochrome c	+0.280

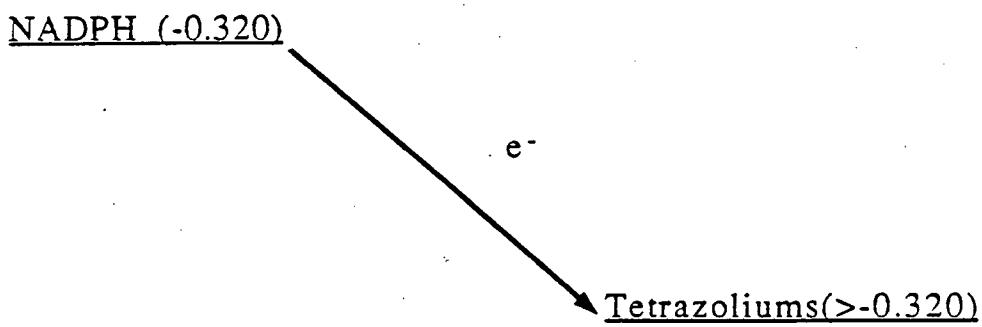
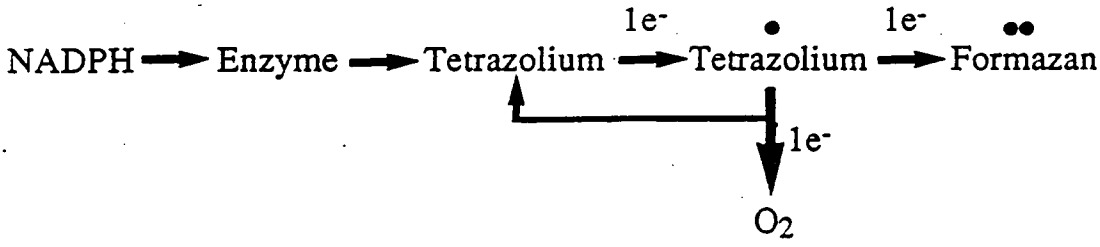


Figure 17b

Effect of Oxygen



all the tetrazoles with redox potentials more positive than that of NADPH (-0.365 V) were reduced. This suggests that competition for electrons can occur between oxygen and the tetrazoliums at the active site. Altman (1976a) has suggested that the effect of oxygen on tetrazolium reactions could also be due to electrons being transferred enzymatically to the tetrazolium one at a time (see figure 17b). The one-electron-reduced tetrazolium is not stable, and, if its redox potential is higher than that of oxygen, it can lose this electron to oxygen in the medium. One-electron transfer requires the presence of a prosthetic group, such as a flavin and/or a metal ion.

The chelators CDTA, EDTA, and cyanide are known to sequester metal ions or at least to inhibit enzymes by forming coordinate bonds with a metal ion at the active site (e.g., cyanide on cytochrome oxidase). Since NADPH-diaphorase histochemistry was unaffected, this enzyme is unlikely to require a heme or an accessible metal ion for catalysis.

Farber et al. (1956) and Nachlas et al. (1958) suggested that diaphorases are flavoproteins. We, however, found various methods of deflavination were without effect. This could be due to the flavin moiety binding covalently to the enzyme during fixation. Flavins are often covalently bound in vivo, as in succinate dehydrogenase (Singer et al., 1956). Therefore, further studies on the purified enzyme would have been necessary to determine whether neuronal NADPH-diaphorase is a flavoprotein.

The NADPH-diaphorase reaction was unaffected by the presence of excess exogenous superoxide dismutase or catalase. This indicated that the diaphorase was unlikely to be reducing tetrazoliums by producing superoxide anions or hydrogen peroxide. The enzyme was therefore not similar to the NADPH oxidases of neutrophils, leukocytes, thyroid follicular cells, or liver cells (Briggs et al., 1975).

The histochemical reaction was very sensitive to thiol reagents. Therefore, it is possible that neuronal NADPH-diaphorase, like some other enzymes possessing diaphorase activity (e.g., lipoamide dehydrogenase), has thiol groups that participate in the enzymatic reaction.

Neuronal NADPH-diaphorase Does Not Use NADH

Neuronal NADPH-diaphorase does not appear to use β-NADH, which agrees with the original observation of Thomas and Pearse (1961, 1964). This suggests neuronal NADPH-diaphorase is not DT-diaphorase (E.C. 1.6.99.2), which can use either NADH or NADPH (Ernster, 1967, Schultzberg et al., 1987). In addition, DT-diaphorase is very sensitive to inhibition by dicumarol and requires an electron mediator such as menadione (Levine et al., 1960, Ernster, 1967, Hall et al., 1972, Martius, 1959). In contrast, neuronal NADPH-diaphorase activity is unaffected by either menadione or dicumarol.

The observation that β-NADH will not serve as a substrate for the histochemical reaction is of significant interest. Davies et. al. (1985) had demonstrated NADPH-diaphorase activity in brain homogenates. However, the activity they described could utilize both NADH and NADPH to reduce NBT. This characteristic is inconsistent with the histochemical properties of neuronal NADPH-diaphorase. Therefore, the first protein described by this group was not the neuronal NADPH-diaphorase observed histochemically.

Many of the diaphorases found biochemically in the brain by Strecker, Giuditta, Flynn and colleagues may also be discounted because they use NADH as an electron donor (Giuditta and Strecker, 1960, 1961, Harper and Strecker, 1962, Levine et al., 1960, Briggs et al., 1975, Farber et al.,1956, Nachlas et al., 1958, Roerig et al., 1972, Giuditta and Aloj, 1965, Vesco and Giuditta, 1966, Williams and Kamin, 1962, Cromlish and Flynn, 1985, Flynn, 1982, Cromlish et

al., 1985). Only those enzymes which use NADPH as the exclusive electron donor remain candidates. These include NADPH-dependent cytochrome P450 reductase (Iscan and Arinc, 1988, Walther et al., 1986, LeGoascogne et al., 1987, Kapitulnik et al., 1987, Naslund et al., 1988), adrenodoxin reductase (Tuls et al., 1987), glutathione reductase (Giuditta and Aloj, 1965), glucuronate reductase (Cromlish and Flynn, 1985, Flynn, 1982, Cromlish et al., 1985), succinic semialdehyde reductase, and prostaglandin-9-reductase. Fortunately, we did not have to examine all these latter possibilities since they did not have the biochemical characteristics, such as molecular size, of the enzyme isolated with our specific biochemical assay for neuronal NADPH-diaphorase.

Kemp et al. (1988) have characterized biochemically a brain NADPH-diaphorase which they have identified as NADPH-dependent cytochrome P450 reductase. They believe their NADPH-diaphorase is the same as that detected histochemically. This enzyme is different from the one originally reported by this group since it does not appear to utilize NADH as a substrate (Davies et al., 1985). As mentioned in the introduction, the distribution of NADPH-dependent cytochrome P450 reductase in brain does not correlate with the distribution of NADPH-diaphorase neurons (Haglund et al., 1984). There was the possibility that the cytochrome P450 reductase of Kemp et al. (1988) was a different isoform from that detected by Haglund et al. (1984). While there are many isoforms of cytochrome P450 there are no reports of isoforms of the reductase. Second, Kemp et al. (1988) found diaphorase activity with triphenyl tetrazolium and neotetrazolium under aerobic conditions, which disagrees with the characteristics of the histochemical NADPH-diaphorase reaction found in this study. Third, Kemp et al. (1988) found their NADPH-diaphorase activity in the kidney and liver. We have not found histochemical NADPH-diaphorase activity in these organs comparable to that in the brain. This

confirms what was described in the introduction that NADPH-dependent cytochrome P450 reductase or an isoform does not contribute to the histochemical neuronal NADPH-diaphorase reaction. Thus the major candidates suggested to be responsible for neuronal NADPH-diaphorase activity, ie. cytochrome P-450 reductase, DT-diaphorase, and NADPH-oxidase, can now be discounted.

Species Distribution

Similar to mammals, neuronal NADPH-diaphorase activity is found in the brains of frogs and turtles. The brains of the hagfish and trout, however, do not contain this activity. The hagfish is a primitive saltwater fish while the trout lives in freshwater. Marine animals do not contain NADPH- or NAD(P)H-dependent diaphorases in the brain (Guiditta and Aloj, 1965). They do, however, contain NADPH-dependent ferricyanide reductase, similar to mammals. If these latter findings can be extended to the hagfish and trout then this would indicate neuronal NADPH-diaphorase and NADPH-dependent ferricyanide reductase activities are not due to the same enzyme.

HAN Antiserum Specifically Detects Neuronal NADPH-diaphorase

For the reasons described above the previously proposed candidates for the identity of neuronal NADPH-diaphorase can be discounted. We still needed a specific biochemical method for detecting neuronal NADPH-diaphorase. The development of HAN antiserum by Michaels and Knigge (1989) allowed us to detect specifically the neuronal NADPH-diaphorase enzyme responsible for the histochemical reaction. Double-labelling studies demonstrated colocalization of NADPH-diaphorase activity with the antigen detected by HAN antiserum. Immunoprecipitation of NADPH-diaphorase activity by HAN

antiserum suggested the bound antigen did not merely colocalize with neuronal NADPH-diaphorase but was indeed the neuronal NADPH-diaphorase of interest. In summary, the NADPH-diaphorase activity immunoprecipitated by HAN antiserum is found immunohistochemically in all NADPH-diaphorase neurons. Therefore, the biochemical diaphorase activity detected by HAN antiserum is responsible for the neuronal NADPH-diaphorase reaction.

NADPH-diaphorase is a Nitric Oxide Synthase

We found that the neuronal NADPH-diaphorase reaction is due to a nitric oxide (NO) synthase. NO synthase forms NO and citrulline from the precursor arginine (Marletta, 1989). This enzyme will be described in more detail later. Evidence suggesting neuronal NADPH-diaphorase is a NO synthase includes: a) copurification of both enzyme activities to apparent homogeneity; b) the purified enzyme has a relative molecular weight of 150 kD on SDS-PAGE, similar to that published for NO synthase (Bredt and Snyder, 1990); and c) the tetrazolium, NBT, competes with arginine for electrons from NADPH. These results, however, merely demonstrate that NO synthase has NADPH-diaphorase activity.

To demonstrate that the NADPH-diaphorase activity of NO synthase was responsible for that seen histochemically, the HAN antiserum was necessary for specific identification. The HAN antiserum specifically labeled a band on Western blots with a molecular weight of 150 kD. NO synthase activity was immunoprecipitated along with NADPH-diaphorase activity by HAN antiserum. This demonstrated that the antigen detected in tissue sections and immunoprecipitated from homogenates by HAN antiserum had the characteristics of a NO synthase. The total amount of NADPH-diaphorase and NO synthase activity immunoprecipitated by the HAN antiserum was small.

Normally the amount of antiserum that was used would be sufficient to immunoprecipitate the antigen quantitatively. However, the HAN antiserum was not made to NADPH-diaphorase directly, so it is most likely that only a small proportion of the antibody population cross-reacted with the enzyme NADPH-diaphorase. This would make it difficult, if not impossible, to have a high enough concentration of antibodies specific for NADPH-diaphorase/NO synthase in the solution to immunoprecipitate the enzyme quantitatively.

Of concern was the lack of requirement for calcium and calmodulin for NADPH-diaphorase activity, even though they are required for NO synthase activity. Chelators sequester calcium and inhibit NO synthase activity but do not inhibit NADPH-diaphorase activity. The lack of structural specificity of tetrazoliums as electron acceptors for NADPH-diaphorase activity suggests the conformation of the enzyme active site is not important for passing electrons to tetrazoliums. In contrast, there is a strong requirement for structural specificity of electron acceptors for NO synthase activity. For example, D-arginine cannot replace L-arginine as a substrate. Therefore, conformation of the enzyme active site is important for NO synthase activity. The necessary conformation is probably regulated by calcium and calmodulin binding. As will be discussed later, NADPH-diaphorase activity probably involves only one component of the multistep reaction of NO synthase.

Subcellular Distribution of NADPH-diaphorase and NO Synthase

The subcellular distribution of NADPH-diaphorase and NO synthase are difficult to correlate. The electron microscopic investigation of Scott et. al. (1987) employed NBT for the cellular location of NADPH-diaphorase in olfactory bulb neurons. Their figures appear to indicate, as do our results with NBT in the striatum, that the reaction product is found as granules distributed

uniformly throughout the cytoplasm. However, NBT may be an inadequate dye for electron microscopy. Its formazan product has a tendency to coalesce into droplets, as shown in this investigation, and is not sufficiently osmiophilic or lipophobic (Kalina et al., 1972). BSPT has been suggested not to have these drawbacks (Kalina et al., 1972). Our results employing BSPT suggest that NADPH-diaphorase is a membrane associated protein with widespread subcellular distribution. In contrast, biochemical NO synthase activity is found primarily in the soluble supernatant of many preparations (see below). It is possible that the electron microscopic results with BSPT represent a fixation artifact or that there is a loose attachment of the enzyme to membranes which is broken during homogenization.

A detailed study of the subcellular distribution of NO synthase activity in bovine aortic endothelial cells employed enzyme markers to characterize the subcellular fractions (Boje and Fung, 1990). They found NO synthase activity associated with membrane fractions. The majority of nitric oxide synthase activity in cultured bovine and porcine aortic endothelial cells was also found in the particulate fraction (Forstermann et al. 1991). This agrees with our electron microscopic results with NADPH-diaphorase which suggest association of the enzyme with membranes. However, Boje and Fung (1990) found NO synthase activity correlates most closely with the plasma membrane enzyme markers which does not agree with our electron microscopic results. The exact subcellular distribution of NO synthase in the brain may be different from that in the aorta.

Citrulline-Immunoreactivity Colocalizes with NADPH-diaphorase

The colocalization of citrulline in NADPH-diaphorase neurons was expected since citrulline is formed along with NO from arginine by NO

synthase (Marletta, 1989). Not all neurons known to contain NADPH-diaphorase activity were observed to display citrulline immunoreactivity. For example, of the many NADPH-diaphorase positive cells in the striatum, only about 60% displayed citrulline immunoreactivity. The lack of detectable citrulline immunoreactivity in many NADPH-diaphorase positive cells may be due to a high turnover rate of citrulline in these cells, such that levels are below those detectable by the immunohistochemical techniques employed.

What becomes of the citrulline produced in the brain? Endothelial cells can maintain a constant level of arginine despite continuous release of nitric oxide (Mitchell et al., 1990), and, when grown in an arginine free medium, can generate arginine from citrulline (Hecker et al., 1990a,b). This suggests that endothelial cells contain a modified urea cycle whereby citrulline formed from arginine by NO synthase is recycled to arginine via argininosuccinate (Hecker et al., 1990a,b). A similar cycle may exist in those neurons possessing NO synthase. This would explain the occurence of citrulline (Sadasivudu and Rao, 1976) and the presence of the enzymes of the latter half of the urea cycle (Ratner et al., 1960) in the brain.

NO Synthase Immunoreactivity

Bredt et al. (1990) have developed an antisera to NO synthase purified from rat cerebellum which they employed immunohistochemically to investigate the distribution of the enzyme in the brain and in peripheral tissues. Similar to NADPH-diaphorase staining, strong immunoreactivity (IR) for NO synthase was found in the olfactory bulb, the islands of Cajellae, the bed nucleus of the stria terminalis, the horizontal limb of the diagonal band of Broca, the supraoptic and paraventricular nuclei of the hypothalamus, and fibers in the posterior pituitary lobe of the pituitary gland. Interestingly, the

cerebral cortex appeared to have little IR while the striatum had none. Further examination of striatal and neocortical tissue sections demonstrated sparsely distributed NO synthase-IR cells with a similar distribution and morphology as that for NADPH-diaphorase neurons.

The basket and granule cells of the cerebellar cortex stained weakly for NADPH-diaphorase activity (Vincent and Kimura, 1991) and did not have any HAN- or citrulline-IR even though the cerebellum contains high levels of NO synthase activity (Forstermann et al., 1990b) and NO synthase-IR (Bredt et al., 1990). Garthwaite et al. (1987) had determined granule cells to be the main source of NMDA-stimulated NO formation (see below), which agrees with the distribution of NADPH-diaphorase staining. The low level of NADPH-diaphorase activity by the NO synthase in basket and granule cells may be due to inhibition during fixation or because of the lack of a significant layer of cytoplasm, where NO synthase is found, surrounding the nucleus. It may also indicate there is more than one form of NO synthase in the brain. The cerebellar form may have little or no NADPH-diaphorase activity. Different forms of NO synthase have already been demonstrated in macrophages and neutrophils and two forms of NO synthase have been described in the cerebellum (East and Garthwaite, 1990). The two cerebellar forms differ in their ability to be inhibited by L-N^G-nitro-arginine (LNNA) with IC_{50} values of 6 and 600 nM.

NO synthase-IR was detected in endothelial cells and in the dense innervating fibers on the adventitia surrounding cerebral blood vessels (Bredt et al., 1990). NADPH-diaphorase activity was weakly detected in blood vessels only after long incubation times. The NADPH-diaphorase activity in blood vessels was probably not due to the same enzyme as found in neurons since NADPH-diaphorase activity was present in blood vessels but not in

neurons when the analog 3'-PO_4-NADPH replaced β-NADPH as the electron donor.

Distribution of NO Synthase Activity

Forstermann et al. (1990b) investigated the distribution of NO synthase activity in the brain by examining the ability of cytoplasmic fractions to activate soluble guanylate cyclase in rat fetal lung fibroblasts. NO is an activator of soluble guanylate cyclase (see below). NO synthase activity was highest in the cerebellum, which agrees with the distribution of NO synthase-IR. The hypothalamus and midbrain-pons regions also had high NO synthase activity which correlates with strong NADPH-diaphorase staining in the paraventricular and supraoptic nuclei and the caudal cholinergic columns respectively. The striatum and neocortex had intermediate levels of activity while the medulla oblongata had the lowest.

While the number of NADPH-diaphorase neurons and fibers in the striatum appear to be greater than that in caudal cholinergic neurons or in the hypothalamus, the biochemical NADPH-diaphorase and NO synthase activity per cell may be greater in the latter two cell groups than in the striatum. Again, the difference between NADPH-diaphorase staining and NO synthase activity in the cerebellum may be due to inhibition of NADPH-diaphorase activity by fixation or to isoforms of NO synthase. Otherwise the distribution of activity found by Forstermann et al. (1990b) agrees with NADPH-diaphorase staining.

Implications of Neuronal NADPH-diaphorase Activity as a Marker for NO Synthase

We conclude that the neuronal NADPH-diaphorase reaction is due to NO synthase. Therefore, NADPH-diaphorase activity is a reliable marker for NO synthase. However, NADPH-diaphorase activity may not detect all forms of NO synthase such as in the cerebellum. As described in the introduction there is a large literature on the distribution of NADPH-diaphorase activity. Therefore, the distribution of NO synthase in the brain can now be determined in considerable detail. Knowledge of the cellular actions of NO synthase and the anatomy of NADPH-diaphorase can also be tied together to suggest functions of NADPH-diaphorase/NO synthase in the brain.

