

Oligodendrocytes, their Progenitors and other Neuroglial Cells in the Aging Primate Cerebral Cortex

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In a previous study it was found that with age there is an increase in the frequency of paranodal profiles of myelinated nerve fibers in the cerebral cortex of monkeys. This indicates that there is an increase in the number of internodal myelin segments, and raises the question of whether additional oligodendrocytes are necessary to generate the increased numbers of internodal myelin segments. The present study shows that in layer 4C β of monkey primary visual cortex there is an age-related increase in the number of oligodendrocytes. When young (4–10 years of age) and old (25–35 years of age) monkeys are compared, the increase is found to be ~50%, and it begins in middle age (12–19 years old). It is also shown that although there is no increase in the population of astrocytes in layer 4C β with age, there appears to be a slight increase in the frequency of microglial cells. As their numbers increase, oligodendrocytes in pairs, rows and groups become more common, which suggests that additional oligodendrocytes are being generated by cell division. Since there is little evidence that mature oligodendrocytes can divide, it is probable that the new oligodendrocytes are generated from progenitor cells which, as many studies have shown, can be labeled by antibodies to NG2, a chondroitin sulfate proteoglycan. By comparing the appearance of these NG2-labeled cells with cells encountered in thin sections of normally prepared tissue, it is shown that the NG2-positive cells have the features of neuroglial cells that were previously described as β astrocytes.

Keywords: NG2 antibody, normal aging, oligodendrocyte progenitors, oligodendrocytes, rhesus monkey, visual cortex

Introduction

In earlier articles we have shown that there are age-related alterations in myelin sheaths within the cerebral cortices of non-human primates (Feldman and Peters, 1998; Peters *et al.*, 2000; Peters and Sethares, 2002). Two of these alterations, namely the localized splitting of sheaths to accommodate electron dense cytoplasm and the blebbing, or ballooning of sheaths, appear to be degenerative changes. In contrast, two other alterations can be attributed to the continued formation of myelin. These latter changes are the formation of redundant myelin, which results in the formation of some sheaths that are much too large for their enclosed axons, and the circumferential splitting of thick sheaths. That myelin formation continues during aging is also evident when the myelin sheaths in the vertical bundles of nerve fibers that pass through primary visual cortex of young monkeys are compared with those from old monkeys. With age, sheaths increase in thickness, and an important contributor to this increase in the mean number of lamellae is an increased frequency of occurrence of sheaths with more than 10 lamellae in old monkeys (Peters *et al.*, 2001). It is these sheaths that most commonly undergo circum-

ferential splitting. In support of the observation that myelin continues to be produced during aging, it might be mentioned that in a recent study of the effects of age on the activity of genes in the hippocampus of rats, Blalock *et al.* (2003) found that with age there is up-regulation in the activity of genes concerned with myelin synthesis.

During electron microscopic studies of the effects of age on sheath thickness (Peters *et al.*, 2001) it was noticed that when cross sections through the vertical bundles of myelinated nerve fibers in area 17 of occipital cortex and in area 46 of prefrontal cortex of young and old monkeys are compared, there is an increase in the frequency of profiles of paranodes (Peters and Sethares, 2003). The evidence suggests that this increase in the frequency of paranodal profiles is due to an increase in the number of internodal lengths of myelin, as would be brought about if some internodal lengths of mature myelin degenerate with age and are replaced by a series of shorter internodes, as is known to occur in remyelination (e.g. Gledhill and McDonald, 1977; Ludwin, 1978, 1995; Hirano, 1989; Kreutzberg *et al.*, 1997; Prineas and McDonald, 1997). In both naturally occurring and experimental demyelination it is generally agreed that during the subsequent remyelination short internodes are formed and the remyelinating axons have sheaths that are inappropriately thin for the sizes of their axons. Indeed, in the cerebral cortex of old monkeys, both short internodes and thin sheaths can be found (Peters and Sethares, 2003). Although it has not been possible to directly ascertain that some of the original myelin internodes are lost, the age-related formation of sheaths containing dark cytoplasm and the formation of balloons are generally considered to indicate that degeneration of myelin is occurring. Such changes also occur in the early stages of demyelination produced by cuprizone poisoning (e.g. Ludwin, 1978, 1995), as well as in other experimental and natural conditions (see Peters and Sethares, 2003). Other evidence that myelin is degenerating with age is that astrocytes in the cortices of old monkeys have been found to contain what is obviously phagocytosed myelin and some of the amorphous inclusions in astrocytes label with antibodies to myelin basic protein (Peters and Sethares, 2003). The conclusion is that with age some internodal lengths of myelin sheaths degenerate and are replaced by newer and shorter internodal lengths.

