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DIFFERENTIAL EXPRESSION OF NADPH DIAPHORASE IN FUNCTIONALLY DISTINCT PREFRONTAL CORTICES IN THE RHESUS MONKEY

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Abstract—The prefrontal cortex of primates is an integrative centre for sensory, cognitive, mnemonic and emotional processes. The cellular features which contribute to the functional specialization of its subsectors are poorly understood. In this study we determined the distribution of nicotinamide adenine dinucleotide phosphate-diaphorase-positive neurons in structurally and functionally distinct prefrontal cortices in the rhesus monkey. This class of neurons express nitric oxide synthase which is necessary for the production of nitric oxide, a novel neural messenger implicated in learning and memory. The density of diaphorase-positive neurons was approximately four times higher in olfactory areas than in eulaminate areas (areas 9, 10, 12, 46, and 8), and two- to three-times higher in the agranular limbic area PAll than in eulaminate areas. Positive neurons were concentrated in a deep band (layers V and VI), a superficial band (layers II and upper III), and were sparsely distributed in the central, thalamic recipient zone (deep layer III, layer IV and upper V). The highest densities of positive neurons were observed in the white matter where their prevalence followed the opposite trend than in the corresponding overlying cortices. The distribution of diaphorase-positive neurons was correlated with the regional anatomic and functional specialization of prefrontal cortices. Thus, diaphorase-positive neurons were most densely distributed in orbital and then medial prefrontal limbic cortices which have a low cell density and widespread connections. In contrast, positive neurons were comparatively sparse in eulaminate cortices, which have a high cell density and more restricted connections.

These findings indicate that the distribution of diaphorase-positive neurons in prefrontal cortices is not random, but is associated with the structural architecture and functional attributes of these cortices. The preponderance of diaphorase-positive neurons in limbic cortices, which have been implicated in learning and memory, is consistent with the idea that nitric oxide may have a role in synaptic plasticity.

Key words: nitric oxide synthase, nitric oxide, limbic cortex, memory.

The prefrontal cortex of the rhesus monkey is a structurally and functionally heterogeneous region (for reviews, see Refs 7, 9, 37, 43 and 92). Prefrontal cortices are connected with every cortical sensory system and the motor cortices, and are thus capable of integrating information for action (for review, see Ref. 38). Among prefrontal cortices, the limbic areas, which are situated on the caudal orbital and medial surfaces, have the most distributed network. Their rich interconnections with diencephalic, temporal and cingulate structures,[8,11,12,32,75,82,88] suggest that they are an integral part of a neural network involved in mnemonic processing.

The cellular features which may contribute to the functional specialization of prefrontal subareas are poorly understood. In the present study, we investigated the organization of neurons which contain nicotinamide adenine dinucleotide phosphate-diaphorase (NADPHd) in the prefrontal cortex. The significance of this class of neurons is based on their ability to synthesize nitric oxide (NO), a novel neural messenger which has been implicated in learning and memory[18,80,99,125] (for review, see Refs 22, 31, 100). NADPHd or nitric oxide synthase (NOS)-positive neurons appear to be resistant to destruction in Alzheimer's and Huntington's disease,[35,59,62,77] to ischemia, aging and exposure to several different toxins[16,17,25,36,53–56,112] (for review, see Refs 28, 30). Neurons which contain diaphorase or NOS thus seem to form a unique subclass with specific properties.

To determine the distribution of NADPHd positive neurons in the prefrontal cortex we used a histochemical procedure. Our approach was based on findings which indicate that in the mammalian CNS, including the cortex, the distributions of neurons which have diaphorase activity and NOS largely coincide.[21,29,45,47,63] In view of the above findings,

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Abbreviations: DMSO, dimethylsulfoxide; NADPH, nicotinamide adenine dinucleotide phosphate; NADPHd, NADPH diaphorase; NO, nitric oxide; NOS, NO synthase.

in recent studies investigators have used the designation diaphorase and NOS interchangeably.[102,122] It should be noted that the distribution of diaphorase-positive neurons in several neural structures had been described, albeit not quantitatively, prior to its association with NOS had been established.[20,27,34,60,61,71–74,76,94–96,101,105,109,117,118] Nevertheless, several questions about the organization of diaphorase-positive neurons remain unresolved. For example, are diaphorase-positive neurons uniformly or differentially distributed in the heterogeneous expanse of the prefrontal cortex? If there are regional variations, are areas with high diaphorase expression unified by some common anatomic or functional attribute?

EXPERIMENTAL PROCEDURES

Data were obtained from brain tissue of five adult rhesus monkeys (*Macaca mulatta*). Experimental procedures were conducted according to the NIH Guide for the Care and Use of Laboratory Animals (NIH pub. No. 80-22, 1987). All efforts were taken to reduce the number of monkeys used and to minimize suffering. The monkeys had received small intracerebral injections of either horseradish peroxidase or fluorescent dyes in connection with other experiments. In the former, perfusion through the heart was initiated with saline, followed by 2 l of fixative (1.25% glutaraldehyde, 1% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) delivered over a 30 min period, followed by perfusion with 2 l of cold (4°C) phosphate buffer. The brain then was removed from the skull, photographed, placed in glycerol phosphate buffer (10% glycerol and 2% dimethylsulfoxide (DMSO) in 0.1 M phosphate buffer at pH 7.4) for one day and then transferred in 20% glycerol phosphate buffer for another two days. In animals injected with fluorescent dyes, perfusion was initiated with saline followed by 4% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4. The brain then was placed in a solution of 4% paraformaldehyde with 10% glycerol and 2% DMSO for one day, and transferred to a solution containing 20% glycerol in 2% DMSO for another two days.