To discuss the functions of NADPH-diaphorase/NO synthase in the brain a more detailed description of the cellular actions of NO synthase is necessary. Many recent reviews on NO and NO synthase have been written (Moncada, 1988a,b, 1989a, Marletta, 1989, Ignarro, 1989a,b, Furchgott and Vanhoutte, 1989). The first part of this review will cover the evidence for NO as a second messenger, including its actions at the cellular level. The model system for these actions is the vascular endothelium. NO also has actions in a wide variety of other tissues. The second part of the description will provide evidence that NO is produced and is active in the brain. This will be followed by background on the enzyme, NO synthase, in the brain.

NO is the Second Messenger Involved In Vascular Smooth Muscle Relaxation

Furchgott and Zawadski (1980) had found that an intact endothelial cell lining was necessary for relaxation of smooth muscle of dissected blood vessels in response to bradykinin, acetylcholine, A23187, thrombin, and ATP (Sakuma

et al., 1988, Furchgott, 1984). They proposed the endothelium released a relaxant factor when stimulated by these agonists. Independently, at a symposium in 1986, Ignarro and Furchgott both proposed that the endothelially-derived relaxant factor (EDRF) was in fact nitric oxide (NO) (Ignarro et al., 1987a,b, Ignarro, 1989a, Moncada et al., 1988a, Palmer et al., 1987, 1988). NO is a small non-polar molecule which can diffuse through cellular membranes.

There are many similarities between EDRF and NO. Both EDRF and NO have a half life of 3-5 seconds. The vasodilator activity of both is inhibited by hemoglobin, which tightly binds NO, and methylene blue. The half-lives of both EDRF and NO and their vasodilator activities can be extended by adding the free radical scavenger, superoxide dismutase. There has also been direct detection of nitric oxide release from the endothelium by measuring the chemiluminescence when NO reacts with ozone, by the characteristic shift of the Soret band upon NO binding to hemoglobin, and by the reaction of the NO breakdown products, nitrite and nitrate, with the Greiss reagent. The bulk of evidence, therefore, supports EDRF being identical to NO. NO has since been found to be responsible for microcirculatory control in various tissues including the lung (Mayer and Bohme, 1989, Archer et al., 1989) and the gastric mucosa (Whittle et al., 1990, Fortes et al., 1989).

It has long been known that nitroso-compounds, such as nitroglycerin, nitroprusside, and NO itself were potent vasodilators (Moncada et al., 1988a, Ignarro et al., 1987a, Ignarro, 1989b, Palmer et al., 1987, Murad et al., 1987). Nitroprusside in solution spontaneously decomposes to release NO. Other nitroso-compounds require a reaction with reduced thiols. The nitrosothiols formed are unstable and spontaneously decompose to release NO.

Some investigators believe EDRF may be a nitrosothiol such as

nitrosocysteine (Ignarro, 1989a,b, 1990b, Myers et al., 1990, Long and Berkowitz, 1989). They provide an alternative mechanism of NO release. Nitrosothiols are relatively stable in acid and could possibly be stored in acidic secretory granules. Release of nitrosothiols into the slightly alkaline extracellular media would cause spontaneous decomposition to release NO. In some tissues, inhibitors of secretion can inhibit vascular smooth muscle relaxation (Ignarro, 1990b). If this mechanism is employed in certain tissues it would still require prior formation of NO from arginine and the active molecule would still be NO (see below). Nitrosothiols could therefore only act as a temporary storage form of NO. Although vesicular release of NO by this mechanism is possible, we assume NO is more likely to be released non-vesicularly by diffusing through the membranes of cells in which it is formed.

Arginine is the Precursor for NO Formation

Production of NO is arginine-dependent, suggesting arginine is the precursor for NO (Moncada et al., 1989b). NO release from vascular endothelium is increased by adding exogenous arginine before or after depletion of arginine stores (Palmer et al., 1988, Gold et al., 1989a,b, Aisaka et al., 1989a). The arginine analogs, N^G-monomethyl-arginine (NMMA) (Aisaka et al., 1989b, Thomas et al., 1989, Amezcua et al., 1989), L-N^G-nitro-arginine (LNNA) (Kobayashi and Hattori, 1990, Ishii et al., 1990, Mulsch and Busse, 1990), and N^G-amino-arginine (Fukuto et al., 1990) are very effective inhibitors of the EDRF/NO effect (Rees et al., 1990, Sakuma et al., 1990). Direct evidence for arginine as the precursor of NO required labelling the guanidino-nitrogen (N^G) of arginine with the isotope ^{15}N (Leaf et al., 1989, Palmer et al., 1988). After activation of endothelial cells, ^{15}N-labelled nitrite and nitrate were

formed with a time course similar to the decrease in labelled arginine. NO is, therefore, derived from the guanidino-nitrogen of arginine.

NO Activates Guanylate Cyclase

Nitric oxide passes through cell membranes to stimulate soluble guanylate cyclase activity in vascular smooth muscle cells leading to an increase in cGMP levels (Ignarro et al., 1987a,b, 1990, Ignarro, 1989a,b, 1990a,b, Murad et al., 1987, Kondo et al., 1989, Fiscus et al., 1984, Archer et al., 1989, Waldman and Murad, 1988). cGMP in the vascular endothelium also increases in response to the vasodilators nitroglycerin and nitroprusside. The cGMP formed most likely leads to activation of cGMP-dependent protein kinase which subsequently phosphorylates proteins involved in mediating the relaxation of vascular smooth muscle (Murad et al., 1987). A more detailed description of soluble guanylate cyclase will follow that for NO synthase.

Arachidonic Acid

Arachidonic acid and its metabolites have also been proposed as candidates for EDRF and to activate guanylate cyclase directly in both the vascular endothelium and other tissues (Ignarro, 1989a,b, 1990b, Moncada et al., 1986, 1988b, Minami and Toda, 1989, Louis et al., 1990, Long and Berkowitz, 1989, Reiser, 1990b). Inhibitors of production of arachidonic acid and its metabolites have shown variable ability to inhibit the activation of guanylate cyclase and EDRF effects depending on the neurohormone and tissue studied. This would suggest arachidonic acid is probably not directly involved in activation of guanylate cyclase and vascular smooth muscle relaxation. Arachidonic acid may instead be involved in the formation and/or release of EDRF/NO in some tissues in response to particular neurohormones (Ignarro,

1989a,b, 1990b).

Macrophages, Neutrophils and Platelets

NO function is found in many tissues other than the vascular endothelium. Macrophages can be activated by bacterial infection, lipopolysaccharides, and γ–interferon to release NO in an arginine-dependent manner (Pellat et al., 1990). While not causing lysis of cellular targets, NO inhibits the mitochondrial respiratory chain by binding heme and non-heme iron, and inhibits cis-aconitase, DNA-synthesis, and other metabolizing enzymes, thus aiding in cell death of tumour and bacterial targets (Hibbs et al., 1987, Pellat et al., 1990, Kostergaard and Leroux, 1989, Stuehr et al., 1989). Neutrophils and mononuclear cells also produce NO which can inhibit platelet aggregation and adhesion, as well as relax vascular smooth muscle (Salvemini et al., 1989, Deana et al., 1989, McCall et al., 1989). The effects of NO release from neutrophils and mononuclear cells involve stimulation of soluble guanylate cyclase in platelets and vascular smooth muscle.

NANC Nerves: NO as a Peripheral Neurotransmitter

Most responses of organs to autonomic stimulation are dependent on noradrenaline or acetylcholine (Gibson et al., 1990). Some inhibitory responses, however, are mediated by a non-adrenergic non-cholinergic (NANC) neurotransmitter (Bult et al., 1990). NO has recently been identified as the neurotransmitter released from NANC nerves to have EDRF-like effects on smooth muscle in the trachea (Tucker et al., 1990), the anococcygeous muscle (Hobbs and Gibson, 1990, Ramagopal and Leighton, 1989, Gillespie et al., 1989, Gibson et al., 1990, Li and Rand, 1989), the gastrointestinal tract (Li and Rand, 1990, Bult et al., 1990, Boeckxstaens et al., 1990, Toda et al., 1990), and the aortic

wall (Stasch et al., 1989, Boje and Fung, 1990, Smith and Lang, 1990). NO can also raise cGMP levels in the kidney (Scroder and Schror, 1989, Schultz et al., 1990, Arnold et al., 1977), the adrenal gland (Palacios et al., 1989, Arnold et al., 1977), liver, skeletal muscle, spleen, and epididymal fat (Arnold et al., 1977). There is NADPH-diaphorase activity in neurons of the gastrointestinal tract and myenteric plexus (Branchek and Gershon, 1989). These neurons may be the NANC nerves mentioned by (Li and Rand, 1990, Bult et al., 1990, Boeckxstaens et al., 1990, Toda et al., 1990). Release of NO from NANC nerve terminals is probably non-vesicular. Calcium influx into the terminals activates NO synthase which stimulates cGMP formation in the post-synaptic compartment. A similar process may occur in the central nervous system.

NO in the Brain

As mentioned above, NO synthase activity (Forstermann et al., 1990) and NO synthase-IR (Bredt et al., 1990) have both been detected in various regions of the brain. The non-enzymatic breakdown products, nitrite and nitrate, have also been detected in various regions of the brain (Schmidt et al., 1990). L-Arginine could enhance the formation of nitrite and nitrate, while NMMA inhibited their formation. Therefore, both NO synthase and NO have been detected throughout the brain. The cerebellum is the model system for investigations of the formation and action of NO in the brain. NO formation and action in other brain regions appears similar to that in the cerebellum so that information derived from the cerebellum is relevant to a discussion of NO and NO synthase in non-cerebellar brain regions.

Arginine-dependent Production of the Endogenous Activator of Guanylate Cyclase in the Brain

It has been known for quite a while that nitroprusside and other nitroso-compounds, including NO, induce large increases of cGMP in the cerebellum (Deguchi and Yoshioka, 1982, Yoshioka et al., 1990). Before 1988, the endogenous activator of soluble guanylate cyclase in the brain was still a mystery. Deguchi and Yoshioka (1982) had found a low molecular weight molecule in the supernatant of acid precipitations of brain cytosol that could stimulate guanylate cyclase. Following purification, the small molecule was identified as L-arginine. By itself, L-arginine could not stimulate partially purified soluble guanylate cyclase, although nitroprusside could still do so. An unknown factor in the acid precipitate, probably the NO-synthesizing enzyme, was necessary for arginine to stimulate the partially purified guanylate cyclase.

The Endogenous Activator of Guanylate Cyclase in the Brain is NO

In 1988, the similarities between cGMP regulation in peripheral tissues and the cerebellum included stimulation of guanylate cyclase by nitroso-compounds (Garthwaite et al., 1987) and a dependence on arginine availability (Deguchi and Yoshioka, 1982). This led to a series of experiments where Garthwaite et al. (1988) tested specifically for NO mediation of the cGMP response to glutamate agonists. NMDA stimulation of guanylate cyclase activity in cerebellar cell suspensions could be increased by the addition of superoxide dismutase and decreased by the addition of hemoglobin or methylene blue. Hemoglobin, incapable of permeating cell membranes, was still capable of inhibiting NMDA-dependent stimulation of guanylate cyclase. This supported the previous proposal by Garthwaite et al. (1987) that cells

stimulated by NMDA do not stimulate their own guanylate cyclase. If these cells did stimulate their own guanylate cyclase then hemoglobin would be incapable of inhibiting this intracellular component. The fact that inhibition by hemoglobin was still so effective suggested NO acts mostly as an intercellular messenger.

To demonstrate further that the mediator was transferable between cells, a cerebellar cell suspension was stimulated by NMDA and the cells were removed by rapid filtration. With a half-life similar to that of EDRF, the medium increased cGMP in cerebellar cell suspensions as well as relaxed rabbit aortic rings (Garthwaite et al., 1988). The ability of the cell suspension to produce an EDRF-like factor was dependent on extracellular calcium, similar to the results with endothelial cells. A similar calcium dependence had been shown for N1E-115 neuroblastoma cell suspensions relaxing rabbit aortic rings (Forstermann et al., 1989). The effect of the cerebellar cell medium on cGMP and aortic relaxation was increased with superoxide dismutase and inhibited by hemoglobin and methylene blue (Garthwaite et al., 1988). The response could be inhibited by the NMDA specific antagonist D,L-2-amino-5-phosphonovalerate (APV). Kainate receptor activation also led to NO release and stimulation of guanylate cyclase (Garthwaite et al., 1989a).

NMDA, kainate, and quisqualate all increased cGMP levels in cerebellar slices (Garthwaite et al., 1989b, Wood, 1991). Similar to the vascular endothelium, the increases in cGMP levels in the cerebellar slice were inhibited by NMMA. This inhibition was reversed by the addition of L-arginine but not by the enantiomer D-arginine. These results indicated that an EDRF-like factor with the characteristics of NO was being released in a calcium-dependent manner in response to excitatory amino acid (EAA) receptor activation. The NO formed then travels between cells to activate

guanylate cyclase and increase cGMP levels.

A large variety of neurotransmitters and pharmacological agents alter cGMP levels in the cerebellum in vivo and in vitro (Wood, 1991). Most appear to act through modulation of EAA input to the cerebellum.

NO Synthase in the Brain

Bredt and Snyder (1990) have recently purified cerebellar NO synthase to homogeneity. The enzyme was a monomer of 150 kD and required calcium, calmodulin, and NADPH. Inhibition by NMMA suggested the enzyme is probably responsible for synthesis of the EDRF/NO substance found by Garthwaite et al. (1987, 1988, 1989b) to mediate the NMDA dependent increase in cerebellar cGMP levels. NO synthase has since been purified by a number of other groups (Forstermann et al., 1991, Mayer et al., 1990).

Synaptosomal NO synthase from the forebrain is entirely dependent on calcium with a half maximal stimulatory effect of approximately 160 nM (Knowles et al., 1989). The enzyme is inactive at resting synaptosomal free calcium concentrations of about 80 nM and fully active at 400 nM. This could be the biochemical mechanism for stimulating the formation of NO. Garthwaite et al. (1988) had previously shown calcium to be necessary for NMDA stimulation of NO formation. Calcium ions may pass through the NMDA channel to activate NO synthase (Wood, 1991).

NO synthase may also be activated via voltage-gated calcium channels. GABA receptor activation decreases cGMP levels in the cerebellum (Wood, 1991). This may be due to a decrease in the activation of voltage-gated calcium channels when cells are hyperpolarized via the GABA receptor. Thus, in the cerebellum, and probably in the rest of the brain, NO synthase activity may be regulated by a balance of EAA versus GABA input which regulates the

membrane potential of a cell and the conductance of its voltage-gated calcium channels.

NO synthase may also be activated by calcium released from intracellular stores. Activation of the metabotropic glutamate receptor stimulates NO synthase and raises cGMP levels in cerebellar slices (Okada, 1991). The metabotropic receptor activates phospholipase C which leads to phosphotidylinositol breakdown and the formation of inositol tris-phosphate (IP_3) (Cubero et al., 1991). IP_3 can then release calcium from intracellular stores and activate NO synthase leading to an increase in cGMP levels.

Enzymatic Mechanism of NO Synthase

The requirement for calcium and calmodulin by the brain form of NO synthase suggests similarities with the enzyme in endothelial cells but not with the macrophage enzyme, which requires magnesium ions (Knowles et al., 1989, Bredt et al., 1990). The NO synthesizing enzymes in all these tissues are soluble, form citrulline as a coproduct of NO, and are inhibited by NMMA (Knowles et al., 1989).

Although activation and cofactor requirements of NO synthase in macrophages is somewhat different than that of the brain enzyme, the enzymatic mechanisms of NO formation appear similar. Both enzymes require arginine, NADPH, a divalent cation, and catalytic amounts of tetrahydrobiopterin (BH_4) for activity (Mayer et al., 1990, Kwon et al., 1989, Tayeh and Marletta, 1989). NADPH is used to recycle BH_4 which goes on to activate an oxygen molecule for monooxygenase activity by NO synthase. The activated oxygen is used to hydroxylate arginine to N^G-hydroxyarginine which then goes on to form citrulline and NO. NADPH-diaphorase activity probably only involves the monooxygenase part of the reaction mechanism.

Rather than passing electrons from NADPH to BH_4 or oxygen the enzyme passes the electrons to a tetrazolium.

Guanylate Cyclase and the cGMP System

The function of NO synthase and thus NADPH-diaphorase depends primarily on the ability of NO to stimulate soluble guanylate cyclase (Ignarro et al., 1987a,b, 1990, Ignarro, 1989a,b, 1990a,b, Murad et al., 1987, Kondo et al., 1989, Fiscus et al., 1984, Archer et al., 1989, Waldman and Murad, 1988). Therefore, information about the possible functions of NADPH-diaphorase/NO synthase in the brain can be determined by relating NADPH-diaphorase anatomy to the anatomy and function of soluble guanylate cyclase, cGMP, and related enzymes. To understand the details of this discussion it is first necessary to describe briefly the biochemistry of these enzymes with emphasis on soluble guanylate cyclase.

Soluble Guanylate Cyclase

In the brain there are three major forms of guanylate cyclase. One form is soluble guanylate cyclase (GC). It was previously thought to be of two forms. Indeed, two different clones have been found in the brain. The molecular weights of the deduced amino acid sequences correspond to the purified 70 and 82 kDa polypeptides of GC (Nakane et al., 1988, 1990, Koesling et al., 1988). Both polypeptides contain a catalytic sequence similar to the carboxy-terminal portion of a particulate guanylate cyclase (Nakane et al., 1990). When each of the clones were transfected separately into cells, no guanylate cyclase activity was detected. When the two clones were transfected together, guanylate cyclase activity was detected, indicating that both polypeptides were necessary to form an active heterodimer corresponding in size to the purified native enzyme of 150 kDa (Nakane et al., 1990, Koesling et

al., 1988, Murad et al., 1987) There is, therefore, only one form of soluble guanylate cyclase. The enzyme has a heme which binds NO and is necessary for enzyme activity (Ignarro, 1990b).

Soluble GC is Activated Primarily by Intercellular NO

The activation of soluble GC by NO can be inhibited in vitro by the addition of hemoglobin to endothelial preparations (Ignarro et al., 1987a,b, Ignarro, 1989b, Moncada et al., 1988a, Palmer et al., 1987, 1988) and cerebellar slices and cell suspensions (Garthwaite et al., 1988, 1989b). Since hemoglobin is impermeable to membranes and cannot enter the intracellular space it cannot inhibit NO from activating GC within the same cells where NO is being produced. Inhibition by hemoglobin implies that NO is primarily an intercellular messenger and is unlikely to activate any GC within the cells where it is produced. Although the mechanism underlying this is unclear at least one mechanism has been suggested. While NO synthase activity is stimulated by calcium, soluble GC is inhibited by calcium (Knowles et al., 1989, Palacios et al., 1989). Soluble GC is inhibited by calcium with a half-maximum of 120 nM and completely inhibited at 1500 nM (Knowles et al., 1989). It, therefore, requires more calcium for the half-maximal activation of NO synthase (160 nM) than for the half-maximal inhibition of GC activity (120 nM). The increased intracellular calcium levels necessary to activate NO synthase simultaneously decrease potential GC activity in the cells actively producing NO. Those surrounding cells which do not have simultaneously high calcium levels have all of their potential GC enzyme activity available for activation by intercellular NO (see figure 18). The result is that NO activates GC primarily in surrounding cells rather than in the cells where it is produced. This would suggest NO acts primarily as a paracrine intercellular messenger

Figure 18

a) Calcium inhibits GC activity. When the intracellular calcium concentration increases in a NO-producing neuron the level of GC enzyme available for activation by NO decreases. There is a balance where calcium activation of NO formation outweighs the inhibitory effects of calcium on GC activity. The result is decreased formation of cGMP in cells activated to produce NO. At higher levels of intracellular calcium (1500 nM) GC activity is completely inhibited.

b) Those surrounding neurons which do not have simultaneously raised intracellular calcium levels also do not have their GC enzyme inhibited. Therefore, the cGMP reponse of surrounding neurons to a given concentration of NO is greater than that of neurons actively producing NO.