Oligodendrocytes are the neuroglial cells that form myelin in the central nervous system and little is known about how they are affected by age in primate cerebral cortex. There was a suggestion from an earlier study of monkey visual cortex that there is an increase in the number of oligodendrocytes with age, but too few monkeys were examined to produce acceptable statistical data (Peters *et al.*, 1991). An increase in the number of oligodendrocytes was also suggested by the obser-

vation that groups and rows of oligodendrocytes become increasingly common in primate visual cortex with age (Peters, 1996). If new oligodendrocytes are produced in the mature brain, they probably originate from progenitors, and it is known that under both normal and pathological conditions oligodendrocytes can be generated from a population of cells that label with the NG2 chondroitin proteoglycan antibody (e.g. Levine and Stallcup, 1987; Levine, 1994; Nishiyama *et al.*, 1996, 1999; Reynolds and Hardy, 1997; Keirstead *et al.*, 1998; Chang *et al.*, 2000; Levine *et al.*, 2001; Jones *et al.*, 2002; Watanabe *et al.*, 2002). The features of these NG2-positive (NG2+) cells have been well defined in light microscope preparations, but few of these cells have been examined by electron microscopy and what the cells look like in thin sections of normal, unlabeled, tissue remains unclear. Although it is agreed that some NG2+ cells are oligodendroglial progenitors, it remains uncertain what proportion of the NG2+ cells in the mature central nervous system are oligodendroglial progenitors, and there is debate about whether the NG2+ cells have other roles to play in the mature brain (e.g. Butt *et al.*, 2002; Nishiyama *et al.*, 2002). It should be pointed out that the possibility exists that not all oligodendroglial progenitor cells express NG2, since Gensert and Goldman (2001) found that in the adult rodent brain some cycling cells express the early oligodendrocyte marker O4, but do not express NG2.

The present study has two goals. The first is to determine if there really is an increase in the frequency of oligodendrocytes with age in monkey cerebral cortex, and the second is to characterize the NG2-labeled cells present in monkey cerebral cortex, with the aim of being able to identify the same cell types in thin sections from tissue that has been routinely prepared and not reacted with an NG2 antibody.

Materials and Methods

Animals Used for Routine Microscopy

Twenty-four rhesus monkeys (*Macaca mulatta*) were used in this study. Seven of the monkeys were young (4–10 years old), six were middle-aged (12–19 years old) and 11 were old (>25 years of age). The ages of the monkeys, given to the nearest year, and their sexes are shown in Table 1. For three of the older monkeys that were not born in captivity, the ages have had to be estimated (est). Details of the method used for fixing the brains of these monkeys for electron microscopic and for semi-thick section light microscopic examination are given in earlier publications (e.g. Peters *et al.*, 1994). The perfusions were carried out in full accord with the approved Institutional Animal Care and Use Committee regulations, and in accordance with the NIH publication *Guide for the Care and Use of Laboratory Animals*. In brief, the animals were pre-anesthetized with ketamine (6.5 mg/kg.). Sodium pentobarbital was then administered intravenously (~35–45 mg/kg) until each monkey was deeply anesthetized and a state of areflexia achieved. The monkeys were then intubated into the trachea and artificially respired with a mixture of CO₂ and O₂. The chest was opened and the monkeys perfused intracardially with a warm solution of 1% paraformaldehyde and 1.25% glutaraldehyde in a 0.1 M cacodylate or phosphate buffer at pH 7.4. Following this perfusion the brains were removed and one hemisphere from each brain was fixed further in a cold solution of 2% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 M buffer of the type used for the perfusion.

Tissue Preparation

Several tissue blocks, ~2 mm thick, were taken from the primary visual cortex, area 17, of each monkey. The blocks were taken from the opercular surface, ~3 mm caudal to the lunate sulcus, where the center of the visual field is represented. The blocks of cortex were

Table 1

Numbers of oligodendrocytes, astrocytes and microglial cells nuclear profiles in layer 4Cb area 17