The brain was frozen in −75°C isopentane[90] and transferred to a freezing microtome. Sections were cut in the coronal plane at 40 μm in 10 series, and collected in a solution of 0.1 M phosphate buffer (pH 7.4). Adjacent series of sections were stained for Nissl bodies, myelin, and acetylcholinesterase to aid in delineating architectonic borders.[41,42]

NADPH diaphorase staining

Recent biochemical and histochemical evidence indicates that, at least in the central nervous system of mammals, NADPHd is a good marker for NOS.[45,47,63] In the Results we use the terms NADPHd or diaphorase for consistency.

Staining for diaphorase was performed by using a slight modification of methods previously described.[34,97,110] Free-floating sections were washed three times (10 min each) at 37°C in 0.1 M Tris–HCl buffer (pH 7.4). The tissue was then incubated at 40°C in 0.1 M Tris–HCl buffer containing 0.8 mM β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, Sigma N-1630), 0.8 mM Nitro Blue Tetrazolium (Sigma N-6876), 0.1% Triton-X, and 0.16% malic acid for 60–90 min with constant agitation. Sections then were washed three times (10 min each) in 0.1 M Tris–HCl buffer, mounted on chrome-alum coated slides and allowed to dry. Sections were then counterstained in 1% Neutral Red solution, dehydrated through graded alcohols (70–100%), cleared in xylene and coverslipped with Permount (Fisher Scientific). In control experiments either NADPH or Nitro Blue Tetrazolium were omitted but all other steps were identical to the experimental.

Data acquisition

Brain sections prepared according to the above method were viewed microscopically under bright-field illumination. Drawings of brain sections through the prefrontal and adjacent cortices, diaphorase-positive neurons, and the site of blood vessels used as landmarks were transferred from the slides onto paper using a digital plotter (Hewlett Packard 7475A) electronically coupled to the stage of the microscope and to a computer (Compaq 386). The analogue signals were converted to digital signals via an analogue-to-digital converter (Data Translation) in the computer. Each diaphorase-positive neuron was recorded by aligning the centre of the cross hair (permanently fixed in one eyepiece of the microscope) with the centre of the labelled neuron and pressing a button. The location of diaphorase-positive neurons was recorded using different symbols and colours to indicate labelled neurons in layers I–III, layers IV–VI, proximal white matter, and deep white matter. Software developed in this laboratory ensured that each labelled neuron was recorded only once.

Data analysis

The frontal cortices were reconstructed serially using the sulci as landmarks and are shown on diagrams of the surface of the cortex. Unfolded maps of the cortex were prepared according to a method described previously.[6,13] References to architectonic areas of the prefrontal cortex are according to a classification described previously.[14]

Areal measures and the number of diaphorase-positive neurons for superficial (layers I–III) and deep (layers IV–VI) cortical layers, proximal white matter, and deep white matter below each architectonic area were counted separately in every section. For purposes of analysis, the white matter was also subdivided into its deep and proximal part (Table 1); the latter lies just below layer VI and corresponds to the region which includes the cortical association fibres.[78] Labelled neurons in the white matter were concentrated either superficially just below layer VI, or in a deep zone which is situated below the proximal white matter zone. A central region in the white matter was devoid of labelled neurons and served as an arbitrary demarcation zone between the superficial and deep white matter. In areas where

Abbreviations used in the table and figures

A	arcuate sulcus	MO	medial orbital sulcus
CC	corpus callosum	OLF	olfactory: olfactory tubercle, anterior olfactory nucleus, frontal prepiriform cortex
Cg	cingulate sulcus		
cl	claustrum	P	principal sulcus
G	gustatory	PAll	periallocortex
Iag	insula, agranular	Pro	proisocortex
IdgI	Insula, dysgranular	ProM	rostral portion of the ventral premotor cortex
LF	lateral fissure	Ro	rostral sulcus
LO	lateral orbital sulcus		

sulci are apposed, the deep white matter was divided equally between the two areas.

Cell counts and areal extent were made using a microscope/computer interface as described previously.[12] Density values were calculated as the number of diaphorase-positive neurons per unit area (mm^2). "Total area" density in the Results refers to the sum of all labelled neurons in each cortical area and the underlying white matter (mm^2). In Table 1 and in Results, "Total cortex" refers to the overall density of labelled neurons/mm^2 in all cortical layers for each area; "Total white matter" refers to the overall density of labelled neurons/mm^2 in the white matter below each area or group of areas (Table 1).

RESULTS

General observations

The results are based on observations made in five adult rhesus monkeys. Diaphorase-positive neurons contained a blue reaction product and were clearly seen against a background of unlabelled neurons counterstained with Neutral Red. Positive staining distinguished the cell body and extensive branching of processes. There was no staining in control sections in which either NADPH or Nitro Blue Tetrazolium were omitted.

The regional distribution of diaphorase-positive neurons was similar among the cases, although the overall density differed to some extent. Detailed quantitative analyses were made in three cases. In case AN the regional density was 1.5–6.69 positive neurons per mm^2, in case AP it was 2.95–13.11, and in case EU it was 0.77–6.88. Differences in the overall density values may be due to different perfusion methods.[24,69,120] However, because we found no consistent differences that could be attributed to the perfusion, overall differences in density may reflect individual variation among monkeys.