Figure 18a

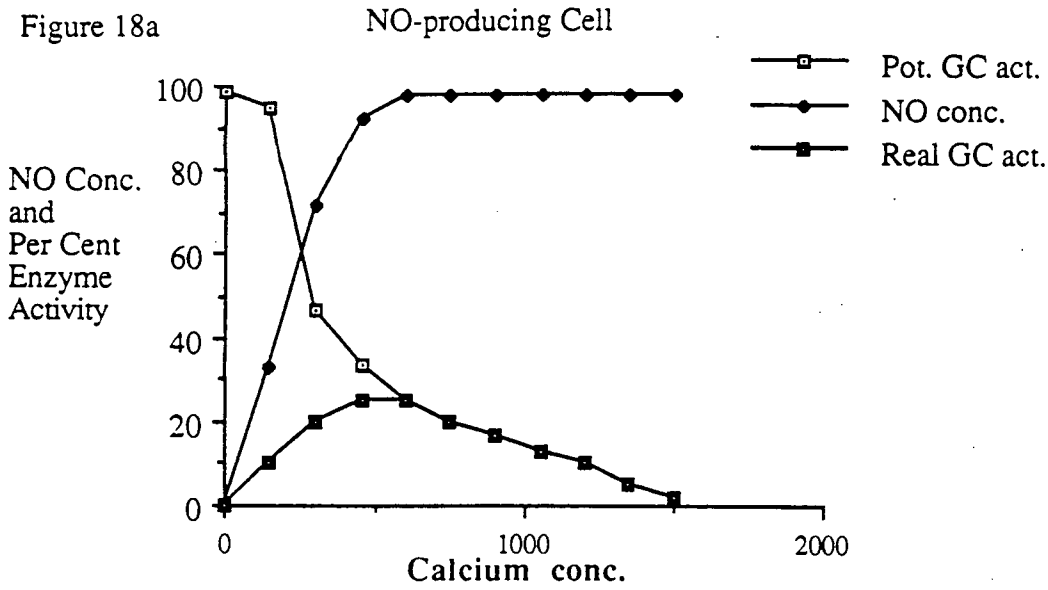
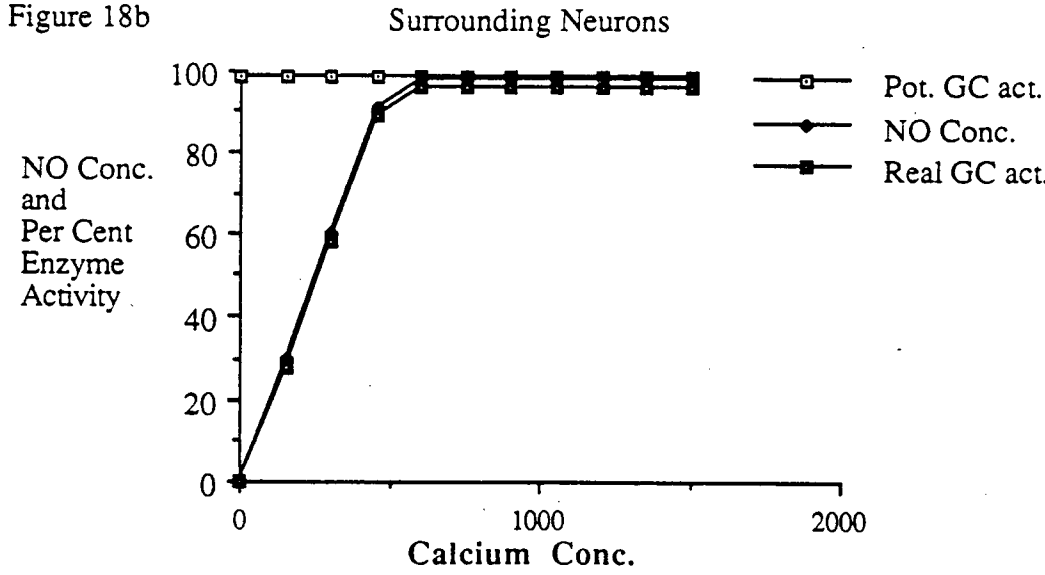


Figure 18b



(Knowles et al., 1989, Collier and Vallance, 1989, Garthwaite et al., 1988, 1989b, Garthwaite, 1991). NO produced in one cell can, however, activate GC in other NO-producing cells as long as the latter cells have not been activated to increase their intracellular calcium levels.

Paradoxically, homogeneous cell lines (Reiser, 1990a,b, Study et al., 1978) and homogenates with exogenously added purifed GC (Mayer et al., 1989, 1990, Palacios et al., 1989) still increased cGMP levels in a calcium-dependent manner. In these studies calcium levels were raised in all cells and throughout the homogenate solutions. cGMP levels still increased because, up to 1500 nM, not all of the GC enzyme was inhibited and could, therefore, still be activated by NO. GC activity, however, would be less than expected because of inhibition by calcium (Palacios et al., 1989). There reaches a point at about 400 nM calcium where no more NO is being produced to counter the inhibition of GC by calcium. This is where calcium no longer stimulates but rather inhibits overall cGMP formation.

Inhibition of GC by calcium may also explain the transient nature of the cGMP response in homogeneous cell lines. When a neurotransmitter or calcium ionophore is applied to these cells, cGMP levels peak within 30 seconds to several minutes and then decrease (Reiser, 1990a,b, McKinney et al., 1990, Studt et al., 1978, Burgess et al., 1989, Pou et al., 1990). When the pharmacological agent is applied to the cells, intracellular calcium levels gradually increase, leading to NO synthase activation and eventually GC activation. As intracellular calcium levels increase, the cGMP response eventually decreases, because GC is gradually being inhibited by higher calcium levels. Most studies of cGMP regulation with cell lines measure the peak response within the first several minutes.

If, as just described, NO can stimulate some cGMP formation in cells actively producing NO then why does hemoglobin inhibit cGMP formation in both slices and homogeneous cell suspensions? With slices, any intracellularly stimulated GC activity in NO-producing cells would be relatively insignificant compared to the uninhibited GC activity in surrounding cells. Thus the majority of GC activity would be inhibited if hemoglobin binds extracellular NO and blocks intercellular stimulation of GC in surrounding cells. Within homogeneous cell suspensions, GC is activated both intracellularly and intercellularly via NO. Taking away the contribution of intercellular NO by adding hemoglobin would decrease overall GC activity. In summary, although NO can stimulate GC in cells actively producing NO, NO primarily activates the GC in surrounding cells. It should be noted that calcium inhibition of GC activity in the brain has so far only been demonstrated by one group (Knowles et al., 1989, Palacios et al., 1989).

Particulate Guanylate Cyclase

A major form of guanylate cyclase is found in the particulate fraction. It is a combined peptide receptor and guanylate cyclase formed by one polypeptide (Garbers, 1989). The most studied form of particulate guanylate cyclase is the receptor for atrial natriuretic peptide (ANP). The ANP receptor-particulate guanylate cyclase is either independent or activated by calcium (Garbers, 1989, Tohda and Nomura, 1990). This particulate form of guanylate cyclase is distinct from the cytoskeletal form found in the retina (Ferrendelli, 1990).

cGMP Phosphodiesterase

The cGMP signal can be terminated by a number of phosphodiesterases

whose relative specificities for cGMP and cAMP and mechanisms of activation are still being worked out (Beavo and Reifsnyder, 1990). The major form of cGMP phosphodiesterase (cGPDE) in the brain is activated by calcium and calmodulin (Greenberg et al. 1978). Thus, cells with raised intracellular calcium levels to activate NO synthase also have activated phosphodiesterase for hydrolyzing cGMP. This makes it even less likely that a relatively significant level of cGMP can occur in cells actively producing NO.

There is another significant cGMP-related phosphodiesterase called phosphodiesterase II which is activated by cGMP to hydrolyze both cGMP and cAMP (Tremblay et al., 1988, Beavo and Reifsnyder, 1990, Whalin et al., 1991). This isoenzyme is widely distributed throughout the brain and could be a major effector mechanism for cGMP action by lowering cAMP levels (Beavo and Reifsnyder, 1990, Whalin et al., 1991).

cGMP-dependent Protein Kinase

Nairn and Greengard (1983) proposed that cGMP has most of its effects by activating a cGMP dependent protein kinase (cGPK). Two different clones for cGPK with mRNA message sizes of 6.2 kbp have been isolated from trachea smooth muscle (Wernet et al., 1989). The two types, Ia and Ib, are functionally identical. They each form a homodimer of 76 kD with two different sites for cGMP binding, an autophosphorylation site in the amino-terminus, and a catalytic site in the carboxy-terminus.

Possible Functions of NO and NO synthase/NADPH-diaphorase in the Brain

The above review covered the cellular functions of NO synthase and thus NADPH-diaphorase in the brain. I will now discuss the possible functions

of NO, NO synthase/NADPH-diaphorase, GC and cGMP in the brain at the level of local circuits. This will tie together the two large literatures on neuronal NADPH-diaphorase and cGMP. At the level of local circuits there are similarities in the activation of NO synthase and the action of NO in the striatum, neocortex, hippocampus, and cerebellum. The possible actions of NO and NO synthase/NADPH-diaphorase in the brainstem, olfactory bulb, retina, and pineal gland will also be discussed. This will be followed by a discussion of the possible involvement of NO in more general brain functions such as arousal, neurotransmitter release, synaptic plasticity, excitotoxicity, activity-dependent blood flow, and astrocyte function.

Local Circuits

Striatum

GC is higher in the striatum and nucleus accumbens than in any other brain region (Walaas, 1981). The striatum also has one of the highest levels of cGPDE (Greenberg et al., 1978, Ariano and Matus, 1981, Ariano, 1983, Ferrendelli, 1990). High levels of both these enzymes probably underlie a relatively rapid cGMP response as compared with the cerebellum which has low levels of cGPDE (see below). Cyclic GMP can be rapidly formed by high GC levels and rapidly hydrolyzed by high cGPDE levels. Inhibition of striatal cGPDE by isobutylmethylxanthine (IBMX) increases the duration of the cGMP signal beyond the control time of 1-2 min, demonstrating the enzyme's ability to control the temporal length of the cGMP response (Hanley and Iverson, 1978, Greenberg et al., 1978). Significant levels of cGPK and it's G-substrate are also found in the striatum, but at 50-100 times lower levels than in the cerebellum (Schlichter et al., 1980, Detre et al., 1984). Thus all the necessary

components for NO and cGMP action are present in the striatum.

The cGMP enzymes have been immunohistochemically localized to the post-synaptic compartment of asymmetric Type I axospinous and axosomatic synapses on medium sized spiny neurons (Ariano and Matus, 1981, Ariano, 1983, 1984). Cholinergic interneurons do not have cGMP-, GC-, or cGPDE-immunoreactivity (IR) (Ariano, 1984). cGMP and associated enzymes are found in aspiny neurons so NADPH neurons may also contain the cGMP system. Double-labelling studies would be necessary to confirm coexistence with NADPH-diaphorase. More detailed electron microscopy studies found GC-IR in the amorphous web of the post-synaptic active region and cGMP-IR, cGPDE-IR, and cGPK-IR in the post-synaptic dendroplasm (Ariano, 1983). Presynaptic terminals were not immunoreactive for the cGMP system (Nakane et al., 1983).

Cholinergic Stimulation of NO Release in the Striatum

Acetylcholine and muscarinic agonists increase cGMP levels in the striatum by stimulating GC (Hanley and Iverson, 1978). Activation of GC probably involves NO and NADPH-diaphorase neurons. Cholinergic innervation of striatal neurons comes from intrinsic large cholinergic interneurons (Ariano, 1983, Izzo and Bolam, 1988, Phelps and Vaughn, 1986). All cholinergic terminals in the striatum form Type II symmetrical synapses so they cannot be directly activating the cGMP system found in Type I asymmetrical synapses (Izzo and Bolam, 1988, Phelps and Vaughn, 1986, Phelps et al., 1985). Cholinergic terminals innervate dendritic shafts most often, followed by dendritic spines and cell bodies. The axosomatic synapses innnervate primarily medium spiny neurons where they provide excitatory input (Gauchy et al., 1991, Phelps et al., 1985, Izzo and Bolam, 1988). Non-cholinergic cell bodies with characteristics unlike medium spiny neurons,

also receive cholinergic terminals (Phelps et al., 1985). These neurons may be NADPH-diaphorase neurons. Dendritic shafts in the neuropil which receive cholinergic terminals may also belong to NADPH-diaphorase neurons. Autoradiographic studies employing ^{3}H-QNB in the striatum have localized muscarinic receptors to substance P-containing medium spiny neurons and somatostatin-containing NADPH-diaphorase neurons (Ariano, 1985, Ariano and Kenny, 1989). Muscarinic receptors were found nearly twice as often associated with somatostatin neurons as with substance P neurons (Ariano and Kenny, 1989). Therefore, it appears NADPH-diaphorase neurons receive a strong cholinergic input via muscarinic receptors.

Cholinergic activation of GC in striatal slices is calcium dependent (Hanley and Iverson, 1978). ACh and muscarinic agonists probably activate NADPH-diaphorase neurons by raising intracellular calcium levels. For the reasons explained above, NO formed in NADPH-diaphorase neurons probably does not stimulate significant levels of their own GC, if GC exists at all in these neurons.

Enkephalins and morphine also increased cGMP levels in neostriatal slices probably via NO (Minneman and Iverson, 1976). It is also likely that glutamate increases and GABA decreases cGMP formation via NO in the neostriatum as they do in the cerebellum (Wood, 1991).

Release of NO in the Striatum

Type I asymmetrical synapses in the striatum receive axons primarily from the neocortex, substantia nigra, and intralaminar nuclei of the thalamus (Ariano, 1983). None of the axons from these regions are from NADPH-diaphorase neurons since these regions either do not contain NADPH-diaphorase neurons or have only local circuit NADPH-diaphorase

interneurons which do not project to other regions. Therefore, the NO which stimulates GC activity in the striatum must come from intrinsic striatal NADPH-diaphorase interneurons.

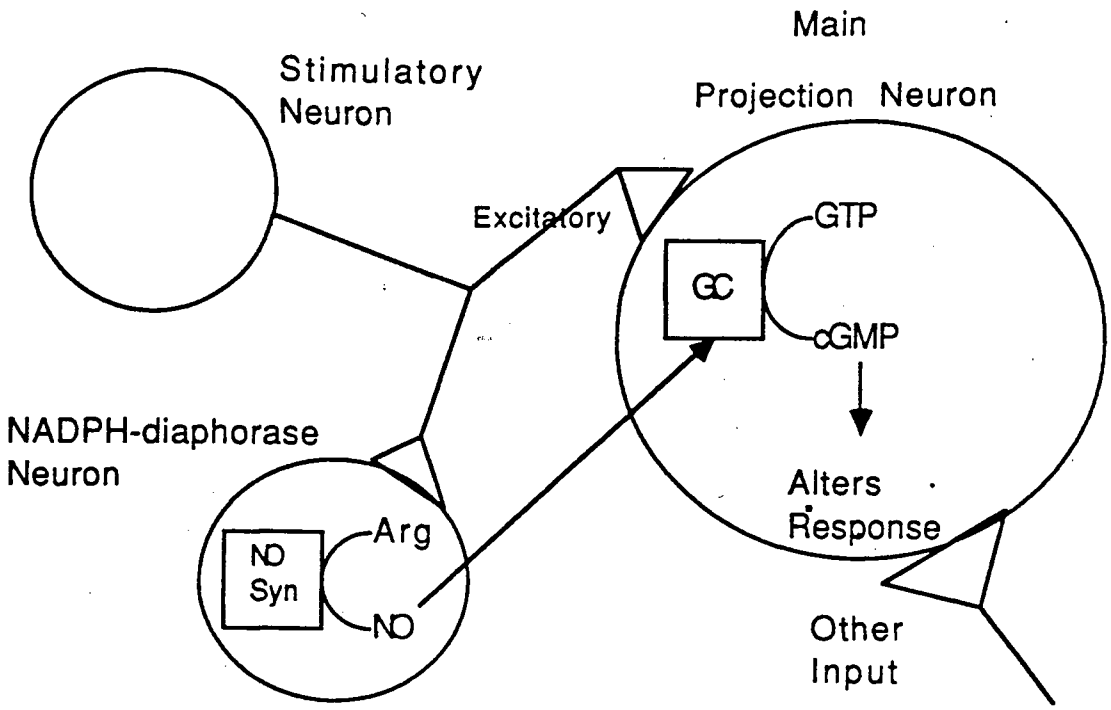
A small proportion of presynaptic terminals on Type I asymmetrical synapses of medium spiny neurons are from intrinsic striatal neurons (Ariano, 1983). It is thus possible for NO to be released directly from presynaptic terminals of NADPH-diaphorase neurons onto GC-containing post-synaptic sites. This mechanism resembles NANC nerves in the periphery in that NO acts like a neurotransmitter released from terminals. As with peripheral NANC nerves, NO would be released non-vesicularly from the presynaptic terminals. However, since intrinsic neurons provide only a small proportion of the terminals of Type I asymmetrical synapses, the majority of GC-containing synapses cannot receive NO input by direct synaptic release.

NO is more likely to be released diffusely to all surrounding GC-containing synapses, thereby serving a three-dimensional region of the striatum with NO input. Since NADPH-diaphorase activity appears throughout the neuron, it may be possible for NO to be released non-vesicularly from various parts of the cell, including terminals, cell bodies, and processes.

Cyclic GMP and thus NO alter post-synaptic responsiveness to further inputs in the neocortex, hippocampus, and cerebellum (see below). A similar action may occur in the striatum. Acetylcholine is excitatory to neurons in the striatum, therefore cholinergic neurons may excite both medium spiny neurons and NADPH-diaphorase neurons via muscarinic receptors (Ariano and Kenny, 1989). NO released from striatal NADPH-diaphorase neurons may then activate GC in the postsynaptic complexes of medium-sized spiny neurons and modify post-synaptic responsiveness to afferent inputs from the neocortex and other brain regions (see figure 19).

Figure 19

The actions of NO may be similar at the local circuit level in the striatum, neocortex, hippocampus, and cerebellum. In the case of the first three, acetylcholine simultaneously excites the main projection neurons of the region while activating NADPH-diaphorase interneurons. The NADPH-diaphorase interneurons release NO which diffuses to the postsynaptic compartments of surrounding neurons, including those of the main projection neurons. Soluble GC in the main projection neurons increases membrane resistance which increases the response to other inputs (Woody et al., 1986, Woody and Gruen, 1987, Doerner and Alger, 1988). The cerebellar cortex uses glutamate or aspartate as the corresponding neurotransmitter. Responsiveness of the cerebellar cortex main projection neurons to other inputs decreases in response to NO unlike that in the other brain regions (Shibuki and Okada, 1991). The stimulatory neurons mentioned in the figure are only those presently shown to lead to cGMP increases in the particular region. It is likely that other inputs, particularly glutamate inputs, to each region can lead to similar NO release and increases in cGMP formation.



