Vascularization in the Primate Visual Cortex during Development

We studied the relationship between vascularization and neuronal activity in the visual cortex during postnatal development in the primate. Analyses were focused on layer IVC that displays a sequential pattern of maturation for the magnocellular- and parvocellular systems in separate sublayers, respectively IVCalpha and IVCbeta. Cytochrome oxidase and endogeneous alkaline phosphatase histochemistry was used to analyse, on the same sections, the laminar patterns of cortical activity and vessel density in the primary visual cortex of the marmoset (Callithrix jacchus). Experiments were carried out in five young and two adult animals. We showed that the temporal pattern of angiogenesis differs in layer IVCalpha and IVCbeta. During the first postnatal month, vessel density is higher in IVCalpha than in IVCbeta and runs parallel to cytochrome oxidase intensity. In 2-month-old animals, both vessel densities and cytochrome oxidase activity are similar in IVCalpha and IVCbeta. In adults, the vessel densities in IVCalpha and IVCbeta are the reverse of those observed during the first postnatal month. Vessel diameter does not account for this evolution in vascular patterns. In the discussion, we suggest that such a developmental time-course of angiogenesis might be linked to the synaptogenesis requirements that proceed differently for the magnocellular- and parvocellular systems in the primate striate cortex.

Introduction

The existence of cortical modules, both neuronal and vascular, has been demonstrated in the supragranular layers of the primary visual cortex (blobs) (Zheng et al., 1991) and in the somatosensory cortex (barrels) (Woosley et al., 1996). However, the mechanisms by which these modules develop have not been addressed in primates. If the anatomical and physiological properties of the primary visual cortex have been largely investigated in several primate species, only a few works studied its vascular architecture (Bell and Ball, 1985, 1990; Zheng et al., 1991). Generally speaking, brain vascular architecture, that determines cerebral blood flow and oxygen utilization by the cerebral tissue, is not extensively analysed. Relationships between blood oxygenation level and neuronal activity are thoroughly investigated (Vanzetta and Grinvald, 1999; Logothetis et al., 1999, 2001) since numerous investigators associate neuronal functional activation with tissue metabolic requirements and temporary modifications in the regional blood flow to study the neuronal basis of cognitive functions in adults. During development, hemodynamic changes have moreover to be considered in a changing space, since brain development still occurs in childhood and adolescence.

To examine the development of cortical modules, we adopt the hypothesis that neural activity drives the maturation of microvascular circuitry in the developing brain. During the postnatal period, brain metabolism is energy-demanding to ensure synaptogenesis and neural maturation. The formation of a given vascular pattern is likely to be controlled by intrinsic mechanisms, neuronal activity and sensory experience. As a direct consequence, alterations in neuronal inputs may prevent the 'normal' vascular pattern from establishing. Sensory deprivation would thus disturb angiogenesis processes and, if irreversible, retardation in angiogenesis might become severe enough to impair neural plasticity. These mechanisms in pathological brain development are rarely considered. In rats, complex environments produce an increase in capillary density in the visual cortex (Sirevaag et al., 1988). Visual deprivation provokes modification of the pial angiarchitecture of the parieto-occipital cortex (Wolff et al., 1992) and delays maturation of the vascular support (Argandoña and Lafuente, 1996). Similar studies have not, to our knowledge, been carried out in the primates and the first step to be considered is to establish the correlation between neural maturation and angiogenesis in the developing brain in control monkeys.

In the present paper, we examine the correlation between vascular organization, analysed by alkaline phosphatase revelation, and cortical metabolism, revealed by cytochrome oxidase activity, in the developing primary visual cortex in the primate Callithrix jacchus. Mitochondrial cytochrome oxidase enzyme activity reveals laminar and modular neuronal activation patterns in the cerebral cortex (Carroll and Wong Riley, 1984; Wong Riley, 1989). Its levels adjust to changes in regional neuronal activity (Wong Riley, 1979; Wong Riley and Welt, 1980). Links between brain oxidative metabolism, assessed by cytochrome oxidase activity and vessel density, have been established in different grey matter structures in the adult rat brain. In the hippocampus and the olfactory cortex there is a clear correspondence between vessel density, glucose utilization and cytochrome oxidase activity (Borowsky and Collins, 1989). In the somatic sensory cortex, differential distribution of microvessels between barrels and the surrounding cortex is correlated with the distribution of mitochondrial enzymes (such as cytochrome oxidase) and with electrical and metabolic neuronal activities (Riddle et al., 1993). In the rabbit parietal cortex, cytochrome oxidase activity and capillary density are not correlated in very young animals, the cytochrome oxidase pattern being more mature compared with that of vessel density, but are closely linked in older rabbits (Tuo et al., 1994).

A possible correlation between neural maturation and angiogenesis in the developing brain needs further investigation in the primate that presents specific features of cortical development, compared to rodents (Kennedy and Dehay, 1993). Development of primate cortex is unique in its early cortical specification, in both areal and laminar definition. Thalamic afferents exert an early influence on the prenatal developing cortex in monkeys, especially on the development of connections. In the rodent cortex, modular features are formed postnatally, whereas the cortex of newborn primates already presents adult-like modules. We focus our study on layer IVC of the primary visual cortex because it represents an adequate model for analysing the
development of a vascular pattern in relation to neural maturation. Layer IVC represents the main target of the visual thalamocortical axons. It is generally described as composed of two distinct layers, layer IVCalpha and layer IVCbeta, that are the respective targets of the magnocellular and the parvocellular systems in the striate cortex (Hubel and Wiesel, 1972; Blasdel and Lund, 1983; Freund et al., 1989). Thalamic afferents are segregated and organized into an adult-like pattern in layer IVCalpha and IVCbeta in newborn macaques (Rakic, 1977; Horton and Hocking, 1996) and very young marmosets (Chapert et al., 2001). Both layers display similarly high cytochrome oxidase staining in adults, but not in juveniles. In young primates, layer IVCalpha shows a dense cytochrome oxidase staining, while layer IVCbeta is only a lightly stained zone (Horton, 1984; Kennedy et al., 1985; Hendrickson et al., 1991; Spatz et al., 1993, 1994; Horton and Hocking, 1996; Fonta et al., 1997). It is proposed that this developmental pattern of cytochrome oxidase in layer IVC corresponds to the differential maturation time-course between the magnocellular and parvocellular pathways. The hypothesis that vascularization of the primary visual cortex is correlated with the differential maturation of the two systems appears pertinent, but had not yet been tested.