Animal no.	Age (years)	Sex	Mean number of profiles per mm ²		
			Oligodendroglia	Astrocytes	Microglia
AM 58	4	M	148.7 ± 13.9	83.8 ± 13.9	19.8 ± 11.2
AM 16	5	M	168.7 ± 32.9	74.6 ± 7.9	13.2 ± 0.0
AM 76	6	F	158.0 ± 13.9	66.6 ± 6.6	15.2 ± 4.0
AM 77	6	F	156.4 ± 27.7	83.8 ± 4.0	17.8 ± 4.0
AM 47	9	M	142.0 ± 10.0	77.2 ± 9.9	13.2 ± 6.6
AM 96	9	F	198.0 ± 33.3	68.0 ± 13.9	13.2 ± 6.6
AM 53	10	M	165.0 ± 34.3	103.0 ± 20.5	28.8 ± 9.9
		Mean	162.4 ± 23.7	79.6 ± 10.9	17.3 ± 6.0
AM 42	12	M	164.0 ± 4.0	95.6 ± 18.7	22.0 ± 4.0
AM 140	13	M	180.0 ± 33.3	100.0 ± 5.0	18.0 ± 4.0
AM 144	15	M	213.3 ± 23.3	75.3 ± 16.7	15.3 ± 10.0
AM143	16	M	178.0 ± 4.0	71.3 ± 8.0	13.3 ± 6.7
AM 101	19	M	166.7 ± 24.0	78.0 ± 7.3	15.3 ± 8.0
AM 133	19	M	283.0 ± 15.3	68.7 ± 10.0	11.3 ± 8.0
		Mean	190.0 ± 32.0	81.5 ± 13.1	15.9 ± 3.7
AM 19	25	F	158.0 ± 7.7	87.8 ± 9.9	15.2 ± 4.0
AM 100	25	F	186.8 ± 33.0	101.0 ± 15.2	24.4 ± 4.0
AM 12	27	F	273.3 ± 126.7	83.8 ± 13.9	17.2 ± 4.0
AM 15	27	F	208.7 ± 25.3	94.4 ± 4.0	15.2 ± 4.0
AM 62	27	M	348.7 ± 44.0	66.6 ± 6.6	28.4 ± 9.9
AM 27	27	M	244.2 ± 73.3	101.0 ± 13.9	21.8 ± 7.9
AM 26	29 (est)	F	278.0 ± 46.7	97.0 ± 20.5	23.8 ± 4.0
AM 17	29 (est)	F	195.3 ± 4.0	68.0 ± 4.0	21.8 ± 7.9
AM 91	32	M	320.0 ± 20.0	72.6 ± 17.2	26.4 ± 17.2
AM 41	32	F	213.0 ± 17.3	83.2 ± 4.0	19.8 ± 6.6
AM 13	35(est)	M	298.0 ± 26.7	68.0 ± 9.9	19.8 ± 6.6
		Mean	247.7 ± 38.6	83.9 ± 10.8	21.3 ± 6.9

osmicated, dehydrated in an ascending series of alcohols and embedded in Araldite.

The blocks of tissue were sectioned vertically, that is normal to the pial surface, and semi-thick sections stained with toluidine blue for light microscopic examination of the quality of preservation of the tissue and to determine the depth of layer 4Cβ. Thin sections were then taken for electron microscopy. These sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 100S electron microscope.

Next the tissue blocks were turned through 90° and trimmed down until layer 4Cβ was reached. In horizontal or tangentially oriented sections, layer 4Cβ can be readily recognized by the fact that the vertically oriented myelinated nerve fibers aggregate into discrete and compact bundles (e.g. Peters and Sethares, 1996; Nielsen and Peters, 2000). Semi-thick and thin sections were then taken for light and electron microscopic examination of the various kinds of neuroglial cells.

Relative Frequency of Neuroglial Cells in Layer 4Cβ

Since our previous studies of the effects of aging on myelinated nerve fibers in visual cortex had been largely carried out in layer 4Cβ (Nielsen and Peters, 2000; Peters *et al.*, 2000, 2001), it was decided to make neuroglial cell counts in this same layer. To make the counts, horizontal, semi-thick (1 μm thick) sections through layer 4Cβ,

stained with toluidine blue, were used, since only in such sections is it possible to identify, and differentiate between, profiles of the three basic types of neuroglial cells and neurons with any certainty. For each monkey, camera lucida drawings were made of sections from two different blocks of tissue. At least three different horizontal sections were used: one section from one of the blocks of tissue and two from the other block. A $\times 40$ objective lens was used and drawings made to show the locations of those profiles of the cell bodies of oligodendrocytes, astrocytes and microglial cells that display nuclei. Each drawing covered an area of 0.15 mm^2 . The numbers of profiles of the three types of neuroglial cells in each drawing were then counted, and the counts pooled to determine the mean number of profiles of each neuroglial cell type per mm^2 .

Since the nuclei have been used as the counting objects, to compare data on neuroglial cell frequency in young and old monkeys it is necessary to ascertain if the sizes of the nuclei change with age. Consequently, vertical semi-thick sections through area 17 of each monkey were examined using a $\times 100$ objective lens, and drawings made of at least 30 randomly selected nuclei of oligodendroglial and astrocytes in layer $4C\beta$. The vertical lengths of the profiles were then measured and the mean lengths of the profiles of the nuclei determined.

Antibody Labeling of Oligodendroglial Precursor Cells

Sections of monkey primary visual cortex were labeled with a polyclonal rabbit antibody to NG2. The antibody was kindly provided by Dr J.M. Levine, State University of New York at Stony Brook. Binding sites of the NG2 antibody were visualized using a silver enhanced DAB reaction product.

Small blocks of tissue were taken from the primary visual cortex of three monkeys, 20, 21 and 27 years old, that had been perfused with a Krebs's buffer. The blocks were lightly fixed by placing them in a solution containing 4% paraformaldehyde in a 0.1 M phosphate buffer solution (PBS) for no longer than 1 h. Next, $50 \mu\text{m}$ sections were cut on a Vibratome, rinsed in 0.1 M PBS and pretreated with 0.3% hydrogen peroxide to quench endogenous peroxidase activity.