Region	Stimulatory Neuron	Main Projection Neuron
Striatum	Cholinergic Interneurons	Medium-sized Spiny Neurons
Neocortex	Basal Forebrain Cholinergic Neurons	Pyramidal Neurons
Hippocampus	Medial Septum and Diagonal Band (Cholinergic)	Pyramidal Neurons
Cerebellum	Climbing Fibers from the Inferior Olive (Glutamate or Aspartate)	Purkinje Neuron

This pattern of NO synthase activation and NO action is repeated in the neocortex, hippocampus, and cerebellum (see Figure 19). Incoming fibers simultaneously activate both the main projection neurons of the region as well as surrounding inhibitory interneurons. The main projection neurons are directly excited while the interneurons are activated to produce NO. NO diffuses to post-synaptic targets in the main projection neurons to regulate their responsiveness to further input (see below).

Cerebral Cortex

Although the cerebral cortex has more GC than the cerebellum, basal and stimulated cGMP levels are lower, due to the much higher levels of the hydrolyzing enzyme cGPDE (Greenberg et al., 1978, Kinscherf et al., 1976, Ferrendelli, 1990). Similar to the striatum, high levels of GC and cGPDE may lead to a relatively rapid cGMP signal. Significant levels of cGPK and its G-substrate have also been detected in the cortex (Schlichter et al., 1980, Detre et al., 1984). Intense cGMP-IR (Greenberg et al., 1978, Ariano et al., 1982, Sakaue et al., 1988), GC-IR (Ariano et al., 1982, Nakane et al., 1983) and GC activity (Davidoff and Dimitrov, 1989) have been detected primarily in post-synaptic compartments, with none detected in presynaptic compartments or in axons.

Similar to the striatum, cGMP levels in the cerebral cortex and hippocampus increase after acetylcholine stimulation of muscarinic receptors (Lee et al., 1972, Ferrendelli et al., 1970, Hoffer et al., 1977). Acetylcholine innervation of the neocortex comes primarily from the basal forebrain nucleus basalis of Meynert (Semba et al., 1990). Muscarinic agonists, in a calcium dependent manner, increase membrane resistance in the pyramidal cells of layers III and V in the motor cortex of awake cats, probably by

decreasing a voltage-dependent K^+ conductance (Woody and Gruen, 1987, Swartz and Woody, 1984, Woody et al., 1986). This effect is inhibited by injection of cGMP antibodies, presumably lowering cGMP levels (Swartz and Woody, 1984). Membrane resistance of these pyramidal neurons is increased by injection of purified cGPK, or by the addition of dibutyryl cGMP to cortical slices (Woody et al., 1986, Woody and Gruen, 1987).

NADPH-diaphorase, and thus NO synthase, is not found in cortical pyramidal neurons. Since soluble GC is probably activated by NO from NADPH-diaphorase neurons, the acetylcholine stimulation of GC in cortical slices should involve NADPH-diaphorase neurons surrounding pyramidal neurons. When muscarinic agonists are applied to cortical slices, pyramidal neurons rapidly hyperpolarize and then undergo a slow depolarization (McCormick and Prince, 1985). The rapid hyperpolarization is due to inhibitory input from GABA interneurons which are rapidly depolarized by muscarinic agonists. Most, if not all NADPH-diaphorase neurons in the neocortex are GABAergic (Aoki and Pickel, 1990, Kowall and Beal, 1988). Thus muscarinic agonists also depolarize NADPH-diaphorase neurons. Assuming this results in an increase in intracellular calcium levels in NADPH-diaphorase neurons, NO synthase will be activated. This is similar to the striatum in that the main projection neurons are excited directly by a neurotransmitter which simultaneously activates surrounding interneurons to produce NO. The NO formed diffuses to the post-synaptic compartment of pyramidal neurons probably to modify post-synaptic responsiveness to other inputs (see Figure 19). It is not known whether the NO-mediated increase in membrane resistance contributes to the slow depolarization seen following addition of muscarinic agonists to cortical slices.

Hippocampus

Cholinergic input to the hippocamus is largely from the medial septum. Hippocampal pyramidal neurons in CA1 slices increase their membrane resistance after application of muscarinic agonists (Cole and Nicoll, 1984). This results in depolarization and increased cell firing. After-hyperpolarization currents (I_{AHP}) are also depressed. Both effects are due to a decrease in a calcium-activated K^+ conductance.

Activation of muscarinic receptors on CA1 pyramidal neurons depresses voltage-dependent Ca^{2+} channels (Doerner and Alger, 1988). This effect can be mimicked by extracellular addition of dibutyryl cGMP or 8-Br-cGMP or by intracellular injection of cGMP. This effect does not involve cGPK since the potent protein kinase inhibitors H7 and H8 do not inhibit this effect. Doerner and Alger (1988) suggest the cGMP-activated phosphodiesterase is stimulated by increased cGMP levels to hydrolyze cAMP. The decrease in cAMP levels results in less activation of the voltage-dependent calcium channel. Less calcium could then decrease the calcium-activated K^+ conductance mentioned above, resulting in increased cell firing and less I_{AHP}.

Neuronal NADPH-diaphorase activity is only moderate in the CAI region of the hippocampus, similar to moderate NO synthase-IR in the region (Bredt and Snyder, 1990). Still, the same mechanism for ACh and NO action in the neocortex may exist in the hippocampus. Acetylcholine may activate muscarinic receptors on both pyramidal neurons and NADPH-diaphorase containing interneurons (McCormick and Prince, 1985). While the pyramidal neurons are directly excited, NADPH-diaphorase neurons are activated to produce NO (see figure 19). NO may also be released from cholinergic NADPH-diaphorase fibers of the septo-hippocampal pathway (Vincent and Pasqualotto, 1991, Kinjo et al., 1989). The NO produced diffuses to postsynaptic

compartments in pyramidal neurons to activate their soluble GC. Increased cGMP increases membrane resistance and decreases after-hyperpolarizations, which leads to increased post-synaptic responsiveness. If the net input to pyramidal neurons is depolarizing, then increased post-synaptic responsiveness would help depolarize pyramidal neurons and increase cell firing as seen after application of muscarinic agonists.

NMDA has also been shown to increase cGMP levels in the hippocampus (Garthwaite, 1991). This increase was blocked by the NO synthase inhibitors NMMA and LNNA.

Cerebellum

The anatomy of the cGMP enzyme system and NO synthase has been most thoroughly investigated in the cerebellum. NADPH-diaphorase activity is not very prominent in the cerebellum. Basal cGMP levels in the cerebellum are ten times higher than in any other brain region (Greenberg et al., 1978, Kinscherf et al., 1976, Ferrendelli, 1990, Lohmann et al., 1981). Potassium-induced depolarization increases cGMP levels in the cerebellum 28 times and only 2.5 to 5 times in all other brain regions (Kinscherf et al., 1976). The cerebellum has only half the GC activity found in the striatum (Greenberg et al., 1978). The cerebellum, however, has very low levels of cGPDE relative to that in the striatum (Greenberg et al., 1978, Ferrendelli, 1990). Low levels of cGPDE would explain the large basal and stimulated cGMP levels found in the cerebellum. Correspondingly, the cGMP response in the cerebellum is probably less rapid than in the neocortex, hippocampus and striatum.

Prominent cGMP-IR (Nakane et al., 1990, Lohmann et al., 1981, Sakaue et al., 1988) and GC-IR (De Vente et al., 1989, Ariano et al., 1982, Zwiller et al., 1981, Nakane et al., 1983) were found in all cell types of the cerebellar cortex,

including Bergman glial cells and astrocytes. GC-immunoreactivity of astrocytes was twice that of Purkinje cells. This agrees with the findings of Garthwaite et al. (1987) that the majority of biochemical GC activity is in glial cells.

Intense cGPK-IR was found in Purkinje cells and perivascular non-neuronal cells. Lower levels of cGPK-IR were detected in all other cells (Walaas et al., 1989, Schlichter et al., 1980). In addition, Purkinje cells contained intense G-substrate-IR (Schlichter et al., 1990, Detre et al., 1984). The G-substrate is a 23 kD soluble protein which is phosphorylated specifically by cGPK. It has similarities to the phosphatase-1 inhibitor which, in other brain regions, is phosphorylated by cAMP-dependent protein kinase. Thus, all cells in the cerebellar cortex may be involved in the action of NO and cGMP.

When climbing fibers are stimulated, they simultaneously excite Purkinje cells in the cerebellar cortex as well as stimulate NO formation (Shibuki and Okada, 1991). The excitatory response of climbing fiber stimulation on Purkinje cells can be inhibited by CNQX while NO release is unaffected. Basket cells and granule cells, but not Purkinje cells, are NO synthase-IR (Bredt et al., 1990) and NADPH-diaphorase positive (Vincent and Kimura, 1991). Therefore, it appears that the mechanism described for NO action in the striatum, neocortex, and hippocampus may be repeated in the cerebellum (see Figure 19). The basket cell interneurons surrounding Purkinje cells are simultaneously activated to release NO which activates the GC in Purkinje cell post-synaptic compartments. This results in an alteration in post-synaptic responsiveness of the Purkinje cell to other inputs. Granule cells are also directly activated by climbing fibers (Wood, 1991). Granule cells can also produce NO in response to climbing fiber stimulation and activate GC in surrounding Purkinje neurons. In the case of the cerebellum there is

actual evidence for a functional alteration of post-synaptic responsiveness, called long term depression (Shibuki and Okada, 1991). Unlike the previously described brain regions, post-synaptic responsiveness in the cerebellum is decreased via NO. This will be discussed further along with general functions of NO action in the brain.

Brainstem

The brainstem has levels of GC activity comparable to that in the neocortex and hippocampus (Greenberg et al., 1978). Like the striatum, it also has some of the highest levels of cGPDE. Again this would allow for relatively rapid cGMP signalling. The cholinergic NADPH-diaphorase neurons of the brainstem innervate the thalamus (Vincent and Kimura, 1991, Kayama and Ogawa, 1987, Mesulam et al., 1989). NO released from these neurons may regulate cGMP in the thalamus. Atrial natriuretic peptide (ANP) released from these same neurons may also regulate cGMP in the thalamus via particulate guanylate cyclase (Standaert et al., 1986). NO released from NADPH-diaphorase neurons in the brainstem may affect surrounding neurons such as the locus ceruleus. This possibility requires an examination of GC-IR in neurons of the locus ceruleus and the rest of the brainstem.

Olfactory Bulb

Unfortunately, there is no literature on cGMP in the olfactory bulb. There is both strong NADPH-diaphorase activity and NO synthase-IR in this region (Bredt et al., 1990). NO synthase-IR was highest in the granule cell layer, which is consistent with the presence of NADPH-diaphorase-containing deep short axon neurons and their fibers in the granule cell layer (Scott et al., 1987, Gall et al., 1986). The more superficial NADPH-diaphorase-containing

short axon neurons send a dense fiber network to surround granule cells. Thus, at the light microscopic scale, most of the NADPH-diaphorase activity and NO synthase-IR should appear in the granule cell layer. This also suggests granule cells should have a prominent cGMP system. Therefore, NO may modulate granule cells and olfactory bulb output.

Retina

The cGMP system of the retina is of two forms (Ferrendelli, 1990). The first system in the rod outer segments employs a light-activated membrane-bound cGPDE not found in the rest of the brain. Lower cGMP levels during light activation decrease tonic cGMP-dependent activation of depolarizing Na^+ conductances, which effectively hyperpolarizes the cell. These photoreceptors do not contain soluble GC, but do contain a cytoskeletal form which is not sensitive to NO. NADPH-diaphorase, and thus NO synthase, is found in human cone photoreceptors (Provis and Mitrofanis, 1990). NO released from these cells may stimulate soluble GC in either the outer plexiform layer of the retina (see below) or the adjacent retinal epithelium.

The cGMP system in the inner retina is different from that in photoreceptors. Soluble GC provides the majority of GC activity in the inner retina (Ferrendelli, 1990). All layers of the retina contain GC-IR, particularly the inner plexiform layer where one also finds NADPH-diaphorase positive amacrine cells (Ferrendelli and De Vries, 1983, Berkelmans et al., 1989). After application of sodium nitroprusside to the retina, the largest cGMP-IR increases are found in amacrine cells. It is not known if these include NADPH-diaphorase containing cells. Large increases were also seen in the inner nuclear layer, and inner and outer plexiform layers. Since GC-IR and cGMP-IR are highest in the inner plexiform layer, NO released from NADPH-diaphorase

amacrine cells may modulate post-synaptic responsiveness of ganglion cells to bipolar neuron input, similar to the mechanism suggested for the neocortex and striatum.

Photoreceptor cells decrease cGMP levels in response to light while the inner retina increases cGMP levels (Ferrendelli, 1990, Ferrendelli and De Vries, 1983, Nawy and Jahr, 1990). However, depolarizing bipolar cells, which are inhibited by glutamate, decrease cGMP in response to light (Nawy and Jahr, 1990). Glutamate application onto these cells activates a phosphodiesterase which hydrolyzes cGMP leading to deactivation of a tonically inward current. This is similar to the effect of light activation in photoreceptors. Depolarizing bipolar neurons are thus not likely to be affected by NO from NADPH-diaphorase positive amacrine cells.

Pineal Gland

In addition to the cerebellum, the pineal gland provides another example where the distribution of GC activity does not correlate well with that of NADPH-diaphorase staining. NADPH-diaphorase activity was not found in rat pineal glands by Sato (1990a). There was also no mention of NO synthase-IR in the pineal gland (Bredt et al., 1990). Vincent and Kimura (1991), however, found a few scattered NADPH-diaphorase positive fibers in the pineal gland, possibly from sensory or sympathetic ganglia. Additionally, cGMP levels increase in the rat pineal gland during adrenergic stimulation (Seidel et al., 1990). cGMP levels are highest when the rat is awake and lowest during slow-wave sleep, which agrees with the changes in cGMP levels in the rest of the brain (Ogasahara et al., 1981). SNP stimulates GC in the gland which suggests NO may be mediating the adrenergic stimulation and diurnal regulation of cGMP levels (Berkelmans et al., 1989).

General Functions of NO in the Brain

Acetylcholine, NO, NADPH-diaphorase, and cGMP in Arousal.

Acetylcholine release and cGMP levels in the neocortex and brainstem change during the sleep-wake cycle (Ogasahara et al., 1981). Acetylcholine and cGMP levels are low during slow-wave sleep while levels are high during wakefulness. This may relate to the function of caudal cholinergic/NADPH-diaphorase neurons in the ARAS, which is involved in arousal. In addition to innervating the thalamus, the caudal cholinergic neurons innervate the basal forebrain cholinergic neurons which provide the majority of cholinergic innervation to the neocortex (Kowall and Mueller, 1988, Mesulam et al., 1989, Consolo et al., 1990, Semba et al., 1990). As mentioned in the introduction, the basal forebrain neurons may be a rostral extension of the ARAS found in the brainstem. The basal forebrain cholinergic neurons could activate neocortical muscarinic receptors leading to NO production. The subsequent increase in cGMP levels would increase membrane resistance and excitability of pyramidal neurons. The neurons could thus be "aroused" to be more responsive to afferent input. The NO component of arousal may complement both the sensory facilitation and the blockade of thalamocortical activity, which leads to desynchronization of cortical EEG, that occur during stimulation of the caudal cholinergic neurons (Semba et al., 1990, Kayama et al., 1986). NO and ANP released from the brainstem cholinergic NADPH-diaphorase neurons probably regulate cGMP levels in the thalamus (see introduction) and may be involved in the blockade of thalamocortical activity.

NO would be a good messenger for a generalized phenomenon such as arousal since its release is not tightly localized like most neurotransmitters but can spread to activate GC in a large number of surrounding neurons. This

would decrease the need for a large number of cholinergic terminals to perform the same job. Activated NADPH-diaphorase interneurons in the neocortex may be responsible for "arousing" their own local group of surrounding pyramidal neurons.

EAA input to the neocortex, or any other brain region, normally activates a brain region while GABA inhibits. If these neurotransmitters regulate NO and cGMP in the neocortex, as they do in the cerebellum (Wood, 1991), then an increase in neuronal activity due to EAA should correspond with an increase in NO release and a decrease in neuronal activity due to GABA should correspond with a decrease in NO release. Again membrane resistance and excitability within an activated region should increase with increased formation of NO and cGMP and increase responsiveness to further input.

Long Term Potentiation (LTP)

Stevens (1988) had proposed NO may be involved in LTP. There are two temporal stages in LTP. The first stage is a transient component lasting about one-half hour and is dependent on presynaptic changes while the second stage is longer lasting and dependent on post-synaptic changes (Stevens, 1989). The presynaptic changes of the first stage can occur by applying NMDA agonists to the post-synaptic complex without stimulation of the afferent axon and pre-synaptic complex. Apparently, a retrograde signal from the post-synaptic complex can facilitate pre-synaptic responses to axonal stimulation. The mechanism proposed (Stevens, 1988) is based on the demonstration by Garthwaite et al. (1988) of EDRF/NO release upon NMDA stimulation. Activated NMDA receptors, long known to be involved in LTP, allow calcium to enter the post-synaptic compartment and activate NO synthase. The NO formed is capable of diffusing back to the pre-synaptic complex where it can facilitate

pre-synaptic responsiveness by an unknown mechanism. This mechanism, however, requires soluble GC in presynaptic terminals which do not appear to contain GC (see above). As well, the classic model of LTP in the hippocampus would require NO synthase in CA1 and CA3 pyramidal cells. NADPH-diaphorase activity (Vincent and Kimura, 1991) and NO synthase-IR (Bredt et al., 1990) are not found in these neurons. Therefore, NO is unlikely to be involved in presynaptic facilitation during LTP. Arachidonic acid appears to be a better candidate for the retrograde signal (Gally et al., 1990).

Long Term Depression (LTD)

There is better evidence for the involvement of NO in LTD in the cerebellar cortex (Shibuki and Okada, 1991). When climbing fibers from the inferior olive are stimulated they release NO that can be detected by an electrochemical probe in the molecular layer. When parallel fibers are activated at nearly the same time as climbing fibers, the quisqualate receptors of the parallel fiber-Purkinje cell synapses are desensitized. Desensitization is inhibited by hemoglobin and NMMA. SNP applied to the cerebellar slice preparation substitutes for climbing fiber activation in LTD. Injection of EGTA, a calcium chelator, into Purkinje cells blocks LTD (Ito, 1989) suggesting there is a NO synthase in Purkinje cells. However, NO synthase-IR is not detected in Purkinje cells but rather in stellate and basket cells (Bredt et al., 1990). Garthwaite et al. (1987) also found Purkinje cells in suspension do not produce NO. CNQX, a quisqualate receptor antagonist, inhibits excitatory postsynaptic currents in response to climbing fiber stimulation but does not inhibit LTD (Shibuki and Okada, 1991). Shibuki and Okada (1991) propose climbing fibers activate basket cells, as well as directly excite Purkinje cells, to produce NO which activates the GC found prominently in dendritic spines of

Purkinje cells (see Figure 19). Raised cGMP levels lead to desensitization and LTD via an unknown mechanism. The blocking of LTD by the injection of EGTA into Purkinje cells may indicate that another calcium-dependent, but NO-independent step, is involved in LTD.