The present work analyses laminar vessel density in the primary visual cortex of the New World monkey Callithrix jacchus, during early postnatal life and in the adult. Analysis focuses on the two sublayers of layer IVC that display a sequential maturation and a high sensitivity to visual deprivation in the first two postnatal months (Spatz et al., 1993; Fonta et al., 1997, 2000). The anatomical identification of a vascular territory can be made by various techniques, some being direct, such as transillumination (Zheng et al., 1991), but not always adequate to detect in parallel other markers by immunohistochemistry or histochemistry (e.g. intravascular contrasting agents). We obtained good quality resolution of vessel distribution by histochemical detection of an endogenous endothelial enzyme, alkaline phosphatase. This method had been previously used to visualize adult brain vascular architecture in humans and macaques (Friede, 1966; Bell and Scarrow, 1984; Bell and Ball, 1985, 1990). Cytochrome oxidase activity was used to visualize, on the same sections, the relative levels of laminar cortical activity. The results show that angiogenesis occurs in parallel to neural maturation in layer IVC.

Material and Methods
The animals were born in a marmoset rearing facility maintained at the Centre de Recherche Cerveau et Cognition, Toulouse, France. Seven marmosets were used: two adults, one 7 years old (98.4) and one 11 years old (97.7); two animals 60 postnatal days old (PND) (99.9 and 00.5); one animal 24 PND (97.12); one 17 PND animal (97.9); and one neonate, a few hours old (00.3).

Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg) with a rinse of NaCl 0.9% with heparin (4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4). The brain was then perfused with paraformaldehyde for 2 h and cryoprotected overnight with 30% sucrose in 0.1 M PB. One animal (97.9) was perfused with 10% formalin.

Cortical blocks were sectioned at a thickness of 30 µm with a cryostat. Experiments were carried out on free-floating frontal sections.

All animal experiments were carried out in accordance with The National Institute of Health guidelines.

Histochemistry
Double staining was carried out on sections devoted to quantitative analyses. The sections were stained first for cytochrome oxidase, then for alkaline phosphatase. In these double-stained sections, the cytochrome oxidase brown background was used to distinguish borders of layers. Vessels, revealed by endothelial alkaline phosphatase, were easily identified as dark blue or black areas. Single staining (cytochrome oxidase or alkaline phosphatase) was performed on the other sections.

For cytochrome oxidase histochemistry, sections were incubated overnight in a solution containing 50 mg 3′,3′-diaminobenzidine tetra-chloride (DAB), 35 mg cytochrome C, 20 mg catalase and 4 g D(+)-sucrose per 100 ml PB (0.1 M, pH 7.4) at room temperature in the dark with agitation. Sections were then rinsed in several PB baths before staining of endothelial cells.

In some cases (if sections were not treated additionally for alkaline phosphatase staining), cytochrome oxidase reaction was preceded by an incubation in a cobalt chloride Tris solution.

Endogenous alkaline phosphatase reactions were performed by incubating sections in a 100 mM Tris–HCl pH 9.5 solution containing 100 mM NaCl, 50 mM MgCl2, 0.55 mM 4-nitro blue tetratolium chloride (NBT) and 0.38 mM 5-bromo-4-chloro-3-indolyl-phosphate-4-toluidine salt (BCIP). The reaction was carried out for 10 min at room temperature. The reaction was stopped by transferring sections to a 10 mM Tris pH 7.5 solution containing 1 mM EDTA and 10 mM levamisole. Sections were then rinsed in Tris and mounted.

Quantitative Analyses of Vessel Density
Mounted sections were examined and selected slides were digitized with a Leica microscope equipped with a 3 CCD camera (Sony DXC930P), interfaced with an IBM-compatible computer. Imascan Board (Imagraph) and Optimas software (Media Cybernetics). Morphometric analyses were performed with Optimas image analysis software. Images were first converted into grey values.

Measurements were made on the sampled images in 1 mm wide stripes extending from layer I to white matter on coronal sections. Two sets of measurements were performed. One set was made in the upper and lower banks of the calcarine sulcus, representing the peripheral visual field (Fritsches and Rosa, 1996). Ten or eleven stripes were analysed in two or three sections from the same hemisphere for each animal. The second set concerned the dorsal and dorso-lateral surfaces of the occipital lobe, corresponding to part of area 17 representing the central visual field (Fritsches and Rosa, 1996). Ten stripes were defined in two sections from the same hemisphere for each animal studied. Ventral and dorsal parts were equally represented in each set of measures.

The same side was investigated for age-matched animals to avoid possible effects of left/right asymmetry, even if cerebral dominance is generally not significant in adult macaques as far as density of neurons and cortical morphometric features are concerned. In any case, there seems to be no hemispheric specialization (O Kusky and Colonnier, 1982b; Gottlieb et al., 1985; Suner and Rakic, 1996).

