Non-uniformity of Neocortex: Areal Heterogeneity of NADPH-diaphorase Reactive Neurons in Adult Macaque Monkeys

We have examined the distribution of cortical neurons in adult monkey cortex which stain for nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), an enzyme which is involved in the synthesis of nitric oxide. In order to compare distributions across areas we employed a cortical unit defined as the radial column, which refers to the volume of cortex below 1 mm² of cortical surface. Numbers of labeled neurons per radial column generate areal density measurements either for the full thickness of the cortex or for individual layers. Measurements were made in six cortical regions (areas V1, V2, STS, auditory cortex, area 4 and area 6). NADPH-d stains nonpyramidal neurons which can be divided into two major groups. Type 1 neurons have large soma diameters, stain densely for NADPH-d and show few morphological variations both within and across areas. Type 2 neurons have small somata and short processes, and can be subdivided on the basis of soma size into dense and light staining categories. Both subcategories of type 2 neurons show significant areal variations in size. In each cortical area the majority of type 1 neurons are located in the white matter. Areal densities of type 1 neurons are minimal in areas V1 and V2, and twice as dense in the frontal cortex. Pairwise comparisons of areal densities among the six areas examined show that in a radial column throughout the full thickness of cortex, areas differ significantly from each other in 12/15 comparisons. Consideration of individual layers shows significant differences in 13/15 comparisons. Type 2 neurons are exclusively located in the cortical gray matter, and in all areas are considerably more numerous than type 1 neurons. Area V1 is unique in that it has up to three times the areal density found in any other cortical area. With reference to published laminar cell density counts our results show that the percentage of labeled NADPH-d neurons in individual layers of area V1 are significantly higher than in the other areas. The laminar distributions of type 1 and type 2 neurons show that each area has a unique profile of NADPH-d expression. The modular or columnar organization of the cortex, also referred to as the radial column hypothesis, is important for understanding both the development and function of the cortex. The present results show that radial columns in individual cortical areas possess distinctive patterns of NADPH-d expression. This important degree of areal heterogeneity of NADPH-d neurons has far reaching implications for both the development and functions of neocortical areas.

Nitric oxide (NO) is implicated in numerous aspects of the physiology of the CNS including vasodilatation, neurotransmission, synaptic plasticity and neuronal death (Gally et al., 1990; Nowicki et al., 1991; Adachi et al., 1992; Dawson et al., 1992; Prado et al., 1992; Vincent and Hope, 1992; Aoki et al., 1993; Jadedola, 1993; Pucino and Enilokopov, 1993). It has now been established that neurons containing nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) synthesize NO and can be accurately localized using NADPH-d histochemistry (Hope et al., 1991; Estrada and DeFelice, 1998). Most reports show a nearly complete co-localization of NO synthase (NOS) and NADPH-d in rat (Khazaria et al., 1994), monkey (Bredt et al., 1991; Aoki et al., 1993; Hashikawa et al., 1994) and human cortex (Lith et al., 1994). Only a small fraction (<2%) of NADPH-d cells are found to lack NOS (Kharazia et al., 1994).

NADPH-d-positive neurons in the primate cerebral cortex are of particular interest due to the involvement of these neurons in cognitive disorders and neurodegenerative diseases, and their resistance to excitotoxicity-mediated cell death and ischemic brain damage (Ferriero et al., 1988; Koh and Choi, 1988; Kowall and Beal, 1988; Uemura et al., 1990; Dawson et al., 1991; Unger and Lange, 1992; Akbarian et al., 1993a,b).

In the cerebral cortex of nonprimates NADPH-d is characteristically expressed in a population of large, densely stained nonpyramidal neurons located principally in the white matter (Kowall and Beal, 1988; Mizukawa et al., 1988a,b; Vincent and Kimura, 1992; Valtschanoff et al., 1993; Yan et al., 1994). Neurons with a similar morphology are found in the cortex of the monkey and have been designated type 1 neurons. These neurons are distinguished from a more numerous lightly labeled population exclusively found in the cortical gray matter and which have been designated type 2 neurons (Sandell, 1986; Aoki et al., 1993; DeFelice, 1993; Hashikawa et al., 1994; Lith et al., 1994; Yan et al., 1996a). In a recent study it has been shown that primate cortex is characterized by a larger proportion of type 2 neurons than that of nonprimates (Yan and Garey, 1997). These phylogenetic differences in the proportions of type 1 and type 2 neurons may be related to differences in their physiological roles since although both types express GABA, all type 2 (and only a few type 1) neurons express calbindin (Yan et al., 1996a).

Despite the wide interest in NADPH-d-positive neurons, it remains unclear whether there are significant variations in their areal density across the primate cerebral cortex. Recent studies of NADPH-d labeled neurons in macaque and human cortex have concluded that there is an overall uniform distribution (Egberongbe et al., 1994; Hashikawa et al., 1994; Yan et al., 1996a). Similar claims have been made in other species (Vincent and Kimura, 1992; Derer and Derer, 1993; Giulii et al., 1994; Kuchiwa et al., 1994; Rodrigo et al., 1994). These findings, largely unsupported by quantitative data, are unexpected on several counts. Firstly, a quantitative study of the distribution of NADPH-d neurons in functionally distinct prefrontal areas in rhesus monkey showed important areal variations (Dombrowski and Barbos, 1996). Secondly, the distribution of NADPH-d-positive neurons have been shown to be lamina-specific in rats, humans and monkeys (Yan et al., 1994, 1996a,b; Dombrowski and Barbos, 1996). Thirdly, NADPH-d-positive neurons are restricted to the septa in the rat somatosensory barrel cortex (Valtschanoff et al., 1993). Fourthly, an extensive quantitative study in the rat showed important areal variations of NADPH-d-positive neurons in individual areas located in the frontal, temporal and occipital lobes (Bidmon et al., 1997).
The modular or columnar organization of the neocortex is a conspicuous functional and anatomical feature (Mountcastle, 1957, 1997; Hubel and Wiesel, 1977) and the radial column is a basic ontogenic unit (Rakic, 1988, 1995). Hence variation in the numbers of neurons of a given phenotype in a radial column in different areas is important for understanding both the development and function of the neocortex.