Areal distribution of diaphorase-positive neurons

The areal distribution of positive neurons is shown in two cases on reconstructed maps of the frontal cortices and in coronal sections in Figs 1 and 2. The density of labelled neurons is shown in Table 1. The overall density of positive neurons was highest in

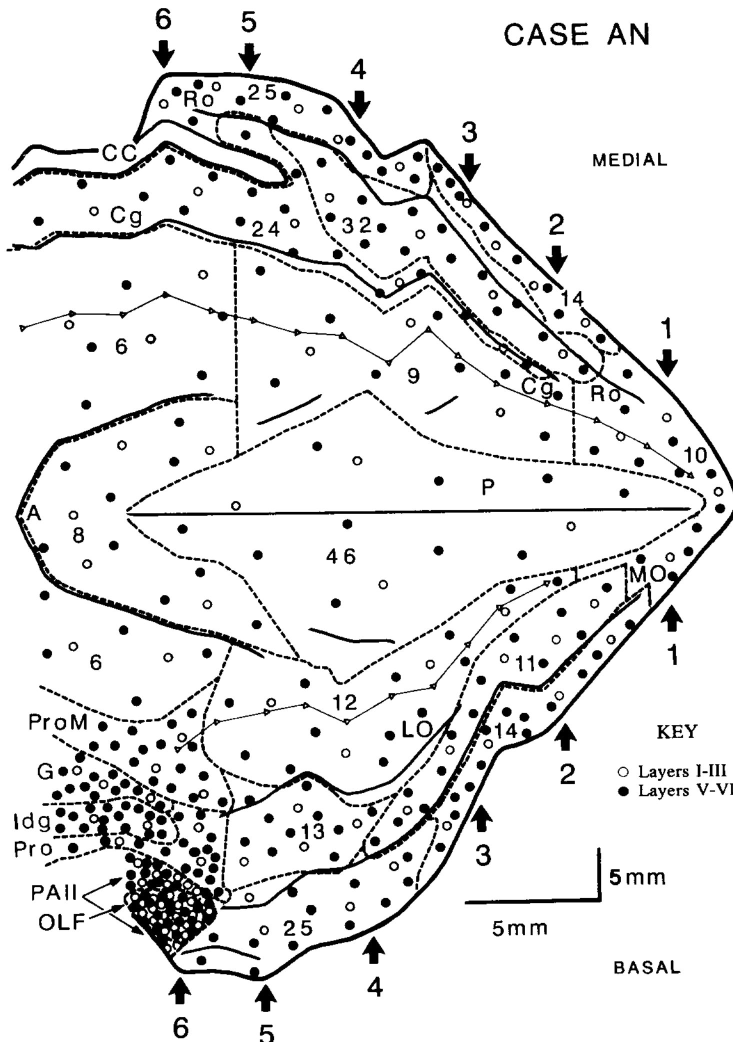

Fig. 1(a) *Caption on page 53.*

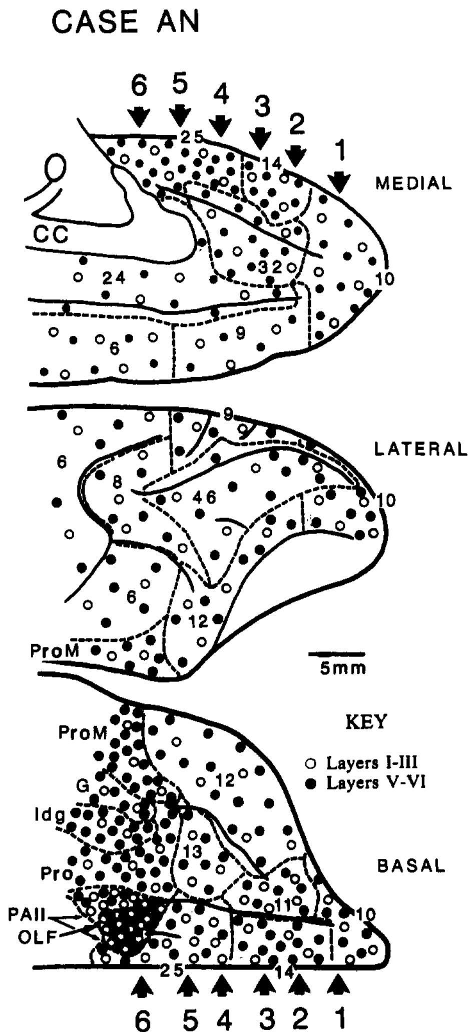

Fig. 1(b) *Caption opposite.*

caudal orbital areas and the adjacent insular areas, followed by rostral orbital and medial areas, and was lowest in lateral prefrontal areas (Table 1; Figs 1–3).

Among caudal orbital areas, labelled neurons were most prevalent in the primary olfactory areas (Table 1; Figs 1A bottom; 1B bottom; 1C section 6; 2 bottom; 3A, D), followed by areas PAll, Pro, the dysgranular insula (Idg) and the gustatory (G) area (Table 1). In rostral orbital areas, the density of diaphorase-positive neurons was somewhat lower than in the caudal areas (Figs 1A bottom; 1B bottom; 1C; 2 bottom).

Medial prefrontal cortices showed the next highest regional density of positive neurons (Figs 1A top; 1B top; 1C; 2 top; 3B, E). Among medial areas, the highest density of positive neurons was observed in the medial part of area 25, followed by areas 24, 32, and 14 and then the medial parts of areas 10 (M10) and 9 (M9; Figs 1A top; 1B top; 2 top).

The incidence of positive neurons was lowest in lateral prefrontal areas, particularly in lateral areas 10 (L10) and 12 (L12), and dorsal areas 10 (D10) and 9 (D9; Figs. 1A centre; 1B centre; 1C; 2 centre; 3C, F). Among lateral cortices, areas 46, 8, and 6 had an intermediate density of positive neurons.

Regional distribution of diaphorase-positive neurons

We combined data from prefrontal areas with similar laminar definition to determine whether regional differences in the distribution of labelled neurons were related to differences in laminar characteristics noted previously.[14] Categories were constructed on the basis of number of layers and laminar definition, and are shown in Table 1. At one extreme olfactory paleocortical areas have either a nuclear appearance (e.g., anterior olfactory nucleus) or are organized into three layers (e.g., prepiriform cortex). The second category included the agranular area PAll, and the third category included all dysgranular areas. Agranular and dysgranular association cortices are collectively called limbic. The following three categories included eulaminate areas, which have six layers, but nevertheless show small differences in laminar distinction. They were grouped in an ascending order for laminar definition. The distribution of positive neurons in premotor area 6 was similar to the last group and is illustrated in Figs 1 and 2. The values for area 6 are not included in Table 1 because they were not computed beyond the caudal limit of the prefrontal cortex at the spur of the arcuate sulcus.