Sections were placed in a pre-incubation mixture of 1% normal goat serum, 0.1% sodium azide and 0.01% Triton-X, in 0.1 M PBS for 1 h, and then transferred to a solution containing the antibody to NG2, diluted to 1:500 in the same mixture. The sections were incubated overnight in the primary antibody, rinsed, and placed in 0.1 M PBS containing biotinylated secondary goat anti-rabbit F(ab')₂ (cat. no. 111-066-006, Jackson ImmunoResearch, West Grove, PA) for 1 h. After rinsing, sections were placed in an avidin-biotin complex (ABC Elite kit, Vector Laboratories, Burlingame, CA), diluted to half the concentration recommended by the manufacturer (Ellis and Halliday, 1992), for 1 h.

The antibody was visualized by immersing the sections in a solution of hydrogen peroxide and DAB (product no. 54-10-00, Kirkegaard and Perry, Gaithersburg, MD). The reaction was monitored by light microscopy. The DAB reaction was enhanced by a silver intensification technique (Göracs *et al.*, 1986).

As a control, alternate sections were processed using the same solutions as described above, but omitting the primary antibody. No labeling occurred.

Sections mounted in glycerine for light microscopy were photographed using a digital camera. Sections prepared for electron microscopy were osmicated, dehydrated in an ascending series of ethanol solutions, embedded in araldite resin, sectioned at 50–70 nm on an ultramicrotome, mounted on grids, stained with uranyl acetate and lead citrate, and viewed with a JEOL 100S electron microscope.

Data Analysis

The linear associations in these data were assessed using Pearson correlation. Both the correlation coefficients and the *P*-values derived from the analyses are given. The analyses were performed using SAS for Windows, version 8.2 (SAS Institute, Cary, NC). The graphs showing correlations between data were drawn using the program Cricket graph.

Results

Morphology of Neuroglial Cells

In electron microscopic preparations (Fig. 1), oligodendrocytes have a dark cytoplasm that contains short cisternae of granular endoplasmic reticulum, polyribosomes, rather short mitochondria and profiles of the Golgi apparatus. The profiles of nuclei of oligodendrocytes are generally round to oval, but they may sometimes have a slightly irregular shape, and they are delineated by a rather wide nuclear cistern. Dense patches of chromatin occur throughout the nucleus and there is a dense, uneven layer of chromatin beneath the nuclear envelope. This layer is interrupted where nuclear pores occur. In older monkeys many of the cells have dense inclusion bodies, which are generally aggregated and located at the pole of the cell opposite to the eccentrically located nucleus (Fig. 1B). These dense inclusions also occur in swellings that are present along many of the processes emanating from the oligodendrocytes (see Peters, 1996, 2002).

The features that allow oligodendrocytes to be recognized in semi-thick plastic sections stained with toluidine blue are the round, dark nuclei, which are surrounded by a darkly staining cytoplasm (Fig. 2). In contrast astrocytes have pale, round or oval nuclei, which have a thin dark rim of chromatin beneath the nuclear envelope. Overall the chromatin of astrocytes stains more homogeneously than that of neurons, which also have pale and larger nuclei. Like oligodendrocytes, microglial cells also have dark nuclei, but they are elongate and generally smaller than those of oligodendrocytes, while the cytoplasm of microglial cells is paler than that of oligodendrocytes.

Frequency of Neuroglial Cells

The frequency of oligodendrocytic profiles displaying nuclei has been determined by light microscopy, using semi-thick plastic sections taken in the horizontal plane through layer $4C\beta$. This is the same layer in which previous studies of alterations in myelin sheaths (Peters *et al.*, 2000) and of paranodal frequency (Peters and Sethares, 2003) with age have been examined. To obtain a complete picture of how the neuroglial cell population alters with age, the frequencies of occurrence of profiles of astrocytes and microglial cells with nuclei were also quantified.

To determine the frequency of occurrence of neuroglial cell profiles that display nuclei, camera lucida drawings were made of fields from at least three different semi-thick sections from each monkey and the mean values of neuroglial cells per mm^2 were calculated. The results are given in Table 1 and in Figure 3.

Oligodendrocytes

Essentially, most of the old monkeys have a higher frequency of oligodendrocytic profiles than young monkeys. Middle-aged monkey fall between, some having similar numbers of oligodendrocytes to young monkeys and others having numbers comparable to those in old monkeys. However, as shown in Figure 3, there is a significant correlation between oligodendrocytic profile frequency and age ($r = 0.70$; $P < 0.0001$).