LTD is not limited to the cerebellum (Stevens, 1990). A different form of LTD is found in the hippocampus (Artola et al., 1990). This form does not appear to involve NO but it does suggest LTD may be found in regions other than the cerebellar cortex. In other brain regions LTD may involve NO and thus probably NADPH-diaphorase neurons.

NO and Activity-Dependent Synaptic Competition

Activity-dependent synaptic competition during neural development may involve LTD (Shatz, 1990) and thus NO and NADPH-diaphorase neurons. Gally et al. (1990) have suggested a model for how NO and thus NO synthase and NADPH-diaphorase may be involved in this function.

NO and Autoinhibition of ACh Release

cGMP, and thus also NO and NADPH-diaphorase neurons, may mediate ACh autoinhibition in the neocortex (Yonehara et al., 1980) and hippocampus (Nordstrom and Bartfai, 1981) through activation of muscarinic receptors. Addition of atropine, a muscarinic antagonist, leads to an increase in ACh release even when TTX is added to the slice preparation. There are thus no long inhibitory loops inhibiting ACh release but rather a direct inhibition via presynaptic muscarinic receptors.

A decrease in Ca^{2+} influx may underly autoinhibition of ACh release. Potassium-evoked $^{45}Ca^{2+}$-influx into cortical slices is inhibited by the addition of dibutyryl-cGMP (Ichida et al., 1981). The authors suggest this is due to

decreased Ca^{2+} influx into nerve endings. Decreased levels of presynaptic Ca^{2+} would result in decreased vesicle release. Direct muscarinic activation of GC in presynaptic terminals leading either to ACh autoinhibition or Ca^{2+} influx is unlikely, however, since little, if any, of the cGMP system is found in presynaptic terminals (Davidoff and Dimitrov, 1989). Therefore, it is difficult to understand how cGMP, and indirectly NO synthase/NADPH-diaphorase, can be involved in ACh autoinhibition and calcium influx into nerve endings.

NO May Regulate Tyrosine Hydroxylase Activity

Sodium nitroprusside (SNP) can activate tyrosine hydroxylase (TH) activity in rat striatal synaptosomes (Roskowski and Roskowski, 1987, Roskowski et al., 1987). cGPK phosphorylates the same site on TH that is phosphorylated by cAMP-dependent protein kinase (PKA). Since the protein inhibitor of PKA does not inhibit cGMP-dependent phosphorylation of TH or the subsequent increase in TH activity seen in PC12 cells, PKA is not involved (Roskowski and Roskowski, 1987). This may underly the acetylcholine-dependent increase in dopamine release in the striatum. Acetylcholine interneurons may activate NO synthase in NADPH-diaphorase neurons to raise striatal cGMP levels to regulate dopamine synthesis and release. However, since GC- and cGPK-IR are not found in presynaptic terminals (Ariano, 1983, Nakane et al., 1983) it is difficult to see how cGPK may directly phosphorylate TH in dopaminergic terminals and regulate enzyme activity in vivo.

Excitotoxicity

Nitric oxide, released from NADPH-diaphorase neurons may be involved in the neuronal excitotoxicity mentioned in the introduction. Excessive stimulation by glutamate or its analogs can lead to cell death which can be

blocked by glutamate antagonists such as APV-7 and MK-801. Since excitotoxicity is calcium-dependent, excitotoxicity has been hypothesized to involve excessive receptor activation causing an excessive inflow of calcium (Choi, 1988). This would lead to activation of autolytic calcium-dependent proteases, deregulation of the calcium/calmodulin signaling pathway because of calcium saturation, and general ionic imbalances.

NO synthase in the brain is activated by calcium. As with the NO released from macrophages and polymorphonuclear cells, excessive NO formation in the brain could lead to NO binding to metabolic enzymes leading to cell death. Indeed, kainic acid-induced excitotoxicity in cerebellar granule cell cultures was partially inhibited by the NO synthase inhibitor, NMMA, while nitroprusside potentiated the toxic effect (Lyons et al., 1990). If NO does have a toxic effect in the brain it is difficult to understand how the NADPH-diaphorase neurons which produce NO are relatively spared from its toxicity. Moncada et al. (1988, 1989) suggest NO can react with the toxic oxygen radicals formed during excitotoxicity and thus protect cells. Alternatively, since NO is a free radical the cells producing it may have metabolic enzymes for dealing with excess free radical generation. This seems likely since superoxide dismutase, which plays a part in metabolizing free radicals, has been localized immunohistochemically to both somatostatin neurons, which contain NADPH-diaphorase, and cholinergic neurons of the striatum (Inagaki et al., 1991). Both sets of striatal neurons are relatively spared from cell death during excitotoxicity and Huntington's disease (Beal et al., 1991).

Astrocytes

Glial cells contain much of the GC in the cerebellar cortex (Garthwaite et al., 1987). cGMP- and GC-IR are found in Bergmann glia surrounding

Purkinje cells and in astrocytes of the cerebellar cortex (Ariano, 1981, De Vente et al., 1989, Chan-Palay and Palay, 1979, Cumming et al., 1979, Zwiller et al., 1981, Nakane et al., 1983). NO released from the cerebellar cortex raises cGMP levels primarily in glial cells (Garthwaite et al., 1987, 1988). Astrocytes in the striatum, neocortex, and corpus callosum also contain cGMP- and GC-IR (Ariano and Matus, 1981, Cumming et al., 1979, Nakane et al., 1983, Davidoff and Dimitrov, 1989). NADPH-diaphorase neurons are found in these regions so it is likely that NO produced in NADPH-diaphorase neurons can activate GC in surrounding astrocytes. It is not known which glial cell functions are mediated by this mechanism.

Astrocytes in culture also produce NO (Murphy et al., 1990). When these astrocytes were stimulated by bradykinin, A23187, quisqualate, or norepinephrine, NO release was detected by its chemiluminescence with ozone. The factor from astrocytes caused relaxation of arterial rings which could be inhibited by the addition of hemoglobin or the arginine analogs, LNNA and NMMA. Therefore, astrocytes may interact with the cGMP signalling system of neurons and play an active role in neurotransmission by releasing the second messenger NO. It should be noted, however, that neither NO synthase-IR (Bredt et al., 1990) nor NADPH-diaphorase activity appears in glial cells. Therefore, the NO synthase in astrocytes must be different from that in neurons.

Blood Vessels

Cerebral blood vessels may also be regulated by NO. Ten to fifteen percent of cerebral blood vessels in the rat brain have smooth muscle in their walls (Toda and Okamura, 1990). Soluble GC-IR, cGMP-IR (Ariano, 1983), and cGPK-IR (Lohmann et al., 1981), were all found in smooth muscle cells while

none were found in endothelial cells. The distribution of NO synthase-IR in cerebral endothelial cells suggests NO may regulate blood flow in the brain, as it does in the periphery (Bredt et al., 1990). NADPH-diaphorase activity, however, was weak in cerebral blood vessels and appeared to be different from that found in neurons.

Alternatively, NO may be released from NANC nerves onto cerebral blood vessels to regulate blood flow. Activity-dependent relaxation of cerebral arteries occurs after electrical stimulation of NANC nerves in the brain (Toda and Okamura, 1990, Toda et al., 1990b). Cholinergic and noradrenergic antagonists did not attenuate this response. Hemoglobin, methylene blue, NMMA, and LNNA inhibited relaxation. Derivatives of NO were detected during electrical stimulation of the nerves. Denudation of endothelial cells did not diminish arterial relaxation while TTX did inhibit relaxation. This clearly supports NO being released from active nerve terminals onto blood vessels to relax vascular smooth muscle. NO may therefore mediate activity-dependent cerebrocortical blood flow.

Blood flow has been measured in the basal forebrain during intravenous infusion of the NO synthase inhibitor, LNNA (Arneric et al., 1990). Under resting conditions the inhibitor had no significant effect, but when basal forebrain neurons were electrically stimulated, there was a frequency dependent increase in blood flow which could be attenuated by LNNA.

The moderate amount of NADPH-diaphorase staining of blood vessels may be related to terminals surrounding the vessels or to NO synthase in the endothelial cells themselves. In any case, it is possible that neural activity in a particular brain region also activates NADPH-diaphorase neurons. NADPH-diaphorase neurons then release NO which relaxes surrounding blood vessels to increase local blood flow.

cGMP and the Blood Brain Barrier

Endothelial cells are the main site of the blood-brain barrier (Karnushina et al., 1980). Unlike the endothelium in other organs, the endothelial cells form tight junctions to each other and do not normally undergo pinocytosis. Cyclic GMP increases permeability of this barrier to macromolecular transport by increasing pinocytosis (Joo et al., 1983). The guanylate cyclase of endothelial cells is not the soluble form (Ariano, 1983) and is activated by calcium (Joo et al., 1983). ANP is capable of regulating cGMP in isolated cerebral microvessels so it is likely the endothelial guanylate cyclase is the particulate ANP receptor-guanylate cyclase (Homayoun et al., 1989). Therefore, it is unlikely that NADPH-diaphorase neurons regulate endothelial permeability via NO.

Summary

NADPH-diaphorase is a histochemical marker for a NO synthase. NADPH-diaphorase now has a function and NO synthase now has a known detailed anatomical distribution. Until now, NO only had a function at the cellular level, but our results allow us to speculate about its function at the local circuit level and in the brain as a whole. Neurotransmitter stimulation raises calcium levels in NADPH-diaphorase neurons to activate NO synthase. The second messenger, NO, diffuses through cell membranes to stimulate soluble guanylate cyclase and cGMP-stimulated phosphodiesterase in the post-synaptic compartments of surrounding neurons. In this way NADPH-diaphorase neurons may provide a localized region with NO input. NO probably alters post-synaptic responsiveness of surrounding neurons, including short-term changes in membrane conductances and long-term changes in synaptic efficacy.

The ability of NADPH-diaphorase neurons to produce NO could make them the "switch" that activates localized regions for action, including routing of blood flow to the region and increasing the effectiveness of inputs to surrounding neurons. Without these important functions the brain could not operate efficiently. It therefore appears that NADPH-diaphorase plays a vital role in brain function.

REFERENCES

Abe, T., Yamada, Y., Hashimoto, P.H., Shimuzu, N. (1963) Histochemical study of glucose-6-phosphate dehydrogenase in the brain of normal adult rat. Med. J. Osaka Univ. **14**:67-78.

Adler, E., Von Euler, H., Gunther, G. (1939) Diaphorase I and II. Nature **143**:641-642.

Aimi, Y., Fujimura, M., Vincent, S.R., Kimura, H. (1991) Localization of NADPH-diaphorase containing neurons in sensory ganglia of the rat. J. Comp. Neurol. **306**:382-392.

Aisaka, K., Gross, S.S., Griffith, O.W., Levi, R. (1989a) L-arginine availability determines the duration of acetylcholine-induced systemic vasodilation in vivo. Biochem. Biophys. Res. Comm. **163**:710-717.

Aisaka, K., Gross, S.S., Griffith, O.W., Levi, R. (1989b) N^G-methylarginine, an inhibitor of endothelium-derived nitric oxide synthesis, is a pressor agent in the guinea pig: does nitric oxide regulate blood pressure in vivo. Biochem. Biophys. Res. Comm. **160**:881-886.

Altman, F.P. (1969) The use of eight different tetrazolium salts for a quantitative study of pentose shunt dehydrogenation. Histochemie **19**:363-374.

Altman, F.P. (1974) Studies on the reduction of tetrazolium salts. III. The products of chemical and enzymatic reduction. Histochemistry. **38**:155-171.

Altman, F.P. (1976a) Tetrazolium salts and formazans. Progr. Histochem. Cytochem. **9**:1-56.

Altman, F.P. (1976b) The quantification of formazans in tissue sections by microdensitometry. II. The use of BPST, a new tetrazolium salt. Histochem. J. **8**:501-506.

Altman, F.P. (1976c) The quantification of formazans in tissue sections by microdensitometry. III. The effect of objective power and scanning spot size. Histochem. J. **8**:507-511.

Altman, F.P. (1977) Quantitative microscopy of enzyme reactions in tissue sections. Microsc. Acta **79**:327-338.

Amezcua, J.L., Palmer, R.M.J., DeSouza, B.M., Moncada, S. (1989) Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. Br. J. Pharmacol. **97**:1119-1124.

Aoki, C. and Pickel V.M. (1990) Neuropeptide Y in cortex and striatum-Ultrastructural distribution and coexistence with classical neurotransmitters and neuropeptides. Ann. N.Y. Acad. Sci. **611**:186-205.

Araki, M., McGeer, P.L., McGeer, E.G. (1985) Differential effect of kainic acid on somatostatin, GABAergic, cholinergic neurons in the rat striatum. Neurosci. Lett. **53**:197-202.

Archer, S.L., Tolins, J.P., Raij, L., Weir, E.K. (1989) Hypoxic pulmonary vasoconstriction is enhanced by inhibition of the synthesis of an endothelium derived relaxing factor. Biochem. Biophys. Res. Comm. **164**:1198-1205.

Ariano, M.A. (1983) Distribution of components of the guanosine 3',5'-phosphate system in rat caudate-putamen. Neuroscience **10**:707-723.

Ariano, M.A. (1984) Rat striatal cyclic nucleotide-reactive cells and acetylcholinesterase reactive interneurons are separate populations. Brain Res. **296**:160-163.

Ariano, M.A. (1985) Muscarinic acetylcholine receptors are localized on striatal cyclic GMP-containing neurons. Brain Res. **334**:152-156.

Ariano, M.A. and Kenny, S.L. (1989) Striatal muscarinic receptors are associated with substance P and somatostatin containing neurons. Brain Res. **497**:51-58.

Ariano, M.A. and Matus, A.I. (1981) Ultrastructural localization of cyclic GMP and cyclic AMP in rat striatum. J. Cell Biol. **91**:287-292.

Ariano, M.A., Lewicki, J.A., Brandwein, H.J., Murad, F. (1982) Immunohistochemical localization of guanylate cyclase within neurons of rat brain. Proc. Natl. Acad. Sci. USA **79**:1316-1320.

Arneric, S.P., Linville, D.G., Kerwin, J.F., Murad, F. (1990) Nitric oxide (NO): Its role in mediating increases in cortical cerebral blood flow (CBF) elicited by electrical stimulation of the basal forebrain (BF). Neurosci. Abstr. **16**:129.11.

Arnold, W.P., Mittal, C.K., Katsuki, S., Murad, F. (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc. Natl. Acad. Sci. USA **74**:3203-3207.

Artola, A., Brocher, S., Singer, W. (1990) Different voltage-dependent thresholds for inducing long-term and long-term potentiation in slices of rat visual cortex. Nature **347**:69-72.

Barrnett, R.J. and Seligman, A.M. (1954) Histochemical demonstration of sulfhydryl and disulfide groups of protein. J. Nat. Canc. Inst. **14**:769-803.

Beal, M.F., Ferrante, R.J., Swartz, K.J., Kowall, N.W. (1991) Chronic quinolinic acid lesions in rats closely resemble Huntington's disease. J. Neurosci. **11**:1649-1659.

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

Beal, M.F., Kowall, N.W., Swartz, K.J., Ferrante, R.J. (1990) Homocysteic acid lesions in rat striatum spare somatostatin-neuropeptide Y (NADPH-diaphorase) neurons. Neurosci. Lett. **108**:36-42.

Beal, M.F., Kowall, N.W., Swartz, K.J., Ferrante, R.J., Martin, J.B. (1988) Systemic

approaches to modifying quinolinic acid striatal lesions in rats. J. Neurosci. 8:3901-3908.

Beal, M.F., Kowall, N.W., Swartz, K.J., Ferrante, R.J., Martin, J.B. (1989) Differential sparing of somatostatin-neuropeptide Y and cholinergic neurons following striatal excitotoxin lesions. Synapse 3:38-47.

Beavo, J.A. and Reifsnyder, D.H. (1990) Primary sequence of nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharm. 11:150-155.

Becker, N.H. (1961) The cytochemistry of anoxic and anoxic-ischemic encephalopathy. Am. J. Path. 38:587-594.

Berkelmans, H.S., Schipper, J., Hudson, L., Steinbusch, H.W.M., De Vente, J. (1989) cGMP immunocytochemistry in aorta, kidney, retina and brain tissues of the rat after perfusion with nitroprusside. Histochemistry. 93:143-148.

Bhattacharjee, J. (1977) Sequential differentiation of retinal cells in the mouse studied by diaphorase staining. J. Anat. 123:273-282.

Bishop, G.A., Chang, H.T., Kitai, S.T. (1982) Morphological and physiological properties of neostriatal neurons: An intracellular horseradish peroxidase study in the rat. Neuroscience 7:179-191.

Boeckxstaens, G.E., Pelckmans, P.A., Bult. H., De Man, J.G., Herman, A.G., Van Maercke, Y.M. (1990) Non-adrenergic non-cholinergic relaxation mediated by nitric oxide in the canine ileocolonic junction. Eur. J. Pharmacol. 190:239-246.

Boegman, R.J. and Parent, A. (1988) Differential sensitivity of neuropeptide Y, somatostatin and NADPH-diaphorase containing neurons in rat cortex and striatum to quinolinic acid. Brain Res. 445:358-362.

Boje, K.M. and Fung, H.-L. (1990) Endothelial nitric oxide generating enzyme(s) in the bovine aorta: Subcellular localization and metabolic characterization. J. Pharm. Exp. Therap. 253:20-26.

Branchek, T.A. and Gershon, M.D. (1989) Time course of expression of neuropeptide Y, calcitonin gene-related peptide, and NADPH-diaphorase activity in neurons of the developing murine bowel and the appearance of 5-hydroxytryptamine in mucosal enterochromaffin cells. J. Comp. Neurol. 285:262-273.

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., Snyder, S.H. (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347:768-770.

Briggs, R.T., Drath, D.B., Karnovsky, M.L., Karnovsky, M.J. (1975) Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. J. Cell Biol. **67**:566.

Brune, B. and Lapetina, E.G. (1989) Activation of a cytosolic ribosyltransferase by nitric oxide-generating agents. J. Biol. Chem. **264**:8455-8458.

Bult, H., Boeckxstaens, G.E., Pelckmans, P.A., Jordaens, F.H., Van Maercke, Y.M., Herman, A.G. (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature **345**:346-347.

Burgess, G.M., Mullaney, I., McNeill, M., Coote, P.R., Minhas, A., Wood, J.N. (1989) Activation of guanylte cyclase by bradykinin in rat sensory neurones is mediated by calcium flux: possible role of the increase in cyclic GMP. J. Neurochem. **53**:1212-1218.

Butcher, R.G. and Altman, F.P. (1973) Studies on the reduction of tetrazolium salts. II. The measurement of the half reduced and fully reduced formazans of neotetrazolium chloride in tissue sections. Histochemie **37**:351-363.