We first examined whether regional variations of labelled neurons were consistent among cases. The regional distribution of diaphorase-positive neurons among cases was highly correlated (rank order; $P < 0.02$). As shown in Fig. 4A, where the six cortical categories were ranked by density of labelled neurons, the plots among cases were virtually identical. This indicates that the regional distribution of labelled neurons was similar among cases. We then examined whether the regional differences in the distribution of positive neurons were statistically significant. An analysis of variance showed overall significant differences in total cortical density of NADPHd-positive neurons among the cortical categories ($F = 5.27$, $P < 0.01$). The regional distribution of diaphorase-positive neurons (Table 1, "Total cortex") was approximately four times higher in olfactory areas (category 1) than in eulaminate areas (categories 5 and 6), and their density in the agranular area PAll (category 2) was 2–3 times higher than in eulaminate areas (Table 1). The density of labelled neurons in dysgranular areas (category 3) fell between the above two extremes.

Diaphorase-positive neurons in the white matter

Numerous positive neurons were noted in the white matter below all cortical areas (Figs 1C; 3; 5). The white matter had an overall higher density of labelled neurons than the overlying cortical areas, with the exception of the olfactory cortex and area PAll,

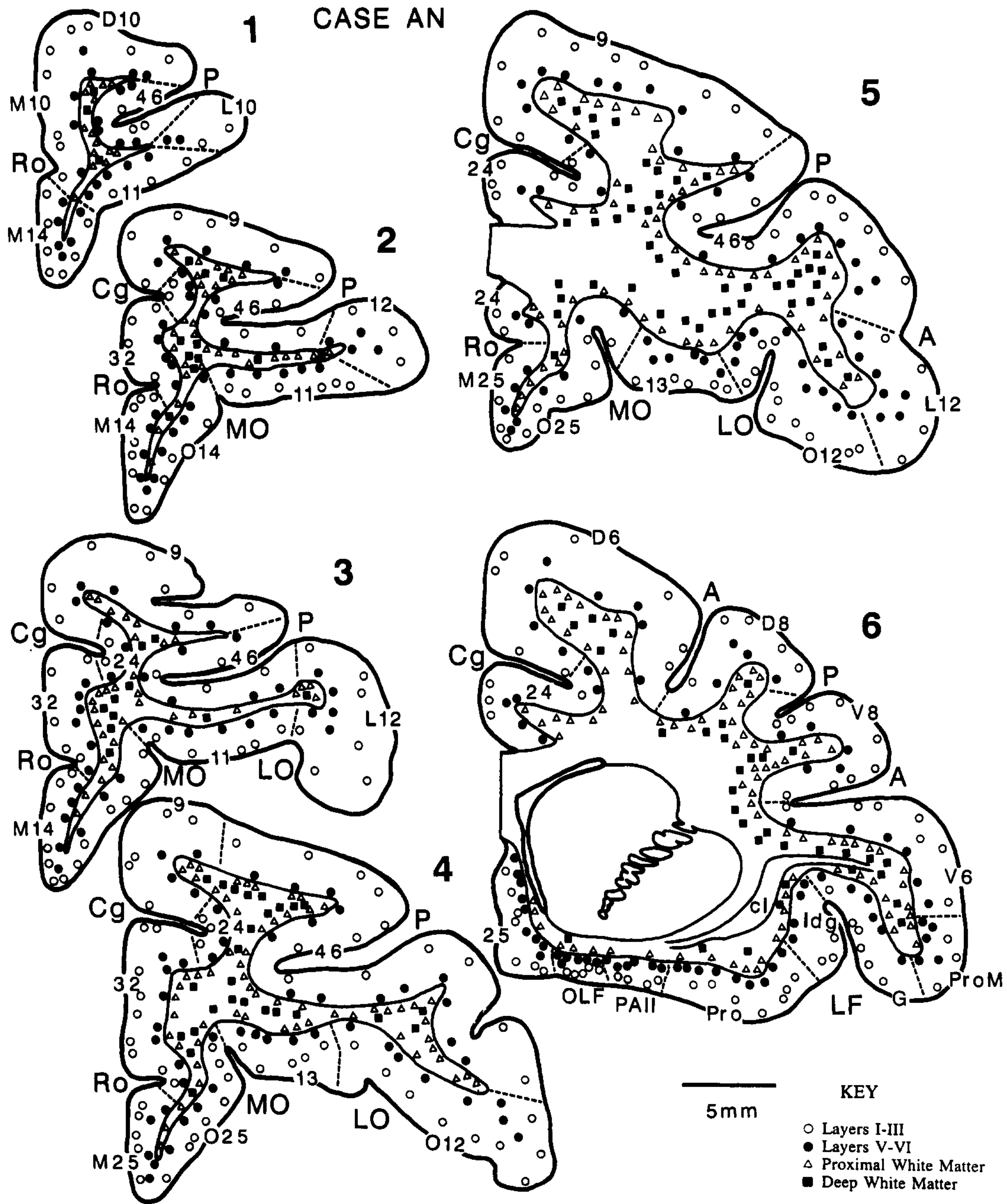

Fig. 1(c).