The frequency of occurrence of oligodendrocyte profiles that display nuclei is a function of the sizes of the nuclei. To determine if there is a change in the sizes of the nuclei with age, at least 30 profiles of the nuclei of oligodendrocytes in



Figure 1. Electron micrographs of oligodendrocytes in layer 4C of monkey primary visual cortex. (A) Two oligodendrocytes from a young (5-year-old) monkey; AM 16. (B) A group of three oligodendrocytes from an old (28-year-old) monkey; AM 15. Note the dense inclusion bodies (i) in the cytoplasm. Scale bars = 2 μ m.

layer 4C β of each monkey were drawn from vertically oriented semi-thick plastic sections and mean vertical diameters calculated. The total mean diameters of nuclear profiles for young monkeys is $5.5 \pm 0.8 \mu\text{m}$, for middle-aged monkeys it is $5.6 \pm 0.8 \mu\text{m}$ and for the old monkeys it is $5.7 \pm 0.8 \mu\text{m}$. Thus there is no significant difference in the sizes of oligodendrocytic nuclei from young, middle aged and old monkeys. Consequently, the increased frequency of oligodendrocytic profiles in layer 4C β with age reflects a real increase in the number of oligodendrocytes.

Astrocytes

Unlike the oligodendrocytes, there is no significant increase in the frequency of occurrence of astrocytic profiles with age (Fig. 3). The total mean value for the number of astrocytic profiles in young monkeys is 76.9 ± 10.9 per mm^2 , for middle-aged monkeys it is 81.5 ± 13.1 mm^2 and for the old monkeys it is 83.9 ± 10.8 per mm^2 . The mean vertical sizes of the nuclear profiles of astrocytes were measured in six young, six middle aged and six old monkeys. In the young monkeys the mean vertical size is $7.7 \pm 1.3 \mu\text{m}$, in middle-aged monkeys it is $7.9 \pm$

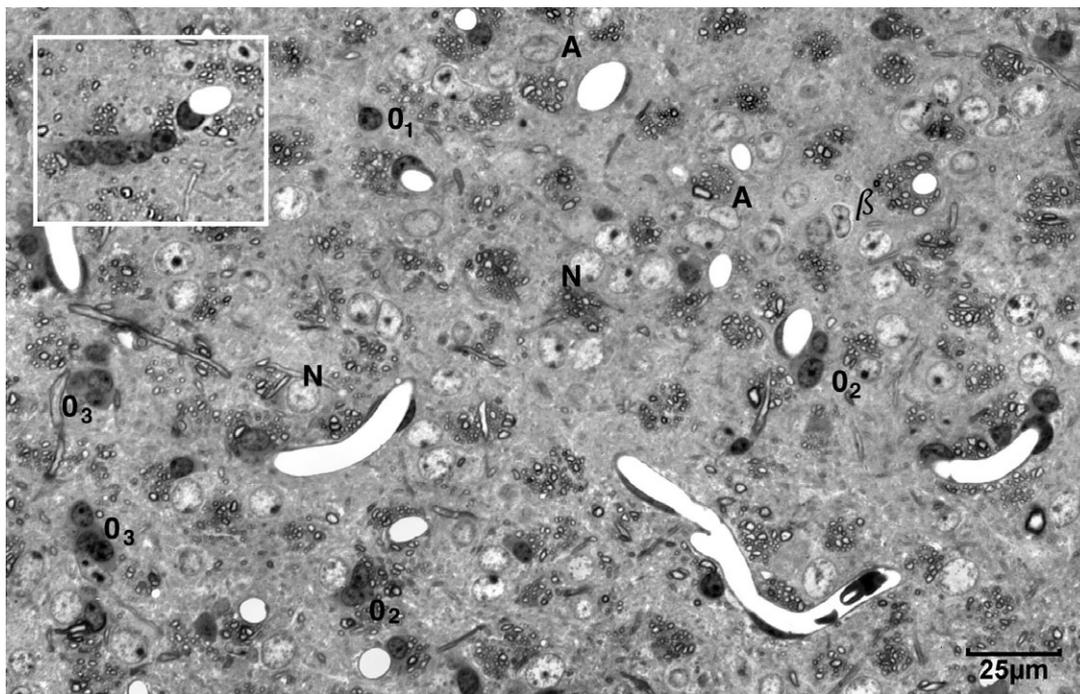


Figure 2. Light micrograph of a semi-thick horizontal section through layer 4C β of a 29-year-old monkey to show the distinctions between the appearances of the various types of cells. Oligodendrocytes have dark rounded nuclei and they may occur singly (O₁), in pairs (O₂) or in groups of three (O₃). Astrocytes (A) have pale nuclei that are not as large and round as those of neurons (N). Note the bean shaped nucleus of a β astrocyte (β). The insert shows a row of four oligodendrocytes in a 27-year-old monkey. Scale bar = 25 μ m.