A number of studies have shown that with the exception of area 17 there is a relative degree of homogeneity in the organization of the cortex in terms of the number of neurons in a radial column (Rockel et al., 1974, 1980; O’Kusky and Colonnier, 1982; Peters, 1987; Beaulieu, 1993; Skoglund et al., 1996). Few studies have directly addressed the issue of heterogeneity in term of numbers of a given phenotype in a radial column (Jones, 1990). A noticeable exception is the study of Jones and his co-workers on GABA-positive neurons; they found equal proportions of GABA-positive neurons in areas V1 and V2 and the motor cortex (Hendry et al., 1987). Heterogeneity has been indicated by areal variations in the numbers of neurons expressing a particular neuropeptide (Campbell et al., 1987a). Quantitative studies on the distribution of NADPH-d-positive neurons have until now not examined the numbers of these neurons in individual radial columns but instead have documented area differences in numbers of neurons in constant volumes of cortex (Dombrowski and Barbas, 1996; Bidmon et al., 1997). In the present study we have examined the area variation of the numbers of type 1 and type 2 NADPH-d-positive neurons under 1 mm² of cortical surface of individual cortical areas in the frontal, temporal, parietal and occipital lobes of the macaque. These results show a hitherto unsuspected areal heterogeneity of NADPH-d-positive cells in primate cortex. There are important areal differences in the numbers of type 1 neurons similar to those in the rodent, with peak levels in both orders being found in the white matter of the frontal cortex (this study) (Bidmon et al., 1997). Type 2 neurons are characteristic of the primate and show important differences from the type 1 neurons since they peak in the occipital cortex and are very numerous in the supragranular layers in all sensory areas. Pair-wise comparisons of areal densities in individual areas show that type 1 neurons differ significantly in 13/15 pairs. Combined, type 1 and type 2 comparisons across layers show that each cortical area has a unique profile of NADPH-d expression.

Material and Methods

Surgical Procedure

Five adult monkeys were used to study the expression of NADPH-d in the neocortex. The animals were deeply anaesthetized before being perfused transcardially with 200 ml of 2.7% saline, 1–3 I of paraformaldehyde solution (4–8%) in 0.1 M phosphate buffer, 0.5 l of 8% sucrose, 0.5 l of 20% sucrose and 0.5 l of 30% sucrose in phosphate buffer. The brains were immediately removed and 40 µm thick sections cut on a freezing microtome in the parasagittal or tangential plane.

Histological Processing

All animals had identical histological treatment and NADPH-d histochemistry was performed according to the protocol of Weinberg et al. (Weinberg et al., 1995). Briefly, floating sections were rinsed in phosphate buffer and incubated at 37°C for 1–5 h in a phosphate buffer solution containing 0.1% NADPH, 0.02% NTB and 0.3% Triton X. Adjacent sections were counterstained for Nissl and processed for cytochrome oxidase (CO) (Silverman and Tootell, 1987) and acetylcholine esterase (AchE) histochemistry using a modified protocol of Hardy (Hardy et al., 1976) or Mesulam (Mesulam and Geula, 1994).

Data Acquisition

Sections were processed for NADPH-d histochemistry at regular intervals throughout the medio-lateral extent of the cortex. Between five and ten sections were chosen for each cortical area where the plane of section was optimally perpendicular to the cortical surface. Individual sections were observed under bright- or darkfield illumination and an x-y plotter electronically coupled to the microscope stage was used to trace out contours as well as to record the positions of labeled neurons. Analysis of the distribution of cells was performed in six neocortical areas located in the occipital, parietal, temporal and frontal lobes. In the occipital cortex the analysis included visual areas V1 and V2. In the parietal lobe we analyzed the visual region located in the medial part of the posterior bank of the superior temporal sulcus which corresponds to the areas MT and FST (Van Essen et al., 1981; Desimone and Ungerleider, 1986). In the temporal lobe counts of positive cells were performed in the auditory cortex on the anterior bank of the superior temporal sulcus. This region was distinguished using AchE and CO histochemistry, and includes the auditory areas AI and R (Morel et al., 1993; Jones et al., 1995). In the frontal lobe we analyzed motor area 4 located in the anterior bank of the central sulcus and the lateral part of premotor area 6 in the posterior bank of the arcuate sulcus (Matelli et al., 1985). In both cases counts were not performed on the precentral gyrus where the two areas join because of the white matter configuration. These cortical areas were chosen because they represent different functional modalities in widely separated regions of the cortex.