Inside each 1 mm wide stripe, quantification of vascular parameters was performed within each layer or sublayer, defined by its cytochrome oxidase staining. The numbers assigned to the layers conform to the nomenclature used by Brodmann.

For all the animals, common areas of interest were as follows.

1. The supragranular layers corresponding to layers II and III (a depth of 200–400 µm below layer I was investigated).
2. Sublayers IVCalpha and IVCbeta — in the youngest animals (first postnatal month, i.e. animals 00.3, 97.9 and 97.12), dense cytochrome oxidase staining was restricted to layer IVCalpha. In 2-month-old animals (99.9 and 00.5), layer IVC displayed an homogeneous staining; however, a thin pale band separated the outer (IVCalpha) and inner (layer IVCbeta) parts of layer IVC. This band was more obvious with alkaline phosphatase staining. Therefore, the two sublayers of IVC could be identified, at high magnification, in juveniles. In the adults (97.7 and 98.4), the inner part (layer IVCbeta) showed a stronger cytochrome oxidase signal than the outer part of layer IVC. However, the boundary between the two sublayers was blurred. The innermost part of layer IVC (100–200 µm) was considered as layer IVCbeta. The 100–200 µm above, presenting a weaker cytochrome oxidase staining, were attributed to layer IVCalpha.
3. Layer V.
4. Layer VI — in 2-month-old animals, cytochrome oxidase staining enabled the two sublayers of layer VI to be clearly distinguished (with an outer sublayer darker than the inner sublayer).
When defining the individual layers, the extreme borders were discarded from the selection, as was the intermediate zone between IVCalpha and IVCbeta, that appears lightly labelled in cytochrome oxidase and more obviously in alkaline phosphatase staining, in 2-month-old animals. In fact, neurons located in this transition zone present connections different from those of neurons in layers IVCalpha and IVCbeta and they may, therefore, represent a separate entity (Boyd et al., 2000).

Figure 1. Double staining [cytochrome oxidase (CO) in brown; alkaline phosphatase (AP) vessels in blue] in the primary visual cortex of a newborn marmoset (coronal section). Layer IVCalpha and layer Va are more CO reactive than layer IVCbeta. AP strongly stains the white matter (wm). (B) Magnified part of the ventral calcarine cortex in (A); cs, calcarine sulcus.
Two parameters were quantified: (i) vascular density defined as the area occupied by vessel profiles per unit area of cortex and (ii) vessel diameter.

Vascular density was analysed at a final magnification of 180× by an automatic sequence of commands created to identify and measure all field areas with grey levels within a range of grey values indicative of vessel staining. This range was manually adjusted for each analysis because staining intensities might provide different grey ranges from one section to another. Thus, a semi-automatic procedure separated stained areas from background. The selection was visually checked and corrections

Figure 2. Cytochrome oxidase (CO, in A) and alkaline phosphatase (AP, in B) staining in the 24 PND animal. CO activity is stronger in layer IVCalpha than in layer IVCbeta. Apart from staining endothelial cells, AP produces a blue background in layer IVC and in the white matter (wm). In both stainings, layer IVCalpha is more labeled than layer IVCbeta.
made to discard selection of artefacts or to achieve a better selection. For this purpose, a higher magnification could be obtained using a zoom function. The area of interest (layer or sublayer) was delimited. Finally, the individual values of the selected areas and the value of the area of interest were extracted and stored in a database (Excel). The total vessel area was expressed as a percentage of the reference area (area of interest).

Data were analysed using non parametric tests (Wilcoxon test for paired samples).

Vessel diameter was defined at a final magnification of 360×. Measurements were performed in the same area of interest used for vascular density analyses in layer IVC. The diameter was measured perpendicular to the long axis of the vessel profile. The same vessel could provide several measures: (i) if it could be traced without branching and if its diameter varied along this distance, two measurements of diameter were made, or (ii) if it branched, measurements were made between two successive branching points. Diameter measures were sorted into classes of 1 μm increment. Diameter distributions were compared by means of a non-parametric test (Kolmogorov–Smirnov test).

Values in the tables are indicated as mean and standard error of the mean (SEM) for each parameter. In statistical analyses, made with SPSS 9.0 software, $P < 0.05$ was regarded as significant.

Results

Metabolic and Vascular Stainings

Cytochrome oxidase staining presents the characteristic laminar patterns previously described in marmosets (Spatz et al., 1993, 1994; Fonta et al., 1997).

In the very young animals (during the first postnatal weeks), dense cytochrome oxidase staining was restricted to the outer part of layer IVC, layer IVCalpha. A narrow band of dense staining was observed at the inner part of layer IVC, named Va (Spatz et al., 1993) (Figs 1, 2A). In 2-month-old marmosets, layers IVCalpha and IVCbeta were heavily stained (Fig. 3). Layer VI was also labeled, more strongly in its upper part. Layer V was faintly stained. Layers II and III were moderately labeled. In adults the lower part of IVC appears darker (Fig. 4).

Endothelial alkaline phosphatase clearly outlines vessels in all the animals studied. But in juveniles, a diffuse background could also be observed in layer IVC (Fig. 5). This staining provided a strong labeling of white matter (Fig. 1A) and layer IVCalpha in the youngest animals. Layer IVCbeta presented a lighter background staining (Fig. 2B). In the 2-month-old marmosets, a strong background was seen in layer IVC and, to a lesser extent, in layers IVA and VI (Fig. 3B). White matter was devoid of any background staining in 2-month-old animals and in adults (Figs 3B, 4). The background staining was weaker over the innermost part of layer IVCbeta in adult preparations (Fig. 6A, B).