Fig. 1. The distribution of diaphorase positive neurons in the cortex in case AN is shown on an unfolded map of the frontal cortex (a); on the medial, lateral, and basal surfaces of the cortex (b); and in diagrams of coronal sections (c) in rostral (1) through caudal (6) prefrontal levels taken at the levels indicated by arrows in parts a and b. Medial is to the left. The symbols here and in Fig. 3 are as follows: open circles represent labelled neurons in layers I–III, filled circles in layers IV–VI, triangles in proximal white matter, black squares in the deep white matter. The density of symbols in the maps is proportional to the overall density of diaphorase-positive neurons in each cortical area, or in the white matter below each area (see Table 1). Architectonic areas are separated by dashed lines. Medial, lateral, and basal surfaces in part a are separated by a thin line with triangles.

where the inverse relationship was observed (Table 1, compare "Total cortex" with "Total white matter"). The density of diaphorase-positive neurons in the underlying white matter ranged from 4.56 to 9.42/mm^2 in case AN, and 1.05 to 6.07/mm^2 in case AP (Table 1). Within the white matter the highest density of labelled neurons was noted below areas 46, 8, lateral areas 10 and 12, and dorsal areas 10 and 9

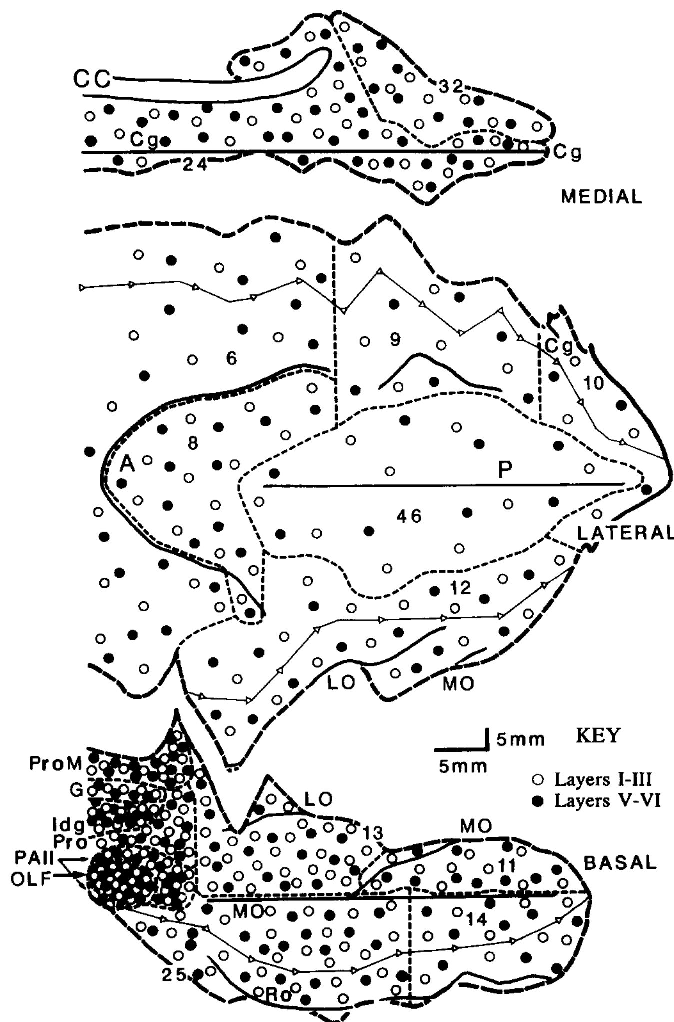

Fig. 2. The distribution of diaphorase-positive neurons in case AP is shown on the medial, lateral, and basal surfaces. Each surface was reconstructed separately to minimize angular distortion. The straight line along a sulcus in each surface shows the reference for each view. The medial parts of areas 10 and 9 and the orbital part of area 12 have been included in the lateral surface in order to preserve the continuity of the areas. Similarly, medial parts of areas 25 and 14 have been included with the basal surface.

(Table 1; Figs 1C; 3C, F; 5). The next highest density of positive neurons was seen below rostral orbital and medial areas (areas 11, 13, 24, 32, 14 and 25; Table 1; Figs 1C; 3B, E), and the lowest density was noted below the olfactory cortex, areas PAll, Pro, and the agranular and dysgranular parts of the insula (Table 1; Figs 1C; 3A, D). As shown in Fig. 4B, the regional density of labelled neurons in the white matter was inversely related to its regional distribution in the overlying cortex.

Laminar and white matter distribution of diaphorase-positive neurons

In eulaminate and dysgranular areas the upper layers include layers I–III, and the deep include layers IV–VI. In the agranular area PAll and the olfactory prepiriform area, which have only three distinguishable layers,[14] the superficial zone includes layers I and II and the deep zone includes layer III. In some olfactory areas, such as the olfactory tubercle, where no clear lamination can be observed in the rhesus monkey, the division into upper and lower layers was made by bisecting each area.

In most areas the relative distribution of diaphorase-positive neurons in the cortex was higher in the deep layers than in the upper layers (Table 1, layers IV–VI and layers I–III). The above observation may be attributed to the inclusion of the cell-sparse layer I with the upper layers. However, our findings indicated that excluding layer I did not change significantly the overall trend of densities by layers in most areas. In the white matter, the distribution of

diaphorase-positive neurons was higher in its proximal than in its deep component (Table 1; Figs 1C; 5).

Positive neurons in the cortex were arranged into two distinct bands: one was concentrated in the deep part of the infragranular layers, and the other occupied layers II and the upper part of layer III. The arrangement of positive neurons into a deep and a superficial band left a central zone where positive neurons were comparatively sparsely distributed (Fig. 1C). The latter included the deep part of layer III, granular layer IV, and the upper part of layer V.

Other labelling

We made a few observations on overall background activity and fibre distribution in the cortex. There was a dense and diffuse plexus of positive activity in the olfactory cortex which included a superficial and a deep component (Fig. 3A). Similarly, the indusium griseum showed dense background activity. We could not determine whether this activity was a result of extensive dendritic or axonal branching or even if it occupied extracellular as well as intracellular compartments. This pattern of overall background labelling was unique to the above structures among those examined.