Quantitative Analysis of Areal Differences in Numbers of NADPH-d-positive Cells

Cortical volumes were measured using a digitalized tracing table. The number of NADPH-d-positive neurons under 1 mm² of cortex and throughout the full thickness of the cortex gives the density of neurons under a unity of surface area (1 × 0.040 mm) which is adjusted to give the number of labeled neurons under 1 mm². This corresponds to the number in a radial column and is referred to as the areal density. Areal densities for type 1 neurons were calculated from individual plots of labeled neurons over 5–10 mm of cortex. Hence the number of observations for type 1 neurons in Table 1 refers to the number of sections. Type 2 neurons were considerably more numerous and their areal density was computed by counting the number of NADPH-d neurons in a 0.055 mm² grid which was moved through the full thickness of the cortex. Hence the number of observations for type 2 neurons in Table 1 refers to the number of counts made over 2–3 nonadjacent sections in each animal.

In all areas, numbers of type 1 neurons were estimated in a deep compartment (the white matter), an upper compartment (layers 1–4) and a lower compartment (layers 5–6). As type 1 neurons are very rare in layer 4 these neurons were allocated to the upper compartment. Because white matter type 1 neurons tend to lie immediately below the cortical gray matter we encountered little difficulty in allocating the neurons in individual areas. The few deep white matter neurons under areas V1 and V2 were shared between these two areas by bisecting the white matter.

The number of NADPH-d-positive cells was estimated using the Abercrombie correction (Abercrombie, 1946). In one monkey, the size of individual cells in each area was computed using an interactive plotting system piloted by Histowid software (Biocom ﬂ®). This made it possible to chart the location and equivalent mean diameter of a large population of neurons in each of the six areas. The equivalent mean diameter was used to obtain the correction factor for the splitting error according to the equation: $N' = n \times (T + D)$, where $N'$ is the corrected cell number, $n$ the neuron count, $T$ the section thickness and $D$ the calculated mean diameter. Statistical analysis of areal variations in numbers were performed using an analysis of variance (ANOVA). Fisher post hoc tests were used for individual comparisons between areas.

Results

Morphological features of NADPH-d-positive cells

Two morphologically distinct classes of neurons express NADPH-d activity in the cerebral cortex of the adult monkey (Sandell, 1986; Aoki et al., 1993; Hashikawa et al., 1994; Yan et al., 1996a). Type 1 neurons (Fig. 1) are characterized by a dense
Golgi-like labeling and a nonpyramidal morphology. These neurons present a large soma and multiple beaded processes. Type 1 neurons are observed in large numbers in all regions of the neocortex, including sensory (visual, auditory, somatosensory), motor and associative areas.

Many type 1 neurons are mainly located in the white matter, where they tend to be concentrated in a thin band immediately below the cortex. In all areas, type 1 neurons in the white matter share common features, including a bipolar morphology (Fig. 1A,B) with arborizations that run mainly along the lower limits of the gray matter. As described by others (Sandell, 1986), these processes can be followed over long distances. However, frequently type 1 neurons in the white matter send processes into the cortex (Fig. 1B), where they can be traced as far as layer 4.

In all areas, variable numbers of type 1 neurons are present in the cortical gray matter (Fig. 1C–G), where they are encountered in all laminae. In layer 1, NADPH-d-positive cells have processes that run over long distances inside layer 1 parallel to the cortical surface although some processes are directed into the supra-granular layers (Fig. 1E). In the sensory cortex, such as the visual or auditory areas, very few type 1 neurons are observed in layer 4, the majority being located in either infra- or supra-granular layers according to an area-specific pattern. Type 1 neurons typically have processes that cross several cortical layers. The processes of infragranular labeled neurons extend into layer 4 as well as deep into the white matter. Similarly, type 1 neurons in the supragranular layers frequently have processes that extend into layers 1 and 4.

There is extensive evidence that NO is involved in the regulation of cerebral blood flow (Iadecola, 1993; Estrada and DeFelipe, 1998). NOS has been reported to be present in endothelial cells (Bredt et al., 1990), and in our material NADPH-d histochemistry stains a dense network of cerebral blood vessels (see Figs 1 and 2). Furthermore, we frequently observed type 1 cells rolled around a blood vessel (Fig. 1G) or having processes in close relation with small arterioles (Fig. 1F).

In the primate NADPH-d is expressed in a second category of neurons, the type 2 cells (Aoki et al., 1993; DeFelipe, 1993; Lüth et al., 1994; Yan et al., 1996a,b). Type 2 neurons constitute a much larger population than does type 1 neurons. Type 2 neurons are characterized by small somata with only short processes and with variable staining for NADPH-d (Fig. 1C,D).

In all cortical areas, two subclasses of type 2 cells can be distinguished on the basis of their staining intensity. The more prevalent category is characterized by a lightly stained soma with reaction product displaced from the nucleus (Figs 1C, 2A). In this category usually only one short process can be distinguished. In the visual cortex the lightly stained category is predominantly in layer 4 (Fig. 2A). A second category of type 2 neurons is characterized by dense NADPH-d staining (Fig. 1C,D, 2B,C) with short processes organized in a radial pattern. These strongly stained type 2 neurons are located almost exclusively in the upper part of layer 2 (Fig. 5). The fact that the laminar location of these two categories of type 2 neurons differ suggests that they constitute distinct functional categories.

### Table 1

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<thead>
<tr>
<th>Number of animals, details of sampling (numbers of sections and neurons counted), areaal densities</th>
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<tr>
<td><strong>Type 1 cells</strong></td>
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The distribution of type 1 neurons in representative sections of the visual, auditory and frontal cortices is shown in Figure 3. These plots suggest that each area is characterized by its proportion of type 1 neurons in the white and gray matter, and further that there may be differences in the areal densities of these neurons in individual areas. Note that type 1 neurons in the white matter of area 6 are spread out over a greater depth than in the visual and auditory cortices (Fig. 3).