Thus, in young animals, the strong cytochrome oxidase staining and the high background level of alkaline phosphate staining in layer IVC matched (Fig. 3). However, a transition band with pale staining in the middle part of layer IVC was clearly apparent with alkaline phosphatase detection and not with cytochrome oxidase staining in 2-month-old marmosets (Fig. 5).

Vessel Density Analyses

We first concentrated the analyses on the calcarine visual cortex that had been investigated for quantitative analyses of the areal, cellular and synaptic components during postnatal development in marmosets (Missler et al., 1993a,b). We then analysed vascular density in the dorsolateral part to compare the development of vascularization in the peripheral-field and central-field striate cortex.

The mean vascular density increases as a function of age (∼10% of the cortical surface in the neonate and ∼22.5% in the adult). We observed differences between animals of a same age (Table 1). Individual specificities or experimental factors (such as depth of anesthesia, level of anoxia, inadequate pressure during perfusion resulting in collapsed blood vessels) may explain such differences. However, the relative vascular densities in the different cortical layers are reproducible between age-matched animals (e.g. 2-month-old marmosets 99.9 and 00.5; Fig. 7).

Figure 7 details the relative percentages of vessel density compared to layer IVCalpha, for each animal. Data were normalized by expressing values as percentages of layer IVCalpha vessel density. For each animal, statistical analyses were performed on raw data (i.e. vessel density) to compare vessel densities between two different laminae.

Whatever the age of the animal, vessel density is always higher in the two sublayers of layer IVC than in the infra- ($P < 0.03$) and supragranular ($P < 0.033$) layers. Layer IVCbeta displays a specific evolution relative to layer IVCalpha. In the newborn marmoset (003) and in the 3-week-old marmosets (97.9 and 97.12), vessel density is higher in layer IVCalpha than in layer IVCbeta ($P < 0.005$). There is a 20–30% difference between
IVCalpha and IVCbeta vessel densities. In 2-month-old animals (99.9 and 0.5), vessel densities in IVCalpha and IVCbeta do not present any significant difference. In adults, vessel density is significantly higher in layer IVCbeta than in IVCalpha [P = 0.02 (98.4) and P = 0.005 (97.7)]. Vessel density is 15% higher in IVCbeta. The densities of vessels in the two sublayers of layer IVC appear, then, to follow the intensity of cytochrome oxidase staining (cf. Figs 1–4).

Figure 4. Double staining [cytochrome oxidase (CO) in brown; alkaline phosphatase (AP) vessels in blue] in the primary visual cortex of an adult marmoset. (B) Magnified part of the dorsal calcarine cortex in (A). CO activity is stronger in the lower tier of layer IV; wm, white matter.
Vessel densities in layers VI and V are similar, except in the 2-month-old animals (Table 1). In these animals, layer VI can be divided into two parts that differ in their cytochrome oxidase staining. The outer part, layer VIA, with a stronger staining, presents a significantly higher vascular density than the inner part, layer VIB \( P = 0.037 \) (9.99) and \( P = 0.026 \) (0.05). Vascular densities in layers VIB and V are similar.

At birth (marmoset 003), the vascular density in layers II and III (9.1%) is not significantly different from those in layers V and VI (8.5 and 9.4%, respectively; Table 1). In 3-week-old juveniles, vascular density is higher in the supragranular layers than in the infragranular layers \( P \leq 0.007 \). In 2-month-old animals, layers II/III and V show similar vascular densities that are significantly lower than density in layer VI \( (0.006 \leq P \leq 0.057) \). In adults, vascular densities, although higher in supragranular layers, are not significantly different from densities in infragranular layers.

The difference in vascular density between IVCalpha and IVCbeta is also found in the dorsal external part of the occipital pole (Table 2). Vascular density is significantly higher in layer IVCalpha than in IVCbeta in the neonate \( (P = 0.007) \), as in the 3-week-old animals \( (P = 0.005) \). The relative vessel density of layer IVCbeta compared to IVCalpha is not different in the calcarine cortex and in the dorsolateral cortex, with a 19–23% difference between the two layers.

Cytochrome oxidase activity also showed a partition of layer IVC in the dorsolateral visual cortex, with dark staining only in IVCalpha (not shown).

**Figure 5.** Alkaline phosphatase staining in the primary visual cortex of a 60 PND-old marmoset. The enzyme is revealed in the vessel walls and in the surrounding cortical tissue in layer IVC. A narrow band is devoid of strong background staining between layer IVCalpha and IVCbeta.

**Figure 6.** Alkaline phosphatase staining in the primary visual cortex of an adult animal. Background staining is absent in the white matter (wm) and appears lightly in IVCbeta. In (A), asterisks indicate the position of the area enlarged in (B), with a 90° clockwise rotation.

**Vessel Diameter Analyses**

Considering that the two sublayers of IVC present the same growth rate during postnatal development (Missler et al., 1993a), the difference in evolution of vessel densities in IVCalpha and IVCbeta could be due to a difference in the number and/or in the size of vessels. We measured the vessel diameters in these two sublayers in the calcarine cortex. Measurements were made in all seven marmosets (Table 3).

The distributions of vessel diameters, sorted into classes of 1 μm, appear similar in layers IVCalpha and IVCbeta (Fig. 8). They were not significantly different except in one adult – the oldest marmoset (97.7), where vessels in layer IVCbeta were slightly larger than those in IVCalpha \( (P = 0.004) \).