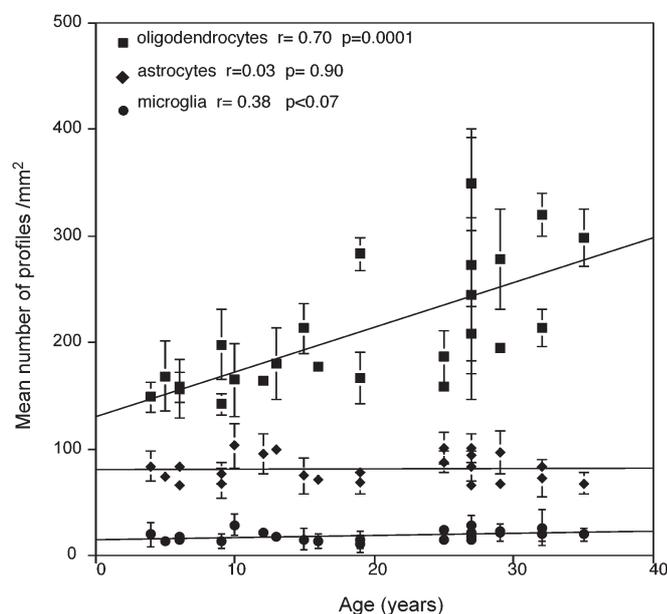


Figure 3. Plot of mean numbers of profiles of oligodendrocytes, astrocytes and microglial cell profiles that display nuclei, per mm² of semi-thick, horizontal semi-thick sections through layer 4C β of area 17 versus age.

1.4 μ m and in the old monkeys it is 7.9 \pm 1.6 μ m, indicating that the sizes of the astrocytic nuclei do not alter with age. Consequently, it can be concluded that there is no change in the frequency of astrocytes in layer 4C β of striate cortex with age.

Microglia

With age there is a slight, but not highly significant increase ($r = 0.38$; $P < 0.07$) in the frequency of microglial profiles. The

total mean value for young monkeys is 17.3 \pm 6.0 profiles per mm² and for old monkeys it is 21.2 \pm 6.9 per mm². Since the microglia only account for ~6% of the total number of neuroglial cell profiles with nuclei, they are so infrequent that no attempt was made to ascertain if the sizes of their nuclei alter with age.

In Summary

In terms of the profiles of neuroglial cells in layer 4C β that display nuclei, when young and old monkeys are compared it is found that there is a 53% increase with age in the numbers of oligodendrocytes, while there is no change in the frequency of astrocytes, and possibly a small increase in the number of microglial cells.

Frequency of Oligodendrocytes in Pairs and Groups

With age it becomes increasingly common to encounter oligodendrocytes in pairs, rows and groups, in which cells abut each other. Examples of such pairs and groups of oligodendrocytes are shown in Figures 1B and 2. To determine if this is related to the increased frequency in oligodendrocytes with age, the camera lucida drawings of the horizontal sections through layer 4C β were re-examined to determine the percentage of oligodendrocytes that occur in pairs, groups or rows (see Fig. 2). The result is shown in Figure 4, in which it is apparent that as the numbers of profiles of oligodendrocytes with nuclei per mm² increases, the percentage of them that are in pairs, groups and rows also increases ($r = 0.72$; $P < 0.0001$). It will also be noticed that in those old and middle-aged monkeys that have similar numbers of oligodendrocytic profiles per mm² to young monkeys, the frequencies of occurrence of contiguous oligodendrocytes are similar. This is

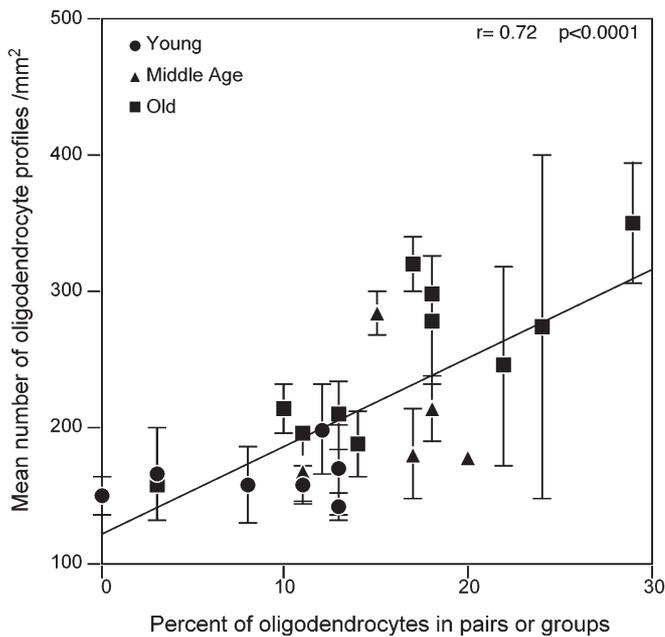


Figure 4. Plot of the percentage of oligodendrocyte profiles that occur in pairs, groups and rows versus the mean number of oligodendrocyte profiles per mm^2 . Based on semi-thick, horizontal sections through layer 4C β of area 17.

compatible with the interpretation that the pairs, groups and rows of oligodendrocytes are generated by cell division.

Correlation between Oligodendrocyte and Paranode Frequencies

Paranodes occur at both ends of myelin segments, and with age there is an increase in the frequency of paranodal profiles in the vertically oriented bundles of myelinated fibers in monkey visual cortex, which suggests that with age there is an increase in the numbers of internodal segments of myelin (Peters and Sethares, 2003). The data from the present study show that there is also an increase in the frequency of occurrence of oligodendrocytes with age. When the data on the frequency of occurrence of paranodal profiles (given in table 1 of Peters and Sethares, 2003) is plotted against the frequency of occurrence of oligodendrocyte profiles in the same monkeys (Fig. 5) it is seen that there is a significant correlation between the two measures ($r = 0.53$; $P < 0.009$).