As a prerequisite to the analysis of areal variations in neuron numbers we first determined the soma size of NADPH-d-positive neurons in each area (see Materials and Methods). By way of an example the distribution of sizes in areas V1 and 6 are shown in Figure 4. When neurons from each layer within an area are pooled there is no significant difference in soma size across areas (F = 1.82; P > 0.05). However when soma sizes within individual laminar compartments are compared in different areas (Fig. 4A) there are small but nevertheless significant variations between areas (upper compartment: F = 4.82, P < 0.001; lower compartment: F = 2.86, P < 0.05; white matter compartment: F = 5.19, P < 0.001). The majority of gray matter neurons are located in the upper compartment (see below) and tend to be smaller than the large population of white matter type 1 neurons.

The areal density of type 1 neurons is minimum in the
occipital cortex and maximum in the frontal cortex (Fig. 4B). An analysis of variance reveals that these areal differences are highly significant ($F = 54.8; P < 0.0001$, Table 2). The tendency for higher areal densities in rostral areas is also present within the visual and motor systems so that the areal density in the visual STS region is ~30% higher than that observed in area V1 and likewise the areal density in area 6 is significantly higher than in area 4. The STS region analyzed in this study includes the two

Figure 1. Type 1 neurons. (A) High power microphotography of type 1 neurons in the white matter of V1 showing beaded processes. (B) Type 1 neurons in the white matter of V1 just below the cortical gray matter with processes entering the infragranular layers (arrow heads). (C, D) Examples of supragranular type 1 neurons. (C) area V1, (D) STS region. Some type 2 neurons are indicated by arrows. Straight arrows indicate the large densely stained type 2 neurons, curved arrows the lightly staining small type 2 neurons (see text). (E) Example of one of the few type 1 neurons in area V2 located in the upper part of layer 1 with processes descending into the supragranular layers (arrowheads). (F, G) Type 1 neurons in the cortical gray matter in close relationship with cerebral blood vessels (star). F shows a type 1 neurons with processes (arrow-head) directed toward a blood vessel, G a type 1 neuron rolled around a blood vessel. Scale bars: 0.05 mm.
visual areas MT and FST. In an attempt to differentiate NADPH-d expression in these individual areas, the STS region was divided into a dorsal and a ventral part. Individual counts of type 1 density did not reveal a difference between these two subregions in any laminar compartment (Mann–Whitney test, \( P > 0.05 \)).

There is a remarkable areal heterogeneity in the areal density of type 1 neurons (Fig. 4C). Table 2 shows that for the 15 possible pairwise comparisons, 12 show a significant difference.

In all areas, type 1 neurons are principally located in the white matter (range: 51% in auditory cortex to 67% in STS) and it is the variations of these white matter neurons (\( F = 61.29; \ P < 0.0001 \), Table 2) which makes the largest contribution to changes in areal densities in Figure 4B.

The upper laminar compartment (layers 2/3) has a higher areal density of type 1 neurons than does the lower compartment (layers 5/6). With the exception of area 4, there is a remarkably constant areal density of type 1 neurons in the upper layers across areas (\( F = 1.87; \ P > 0.05 \)) suggesting that these neurons might have a common role in the different areas.

Except for area 6 and the auditory cortex, the lower laminar compartment contains the lowest areal density of type 1 neurons and outside of these two areas there is little variation in density in this compartment.

Pairwise comparisons across areas and compartments shows significance in 15/15 comparisons (Table 2).

Areal Densities of Type 2 Neurons

The distribution of type 2 neurons in 500 \( \mu \)m wide columns is illustrated in Figure 5. In all areas, the darkly stained category of type 2 neurons (indicated by crosses) are concentrated in the upper part of layer 2. Area V1 shows high numbers of type 2 neurons in layer 4 as well as in layers 2/3. In the other areas layer 4 appears to contain relatively fewer type 2 neurons.

Equivalent diameter distributions of type 2 neurons for areas V1 and the auditory cortex are illustrated in Figure 6A. The type 2 neuron diameters range from 5.8 to 13.5 \( \mu \)m and are approximately half the value of type 1 neurons (range 8.3–23.4 \( \mu \)m). The mean value of cell diameter for individual areas show significant variations (\( F = 151.6; \ P < 0.0001 \)) and there is a continuum of increasing size going from area V1 to area 6 (Figure 6B). In each area, cell size varies significantly according to the laminar location (Figure 6C). This is particularly true for the dense staining category of type 2 neurons, which have soma diameters that are \(~25\%\) larger than the lightly staining category.

Areal counts reveal significant variations in the areal densities of type 2 neurons (Fig. 6D; \( F = 3.89; \ P < 0.05 \)). Areal density is up to three times higher in area V1 compared with other areas. Outside area V1 the areal density of type 2 neurons is similar across areas (\( F = 0.3 ; \ P > 0.05 \)). Pairwise comparisons of the areal density of type 2 neurons show 9/15 possible comparisons are significantly different.

Area V1 is unique because of the high areal density of type 2 neurons in layer 4 and in the supragranular layers, which together have 2–10 times the values observed in other areas (Fig. 6E). Across all areas including area V1 there is no significant variation in the areal densities of the infragranular layers (\( F = 1.99; \ P > 0.05 \)). Outside of area V1, supragranular layers contain higher densities than either infragranular layers or layer 4, and there is some weak but significant differences between areas (Table 3).