Differences between age-matched animals (e.g. 00.5 and 99.9) clearly appear. Vessel diameter and density were both lower in marmoset 00.5 than in marmoset 99.9. It can be suggested that the differences in vessel density observed between the two animals (cf. Table 1) were supported by
differences in vessel sizes that are highly dependent on physiological factors (level of anoxia, anesthesia).

Discussion

The present data show that vascular density in the primary visual cortex of marmosets is higher in layer IVC than in the other layers. This has also been shown in other primates (Bell and Ball, 1985; Zheng et al., 1991). This coarse laminar pattern is already present at birth, but layer IVC cannot be considered as a single fully determined entity. Angiogenesis, as neural maturation and aging, displays a different time-course in layers IVCalpha and IVCbeta. We assume that, in marmosets, layers IVCalpha and IVCbeta are, respectively, the targets of the magno- and parvocellular thalamic axons, as in other monkey species (Fitzpatrick et al., 1983; Lund, 1988; Freund et al., 1989).

Therefore, we showed that angiogenesis in layers IVCalpha and IVCbeta runs parallel to a sequential maturation of the magno- and parvocellular systems during the first 2 postnatal months. At the age of 2 postnatal months, the two parts of layer IVC present similar levels of neuronal activity and vessel densities. This pattern changes in adults and a more pronounced cytochrome oxidase activity, as well as a higher density of vessels, is found in the parvocellular part. Vessel size does not account for developmental changes in the vascular pattern, but might explain aging modification in the older animals.

The difference in relative vessel density between IVCalpha and IVCbeta is maintained in a similar fashion in the peripheral and the central visual field cortex during the first postnatal month. Growth indicators and neural density do not present differences in cortical regions subserving central versus peripheral visual fields.

Table 1

<table>
<thead>
<tr>
<th>Age</th>
<th>Layer</th>
<th>IVCalpha</th>
<th>IVCbeta</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II–III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 PND (00.3R) (n = 11)</td>
<td>9.14 ± 0.34</td>
<td>13.9 ± 0.48</td>
<td>10.69 ± 0.56</td>
<td>8.52 ± 0.51</td>
<td>9.41 ± 0.56</td>
</tr>
<tr>
<td>17 PND (97.9L) (n = 10)</td>
<td>15.27 ± 0.57</td>
<td>21.78 ± 0.97</td>
<td>17.54 ± 1.01</td>
<td>11.97 ± 0.69</td>
<td>11.53 ± 0.83</td>
</tr>
<tr>
<td>24 PND (97.12L) (n = 10)</td>
<td>15.89 ± 0.7</td>
<td>25.7 ± 1.16</td>
<td>17.92 ± 1.09</td>
<td>12.24 ± 0.67</td>
<td>11.95 ± 0.47</td>
</tr>
<tr>
<td>60 PND (99.9L) (n = 10)</td>
<td>13.34 ± 0.61</td>
<td>21.62 ± 1.11</td>
<td>21.74 ± 0.86</td>
<td>13.52 ± 0.51</td>
<td>16.3 ± 0.58</td>
</tr>
<tr>
<td>60 PND (00.5L) (n = 11)</td>
<td>8.94 ± 0.29</td>
<td>13.22 ± 0.5</td>
<td>13.68 ± 0.54</td>
<td>8.57 ± 0.43</td>
<td>11.48 ± 0.46</td>
</tr>
<tr>
<td>7 years (98.4R) (n = 10)</td>
<td>21.62 ± 0.84</td>
<td>26.89 ± 1.42</td>
<td>30.75 ± 1.78</td>
<td>20.95 ± 1.54</td>
<td>19.67 ± 1.25</td>
</tr>
<tr>
<td>11 years (97.7R) (n = 10)</td>
<td>19.0 ± 0.89</td>
<td>25.52 ± 1.03</td>
<td>29.69 ± 1.09</td>
<td>17.99 ± 0.81</td>
<td>18.23 ± 0.62</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Age</th>
<th>Periphery</th>
<th>Central</th>
<th>IVCalpha</th>
<th>IVCbeta</th>
<th>IVCalpha</th>
<th>IVCbeta</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 PND (00.3)</td>
<td>13.9 ± 0.48</td>
<td>10.69 ± 0.56</td>
<td>16.39 ± 1.04</td>
<td>12.57 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 PND (97.9)</td>
<td>21.78 ± 0.97</td>
<td>17.54 ± 1.01</td>
<td>22.16 ± 1.2</td>
<td>16.89 ± 1.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 PND (97.12)</td>
<td>25.7 ± 1.16</td>
<td>17.92 ± 1.09</td>
<td>23.23 ± 0.61</td>
<td>18.58 ± 0.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Vessel density = area occupied by vessels profiles per unit area of cortex.

PND: postnatal days; n, number of measures; in brackets: animal and hemisphere identification (R, right; L, left).
Paper and Elsewhere

Spatz et al. compared to IVCalpha, observed in marmosets in the present study. The higher cytochrome oxidase reactivity of layer IVCbeta, (Hendrickson et al., 1993) and reaching from the fourth postnatal month in humans (Wong-Riley et al., 1981; Gottlieb et al., 1993) and from the second postnatal month in marmosets. The adult pattern is observed between the newborn and the adult-like patterns occurring during the second postnatal month in marmosets. The adult pattern is reached from the fourth postnatal month in humans (Wong-Riley et al., 1993) and from the second postnatal month in macaques (Hendrickson et al., 1993; Endo et al., 2000). In the adult, the higher cytochrome oxidase reactivity of layer IVCbeta, compared to IVCalpha, observed in marmosets in the present paper and elsewhere (Spatz et al., 1994) has also been described in macaques (Wong-Riley and Carroll, 1984) and humans (Wong-Riley et al., 1993).