We also noted a horizontally oriented strip of positive fibres in the central part of layer I, which was particularly prominent in eulaminate areas 8 and 46 (Figs 3C; 6A). In contrast, in limbic areas positive processes in layer I were diffusely distributed and less prominent (Figs 3B; 6B). Fibre labelling in layer I was sparse or absent in the olfactory cortex (Fig. 3A).

DISCUSSION

Technical considerations

Previous studies have shown that diaphorase label is sensitive to fixation treatments.[33,67,111] The overall differences in label that we noted among cases may be attributed to inadvertent differences in fixation, or may reflect individual differences among animals (Table 1). In spite of the differences in the overall density, the pattern of the regional distribution of positive neurons across architectonic areas and in the white matter was strikingly similar in different animals (Fig. 4A). These findings suggest that in normal monkeys, while the overall number of positive neurons varies among animals, the pattern of their regional distribution appears to be strictly regulated.

Diaphorase expression is high in limbic cortices

The distribution of diaphorase-positive neurons differed considerably within prefrontal areas in a strikingly similar pattern in all animals. Olfactory and limbic prefrontal cortices had the highest density and eulaminate cortices had the lowest density of diaphorase-positive neurons. The results suggest that the differences in the expression of diaphorase coincide broadly with the structural differences noted in the prefrontal cortices of the rhesus monkey.[7,9,14,15] Thus, as the degree of laminar definition increases from agranular limbic to dysgranular limbic and then to eulaminate areas, the density of diaphorase-positive neurons decreased.

Our results indicated that positive neurons in the cortex were concentrated in a superficial band (layers II, the upper part of layer III) and a deep band (deep part of layer V and layer VI), and were sparsely

Table 1. Density° of NADPH diaphorase positive neurons in layers I–III, IV–VI and the white matter of prefrontal and adjacent cortices**

CASE: AN Area	Cortex I–III	 IV–VI	White matter Proximal	 Deep	Total cortex	Total white matter	Total area
1. OLF*	4.35	8.41	4.25	0.47	6.69	4.72	6.35
2. PAll*	3.69	6.6	3.6	0.96	5.43	4.56	5.21
3. Pro, ProM, G, Idg, 25, 13, 24, 32	0.95	3.45	6.07	2.5	1.91	8.57	3.62
4. 14, O12, 11, M10, M9	0.99	3.41	4.76	1.91	1.82	6.67	2.81
5. L10, L12, R46, D9, D10	0.93	2.45	6.63	2.79	1.5	9.42	2.59
6. C46, V8, D8	1.18	2.33	5.11	2.37	1.65	7.48	3.15
CASE: AP Area	**Cortex I–III**	** IV–VI**	**White matter Proximal**	** Deep**	**Total cortex**	**Total white matter**	**Total area**
1. OLF*	19.15	8.81	1.05	0	13.11	1.05	9.39
2. PAll*	6.65	7.12	4.17	0	6.89	4.17	6.5
3. Pro, ProM, G, Idg, 25, 13, 24, 32	5.46	5.18	4.89	0.75	5.35	5.64	5.42
4. 14, O12, 11, M10, M9	3.24	3.51	4.64	4.13	3.36	6.07	3.92
5. L10, L12, R46, D9, D10	2.59	3.47	3.56	1.54	2.95	5.1	3.37
6. C46, V8, D8	3.94	4.34	3.47	1.11	4.14	4.57	4.27

°Density = neurons/mm^2.

*Olfactory cortices and area PAII have only three layers. The superficial layers (I and II) are represented under the column CORTEX I–III and the deep layer (III) under the column CORTEX IV–VI.

**Areas were grouped into six categories according to the degree of their laminar definition. Category 1 represents olfactory areas and category 6 the best delineated eulaminate areas.

A letter before an architectonic area indicates: C, caudal; D, dorsal; L, lateral; M, medial; O, orbital; R, rostral; V, ventral.

distributed in deep layer III and layer IV, which are recipient of thalamic projections (for review, see Ref. 52). These results are consistent with the observations of other investigators.[45] We also noted that positive fibres in layer I of eulaminate areas formed a horizontal strip which contrasted sharply with their scattered ramification in limbic cortices (Figs 3B, C; 6). The above observations suggest that there are differences in the pattern of branching of processes of diaphorase-positive neurons in limbic and in eulaminate areas.

Relationship of diaphorase-positive neurons in the cortex and white matter

Diaphorase-positive neurons were densely distributed in the white matter below layer VI. In fact, in most areas the overall density of positive neurons was higher in the white matter than in the overlying cortex. There are several explanations for the strong presence of diaphorase-positive neurons in the white matter in adult primates. First, their presence in the proximal white matter, in a position previously held