Compared with type 1 neurons, areal differences show the opposite trend: areal densities of type 1 cells are low in area V1 and high in the frontal cortex and the opposite is true for type 2 cells. Type 2 neurons show less significant variations of laminar distributions than do type 1 neurons (Fig. 6E) since only 9/15 possible comparisons are statistically significant (compare Tables 2 and 3).

These results show that area V1 has a significantly higher areal density of type 2 neurons than other cortical areas. This could simply be due to the higher number of neurons in a radial column of area V1 (Rockel et al., 1980). To examine if this is the case we estimated the percentage of labeled type 2 neurons within each compartment using the published lamina cell counts of Hendry et al. (Hendry et al., 1987). This shows that the proportions in all laminar compartments of area V1 are nearly twice that observed in the adjacent area V2 and further shows...
that there is a significant decrease in supragranular layers between areas V2 and 6 (insert in Fig. 6C).

NADPH-d-labeled Fibers

Dark-field illumination shows that the cortical layers show characteristic differences in the density of NADPH-d-positive fibers (Fig. 7). In all areas these fibers form a dense lattice just below the cortical gray matter. Fiber density is relatively lower in the gray matter of area V1, making the white matter band of fibers in this area relatively more prominent.

In area V1, NADPH-d-positive fibers in gray matter are organized in a bilaminar fashion: a deep lamina fiber band is centered on layer 5, and a wide superficial band goes from the top of layer 4A up to and including layer 1. The overall fiber pattern is similar in visual areas, as indicated in Figure 7 by the labeling pattern in STS. The anterior cortical areas 4 and 6 contain a richer density of fibers which are more homogeneously distributed across the thickness of the cortex.

Discussion

The present findings show that there are important differences in the areal and laminar distribution of NADPH-d-positive neurons. Two major trends are (i) the large numbers of type 1 neurons in rostral areas located in the output layers to subcortical structures; and (ii) the large numbers of type 2 neurons located in layer 4 and supragranular layers of area V1. When the different NADPH-d types are considered jointly each cortical area examined in this study appears to have a unique profile of NADPH-d neurons. This is mainly due to variations of type 1 neurons in infragranular layers and white matter coupled with variations of type 2 neurons in supragranular layers and layer 4. Across areas, type 1 neurons tend to be relatively constant in the supragranular layers and type 2 neurons relatively constant in the infragranular layers. Before examining the theoretical significance of these findings we shall first address certain technical issues.

Reliability of the Quantification Made in the Present Study

The present results indicating important areal variations in the distribution of NADPH-d neurons are obtained from neuron counts performed on representative sections. These cell counts include counts of sectioned neuronal profiles (i.e. parts of individual cells), resulting in an overestimation of numbers of neurons. The magnitude of this overestimation depends on variables including the shape and the size of the neurons as well as section thickness. We have therefore used the Abercrombie correcting method (Abercrombie, 1946) to estimate the number of NADPH-d-positive neurons. However, this method is known to overestimate numbers of neurons, the magnitude of the error depending on the section thickness and its relation to the cell size (Clarke, 1992). Given the section thickness and cell sizes in the present study, one can predict that type 1 and type 2 neuron
Figure 4. Dimensions and areal distribution of type 1 neurons. (A) Left, distribution of type 1 neuron diameters in area V1 and area 6. Distributions are centered on the means, which are virtually identical. Right, small variations in mean diameter (open bars) are observed across cortical areas. The means for each laminar compartment are indicated. (B) Total number of type 1 neurons under 1 mm². (C) Numbers of type 1 neurons under 1 mm² in individual compartments. Abbreviations: Upper, upper compartment (layers 2–3–4); Lower, lower compartment (layers 5–6); WM, white matter compartment.
Comparison between identical compartments across areas.

Within each category we show heterogeneity by providing a comparison of proportions of type 1 and type 2 neurons. Further, because the final analysis compares neuron populations which are sufficiently similar, the precision of the Abercrombie correcting factor is adequate for documenting the heterogeneity of neuron dimensions of the populations being compared areal density values in more rostral areas. However, these variations do not differ significantly from zero.

Table 2

| Total | ANOVA \( F = 54.82 \) \( P < 0.0001 \)
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<td>Area 6</td>
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WM ANOVA: \( F = 61.29 \) \( P < 0.0001 \)

| | Area V1 | Area V2 | STS | Auditory | Area 4 |
| Area V2 | * |
| STS | *** |
| Auditory | *** |
| Area 4 | *** |
| Area 6 | *** |

Upper ANOVA: \( F = 49.31 \) \( P < 0.0014 \)

| | Area V1 | Area V2 | STS | Auditory | Area 4 |
| Area V2 | 0.61/h.s.*** |
| STS | * |
| Auditory | 0.06/h.s.**** |
| Area 4 | 0.19/h.s.**** |
| Area 6 | 0.20/h.s.**** |

Lower ANOVA: \( F = 43.82 \) \( P < 0.0001 \)

| | Area V1 | Area V2 | STS | Auditory | Area 4 |
| Area V2 | 0.43/h.s.*** |
| STS | 0.67/h.s.*** |
| Auditory | 0.10/h.s.*** |
| Area 4 | 0.40/h.s.*** |
| Area 6 | *** |

An analysis of variance (ANOVA) across areas was performed for all NADPH-d-positive neurons, and separately for neurons located in the white matter and in the upper and lower layers (see text). In each case, the F value and the probability (P) are provided. A Fisher post hoc test was used for individual comparisons.