We noticed that layer VI was more cytochrome oxidase reactive in its outer part than in its inner part in 2-month-old marmosets. This could be linked to the concentration of thalamo-cortical terminals in the upper part of layer VI for New World primates (Hendrickson et al., 1978; Spatz, 1979; Fitzpatrick et al., 1983). The matching of intracortical and cortico-thalamic connections for neurons in the upper and lower layers of layer VI with the parvo- and magnocellular system, respectively, is documented in macaques (Fitzpatrick et al., 1985; Wiser and Callaway, 1996). To our knowledge, partition of layer VI into sublayers associated with the magno- or the parvocellular system has not been described in New World monkeys.

**Alkaline Phosphatase Staining**

With the alkaline phosphatase method, apart from staining endothelial cells, we obtained a lamina-specific background staining that is found mainly in juvenile preparations. Interestingly, this background staining is found in the layers that are the targets of thalamic afferents (layers IV and VI) and in the white matter where axons from the thalamus (LGN) accumulate before birth. Moreover, in layer IVC, alkaline phosphatase background intensity parallels the evolution of cytochrome oxidase activity, being high first in IVCalpha, then in IVCbeta. Several explanations can be proposed, as background staining can be due either to endothelial cells themselves, or to other cortical elements. Artefactual presence of alkaline phosphatase outside vessels due to physical or chemical alterations of the vessels can be discarded, since background staining is not general. For the same reasons, enzyme leakage in the vessel vicinity by immaturity and absence of blood–brain barrier properties seems unlikely. We propose, instead, that alkaline phosphatase reveals neuronal or glial properties involved in postnatal development of thalamo-cortical afferents. Alkaline phosphatase activity in neocortical neuropile, especially in the vicinity of the cell body, is linked to the formation of neuronal connections in the central nervous system (Marani et al., 1995), cell differentiation and onset of synaptic development in the retina (Arai and Saito, 1986). This enzyme has been localized on the pre- and postsynaptic membranes in the rat central nervous system (Sugimura and Mizutani, 1979) and its presence is related to the formation of neuronal connections in the central nervous system (Marani et al., 1995), cell differentiation and onset of synaptic development in the retina (Arai and Saito, 1986).

**Cytochrome Oxidase Staining**

The laminar pattern of cytochrome oxidase activity in the primary visual cortex of young and adult marmosets, already described (Spatz et al., 1993, 1994; Fonta et al., 1997), resembles that found in the macaque (Horton, 1984; Kennedy et al., 1985; Hendrickson et al., 1991) and human (Wong-Riley et al., 1993) developmental patterns. Dense cytochrome oxidase staining is restricted to layer IVCalpha and to a thin layer at the boundary between layers IVCbeta and V in neonates. In adult animals, layers IVCalpha and IVCbeta are both highly cytochrome oxidase reactive. We presently show that the evolution between the newborn and the adult-like patterns occurs during the second postnatal month in marmosets. The adult pattern is reached from the fourth postnatal month in humans (Wong-Riley et al., 1993) and from the second postnatal month in macaques (Hendrickson et al., 1991; Endo et al., 2000). In the adult, the higher cytochrome oxidase reactivity of layer IVCbeta, compared to IVCalpha, observed in marmosets in the present paper and elsewhere (Spatz et al., 1994) has also been described in macaques (Wong-Riley and Carroll, 1984) and humans (Wong-Riley et al., 1993).

We noticed that layer VI was more cytochrome oxidase reactive in its outer part than in its inner part in 2-month-old marmosets. This could be linked to the concentration of thalamo-cortical terminals in the upper part of layer VI for New World primates (Hendrickson et al., 1978; Spatz, 1979; Fitzpatrick et al., 1983). The matching of intracortical and cortico-thalamic connections for neurons in the upper and lower layers of layer VI with the parvo- and magnocellular system, respectively, is documented in macaques (Fitzpatrick et al., 1985; Wiser and Callaway, 1996). To our knowledge, partition of layer VI into sublayers associated with the magno- or the parvocellular system has not been described in New World monkeys.

**Alkaline Phosphatase Staining**

With the alkaline phosphatase method, apart from staining endothelial cells, we obtained a lamina-specific background staining that is found mainly in juvenile preparations. Interestingly, this background staining is found in the layers that are the targets of thalamic afferents (layers IV and VI) and in the white matter where axons from the thalamus (LGN) accumulate before birth. Moreover, in layer IVC, alkaline phosphatase background intensity parallels the evolution of cytochrome oxidase activity, being high first in IVCalpha, then in IVCbeta and beta. Several explanations can be proposed, as background staining can be due either to endothelial cells themselves, or to other cortical elements. Artefactual presence of alkaline phosphatase outside vessels due to physical or chemical alterations of the vessels can be discarded, since background staining is not general. For the same reasons, enzyme leakage in the vessel vicinity by immaturity and absence of blood–brain barrier properties seems unlikely. We propose, instead, that alkaline phosphatase reveals neuronal or glial properties involved in postnatal development of thalamo-cortical afferents. Alkaline phosphatase activity in neocortical neuropile, especially in the vicinity of the cell body, is linked to the formation of neuronal connections in the central nervous system (Marani et al., 1995), cell differentiation and onset of synaptic development in the retina (Arai and Saito, 1986). This enzyme has been localized on the pre- and postsynaptic membranes in the rat central nervous system (Sugimura and Mizutani, 1979) and its presence is related to the formation of neuronal connections in the central nervous system (Marani et al., 1995), cell differentiation and onset of synaptic development in the retina (Arai and Saito, 1986). In vitro studies suggest that the putative role of alkaline phosphatase in tissue formation may be through cell–cell interaction and proliferation (Hui et al., 1993). These different findings document the possible activity of alkaline phosphatase in developmental neural circuitry; however, its cellular substrates have not yet been identified.