Cells Labeled by the NG2 Antibody

Light Microscopy

NG2 antibody labels cells throughout the depth of the cerebral cortex. Some of the labeled cells are obviously oligodendrocytes, which display round nuclei, surrounded by a rim of labeled cytoplasm from which a few thin processes may arise (Fig. 6A). Labeled oligodendrocytes are common in layer 4. In the more abundant population of other cells labeled by the NG2 antibody a variety of shapes is evident (Fig. 6). Their cell bodies are small, being ~ 12 – $15 \mu\text{m}$ in diameter, and the nuclei, which are sometimes visible as pale oval or bean-shaped profiles surrounded by a thin rim of labeled cytoplasm, are 5 – $10 \mu\text{m}$ in diameter. A few of the cells have numerous, rather thick, branching processes radiating out from their perikarya

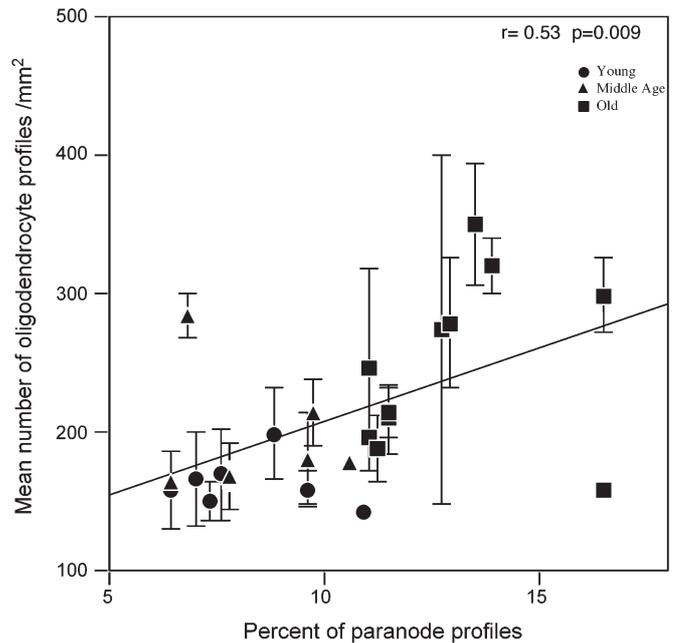


Figure 5. A plot of the percentage of paranodal profiles in transverse thin sections through the myelinated nerve fiber bundles in layer 4C β versus the mean number, per mm^2 , of oligodendroglial profiles that display nuclei.

(Fig. 6A), but most cells have five or six thin processes that branch only once or twice (Fig. 6B–D). The processes have rather irregular and somewhat indefinite outlines and bear small protrusions. Since the antibody reaction does not extend through the section, it is difficult to assess the true lengths of the processes, but the longest ones can be seen to extend for $\sim 80 \mu\text{m}$.

Electron Microscopy

In this material, in which the fixation was less than optimal and the antibody labeling was accentuated by use of silver intensification, all of the cells with the exception of the NG2-labeled cells were reasonably well preserved. The labeled cells are particularly fragile. Their cell membranes usually show some disruption, so that the outlines of the cells are not always clear, making it difficult to determine if the antibody reaction is at the surface of the cells or within the periphery of the cytoplasm (Fig. 7).

The NG2-labeled cells are often adjacent to neurons (Fig. 7A), blood vessels (Fig. 7C), or oligodendrocytes. Their most striking and best-preserved feature are their nuclei, which are commonly elongate or bean-shaped, with slightly irregular outlines and an uneven rim of heterochromatin beneath the nuclear envelope. Heterochromatin is also present in patches throughout the nucleus and some nuclear profiles show a large nucleolus. The cells have somewhat irregular outlines and as would be expected from the light microscopic observations, indistinct processes may be sometimes seen to extend into the surrounding neuropil. The cytoplasm usually forms a thin rim around the nucleus and may be more voluminous at one pole of the cell body. Where the cytoplasm is intact it shows a few small cisternae of rough endoplasmic reticulum and rather stubby or round mitochondria. One or two inclusions may also occur in the cytoplasm, but no bundles of filaments have been encountered.