**P < 0.001; ***P < 0.01; *P < 0.05.

Corrections of neuron number were made using the neuron soma sizes obtained from samples in each area. We have shown that type 1 neurons present similar dimensions across areas, and consequently similar correcting factors are applied to each areal count. Type 2 neurons show regional variations in soma sizes, with smaller values in the occipital cortex and larger values in more rostral areas. However, these variations do not exceed 21%. Consequently, the range of correcting factors between areas is small \( \frac{T}{T + D} \); range 0.79–0.84]. Given that in the present study we are examining changes in proportions (and not absolute numbers) of NADPH-d neurons and given that the neuron dimensions of the populations being compared are sufficiently similar, the precision of the Abercrombie correcting method is adequate for documenting the heterogeneity of NADPH-d neurons in monkey cortex. It could be objected that conventional profile counting methods are also subject to errors if cell morphologies vary (Williams and Rakic, 1988). In the present study this will not introduce a large degree of error because the final analysis compares neuron populations which have similar morphologies, so, for example, we do not attempt to compare proportions of type 1 and type 2 neurons. Further, within each category we show heterogeneity by providing a comparison between identical compartments across areas.

Hence, for example, we are able to show that white matter type 1 neurons with similar morphologies are twice as numerous in the frontal cortex than in the occipital cortex (Fig. 4).

In the present study we have chosen sections which are cut perpendicular to the cortical surface. However, it could be argued that small variations in the plane of section resulting in variations in cortical thickness could be a contributory factor to interareal differences in numbers of NADPH-d neurons in radial columns. Although this objection cannot be entirely ruled out, it cannot account for the massive differences between area V1 and the more anterior cortical areas. Variations in the plane of section could contribute to some of the area differences seen amongst areas V2, STS, the auditory cortex, area 4 and area 6. The fact that outside area V1 we find a constant level of areal density of type 1 neurons in the supragranular layers across areas, coupled with a constant density of type 2 neurons in the infragranular layers, shows that variations in the plane of section cannot account for all the observed interareal variations.

Cortical Heterogeneity of Type 1 Neurons

Outside of primates, NADPH-d expression occurs largely in neurons that are similar in morphology to type 1 neurons of the monkey (Vincent and Kimura, 1992; Valtschanoff et al., 1993; Rodrigo et al., 1994; Gabbott et al., 1997). The areal differences which we found in type 1 neurons in the primate resemble those in the rat cortex, where there are low values in primary sensory areas and high values in associative, frontal and limbic areas (Bidmon et al., 1997).

In monkeys, the majority of type 1 neurons are located in the white matter in all areas. Within the gray matter, type 1 neurons are preferentially located in the upper layers in visual areas V1, V2 and STS, while in the auditory cortex and area 6, type 1 neurons are relatively more numerous in lower layers. These laminar distributions are consistent with qualitative observations in the visual and auditory cortices of humans and monkeys (Sandell, 1986; Gipponi and Pandya, 1991; Lüth et al., 1994). A major difference between rodents and primates concerns the laminar location of type 1 neurons. The predominance of gray matter type 1 neurons in the upper cortical layers of the primate (this study) (Yan et al., 1996a) differs significantly from that in rodents, where the majority of NADPH-d-positive cells which correspond to type 1 neurons are located in infragranular layers (Yan et al., 1994; Xiao et al., 1996; Gabbott et al., 1997).

Type 1 cells in the white matter could constitute a vestige of the subplate, which is a largely transient embryonic structure located below the cortical plate and formed by the earliest generated neurons (Kostovic and Rakic, 1980; Luskin and Shatz, 1985; Chun and Shatz, 1989). In the monkey the subplate reaches its maximum size at mid-gestation and is thought to largely disappear by birth (Kostovic and Rakic, 1990). Regional differences in areal density of type 1 cells might be due to a higher rate of elimination of subplate neurons in the visual areas. Areal differences in the numbers of white matter type 1 neurons could reflect regional differences in numbers of white matter neurons (Meyer et al., 1992).

Cortical Heterogeneity of Type 2 Neurons

Counts of type 2 neurons reveal disparities across areas, with a particularly high areal density in the primary visual area V1. Differences in areal density are also present outside of V1 but are less pronounced – especially in the infragranular layers, which are characterized by low numbers in all areas examined. Type 2 neurons have been described in the visual cortex of humans.
and monkeys, and the laminar and areal differences established in the present study are consistent with earlier qualitative descriptions (Aoki et al., 1993; Lüth et al., 1994; Yan et al., 1996a).

In the present study we have used data on neuron numbers published by Hendry et al. (Hendry et al., 1981) to determine whether the differences in areal density of type 2 are due in part to differences in the proportions of these neurons or whether they merely reflect overall changes in neuron number in individual layers. While it is clearly preferable to use neuron counts from the same section, this approach is valid for detecting large changes in proportions of neurons. This is indeed the case for type 2 NADPH-d neurons, which we found to exist in much larger proportions in area V1 than in area V2, both occipital areas having considerably higher proportions than that found in the frontal cortex (see insert Fig. 6E).