**Table 3**

<table>
<thead>
<tr>
<th>Age</th>
<th>0 PND (00.3)</th>
<th>17 PND (97.9)</th>
<th>24 PND (97.12)</th>
<th>60 PND (99.9)</th>
<th>60 PND (00.5)</th>
<th>7 years (98.4)</th>
<th>11 years (97.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>7.44</td>
<td>7.12</td>
<td>8.69</td>
<td>8.56</td>
<td>10.51</td>
<td>10.19</td>
<td>10.51</td>
</tr>
<tr>
<td>Beta</td>
<td>7.12</td>
<td>7.44</td>
<td>8.56</td>
<td>8.69</td>
<td>10.19</td>
<td>10.51</td>
<td>10.40</td>
</tr>
<tr>
<td>SEM</td>
<td>0.11</td>
<td>0.10</td>
<td>0.12</td>
<td>0.13</td>
<td>0.17</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>n</td>
<td>736</td>
<td>554</td>
<td>901</td>
<td>701</td>
<td>813</td>
<td>658</td>
<td>774</td>
</tr>
<tr>
<td>SEM</td>
<td>0.10</td>
<td>0.12</td>
<td>0.13</td>
<td>0.17</td>
<td>0.16</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>n</td>
<td>1003</td>
<td>1057</td>
<td>849</td>
<td>929</td>
<td>1020</td>
<td>964</td>
<td>1044</td>
</tr>
</tbody>
</table>

n: number of measures.

**Figure 8.** Distributions of vessel sizes in layer IVCalpha and IVCbeta, in the different marmosets studied. Diameters have been sorted into classes of 1 µm increment. Numbers of measures realized in each case are presented in Table 3.

Peripheral visual fields in macaques (Powell and Hendrickson, 1981; Gottlieb et al., 1985). Maturation of both magno- and parvocellular retinogeniculate axon arbors occurs 1 week ahead for central vision compared to peripheral vision in the galago (Lachica and Casagrande, 1988). As we sampled animals at ages spaced 1 month apart, a timing difference of a week cannot be detected.
Angiogenesis and Neuronal Development

The present study shows that vascular density increases as a function of age from birth to adulthood. However, this evolution may result from two different processes. First, during postnatal development, angiogenesis occurs and supplies an increasing and maturing neuropile. In marmosets, the size of the primary visual cortex increases after birth and shows an overshoot at +3 postnatal months (Missler et al., 1993a). Then, as the cortical neuropile is reduced in adults, blood vessels concentrate. The size of the visual cortex decreases from the third month until 9–12 months after birth, to reach adult values that are not much different from those of newborns—105% for the cortical thickness and 112% for the volume (Missler et al., 1993a).

The laminar distribution of cortical vessels, with a strikingly high density in layer IVC, that abruptly ends at the boundary between the primary (V1)/secondary (V2) visual cortex as in adult human vascular architecture (Bell and Ball, 1985), may reflect the cytoarchitectonic features of the cortex. Density of neuron somata is higher in layer IVC, in the adult as in the young marmoset (Fritschy and Garey, 1986). However, the developmental variation in vessel density cannot reflect modifications in the numerical density of neurons in individual laminae: neuron number does not vary greatly during postnatal development, as there is no evidence for important neuronal loss or mitosis during postnatal development in marmoset visual cortex (Fritschy and Garey, 1986). Moreover, neuron density is higher in layer IVCb than in layer IVCalpha in both young and adults [in macaques (O’Kusky and Colonnier, 1982a,b)], while vessel density is first higher only in layer IVCalpha.

The differential increase in vascular supply in layers IVCalpha and IVCbeta during postnatal development is more likely to be associated with corresponding different metabolic requirements in neurites. As maturation or changes in cortical function are generally associated with modifications in synaptic circuits, angiogenesis and synaptogenesis might be closely linked. In rats, vascular proliferation takes place during synaptogenesis in the visual cortex (Rowan and Maxwell, 1981; Bakkum et al., 1991). In the marmoset visual cortex, a high rate of synaptogenesis occurs during the first three postnatal months (Missler et al., 1993b). Unfortunately, the authors of that study did not show the data for individual layers. In macaques, an overproduction of synapses is reached at three postnatal months, followed by a plateau of high synaptic density (Bourgeois and Rakic, 1993). These last authors did not distinguish layers IVCalpha and IVCbeta, but others (O’Kusky and Colonnier, 1982a) have found a higher synaptic density in IVCbeta than in IVCalpha at six postnatal months. Data on the time-course of synaptogenesis during the first three postnatal months are unfortunately unavailable.

In the adult, the total density of synapses (O’Kusky and Colonnier, 1982a,b), both asymmetric and symmetric (Latawiec et al., 2000), is maintained higher in IVCbeta than in IVCalpha. This result can parallel the higher density of capillaries presently established in adult marmoset layer IVCbeta.

The postnatal development of dendritic spines is better documented than that of synapses. In macaques, dendritic spine density on layer IVC spiny stellate neurons, that represent 95% of layer IVC neuropile (Mates and Lund, 1983), shows a marked increase during the first two postnatal months, spine frequency and spine maturation displaying different time-courses in IVCalpha and IVCbeta (Boothe et al., 1979; Lund and Holbach, 1991). The majority of new synapses during postnatal development form on dendritic spines and represent 75% of the synapses in the macaque (Bourgeois and Rakic, 1993). From this pool of data we may then expect a parallel between development of dendritic spines and synapse densities during the first postnatal months. Therefore, if synaptogenesis is shown to occur differently in IVCalpha and IVCbeta, it can be hypothesized that synaptogenesis produces angiogenesis in layer IVC and that this angiogenesis proceeds asynchronously in layers IVCalpha and IVCbeta, because of different evolution in the maturation in these two layers.