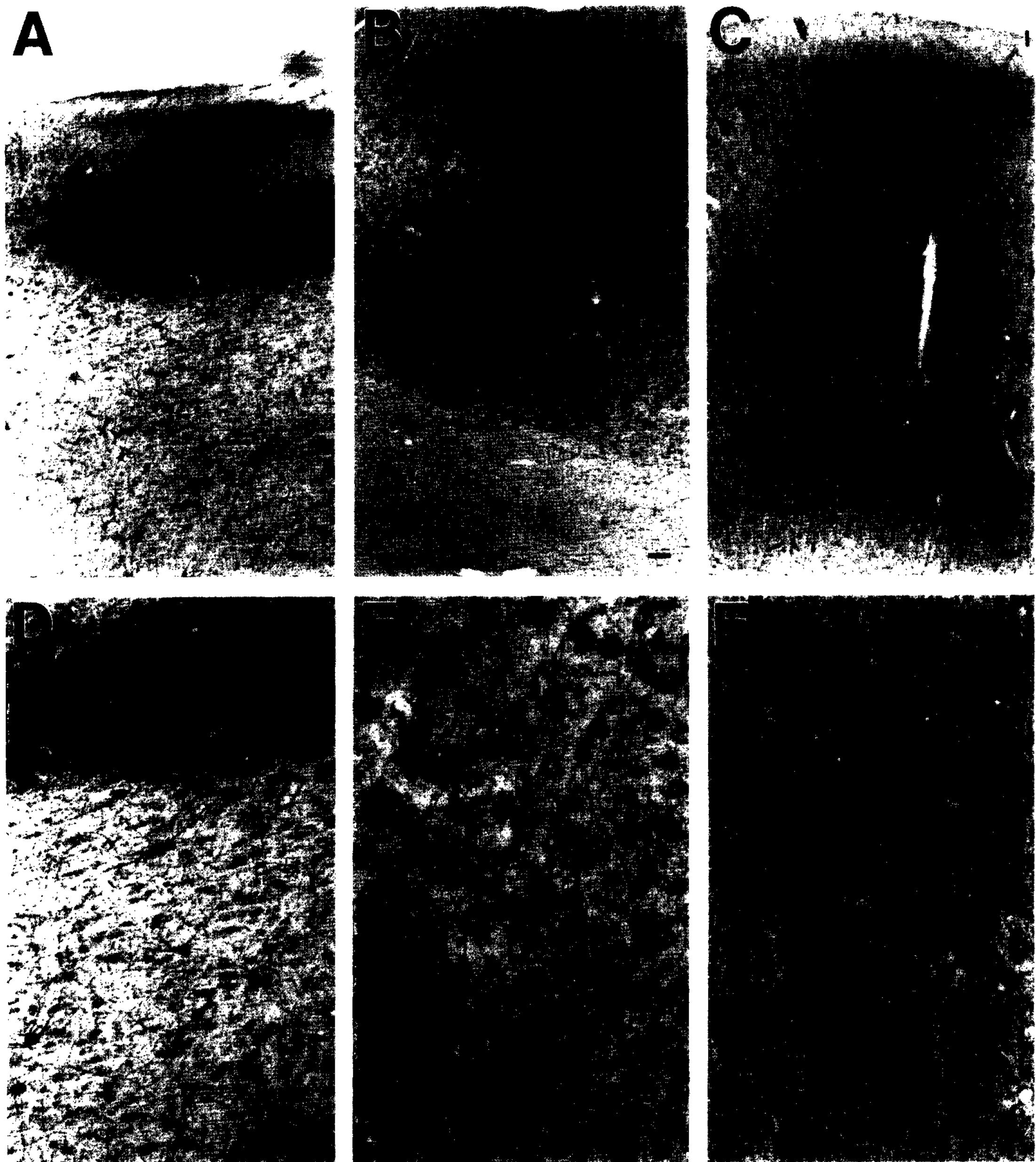

Fig. 3. Bright-field photomicrographs showing diaphorase-positive neurons in the olfactory cortex (A), dysgranular area 24 (B), and eulaminate area 46 (C). Frames D, E, and F show the cortices depicted respectively in A, B, C at higher magnification. Scale bars = 100 μm.

by the subplate during development, suggests that they may be vestigial, having survived subplate elimination in postnatal life.[26,64,116] Diaphorase co-localizes in neurons with somatostatin and neuropeptide Y[29,46,118] which are major components of the transient subplate and are generated early in cortical development.[46,57,64,103] The prevalence of diaphorase-positive neurons in the deep cortical layers which develop first[65,66,68,83–87] suggests that diaphorase may be expressed early in ontogeny as well.

Recent studies have provided evidence that diaphorase or NOS-positive neurons have a role in development. Prominent expression of NOS or diaphorase activity appears in the cortical plate of the developing rat and decreases substantially by birth.[23,124] In addition, the expression of diaphorase coincides with the time of peak refinement of axonal projections during development. Moreover, there is a concomitant decline in the expression of diaphorase (or NOS) as axons are eliminated during maturation.[93,122] Finally, inhibition of NOS during development reduces the elimination of ipsilateral retinotectal projections within the visual system of the chick.[123] The above evidence suggests that diaphorase neurons may have an active role in shaping the cortex.

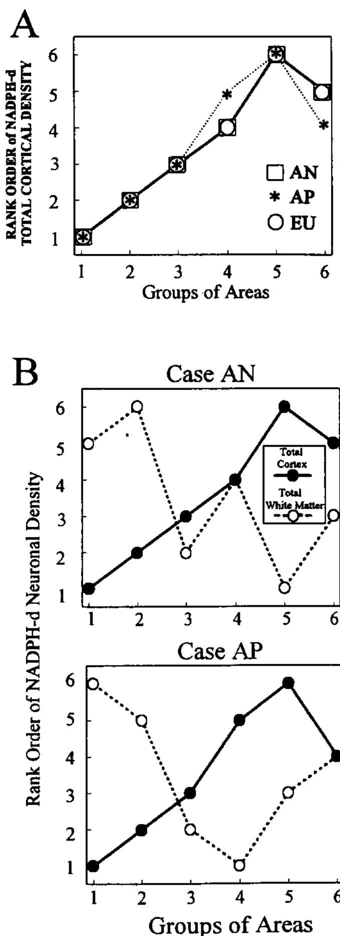

Fig. 4. (A) Line graphs showing the rank order of the regional distribution of diaphorase-positive neurons in three cases. The areas in each cortical category are listed in Table 1 and are arranged according to their laminar definition (1 olfactory, 6 best delineated eulaminate). Rank order depicts the density of NADPH diaphorase-positive neurons (1 highest density, 6 lowest density). The rank order of cortical categories in the three cases is highly correlated ($P < 0.02$). (B) The density of positive neurons (by rank) in the cortex (solid line) was inversely related to the density of labelled neurons in the underlying white matter (dashed line) as shown for cases AN (top) and AP (bottom).