Type 2 cells express GABA (Yan et al., 1996a; Yan and Garey, 1997), which is a specific marker of interneurons (Van Brederode et al., 1990; Jones, 1993; Jones et al., 1993). Our results on the areal variations of type 2 neurons are compatible with global differences observed in the number of interneurons across the cortex of the monkey revealed with GABA, calcium binding proteins or somatostatin immunoreactivity (Campbell et al., 1987a; Hendry et al., 1987; Kondo et al., 1994; Conde et al., 1996). It has been reported that there are 50% more GABA-positive neurons in area V1 than in area V2 or the motor area (Hendry et al., 1987). Since 100% of NADPH-d-positive type 2 neurons express GABA, the distribution of type 2 neurons might be expected to follow the same trend as GABA-positive neurons. However, the distribution of type 2 neurons shows an important difference to that observed of GABA-positive cells: whereas the proportion of GABA-positive cells with respect to the overall population of neurons in a radial column does not vary across areas (Hendry et al., 1987), the proportion of type 2 neurons is nearly twice as high in area V1 than in area V2, which in turn is significantly higher than in area 6.

Further evidence that type 2 neurons are a particular subpopulation of GABA-positive interneurons comes from deprivation studies. In adult monkey area V1, the expression of GABA and calcium binding protein is dependent on ascending neuronal activity (Jones et al., 1993). Monocular deprivation in the adult induces a rapid down-regulation of GABA and calcium binding protein expression in visually deprived cortical columns in area V1 (Hendry and Jones, 1986; Carder et al., 1996). This down-regulation has been interpreted as subserving plasticity in the visual system of the adult (Jones et al., 1993). However, numbers of NADPH-d neurons were reported not to change in the visual cortex of monocularly deprived adult monkeys (Aoki et al., 1993).

Functional Considerations of Areal Heterogeneity of NADPH-d-positive Neurons

Type 1 and type 2 neurons express GABA, but the major histochemical difference between the two types is that whereas only 4% of type 1 express calbindin, 100% of type 2 neurons do (Yan et al., 1996a). Calbindin- and GABA-positive neurons are differently distributed across the cortex. In the visual system, calbindin-positive neurons are reported to be more numerous in

Figure 5. Distribution of type 2 neurons in 500 µm columns area V1, area V2, auditory cortex, area 6. Crosses: densely labeled category. Black dots: lightly stained category.
Figure 6. Dimensions and distribution of type 2 neurons. (A) Type 2 neurons diameter in area V1 and auditory cortex. Distributions are centered on the mean which varies significantly for each area (area V1: 8.44 µm; auditory cortex: 9.98 µm). (B) Mean diameter of all neurons in individual cortical areas. Neurons in posterior visual areas present a smaller size compared to neurons in anterior cortex. (C) Regional variations in neuron size are conserved when the analysis is carried out separately for the two categories of type 2 neurons in different compartments. (D) Total number of type 2 neurons under 1 mm² of cortex in individual areas of the neocortex. (E) Number of type 2 neurons under 1 mm² of cortex according to their laminar location. The insert in graph E indicates the proportions of type 2 cells with respect to the total number of neurons under 1 mm² of cortical surface (see text).
the temporal visual areas than in area V1 (Kondo et al., 1994), which is a trend opposite to that of the distribution of type 2 neurons. These results suggest that type 2 NADPH-d-positive neurons might correspond to a subpopulation of calbindin-positive neurons. A subpopulation has been described which is a suitable candidate. These are neurons which are lightly stained for calbindin and present a similar laminar distribution to type 2 neurons with a high density in layer 4 of area V1 (Van Brederode et al., 1990) and which tend to decrease going from caudal to rostral areas (Kondo et al., 1994).

Altogether, these observations are compatible with the fact that interneurons are composed of multiple subpopulations of neurons, each of which is characterized by neuropeptide and calcium binding protein content as well as laminar and areal distribution. Type 2 neurons are one of these categories, and their high concentration in area V1 suggests they might play a particularly important role in the physiology of this area.

Type 2 neurons have been reported in numerous cortical regions of the monkey (this study) (Sandell, 1986; Aoki et al., 1993; Yan et al., 1996a) and human (Lüth et al., 1994; Yan et al., 1996b), and are not reported in the cortex of carnivores and rodents (Bredt et al., 1990, 1991; Vincent and Kimura, 1992; Rodrigo et al., 1994; Yan et al., 1994). However, a recent study shows that only rodents do not have type 2 neurons and suggests that primates present the highest density of type 2 cells (Yan and Garey, 1997). Significant species differences exist both in the proportion and laminar distribution of GABA interneurons that express calcium binding proteins (DeFelipe, 1993; Glezer et al., 1993). Thus, NADPH-d expression in GABA-positive neurons is one of the phenotypic changes that influence interneurons and which might be related to specific functional adaptations of the primate.

**NADPH-d-positive Fibers**

In the cortex and the underlying white matter, there is a dense network of NADPH-d-positive fibers which shows regional specific patterns. In sensory areas such as V1 and STS, there are two bands of NADPH-d fibers located in the supragranular layers and in layer 5. In premotor area 6, positive processes are much more uniformly distributed across layers. It has been shown in area V1 that NOS-positive processes are axons forming synaptic junctions with dendrites (Aoki et al., 1993). However, the origin of NADPH-d fibers in the cortex is still not known. The distribution of these fibers is not related to the location of either the type 1 or the type 2 neurons, but it nevertheless remains possible that the cortical NADPH-d-positive fibers do arise from the gray and white matter positive cells (Meyer et al., 1991).