Chan-Palay, V. and Palay, S.L. (1979) Immunocytochemical localization of cyclic GMP: Light and electron microscope evidence for involvement of neuroglia. Proc. Natl. Acad. Sci. USA **76**:1485-1488.

Choi, D.W. (1988) Calcium-mediated neurotoxicity: Relationship to specific channel types and role in ischemic damage. Trends Neur. **11**:465-469.

Cobcroft, M., Vaccaro, T., Mitrofanis, J. (1989) Distinct patterns of distribution among NADPH-diaphorase neurones of the guinea pig retina. Neurosci. Lett. **103**:1-7.

Cohen, M.R., Sridhara, N., Ramchand, C.N. (1987) Kainic acid induced damage to the striatum increases DT diaphorase activity. Res. Comm. Chem. Path. Pharmacol. **57**:239-248.

Cole, A.E. and Nicoll, R.A. (1984) The pharmacology of cholinergic excitatory responses in hippocampal pyramidal cells. Brain Res. **305**:283-290.

Collier, J. and Vallance, P. (1989) Second messenger role for NO widens to nervous and immune system. Trends Pharm. **10**.

Consolo, S., Bertorelli, R., Forloni, G.L., Butcher, L.L. (1990) Cholinergic neurons of the pontomesencephalic tegmentum release acetylcholine in the basal nuclear complex of freely moving rats. Neuroscience **37**:717-723.

Cromlish, J.A. and Flynn, T.G. (1985) Identification of pig brain aldehyde reductases with the high-K_m aldehyde reductase, the low K_m aldehyde reductase and aldose reductase, carbonyl reductase, and succinic semialdehyde reductase. J. Neurochem. **44**:1485-1493.

Cromlish, J.A., Yoshimoto, C.K., Flynn, T.G. (1985) Purification and characterization of four NADPH-dependent aldehyde reductases from pig brain. J. Neurochem. **44**:1477-1484.

Croul-Ottman, C. and Brunjes, P.C. (1988) NADPH diaphorase staining within the developing olfactory bulbs of normal and unilaterally odor-deprived rats. Brain Res. **460**:323-328.

Cubero, A., Martin, M., Ros, M. (1991) Presence of metabotropic glutamate receptors and phospholipase C activity in bovine brain coated vesicles. J. Neurochem. **57(Suppl.)**:S140.

Cumming, R., Arbuthnott, G., Steiner, A. (1979) Characterization of immunofluorescent cyclic GMP-positive fibres in the central nervous system. J. Cyclic Nucl. Res. **5**:463-467.

Davidoff, M. and Dimitrov, N. (1989) Electron microscopical localization of guanylate cyclase activity in the neocortex of the guinea pig. Acta Histochem. **85**:109-116.

Davies, S.W. and Roberts, P.J. (1987) No evidence for preservation of somatostatin-containing neurons after intrastriatal injections of quinolinic acid. Nature **327**:326-329.

Davies, S.W. and Roberts, P.J. (1988) Sparing of cholinergic neurons following quinolinic acid lesions of the rat striatum. Neuroscience **26**:387-393.

Davies, S.W., Kuonen, D.R., Roberts, P.J. (1985) Biochemical demonstration of an enzyme responsible for NADPH-dep diaphorase. Neurosci. Lett. Suppl. **21**:S81.

De Quidt, M.E. and Emson, P.C. (1986) Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system--II. Immunohistochemical analysis. Neuroscience **18**:545-618.

De Vente, J., Bol, J.G.J.M., Steinbusch, H.W.M. (1989a) Localization of cGMP in the cerebellum of the adult rat: An immunohistochemical study. Brain Res. **504**:332-337.

De Vente, J., Schipper, J., Steinbusch, H.W.M. (1989b) Formaldehyde fixation of cGMP in distinct cellular pools and their recognition by different cGMP-antisera. Histochemistry **91**:401-412.

Deana, R., Ruzzene, M., Doni, M.G., Zoccarato, F., Alexandre, A. (1989) Cyclic GMP and nitroprusside inhibit the activation of human platelets by fluoroaluminate. Biochim. Biophys. Acta **1014**:203-206.

Defendi, V. and Pearson, B. (1955) Quantitative estimation of succinic dehydrogenase activity in a single microscopic section. J. Histochem. Cytochem. **3**:61-69.

Deguchi, T. and Yoshioka, M. (1982) L-arginine identified as an endogenous activator for soluble guanylate cyclase from neuroblastoma cells. J. Biol. Chem. **257**:10147-10151.

Deguchi, Y. (1963) A histochemical method for demonstrating protein-bound sulfhydryl and disulfide groups with nitro blue tetrazolium. J. Histochem.

Cytochem. **12**:261-265.

DeSouza, E.B. (1987) Corticotropin-releasing factor receptors in the rat central nervous system: characterization and regional distribution. J. Neurosci. 7:88-100.

DeSouza, E.B., Insel, T.R., Perrin, M.H., Rivier, J., Vale, W.W., Kuhar, M.J. (1985) Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: and autoradiographic study. J. Neurosci. **5**:3189-3203.

Detre, J.A., Nairn, A.C., Aswad, D.W., Greengard, P. (1984) Localization in mammalian brain of G-substrate, a specific substrate for guanosine 3',5'-cyclic monophosphate-dependent protein kinase. J. Neurosci. **4**:2843-2849.

Doerner, D. and Alger, B.E. (1988) Cyclic GMP depresses hippocampal Ca^{2+} current through a mechenism independent of cGMP-dependent protein kinase. Neuron **1**:693-699.

Duckett, S. and Pearse, A.G.E. (1964) The nature of the solitary active cells of the central nervous system. Experentia **20**:259-260.

Duckett, S. and Pearse, A.G.E. (1967) Histoenzymology of the developing human basal ganglia. Histochemie **8**:334-341.

Eadie, M.J., Tyrer, J.H., Kukums, J.R., Hooper, W.D. (1970) Aspects of tetrazolium salt reduction relevant to quantitative histochemistry. Histochemie **21**:170-180.

East, S.J. and Garthwaite, J. (1990) Nanomolar NG-nitroarginine inhibits NMDA-induced cyclic GMP formation in rat cerebellum. Eur. J. Pharmacol. **184**:311-313.

Ellison, D.W., Kowall, N.W., Martin, J.B. (1987) Subset of neurons characterized by the presence of NADPH-diaphorase in human substantia innominata. J. Comp. Neurol. **260**:233-245.

Farber, E., Sternberg, W.H., Dunlap, C.E. (1956) Histochemical localization of specific oxidative enzymes I. Tetrazolium stains for diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diapohorase. J. Histochem. **4**:254-265.

Felgenhauer, K. and Stammler, A. (1962) Das verteilungsmuster der dehydrogenasen und diaphorasen im zentralnervensystem des meerschweinchens. Zeitschrift fur Zellforschung **58**:219-233.

Ferrante, R.J., Kowall, N.W., Beal, M.F., Martin, J.B., Bird, E.D., Richardson, E.P., Jr. (1987) Morphologic and histochemical characteristics of a spared subset of striatal neurons in Huntington's disease. J. Neuropath. Exp. Neur. **46**:12-27.

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

Ferrendelli, J.A. (1990) Distribution and regulation of cyclic GMP in the central nervous system. Adv. Cyclic Nucl. Res. **9**:453-464.

Ferrendelli, J.A. and De Vries, G.W. (1983) Cyclic GMP systems in the retina. Fed. Proc. **42**: 3103-3106.

Ferrendelli, J.A., Kinscherf, D.A., Chang, M.M. (1973) Regulation of levels of guanosine cyclic 3',5'-monophosphate in the central nervous system. Mol. Pharmacol. **9**:445-454.

Ferrendelli, J.A., Steiner, A.L., McDougal, D.B., Jr., Kipnis, D.M. (1970) The effect of oxotremorine and atropine on cGMP and cAMP levels in mouse cerebral cortex and cerebellum. Biochem. Biophys. Res. Comm. **41**:1061-1067.

Ferriero, D.M., Arcavi, L.J., Sagar, S.M., McIntosh, T.K., Simon, R.P. (1988) Selective sparing of NADPH-diaphorase neurons in neonatal hypoxia-ischemia. Ann. Neurol. **24**:670-676.

Ferriero, D.M., Arcavi, L.J., Simon, R.P. (1990) Ontogeny of excitotoxic injury to nicotinamide adenine dinucleotide phosphate diaphorase reactive neurons in the neonatal rat striatum. Neuroscience **36**:417-424.

Findlay, G.H. (1954) The value of some tetrazolium salts as histochemical reagents fo sulfhydryl groups. J. Histochem. Cytochem. **3**:331-338.

Fiscus, R.R., Rapoport, R.M., Murad, F. (1984) Endothelium-dependent and nitrovasdilator-induced activation of cyclic GMP-dependent protein kinase in rat aorta. J. Cyc. Nucl. Prot. Phosph. Res. **3**:415-425.

Flynn, T.G. (1982) Aldehyde reductases: Monomeric NADPH-dependent oxidoreductases with multifunctional potential. Biochem. Pharmacol. **31**:2705-2712.

Forstermann, U., Gorsky, L.D., Pollock, J.S., Ishii, K., Schmidt, H.H.H.W., Heller, M., Murad, F. (1990a) Hormone-induced biosynthesis of endothelium-derived relaxing factor/nitric oxide-like material in N1E-115 neuroblastoma cells requires calcium and calmodulin. Mol. Pharmacol. **38**:7-13.

Forstermann, U., Gorsky, L.D., Pollock, J.S., Schmidt, H.H.W., Heller, M., Murad, F. (1990b) Regional distribution of EDRF/NO-synthesizing enzyme(s) in rat brain. Biochem. Biophys. Res. Comm. **168**:727-732.

Forstermann, U., Ishii, K., Gorsky, L.D., Murad, F. (1989) The cytosol of N1E-115 neuroblastoma cells synthesizes an EDRF-like substance that relaxes rabbit aorta. Arch. Pharmacol. **340**:771-774.

Forstermann, U., Pollock, J.S., Schmidt, H.H.H.S., Heller, M., Murad, F. (1991) Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. Proc. Natl. Acad. Sci. **88**:1788-1792.

Fortes, Z.B., Oliveira, M.A., Scivoletto, R., Carvalho, M.H.C., De Nucci, G., Nigro, D. (1990) Nitric oxide release may be involved in the microcirculatory response to acetylcholine. Eur. J. Pharmacol. **182**:143-147.

Fukuto, J.M., Wood, K.S., Byrns, R.E., Ignarro, L.J. (1990) N^G-amino-arginine: a new potent antagonist of L-arginine-mediated endothelium-dependent relaxation. Biochem. Biophys. Res. Comm. **168**:458-465.

Furchgott, R.F. (1984) The role of endothelium in the responses of vascular smooth muscle to drugs. Ann. Rev. Pharm. Tox. **24**:175-197.

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

Furchgott, R.F. and Zawadski, J.V. (1980) The obligatory role of endithelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature **288**:373-376.

Gall. C., Seroogy, K.B., Brecha, N. (1986) Distributin of VIP- and NPY-like immunoreactivities in rat main olfactory bulb. Brain Res. **374**:389-394.

Gally, J.A., Montague, P.R., Reeke, G.N., Jr., Edelman, G.M. (1990) The NO hypothesis: Possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system. Proc. Natl. Acad. Sci. U.S.A. **87**:3547-3551.

Garbers, D.L. (1989) Cyclic GMP and the second messenger hypothesis. Trends Endo. **1**:64-67.

Garthwaite, J. (1991) Glutamate, nitric oxide and cell-cell signalling in the nervous system. Trends Neur. **14**:60-67.

Garthwaite, J. and Garthwaite, G. (1987) Cellular origins of cyclic GMP responses to excitatory amino acid receptor agonists in rat cerebellum in vitro J. Neurochem. **48**:29-39.

Garthwaite, J., Charles, S.L., 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., Southam, E., Anderton, M. (1989a) A kainate receptor linked to nitric oxide synthesis from arginine. J. Neurochem. **53**:1952-1954.

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

Gauchy, C., Desban, M., Krebs, M.O., Glowinski, J., Kemel, M.L. (1991) Role of dynorphin-containing neurons in the presynaptic inhibitory control of the acetylcholine-evoked release of dopamine in the striosomes and the matrix of the cat caudate nucleus. Neuroscience. **41**:449-458.

Gibson, A., Mirzazadeh, S., Hobbs, A.J., Moore, P.K. (1990) L-NG-monomethyl arginine and L-N^G-nitro arginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus nuscle. Br. J. Pharmacol. **99**:602.

Gillespie, J.S., Liu, X., Martin, W. (1989) The effects of L-arginine and N^G-monomethyl-L-arginine on the response of the rat anococcygeus muscle to NANC stimulation. Br. J. Pharmacol. **98**:1080-1082.

Giuditta, A. and Aloj, E. (1965) Pathways of oxidation of reduced nicotinamide nucleotides in the central nervous system of marine animals. J. Neurochem. **12**:567-579.

Giuditta, A. and Aloj, E. (1967) Soluble NADH and NADPH dehydrogenases in the optic lobes of Sepia officinalis--II. Dicumarol-insensitive enzymes. J. Neurochem. **14**:967-975.

Giuditta, A. and Strecker, H.J. (1961) Purification and some properties of a brain diaphorase. Biochim. Biophys. Acta **48**:10-19.

Glick, D. and Nayyar, S.N. (1956) Studies in histochemistry: XLII: Further studies on the determination of succinic dehydrogenase in microgram amounts of tissue and distribution of the activity in the bovine adrenal. J. Histochem. Cytochem. **4**:389-396.

Gold, M.E., Bush, P.A., Ignarro, L.J. (1989a) Depletion of arterial L-arginine causes reversible tolerance to endothelium-dependent relaxation. Biochem. Biophys. Res. Comm. **164**:714-721.

Gold, M.E., Wood, K.S., Buga, G.M., Byrns, R.E., Ignarro, L.J. (1989b) L-arginine causes whereas L-arginosuccinic acid inhibits endothelium-dependent vascular smooth muscle relaxation. Biochem. Biophys. Res. Comm. **161**:536-543.

Greenberg, L.H., Troyyer, E., Ferrendelli, J.A., Weiss, B. (1978) Enzymatic regulation of the concentration of cyclic GMP in mouse brain. Neuropharmacol. **17**:737-745.

Haglund, L., Kohler, C., Haaparanta, T., Goldstein, M., Gustafsson, J.-A. (1984) Presence of NADPH-cytochrome P450 reductase in central catecholaminergic neurones. Nature **307**:259-262.

Hall, J.M., Lind, C., Golvano, M.P., Rase, B., Ernster, L. (1972) DT-diaphorase--Reaction mechanism and metabolic function. In: A. Akeson and A. Ehreberg (Eds.) **Structure and function of oxidation-reduction enzymes. Wenner-Gren International Symposium.** Elmsford, New York: Pergamon Press: **18**:433-443, .

Hanley, M.R. and Iversen, L.L. (1978) Muscarinic cholinergic receptors in rat corpus striatum and regulation of guanosine cyclic 3',5'-monophosphate. Mol. Pharmacol. **14**:246-255.

Hecker, M., Mitchell, J.A., Harris, H.J., Katsura, M., Thiemermann, C., Vane, J.R. (1990a) Endothelial cells metabolize N^G-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. Biochem. Biophys. Res. Comm. **167**:1037-1043.

Hecker, M., Sessa, W.C., Harris, H.J., Anggard, E.E., Vane, J.R. (1990b) The metabolism of L-arginine and its significance for the biosynthesis of

endothelium-derived relaxing factor. Proc. Natl. Acad. Sci. USA **87**:8612-8616.

Hess, R. and Pearse, A.G.E. (1963) Pathways of reduced pyridine nucleotide oxidation in rat-brain homogenate demonstrated by a tetrazolium method. Biochim. Biophys. Acta **71**:285-294.

Hibbs, J.B. ,Jr., Taintor, R.R., Vavrin, Z. (1987) Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Nature **235**:473-476.

Hobbs, A.J. and Gibson, A. (1990) L-N^G-nitro-arginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus. Br. J. Pharmacol. **100**:749-752.

Hoffer, B., Seiger, A., Freedman, R., Olson, L., Taylor, D. (1977) Electrophysiology and cytology of hippocampal formation transplants in the anterior chamber of the eye. II. Cholinergic mechanisms. Brain Res. **119**:107-132.

Homayoun, P., Lust, W.D., Harik, S.I. (1989) Effect of several vasoactive agents on guanylate cyclase activity in isolated rat brain microvessels. Neurosci. Lett. **107**:273-278.

Husain, M. and Massey, V. (1978) Reversible resolution of flavoproteins into apoproteins and free flavins. In: S. Fleischer, L. Packer (Eds.) **Methods in enzymology.** New York: Academic Press; **53**:429-437.

Hsu, S.-M., Rawo, L., Fanger, H. (1981) Use of the avidin-biotin-peroxidase complex (ABC) in immunperoxidase technique. J. Histochem. Cytochem. **29**:577-580.

Ichida, S., Osugi, T., Noguchi, Y., Yoshida, H. (1981) Potassium stimulated $^{45}Ca^{2+}$ uptake by cortical slices of rat brain: Effects of cyclic nucleotide derivatives. Brain Res. **213**:472-475.

Ignarro, L.J. (1989a) Endothelium-derived nitric oxide: actions and properties. FASEB **3**:31-36.

Ignarro, L.J. (1989b) Endothelium-derived nitric oxide: Pharmacology and relationship to the action of organic nitrate esters. Pharm. Res. **6**:651-659.

Ignarro, L.J. (1990a) Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu. Rev. Pharm. Tox. **30**:535-560.

Ignarro, L.J. (1990b) Haem-dependent activation of guanylate cyclase and cyclic GMP formation by endogenous nitric oxide: A unique transduction mechanism for transcellular signalling. Pharm. Tox. **67**:1-7.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G. (1987a) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA **84**:9265-9269.

Ignarro, L.J., Bush, P.A., Buga, G.M., Wood, K.S., Fukuto, J.M., Rajfer, J. (1990)

Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. Biochem. Biophys. Res. Comm. **170**:843-850.

Ignarro, L.J., Byrns, R.E., Buga, G.M., Wood, K.S. (1987b) Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacolgic and chemical properties identical to those of nitric oxide radical. Circul. Res. **61**:866-879.

Inagaki, S., Suzuki, K., Taniguchi, N., Takagi, H. (1991) Localization of Mn-superoxide dismutase (Mn-SOD) in cholinergic and somatostatin-containing neurons in the rat neostriatum. Brain Res. **549**:174-177.

Iscan, M.Y. and Arinc, E. (1988) Comparison of highly purified sheep liver and lung NADPH-cytochrome P-450 reductases by the analysis of kinetic and catalytic properties. Int. J. Biochem. **20**:1189-1196.

Ishii, K., Chang, B., Kerwin, J.F., Jr., Huang, Z.-J., Murad, F. (1990) N^G-nitro-L-arginine: a potent inhibitor of endothelium-derived relaxing factor formation. Eur. J. Pharmacol. **176**:219-223.

Ito, M. (1989) Long-term depression. Ann. Rev. Neurosci. **12**:85-102.

Izzo, P.N. and Bolam, J.P. (1988) Cholinergic synaptic input to different parts of spiny striatonigral neurons in the rat. J. Comp. Neurol. **269**:219-234.