In our own material we saw a clear relationship in the density of diaphorase-positive neurons in the cortex and white matter. Thus, positive neurons were abundant in the white matter below eulaminate areas where the cortical distribution is low, and they were comparatively sparsely distributed beneath olfactory and limbic cortices where the cortical distribution is high (Fig. 4B). This relationship is consistent with the idea that neuronal migration in limbic prefrontal cortices may precede that of eulaminate areas and may coincide with a time when diaphorase expression is high. Further developmental studies are necessary to address this issue.

The possible significance of diaphorase-positive neurons during development and in the adult cortex has recently been addressed in a *post-mortem* investigation of the brains of patients diagnosed with schizophrenia, which is thought to have a developmental origin.[51,58] Compared to controls, brains of schizophrenic patients showed an overall decrease of diaphorase-positive neurons in areas 9 and 21 and in the subjacent white matter and an increase in the deep white matter.[2,3] In view of our findings which indicate that diaphorase is enriched in limbic cortices, it may be interesting to examine these areas in schizophrenic brains particularly since the disorder affects disproportionally limbic cortices.[89,121]

Functional implications

Recent studies have implicated NO in synaptic plasticity in the hippocampus, cerebellum, and cortex[18,19,33,44,79,80,98,99,104,114,125] (for review, see Refs 22, 100, 106, 107, 127). We have shown that diaphorase-positive neurons are preponderant in agranular and dysgranular areas, which are the limbic component of the cortical mantle. Limbic cortices have an important role in mnemonic process.[5,37,119,126] Moreover, the areas with a high number of diaphorase-positive neurons in the orbital and medial prefrontal cortices are the major recipients of input from other subcortical limbic structures including the amygdala and the hippocampus.[1,4,11] Diaphorase is also enriched in neurons of the amygdala and subicular fields of the hippocampal formation[20,70,81,105,114,115] which issue robust projections to the prefrontal cortex[10,11] (for review, see Ref. 91). Thus, prefrontal limbic areas and subcortical limbic structures which are interconnected constitute a mnemonic network which may be influenced by NO.

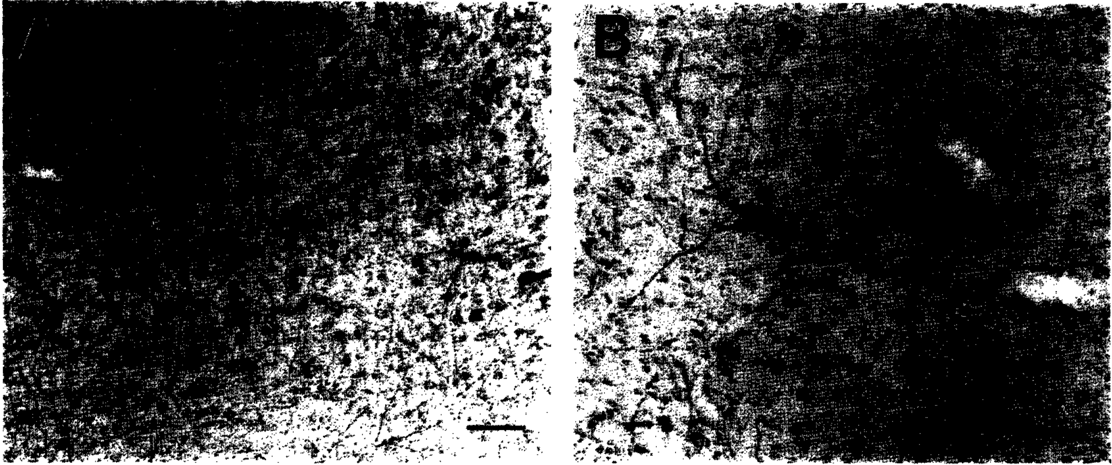

Fig. 5. Diaphorase-positive neurons in layers V–VI of dorsal area 9 (A) and in the proximal white matter underlying dorsal area 9 (B). Scale bars = 100 μm.

Fig. 6. Comparison of diaphorase-positive fibres in layer I of eulaminate area 8 (A) and limbic area 24 (B). Dense fibre labelling is shown in the central part of the top third of layer I (A); in contrast, only sparse labelling is seen in layer I of area 24 (B). Scale bars = 50 μm.

Our results also showed that the density of diaphorase-positive neurons was generally higher in the orbital than in the medial component of the prefrontal segment of the limbic system. The above evidence suggests that the differential expression of diaphorase in the prefrontal cortex may follow functional lines. Basal prefrontal areas are connected with inferior temporal visual cortices[6,8] which have been implicated in the analysis of visual features and their memory.[39,40,48–50,108] Medial cortices are preferentially connected with dorsolateral visual cortices[6] which have been implicated in visuospatial functions (for review, see Ref. 113). Additional studies are necessary to address whether the trend of diaphorase-positive neurons that we observed in the two prefrontal sectors distinguishes functional districts of temporal and dorsolateral visual areas as well.

CONCLUSION

Our findings suggest that the distribution of the class of neurons with diaphorase in prefrontal cortices and in the underlying white matter is not random. Diaphorase-positive neurons are most densely distributed in limbic prefrontal cortices. NO, produced in diaphorase-positive neurons, may have a role in the demonstrated function of prefrontal limbic cortices in memory. Moreover, the prevalence of diaphorase-positive neurons in the white matter appears to be inversely related to their density in the overlying cortex. This observation may reflect temporal differences in the development of distinct prefrontal cortices. Further studies are necessary to determine the function of diaphorase in prefrontal areas, its possible role in development and in the functional architecture of limbic areas in normal and pathological states.

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