From all the elements presented above, it can be proposed that angiogenesis is due to an increased period of synaptogenesis in the cerebral cortex. If this is so, the initial formation of the vascular pattern, as with the initial formation of synapses (Bourgeois and Rakic, 1996), is dominated by intrinsic mechanisms and stimulation from the periphery would have no effect on angiogenesis. However, an early deprivation of retinal input increases the proportion of asymmetrical synapses in layer IVC (Bourgeois and Rakic, 1996). As asymmetrical synapses are putatively excitatory and as the main excitatory (glutamatergic) neurons and asymmetric synapses have a higher oxidative metabolism than inhibitory (GABAergic) neurons and symmetric synapses (Carroll and Wong-Riley, 1984; Hertz et al., 1988), one would expect higher vascularization in layer IVC in the visual cortex of deprived animals than in controls and, in consequence, a higher metabolic activity. This is consistent with positron emission tomography (PET) analyses of visual cortex metabolism in human subjects with early retinal loss, who present a higher glucose utilization than normal subjects (Veraart et al., 1990).

Magnocellular/Parvocellular Maturation

We show in the present paper that vascularization of the primary visual cortex is correlated with the differential maturation of layers IVCalpha and IVCbeta. These two anatomical modules sustain different visual functions. Classically, the occipito-parietal visual stream, dominated by the magnocellular system, deals with spatial localization, motion analyses and luminance sensitivity, and the occipitotemporal stream with pattern discrimination, object recognition and chromatic sensitivity. Data show that these functional pathways present a different time-course of development in both humans and monkeys. The general trend is that the functions involving the magnocellular pathway mature earlier (Mikami and Fujita, 1992; Kovacs et al., 1999; Dobkins et al., 1999). Physiological studies measuring energy metabolism in monkeys by means of 2DG autoradiography reveal that areas of motion analysis (occipitoparietal stream) mature earlier than those of the object recognition pathway (Bacheler et al., 1991; Distler et al., 1996). If we consider the different steps of the anatomical pathways from the retina to the primary visual cortex, it appears that, apart from the retinal ganglion cells step (Meissirel et al., 1997), the magnocellular system matures earlier than the parvocellular system (Gottlieb et al., 1985; Lachica and Casagrande, 1988; Pospichal et al., 1994; Hawken et al., 1997; Kogan et al., 2000). Therefore, convergence of data, through different approaches (anatomical, functional and metabolic), suggests a more precocious magnocellular system.

Glia Partner

Blobs in the primary visual cortex, revealed by a high level of cytochrome oxidase activity, coincide with areas expressing high density of astroglia (Colombo et al., 1999). As blobs are also vascular modules (Zheng et al., 1991), we may suggest that the modular organization of the cortex represents, in fact, the close metabolic coupling between neurons, glial cells and endothelial cells. It is tempting to associate glia, that is a crucial element in
cortical tissue postnatal development, with the neuron-endothelial partnership.

Glia has a pivotal role in coupling neuronal activity to energy metabolism. A pool of recent data show that astrocytes display an active part in processing and conveying neuronal information and may then be involved in plasticity processes. Moreover, astroglia induces blood–brain barrier characteristics, of which alkaline phosphatase is an indicator, in cerebral endothelial cells, through soluble factors or direct contact (Meyer et al., 1991; Takemoto et al., 1994). Reciprocally, glial differentiation into astrocytes or oligodendrocytes may result from interactions with blood vessels in the neocortex during postnatal development (Zerlin and Goldmann, 1997). In rats, gliogenesis, angiogenesis and synaptogenesis occur over the same period – the first three postnatal weeks (Rowan and Maxwell, 1981; Bakkum et al., 1991; Zerlin and Goldmann, 1997). In primates, too, gliogenesis may occur in parallel to synaptogenesis and angiogenesis. Glial cell density fluctuates and glial maturation occurs during early postnatal development in marmosets (O’Kusky and Colonnier, 1982b, Mates and Lund, 1983). If cytological relationships and metabolic coupling between glia and neurons, and glial and endothelial cells are documented, the mechanisms of angiogenesis in the cerebral cortex during postnatal development and the part of neuronal activity in this process are still poorly understood. Reciprocal neuron-endothelial cell interactions during brain development are expected, since one receptor to vascular endothelial growth factor, that stimulates blood vessel growth, was found to be also expected, since one receptor to vascular endothelial growth factor (Fonta et al., 1999). This could represent a coordination system between the developing nervous and circulatory systems.

Notes

We are grateful to Pierre Carrechio for his helpful advice in image analyses. We thank Pascal Barone for his comments on this manuscript, Catherine Chappert, Catherine Marlot and Frédéric Sarrato for their analyses. We thank Pascal Barone for his comments on this manuscript, Catherine Chappert, Catherine Marlot and Frédéric Sarrato for their analyses. We thank Pascal Barone for his comments on this manuscript, Catherine Chappert, Catherine Marlot and Frédéric Sarrato for their analyses. We thank Pascal Barone for his comments on this manuscript, Catherine Chappert, Catherine Marlot and Frédéric Sarrato for their analyses. We thank Pascal Barone for his comments on this manuscript, Catherine Chappert, Catherine Marlot and Frédéric Sarrato for their analyses. We thank Pascal Barone for his comments on this manuscript.

References