Jarrett, A. (1962) A histochemical method for the demonstration of cysteine desulphurase. J. Histochem. Cytochem. **10**:400-401.

Joo, F., Temesvari, P., Dux, E. (1983) Regulation of the macromolecular transport in the brain microvessels: The role of cyclic GMP. Brain Res. **278**:165-174.

Kalina, M., Plapinger, R.E., Hoshino, Y., Seligman, A.M. (1972) Nonosmiophilic tetrazolium salts that yield osmiophilic lipophobic formazans for ultrastructural localization of dehydrogenase activity. J. Histochem. Cytochem. **20**:685-695.

Kapitulnik, J., Gelboin, H.V., Guenerich, F.P., Jacobowitz (1987) Immunohistochemical localization of cytochrome P-450 in rat brain. Neuroscience **20**:829-833.

Karnushina, I., Toth, I., Dux, E., Joo, F. (1980) Presence of the guanylate cyclase in brain capillaries: Histochemical and biochemical evidence. Brain Res. **189**:588-592.

Kauffman, F.C., Pickel, V.M., Sims, K.L., Bloom, F.E. (1974) Localization of nicotinamide adenine dinucleotide phosphate-dependent dehydrogenases in catecholamine-containing neurons of rat brain. J. Histochem. Cytochem. **22**:20-28.

Kawamata, T., Nakamura, S., Akiguchi, I., Kimura, J., Kameyama, M., Kimura, H., Takeda, T. (1990) Effect of aging on NADPH-diaphorase neurons in laterodorsal tegmental nucleus and striatum of mice. Neurobiol. of Aging **11**:185-192.

Kayama, Y. and Ogawa, T. (1987) Electrophysiology of ascending, possibly cholinergic neurons in the rat laterodorsal tegmental nucleus: comparison with monoamine neurons. Neurosci. Lett **77**:277-282.

Kayama, Y., Takagi, M., Ogawa, T. (1986) Cholinergic influence of the lateral tegmental nucleus on neuronal activity in the rat lateral geniculate nucleus. J. Neurophys. **56**:1297.

Kemp, M.C., Kuonen, D.R., Roberts, P.J. (1987) Kinetics of rat brain NADPH-diaphorase. Biochem. Soc. Trans. **15**:501.

Kemp, M.C., Kuonen, D.R., Sutton, A., Roberts, P.J. (1987) Rat brain NADPH-diaphorase utilises cytochrome c as substrate. **9**:695P.

Kemp, M.C., Kuonen, D.R., Sutton, A., Roberts, P.J. (1988) Rat brain NADPH-dependent diaphorase. Biochem. Pharmacol. **37**:3063-3070.

Kinjo, N., Skinner, R.D., Powell, E.W. (1989) A study of NADPH-diaphorase positive septohippocampal neurons in rat. Neurosci. Res. **7**:154-158.

Kinscherf, D.A., Chang, M.M., Rubin, E.H., Schneider, D.R., Ferrendelli, J.A. (1976) Comparison of the effects of depolarizing agents and neurotransmitters on regional CNS cyclic GMP levels in various animals. J. Neurochem. **26**:527-530.

Knowles, R.G., Palacios, M., Palmer, R.M.J., Moncada, S. (1989) Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. Proc. Natl. Acad. Sci. USA **86**:5159-5162.

Kobayashi, Y. and Hattori, K. (1990) Nitroarginine inhibits endothelium-derived relaxation. Japan. J. Pharmacol. **52**:167-169.

Koesling, D., Herz, J., Gausepohl, H., Niroomand, F., Hinsch, K.-D., Mulsch, A., Bohme, E., Schultz, G., Frank, R. (1988) The primary structure of the 70 kDa subunit of bovine soluble guanylate cyclase. FEBS **239**:29-34.

Koh, J.-Y. and Choi, D.W. (1988a) Cultured striatal neurons containing NADPH-diaphorase or acetylcholinesterase are selectively resistant to injury by NMDA receptor agonists. Brain Res. **446**:374-378.

Koh, J.-Y. and Choi, D.W. (1988b) Vulnerability of cultured cortical neurons to damage by excitotoxins: Differential susceptibility of neurons containing NADPH-diaphorase. J. Neurosci. **8**:2153-2163.

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

Kondo, K., Mitchell, J.A., DeNucci, G., Vane, J.R. (1989) Simultaneous measurement of endothelium-derived relaxing factor by bioassay and guanylate cyclase stimulation. Br. J. Pharmacol. **98**:630-636.

Kostergaard, J. and Leroux, M.E. (1989) L-arginine independent macrophage

tumor cytotoxicity. Biochem. Biophys. Res. Comm. **165**:1262-1266.

Kowall, N.W. and Beal, M.F. (1988) Cortical somatostatin, neuropeptide Y, and NADPH-diaphorase neurons: normal anatomy and alterations in Alzheimers disease. Ann Neur. **23**:105-114.

Kowall, N.W. and Mueller, M.P. (1988) Morphology and distribution of nicotinamide adenine dinucleotide phosphate (reduced form) diaphorase reactive neurons in human brainstem. Neuroscience **26**:645-654.

Kowall, N.W., Beal, M.F., Ferrante, R.J., Martin, J.B. (1985) Topography of nicotinamide adenine dinucleotide phosphate-diaphorase staining neurons in rat striatum. Neurosci. Lett. **59**:61-66.

Kowall, N.W., Ferrante, R.J., Beal, M.F., Richardson, R.P., Jr., Sofroniew, M.V., Cuello, A.C., Martin, J.B. (1987a) Neuropeptide Y, somatostatin, and reduced nicotinamide adenine dinucleotide phosphate diaphorase in the human striatum: A combined immunocytochemical and enzyme histochemical study. Neuroscience **20**:817-828.

Kowall, N.W., Ferrante, R.J., Martin, J.B. (1987b) Patterns of cell loss in Huntington's disease. Trends Neur. **10**:24-29.

Kowall, N.W., Pendlebury, W.W., Kessler, J.B., Perl, D.P., Beal, M.F. (1989) Aluminum-induced neurofibrillary degeneration affects a subset of neurons in rabbit cerebral cortex, basal forebrain and upper brainstem. Neuroscience **29**:329-337.

Kuhn, M., Otten, A., Frolich, J.C., Forstermann, U. (1991) Endothelial cyclic GMP and cyclic AMP do not regulate the release of endothelium-derived relaxing factor/nitric oxide from bovine aortic endothelial cells. J. Pharmacol. Therap. **256**:677-682.

Kun, E. and Abood, L.G. (1949) Colorimetric estimation of succinic dehydrogenase by triphenyl tetrazolium. Science **109**:144-146.

Kuonen, D.R., Kemp, M.C., Roberts, P.J. (1988) Demonstration and biochemical characterisation of rat brain NADPH-dependent diaphorase. J. Neurochem. **50**:1017-1025.

Kuwabara, T. and Cogan, D.G. (1959) Tetrazolium studies on the retina: III. Activity of metabolic intermediates and miscellaneous substrates. J. Hitochem. Cytochem. 8:214-224.

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

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

Le Goascogne, C., Robel, P., Gouezou, M., Sananes, N., Baulieu, E.-E., Waterman M (1987) Neurosteroids: Cytochrome P-450$_{scc}$ in rat brain. Science **237**:1212-

1215.

Leaf, C.D., Wishnok, J.S., Tannenbaum, S.R. (1989) L-arginine is a precursor for nitrate biosynthesis in humans. Biochem. Biophys. Res. Comm. **163**:1032-1037.

Lee, T.-P., Kuo, J.F., Greengard, P. (1972) Role of Muscarinic cholinergic receptors in regulation of guanosine 3':5'-cyclic monophosphate content in mammalian brain, heart muscle, and intestinal smooth muscle. Proc. Natl. Acad. Sci. USA **69**:3287-3291.

Levine, W., Guiditta, A., Englard, S., Strecker, H.J. (1960) Brain diaphorases. J. Neurochem. **6**:28-36.

Li, C.G. and Rand, M.J. (1989) Evidence for a role of nitric oxide in the neurotransmitter system mediating relaxation of the rat anococcygeus muscle. Clin. Exp. Pharm. Phys. **16**:933-938.

Li, C.G. and Rand, M.J. (1990) Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. Eur. J. Pharmacol. **191**:303-309.

Lippold, H.J. (1982) Quantitative succinic dehydrogenases histochemistry. Histochemistry **76**:381-405.

Lohmann, S.M., Walter, U., Miller, P.E., Greengard, P., De Camilli, P. (1981) Immunohistochemical localization of cyclic GMP-dependent protein kinase in mammalian brain. Proc. Natl. Acad. Sci. USA **78**:653-657.

Long, C.J. and Berkowitz, B.A. (1989) What is the relationship between the endothelium derived relaxant factor and nitric oxide. Life Sci. **45**:1-14.

Louis, J.C., Basset, P., Revel, M.O., Vincendon, G., Zwiller, J. (1990) Opposite effects of arachidonic acid and of its hydroperoxides on brain soluble guanylate cyclase activity. Neurochem. Int. **18**:131-135.

Lyons, W.E., Puttfarcken, P.S., Coyle, J.T. (1990) Effects of L-N^G-monomethylarginine and sodium nitroprusside on kainate mediated toxicity in rat cerebellar granule cell cultures. Neurosci. Abstr. **16**:89.10.

Mampalam, T.J., Gonzalez, M.F., Weinstein, P., Sharp, F.R. (1988) Neuronal changes in fetal cortex transplanted to ischemic adult rat cortex. J. Neurosurg. **69**:904-912.

Marletta, M.A. (1989) Nitric oxide: biosynthesis and biological significance. Trends Bioch. **14**:488-492

Martin, W., White, D.G., Henderson, A.H. (1988) Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. Br. J. Pharmacol. **93**:229-239.

Martius, C. (1963) Quinone reductases. In: P.D. Boyer, H. Lardy, K. Myrback (Eds.) **The enzymes.** New York:Academic Press; **7**:517-532.

Mayer, B. and Bohme, E. (1989) Ca^{2+}-dependent formation of an L-arginine-derived activator of soluble guanylyl cyclase in bovine lung. FEBS **256**:211-214.

Mayer, B., John, M., Bohme, E. (1990) Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. FEBS **277**:215-219.

Mayer, B., Schmidt, K., Humbert, P., Bohme, E. (1989) Biosynthesis of endothelium-derived relaxing factor: A cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. Biochem. Biophys. Res. Comm. **164**:678-685.

McCall, T.B., Boughton-Smith, N.K., Palmer, R.M.J., Whittle, B.J.R., Moncada, S. (1989) Synthesis of nitric oxide from L-arginine by neutrophils. Biochem. J. **261**:293-296

McCormick, D.A. and Prince, D.A. (1985) Two types of muscarinic response to acetylcholine in mammalian cortical neurons. Proc. Natl. Acad. Sci. U.S.A. **82**:6344-6348.

McKinney, M., Bolden, C., Smith, C., Johnson, A., Richelson, E. (1990) Selective blockade of receptor-mediated cyclic GMP formation in N1E-115 neuroblastoma cells by an inhibitor of nitric oxide synthesis. Eur. J. Pharmacol. **178**:139-140.

Mellgren, S.I. (1973) Distribution succinate-, A-glycerophosphate-, NADH-, and NADPH dehydrogenases (tetrazolium reductases) in the hippocampal region of the rat during postnatal development. Z. Zellforsch. **141**:347-373.

Mesulam, M.M., Geula, C., Bothwell, M.A., Hersh, L.B. (1989) Human reticular formation: Cholinergic neurons of the pedunculopontine and laterodorsal tegmental nuclei and some cytochemical comparisons to forebrain cholinergic neurons. J. Comp. Neurol. **281**:611-633.

Minami, Y. and Toda, N. (1989) Possible involvement of 5-lipoxygenase products in the generation of endothelium-derived relaxing factor. J. Pharm. Exp. **250**:1055.

Minneman, K.P. and Iversen, L.L. (1976) Enkephalin and opiate narcotics increase cyclic GMP accumulation in slices of rat neostriatum. Nature **262**:313-314.

Mitchell, J.A., Hecker, M., Anggard, E.E., Vane, J.R. (1990) Cultured endothelial cells maintain their L-arginine level despite the continuous release of EDRF. Eur. J. Pharmacol. **182**:573-576.

Mitrofanis, J. (1989) Development of NADPH-diaphorase cells in the rat's retina. Neursci. Lett. **102**:165-172.

Mizukawa, K., McGeer, P.L., Vincent, S.R., McGeer, E.G. (1988) Ultrastructure of reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase-positive neurons in the cat cerebral cortex, amygdala and caudate nucleus. Brain Res. **452**:286-292.

Moncada, S., Palmer, R.M.J., Gryglewski, R.J. (1986) Mechanism of action of some inhibitors of endothelium-derived relaxing factor. Proc. Natl. Acad. Sci. **83**:9164-9168.

Moncada, S., Palmer, R.M.J., Higgs, E.A. (1988a) The discovery of nitric oxide as the endogenous nitrovasodilator. Hypertension **12**:365-372.

Moncada, S., Palmer, R.M.J., Higgs, E.A. (1989a) Biosynthesis of nitric oxide from L-arginine. Biochem. Pharmacol. **38**:1709-1715.

Moncada, S., Palmer, R.M.J., Higgs, E.A. (1989b) The biological significance of nitric oxide formation from L-arginine. Biochem. Soc. Trans. **17**:642-644.

Moncada, S., Radomski, M.W., Palmer, R.M.J. (1988b) Endothelium-derived relaxing factor. Biochem. Pharmacol. **37**:2495-2501.

Mufson, E.J., Mash, D.C., Hersh, L.B. (1988) Neurofibrillary tangles in cholinergic pedunculopontine neurons in Alzheimer's disease. Ann. Neur. **24**:623-629.

Mulsch, A. and Busse, R. (1990) N^G-nitro-arginine (N^5-[imino(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. Arch. Pharmacol. **341**:143-147.

Mulsch, A., Bassenge, E., Busse, R. (1989) Nitric Oxide synthesis in endothelial cytosol: Evidence for a calcium-dependent and a calcium-independent mechanism. Arch. Pharmacol. **340**:767-770.

Murad, F., Waldman, S., Molina, C., Bennett, B., Leitman, D. (1987) Regulation and role of guanylate cyclase-cyclic GMP in vascular relaxation. In: M.C. Cabot and W.L. McKeehan (Eds.) **Mechanisms of signal transduction by hormones and growth factors.** New York:Alan R Liss.

Murphy, S., Minor, R.L., Jr., Welk, G., Harrison, D.G. (1990) Evidence for an astrocyte-derived vasorelaxing factor with properties similar to nitric oxide. J. Neurochem. **55**:349-351.

Myers, P.R., Minor, R.L., Jr., Guerra, R., Jr., Bates, J.N., Harrison, D.G. (1990) Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature **345**:161-163.

Nachlas, M.M., Walker, D.G., Seligman, A.M. (1958) The histochemical localization of triphosphopyridine nucleotide diaphorase. J. Biophys. Biochem. Cytol. **4**:467.

Nairn, A.C. and Greengard, P. (1983) Cyclic GMP-dependent protein phosphorylation in mammalian brain. Fed. Proc. **42**:3107-3113.

Nakamura, S. and Vincent, S.R. (1985) Acetylcholinesterase and somatostatin-immunoreactivity coexist in human neocortex. Neurosci. Lett. **61**:183-187.

Nakamura, S., Kawamata, T., Kimura, T., Akiguchi, I., Kameyama, M., Nakamura, N., Wakata, Y., Kimura, H. (1988) Reduced nicotinamide adenine dinucleotide phosphate-diaphorase histochemistry in the pontomesencephalic region of the human brainstem. Brain Res. **455**:144-147.

Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W., Murad, F. (1990) Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase from rat lung. J. Biol. Chem. **265**:16841-16845.

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

Nakane, M., Saheki, S., Kuno, T., Ishii, K., Murad, F. (1988) Molecular cloning of a cDNA coding for 70 kilodalton subunit of soluble guanylate cyclase from rat lung. Biochem. Biophys. Res. Comm. **157**:1139-1147.

Naslund, B.M.A., Glaumann, H., Warner, M., Gustafsson, J.-A., Hansson, T. (1988) Cytochrome P-450 b and c in the rat brain and pituitary gland. Mol. Pharmacol. **33**:31-37.

Nawy, S. and Jahr, C.E. (1990) Suppresion by glutamate of cGMP-activated conductance in retinal bipolar cells. Nature **346**:269-271.

Nordstrom, O. and Bartfai, T. (1981) 8-Br-cyclic GMP mimics activation of muscarinic autoreceptor and inhibits acetylcholine release from rat hippocampal slices. Brain Res. **213**:467-471.

O'Sullivan, A.J. and Burgoyne, R.D. (1990) Cyclic GMP regulates nicotine induced secretion from cultured bovine adrenal chromaffin cells: Rffects of 8-bromo-cyclic GMP, atrial natriuretic peptide, and nitroprusside (nitric oxide). J. Neurochem. **54**:1805-1808.

Ogasahara, S., Taguchi, Y., Wada, H. (1981) Changes in the levels of cyclic nucleotides in rat brain during the sleep-wakefulness cycle. Brain Res. **213**:163-171.

Ogawa, T., Kimoto, M., Sasaoka, K. (1989) Purification and properties of a new enzyme, N^G,N^G-dimethylarginine dimethylaminohydrolase, from rat kidney. J. Biol. Chem. **264**:10205-10209.

Okada, D. (1991) Cyclic GMP production induced by activation of metabotropic glutamate receptors in rat cerebellar slices. J. Neurochem. **57(Suppl.)**:S140.

Otterson, O.P. and Storm-Mathieson, J. (1984) Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immuncytochemical technique. J. Comp. Neurol. **229**:374-392.

Palacios, M., Knowles, R.G., Palmer, R.M.J., Moncada, S. (1989) Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem. Biophys. Res. Comm. **165**:802-809.

Palmer, R.M.J., Ashton, D.S., Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature **333**:664-666.

Palmer, R.M.J., Ferrige, A.G., Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature **327**:524-526.

Pearse, A.G.E. (1952) The histochemical demonstration of cystine-containing structures by methods involving alkaline hydrolysis. J. Histochem. Cytochem. **1**:460-468.

Pellat, C., Henry, Y., Drapier, J.-C. (1990) IFN-γ-activated macrophages: detection by electron paramagnetic resonance of complexes between L-arginine-derived nitric oxide and non-heme iron proteins. Biochem. Biophys. Res. Comm. **166**:119-125.

Phelps, P.E. and Vaughn, J.E. (1986) Immunocytochemical localization of choline acetyltransferase in rat ventral striatum: alight and electron microscopic study. J. Neurocyt. **15**:595-617.

Phelps, P.E., Houser, C.R., Vaughn, J.E. (1985) Immunocytochemical localization of choline acetyltransferase within the rat neostriatum: A correlated light and electron microscopic study of cholinergic neurons and synapses. J. Comp. Neurol. **238**:286-307.

Pou, W.S., Pou, S., Rosen, G.M., El-Fakahany, E.E. (1990) EDRF release is a common pathway in the activation of guanylate cyclase by receptor agonists and calcium ionophores. Eur. J. Pharmacol. **182**:393-394.