Another possibility is that the NADPH-d fiber networks originate from subcortical structures. The thalamus can be excluded as a possible source of NADPH-d-positive fibers because the principal thalamic recipient layers are not densely labeled and there is no NADPH-d-positive cells in the lateral geniculate nucleus (P. Barone and H. Kennedy, unpublished observation) (Aoki et al., 1993; Satoh et al., 1995). This absence of NADPH-d neurons in the lateral geniculate nucleus constitutes a primate feature since NADPH-d-positive neurons are present in

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**Figure 7.** Dark-field microphotographs of NADPH-d expression in different cortical areas. NADPH-d-positive fibers form a dense lattice in the white matter in all areas. In the frontal cortex, the network of positive processes is dense and appears more uniformly distributed across layers than in visual areas. Scale bar: 0.2 mm.
Table 3
Statistical comparisons of type 2 neurons areal density in six different regions of the cortex

<table>
<thead>
<tr>
<th></th>
<th>ANOVA: F</th>
<th>Area V1</th>
<th>Area V2</th>
<th>STS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer 4</td>
<td>1.99</td>
<td>0.08 n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infra cells</td>
<td>37.88</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0001</td>
<td></td>
<td></td>
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<tr>
<td>STS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auditory</td>
<td>0.77 n.s.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supra cells</td>
<td>34.89 n.s.</td>
<td></td>
<td>0.08 n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area V2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auditory</td>
<td>0.26 n.s.</td>
<td>0.31 n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area 4</td>
<td>0.65 n.s.</td>
<td>0.15 n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area 6</td>
<td>0.07 n.s.</td>
<td>0.24 n.s.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An analysis of variance (ANOVA) across areas was performed for all NADPH-d-positive neurons, and separately for neurons located in layer 4 and in the infragranular and supragranular layers. Conventions are as in Table 2.

**P < 0.001; *P < 0.01; *P < 0.05.

the thalamic relay of the rat (Mitrofanis, 1992; Gabbott and Bacon, 1994) and of the developing carnivore (Cramer et al., 1995; Guido et al., 1997). Other subcortical origins of NADPH-d fibers remain possible. The cortex is the target of afferents from dopaminergic, serotonergic and cholinergic systems that originate from the ventral tegmental area, the Raphe nucleus and the basal forebrain respectively. All of these structures have been shown to contain NADPH-d-positive cells (Ellison et al., 1987; Breit et al., 1991; Eggerongbe et al., 1994; Rodrigo et al., 1994; Satoh et al., 1995). Cholinergic, serotonergic and dopaminergic afferents to the cortex of the monkey terminate in a specific areal and laminar organization (Campbell et al., 1987b; Lewis et al., 1987; Berger et al., 1988; Wilson and Milliver, 1991; Voytko et al., 1992; Mrzljak and Goldman-Rakic, 1993). Because none of the cortical fiber patterns of these different systems correspond precisely to the pattern of NADPH-d-positive fibers, it could mean that NADPH-d fibers in the cortex correspond to a subset of these fibers.

Developmental Considerations of Nonuniformity of Neocortex

Although some authors have argued in favor of a uniform immature cortex, there is increasing evidence that the neocortex develops from an early fate map of gene expression in the embryonic germinal zone (Rakic, 1988; Arimatsu et al., 1992; Kennedy and Dehay, 1993; Cohen-Tannoudji et al., 1994). Further, it is known that the connectivity of cortical neurons which are generated early in corticogenesis is determined during their final round of mitosis in the ventricular zone (McConnell and Kazznowski, 1991), and there is evidence that this fate determination contributes to areal differences in neuronal phenotypes (Polleux et al., 1997). Therefore, heterogeneity, including that of NADPH-d-positive neurons in the white matter, could either result directly from the developmental protomap or be the consequence of areal-specific developmental mechanisms relying on extrinsic cues.

In the present study, the principal source of heterogeneity of areal density stems from the type 1 neuron. Type 1 neurons constitutes a specific subclass of interneurons the bulk of which are likely to be born at a restricted period of early cortico-genesis. This contrasts with the homogeneity of GABA-positive neurons (Hendry et al., 1987), which are generated throughout neurogenesis (Cobas and Fairén, 1988). It remains to be seen whether there is a large-scale heterogeneity with respect to other subclasses of interneurons.

Conclusion

The radial column is important for understanding cortical physiology and development (Mountcastle, 1957, 1997; Hubel and Wiesel, 1977; Rakic, 1988). Although the uniformity of the neocortex is often emphasized, the present study, along with the findings of Csilik et al. (Csilik et al., 1998), shows that there are important heterogeneities in terms of NADPH-d expression. Given the involvement of NO in a wide range of physiological responses of the cortex (Gally et al., 1990; Nowicki et al., 1991; Adachi et al., 1992; Dawson et al., 1992; Prado et al., 1992; Vincent and Hope, 1992; Iadecola, 1993; Pneuova and Enikolopov, 1995) and its implication in cognitive disorders (Akbarian et al., 1993a,b), the heterogeneity of NADPH-d could have farreaching consequences. The present results suggest that type 1 and type 2 neurons contribute to the areal specificity of intrinsic connectivity and local circuitry (Kritzer et al., 1992; Amir et al., 1993; Lund et al., 1993; Elston and Rosa, 1997). NO has been shown to modulate the release of other neurotransmitters (Hansbauer et al., 1992; Montague et al., 1994) and to be involved in the modulation of synaptic plasticity (Kara and Friedlander, 1999). Consequently NO might influence the functional properties of individual cortical regions of the primate. If the type 1 and type 2 neurons have different functional roles, these results showing important areal differences in the distribution of these two types would suggest that these functional roles are emphasized differently in individual areas.

Notes

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