Provis, J.M. and Mitrofanis, J. (1990) NADPH-diaphorase neurones of human retinae have a uniform topographical distribution. Visual Neurosci. **4**:619-623.

Ramagopal, M.V. and Leighton, H.J. (1989) Effects of N^G-monomethyl-L-arginine on field stimulation-induced decreases in cytosolic Ca^{2+} levels and relaxation in the rat anococcygeus muscle. Eur. J. Pharmacol. **174**:297-299.

Ratner, S., Morell, H., Carvalho, E. (1960) Enzymes of arginine metabolism. Arch. Biochem. Biophys. **91**:280-289.

Reiser, G. (1990a) Endothelin and a Ca^{2+} ionophore raise cyclic GMP levels in a neuronal cell line via formation nitric oxide. Br. J. Pharmacol. **101**:722-726.

Reiser, G. (1990b) Mechanism of stimulation of cyclic-GMP level in a neuronal cell line mediated by serotonin ($5\text{-}HT_3$) receptors. FEBS **189**:547-552.

Roerig, D.L., Mascario Jr., L., Aust, S.D. (1972) Microsomal electron transport: tetrazolium reduction by rat liver microsomal NADPH-cytochrome c reductase. Arch. Biochem. Biophys. **153**:475.

Roskowski, R., Jr., and Roskowski, L.M. (1987) Activation of tyrosine hydroxylase in PC12 cells by the cyclic GMP and cyclic AMP second messenger systems. J. Neurochem. **48**:236-242.

Roskowski, R., Jr., Vulliet, P.R., Glass, D.B. (1987) Phosphorylation of tyrosine hydroxylase by cyclic GMP-dependent protein kinase. J. Neurochem. **48**:840-

845.

Sadasivudu, B. and Rao, T.I. (1976) Studies on functional and metabolic role of urea cycle intermediates in brain. J. Neurochem. **27**:785-794.

Sagar, S.M. (1986) NADPH diaphorase histochemistry in the rabbit retina. Brain Res. **373**:153-158.

Sagar, S.M. and Ferriero, D.M. (1987) NADPH diaphorase activity in the posterior pituitary: relation to neuronal function. Brain Res. **400**:348-352.

Sakaue, M., Kuno, T., Tanaka, C. (1988) Novel type of monoclonal antibodies against cyclic GMP and application to immunocytochemistry of the rat brain. Japan J. Pharmacol. **48**:47-56.

Sakharova, A.V., Salimova, N.B., Sakharov, D.A. (1979) Peculiar cells notable for very high activity of glucose-6-phosphate dehydrogenase in the mammalian medulla oblangata. A histochemical and electron microscopic study. Neuroscience **4**:1173-1177.

Sakuma, I., Stuehr, D.J., Gross, S.S., Nathan, C., Levi, R. (1988) Identification of arginine as a precursor of endothelium-derived relaxing factor. Proc. Natl. Acad. Sci. USA **85**:8664-8667.

Salvemini, D., DeNucci, G., Gryglewski, R.J., Vane, J.R. (1989) Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. Proc. Natl. Acad. Sci. USA **86**:6328-6332.

Sandell, J.H. (1984) The distribution of hexokinase compared to cytochrome oxidase and acetylcholinesterase in the somatosensory cortex and the superior colliculus of the rat. Brain Res. **290**:384-389.

Sandell, J.H. (1985) NADPH diaphorase cells in the mammalian inner retina. J. Comp. Neurol. **238**:466-472.

Sandell, J.H. (1986) NADPH diaphorase histochemistry in the macaque striate cortex. J. Comp. Neurol. **251**:388-397.

Sandell, J.H., Graybiel, A.M., Chesselet, M.-F. (1986) A new enzyme marker for striatal compartmentalization: NADPH diaphorase activity in the caudate nucleus and putamen of the cat. J. Comp. Neurol. **243**:326-334.

Sato, T. (1990a) Histochemical demonstration of NADPH-diaphorase activity in the pineal organ of the frog (Rana esculenta) but not in the pineal organ of the rat. Arch. Histol. Cytol. **53**:141-146.

Sato, T. (1990b) NADPH-diaphorase positive amacrine cells in the retinae of the frog (Rana eculenta) and pigeon (Columbia livia). Arch. Histol. Cytol. **53**:63-69.

Scherer-Singler, U., Vincent, S.R., Kimura, H., McGeer, E.G. (1983) Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. J. Neurosci. Meth. **9**:229-234.

Schlichter, D.J., Detre, J.A., Aswad, D.W., Chehrazi, B., Greengard, P. (1980) Localization of cyclic GMP-dependent protein kinase and substrate in mammalian cerebellum. Proc. Natl. Acad. Sci. USA **77**:5537-5541.

Schmidt, H.H.W., Wilke, P., Evers, B., Bohme, E. (1989) Enzymatic formation of nitrogen oxides from L-arginine in bovine brain cytosol. Biochem. Biophys. Res. Chem. **165**:284-291.

Schroder, H. and Schror, K. (1989) Cyclic GMP stimulation by vasopressin in LLC-PK1 kidney epithelial cells is L-arginine dependent. Arch. Pharmacol. **340**:475-477.

Schultz, P.J., Schorer, A.E., Raij, L. (1990) Effects of endotheliu-derived relaxing factor and nitric oxide on rat mesangial cells. Am. J. Physiol. **258**:F162-167.

Schultzberg, M., Segura-Aguilar, Lind, C. (1988) Distribution of DT-diaphorase in the rat brain: Biochemical and immunohistichemical studies. Neuroscience **27**:763-776.

Scott, J.W., McDonald, J.K., Pemberton, J.L. (1987) Short axon cells of the rat olfactory bulb display NADPH-diaphorase activity, neuropeptide Y-like activity, and somatostatin-like immunoreactivity. J. Comp. Neurol. **260**:378-391.

Seidel, A., Kantarjian, A., Vollrath, L. (1990) A possible role for cyclic guanosine monophosphate in the rat pineal gland. Neurosci. Lett. **110**:227-231.

Seligman, A.M. and Rutenberg, A.M. (1951) The histochemical demonstration of succinic dehydrogenase. Science **113**:317-320.

Semba, K., Reiner, P.B., Fibiger, H.C. (1990) Single cholinergic mesopontine tegmental neurons project to both the pontine reticular formation and the thalamus in the rat. Neuroscience **38**:643-654.

Shatz, C.J. (1990) Impulse activity and the patterning of connections during CNS development. Neuron **5**:745-756.

Shibuki, K. and Okada, D. (1991) Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. Nature **349**:326-328.

Shimuzu, N. and Abe, T. (1966) Histochemical studies of the brain with reference to glucose metabolism. Progr. Brain Res. **21A**:197-216.

Shu, S., Ju, G., Fan, L. (1988) The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Lett. **85**:169-171.

Sims, K.L., Kauffman, F.C., Johnson, E.C., Pickel, V.M. (1974) Cytochemical localization of brain nicotinamide adenine dinucleotide phosphate (oxidized)-dependent dehydrogenases. J. Histochem. Cytochem. **22**:7-19.

Singer, T.P., Kearney, E.B., Massey, V. (1956) Observations on the flavin moiety of succinic dehydrogenase. Arch. Biochem. Biophys. **60**:255.

Skinner, R.D., Conrad, C., Henderson, V., Gilmore, S.A., Garcia-Rill, E. (1989) Development of NADPH diaphorase-positive pedunculopontine nucleus neurons. Exp. Neurol. **104**:15-21.

Standaert, D.G., Saper, C.B., Rye, D.B., Wainer, B.H. (1986) Colocalization of atriopeptin-like immunoreactivity with choline acetyltransferase- and substance P-like immunoreactivity in the pedunculopontine and laterodorsal tegmental nuclei in the rat. Brain Res. **382**:163-168.

Stasch, J.-P., Kazda, S., Neuser, D. (1989) Different effects of ANP and nitroprusside on cyclic GMP extrusion of isolated aorta. Eur. J. Pharmacol. **174**:279-282.

Sternberg, W.H., Farber, E., Dunlap, C.E. (1956) Histochemical localization of specific oxidative enzymes: II Localization of diphosphopyridine nucleotide and triphosphopyridine nucleotide diaphorases and the succindehydrogenase system in the kidney. J. Histochem. Cytochem. **4**:266-283.

Stevens, C.F. (1988) A relaxing factor in the brain. Nature **336**:308-309.

Stevens, C.F. (1989) Strengthening the synapses. Nature **338**:460-461.

Stevens, C.F. (1990) A depression long awaited. Nature **347**:16.

Stivers, J.A., Skirboll, L.R., Long, R., Crawley, J.N. (1988) Anatomical analysis of frontal cortex sites at which carbachol induces motor seizures in the rat. Pharm. Biochem. Behav. **30**:129-136.

Study, R.E., Breakefield, X.O., Bartfai, T., Greengard, P. (1978) Voltage-sensitive calcium channels regulate guanosine 3',5'-cyclic monophosphate levels in neuroblastoma cells. Proc. Natl. Acad. Sci. USA **75**:6295-6299.

Stuehr, D.J., Gross, S.S., Sakuma, I., Levi, R., Nathan, C.F. (1989) Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. J. Exp. Med. **169**:1011-1020.

Summy-Long, J.Y., Salisbury, R., Marietta, M.P., Hartman, R.D., Weisz, J (1984) Pathways of hydrogen utilization from NADPH generated by glucose-6-phosphate dehydrogenase in circumventricular organs and the hypothalamo-neurohypophysial system: A cytochemical study. Brain Res. **294**:23-35.

Swartz, B.E. and Woody, C.D. (1984) Effects of intracellular antibodies to cGMP on responses of cortical neurons of awake cats to extracellular application of muscarinic agonists. Exp. Neurol. **86**:388-404.

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. (1961) The fine localization of dehydrogenases in the nervous system. Histochemie **2**:266-282.

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 Neuropath. 3:238-249.

Thomas, G., Cole, E.A., Ramwell, P.W. (1989) N^G-monomethyl L-arginine is a non-specific inhibitor of vascular relaxation. Eur. J. Pharmacol. **170**:123-124.

Toda, N. and Okamura, T. (1990) Possible role of nitric oxide in transmitting information from vasodilator nerve to cerebroarterial muscle. Biochem. Biophys. Res. Comm. **170**:308-313.

Toda, N., Baba, H., Okamura, T. (1990a) Role of nitric oxide in non-adrenergic, non-cholinergic nerve-mediated relaxation in dog duodenal longitudinal muscle strips. Japan J. Pharmacol. **53**:281-284.

Toda, N., Minami, Y., Okamura, T. (1990b) Inhibitory effects of L-N^G-nitro-arginine on the synthesis of EDRF and the cerebroarterial response to vasodilator nerve stimulation. Life Sci. **47**:345-351.

Tohda, M. and Nomura, Y. (1990) Serotonin stimulates both cytosolic and membrane-bound guanylate cyclase in NG108-15 cells. J. Neurochem. **55**:1800-1805.

Tremblay, J., Gerzer, R., Hamet, P. (1988) Cyclic GMP in cell function. Adv. Second Messenger Phosphoprotein Res. **22**:319-365.

Tucker, J.F., Brave, S.R., Charalambous, L., Hobbs, A.J., Gibson, A. (1990) L-N^G-nitro arginine inhibits non-adrenergic, non-cholinergic relaxations of guinea-pig isolated tracheal smooth muscle. Br. J. Pharmacol. **100**:663-664.

Tuls, J., Geren, L., Lambeth, J.D., Millett, F. (1987) The use of a specific fluorescence probe to study the interaction of adrenodoxin with adrenodoxin reductase and cytochrome P450 reductase$_{SCC}$. J. Biol. Chem. **262**:20-25.

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

Unger, J.W., McNeill, T.H., Lapham, L.L., Hamill, R.W. (1988) Neuropeptides and neuropathology in the amygdala in Alzheimers disease: relatioship between somatostatin, neuropeptide Y and subregional distribution of neuritic plaques. Brain Res. **452**:293-302.

Van Nooren, C.J.F. and Butcher, R.G. (1984) Histochemical localization of NADP-dependent dehydrogenase activity with four different tetrazolium salts. J. Histochem. Cytochem. **32**:998-1004.

Vaney, D.I. and Young, H.M. (1988) GABA-like immunoreactivity in NADPH-diaphorase amacrine cells of the rabbit retina. Brain Res. **474**:380-385.

Vesco, C. and Guiditta, A. (1966) Tetrazolium reductase activity of the enzymatic systems of oxidation of reduced nicotinamide nucleotides in mammalian brain. Biochim. Biophys. Acta **113**:197-215.

Villalba, R.M., Rodrigo, J., Alvarez, F.J., Achaval, M., Martinez-Murillo, R.

(1989) Localization of C-PON immunoreactivity in the rat main olfactory bulb. Demonstration that the population of neurons containing endogenous C-PON display NADPH-diaphorase activity. Neuroscience **33**:373-382.

Vincent, S.R. (1986) NADPH-diaphorase histochemistry and neurotransmitter coexistence. In: P. Panula, H. Paivarinta, S. Soinila (Eds.) **Neurohistochemistry: Modern methods and applications.** New York: Alan R. Liss; 375-396.

Vincent, S.R. and Johansson, O. (1983) Striatal neurons containing both somatostatin - and avian pancreatic polypeptide (APP)-like immunoreactivities and NADPH-diphorase activity: A light and electron microscopic study. J. Comp. Neurol. **217**:264-270.

Vincent, S.R. and Kimura, H. (1991) Histochemical mapping of nitric oxide synthase in the rat brain. (unpublished).

Vincent, S.R., Johansson, O., Hokfelt, T., Skirboll, L., Elde, R.P., Terenius, L., Kimmel, J., Goldstein, M. (1983a) NADPH-diaphorase: A selective histochemical marker for striatal neurons containing both somatostatin- and avian pancreatic polypeptide (APP)-like immunoreactivities. J. Comp. Neurol. **217**:252-263.

Vincent, S.R., Johansson, O., Skirboll, L., Hokfelt, T. (1982) Coexistence of somatostatin- and avian pancreatic polypeptide-like immunoreactivities in striatal neurons which are selectively stained for NADPH-diaphorase activity. In: E. Costa and M. Trabucchi (Eds.) **Regulatory Peptides: From Molecular Biology to Function**. New York, Raven Press.

Vincent, S.R., Satoh, K., Armstrong, D.M., Fibiger, H.C. (1983b) NADPH-diaphorase: A selective histochemical marker for the cholinergic neurons of the pontine reticular formation. Neurosci. Lett. **43**:31-36.

Vincent, S.R., Staines, W.A., Fibiger, H.C. (1983c) Histocemical demonstration of separate populations of somatostatin and cholinergic neurons in the rat striatum. Neurosci. Lett. **35**:111-114.

Vincent, S.R., McIntosh, C.H.S., Buchan, A.M.J., Brown, J.C. (1985) Central somatostatin systems revealed with monoclonal antibodies. J. Comp. Neurol. **238**:169-186.

Vincent, S.R., Satoh, K., Armstrong, D.M., Panula, P., Vale, W., Fibiger, H.C. (1986) Neuropeptides and NADPH-diaphorase activity in the ascending cholinergic reticular system of the rat. Neuroscience **17**:167-182.

Von Pechmann and Runge (1894) Oxydation der formazylverbindungen II. Ber. Dtsch. Chem. Gesellsch. **27**:2920-2930.

Wagner, H., Arnold, F., Simon, H. (1976) Intralobulare enzymaktivitatsverteilung der SDH in mauselebern. Eine histophotometrische studie. Acta Histochem. **57**:68-77.

Walaas, I. (1981) The effects of kainic acid injections on guanylate cyclase activity in the rat caudatoputamen, nucleus accumbens and septum. J.

Neurochem. **36**:233-241.

Walaas, S.I., Girault, J.-A., Greengard, P. (1989) Localization of cyclic GMP-dependent protein kinase in rat basal ganglia neurons. J. Mol. Neurosci. **1**:243-250.

Waldman, S.A. and Murad, F. (1988) Biochemical mechanisms underlying vascular smooth muscle relaxation: The guanylate cyclase-cyclic GMP system. J. Cardio. Pharm. **12**:S115-S118.

Wallace, M.N. (1986) Spatial relationship of NADPH-diaphorase and acetylcholinesterase lattices in the rat and mouse superior colliculus. Neuroscience **19**:381-391.

Wallace, M.N., Fredens, K. (1989) Relationship of afferent inputs to the lattice aof high NADPH-diaphorase activity in the mouse superior colliculus. Exp. Brain Res. **78**:435-445.

Walther, B., Ghersi-Egea, J.F., Minn, A., Siest, G. (1986) Subcellular distribution of cytochrome P-450 in the brain. Brain Res. **375**:338-344.

Wernet, W., Flockerzi, V., Hofmann, F. (1989) The cDNA of the two isoforms of bovine cGMP-dependent protein kinase. FEBS Lett. **251**:191-196.

Whalin, M.E., Scammell, J.G., Strada, S.J., Thompson, W.J. (1991) Phosphodiesterase II, the cGMP-activatable cyclic nucleotide phosphodiesterase, regulates cyclic AMP metabolism in PC12 cells. Molec. Pharmacol. **39**:711-717.

Whittle, B.J.R., Lopez-Belmonte, J., Moncada, S. (1990) Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. Br. J. Pharmacol. **99**:607-611.

Wilcox., K.S., Grant, S.J., Burkhart, B.A., Christoph, G.R. (1989) In vitro electrophysiology of neurons in the lateral dorsal tegmental nucleus. Brain Res. Bull. **22**:557-560.

Williams, C.H., Jr. and Kamin, H. (1962) Microsomal triphosphopyridine nucleotide-cytochrome c reductase of liver. J. Biol. Chem. **237**:587-595.

Wood, P.L. (1991) Pharmacology of the second messenger, cyclic guanosine 3',5'-monophosphate in the cerebellum. Pharmacol. Rev. **43**:1-25.

Woody, C.D. and Gruen, E. (1987) Acetylcholine reduces net outward cuurents measured in vivo with single electrode voltage clamp techniques in neurons of the motor cortex of cats. Brain Res. **424**:193-198.

Woody, C.D., Bartfai, T., Gruen, E., Nairn, A.C. (1986) Intracellular injection of cGMP-dependent protein kinase results in increased input resistance in neurons of the mammalian motor cortex. Brain Res. **386**:379-385.

Yonehara, N., Matsuda, T., Saito, K., Ishida, H., Yoshida, H. (1980) Effect of cyclic nucleotide derivatives on the release of ACh from cortical slices of the rat brain. Brain Res. **182**:137-144.

Yoshioka, M., Fujimori, H., Deguchi, T., Masayasu, H., Suzuki, K., Inamura, K., Kosasayama, Ishikawa, F. (1990) Effects of arginine derivatives on soluble guanylate cyclase from neuroblastoma N1E 115 cells. Biochem. Pharmacol. **39**:37-47.

Zweig, R.M., Jankel, W.R., Hedreen, J.C., Mayeux, R, Price, D.L. (1989) The pedunculopontine nucleus in Parkinson's disease. Ann. Neurol. **26**:41-46.

Zwiller, J., Ghandour, M.S., Revel, M.O., Basset, P. (1981) Immunohistochemical localization of guanylate cyclase in rat cerebellum. Neurosci. Lett. **23**:31-36.