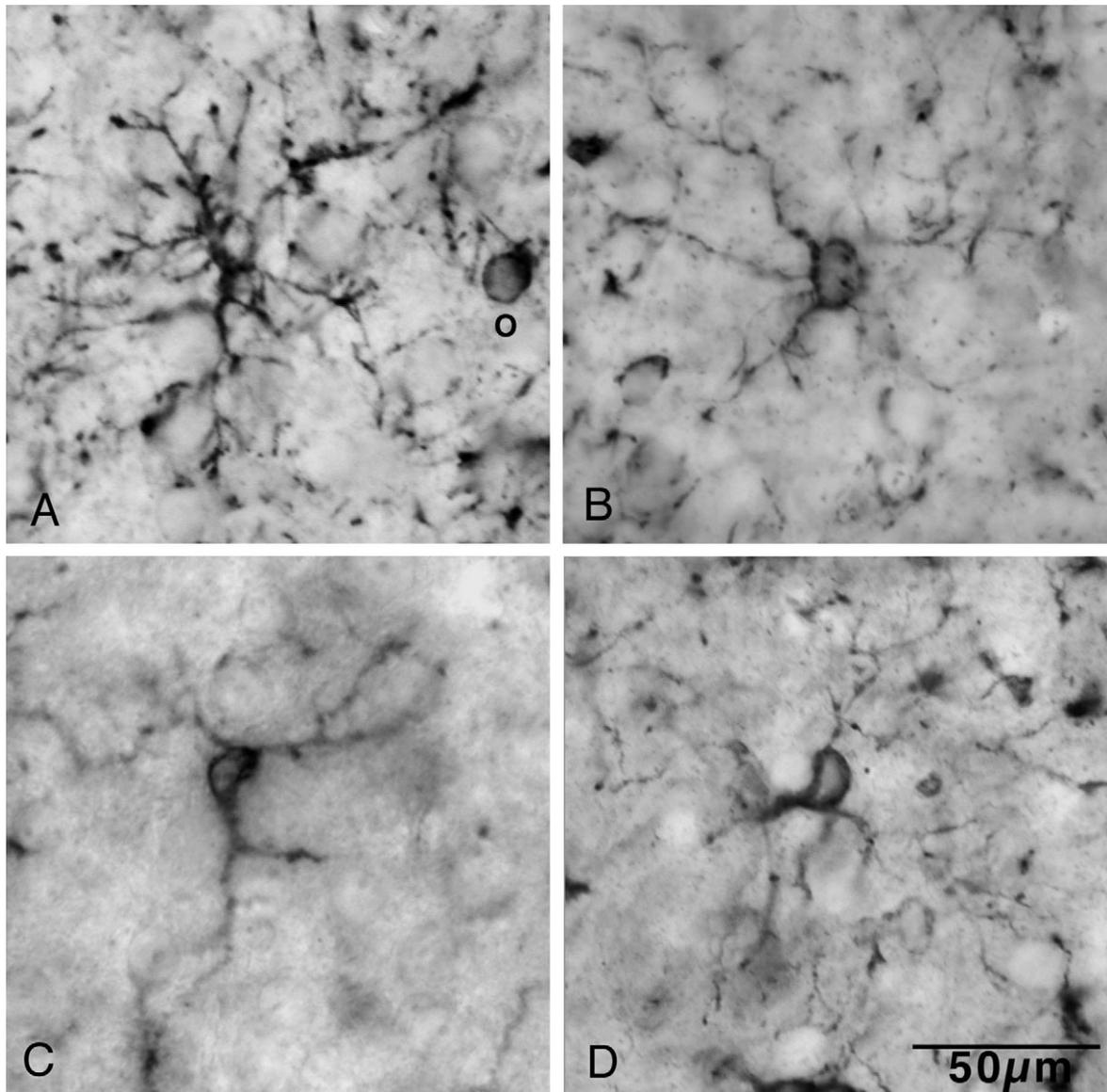


Figure 6. Light micrographs of cells labeled by an antibody to NG2. (A) This cell has many processes that radiate from the cell body, branch and have short extensions passing into the surrounding neuropil. Note the labeled oligodendrocyte (O). (B) This cell has thin, irregular processes with thin extensions. (C) A cell with few processes. Its cell body is elongate and the nucleus is seen as a pale oval in the middle of the cell body. (D) This cell also has an oval cell body that shows the pale nucleus. Thin processes extend from both poles of the cell body. Scale bar = 50 μm .

As pointed out, the NG2 antibody also labels some oligodendrocytes. Their labeling is generally confined to the outer zone of the perikaryon, beneath the cell membrane. The labeled oligodendrocytes are often in pairs, and only one member of the pair may be labeled.

The Identity of the NG2-labeled Cells in Tissue Prepared for Routine Electron Microscopy

When routinely prepared, well-fixed, material is examined by electron microscopy a cell type can be found that has the features of those cells labeled with the NG2 antibody (Figs 8 and 9). Because these cells do not yet have a name, for present purposes they will be referred to as oligodendroglial progenitors, even though it is realized that not all NG2+ cells may have

this function. Such progenitor cells are present in the cortices of both young (Fig. 8A) and old monkeys (Figs 8B and 9). Like the ones labeled with the NG2 antibody, these cells often occur adjacent to neurons (Figs 8A and 9), or blood vessels (Fig. 8B). They have elongate, often bean-shaped nuclei with rather irregular contours. There is a thin layer of heterochromatin beneath the nuclear envelope and heterochromatin occur throughout the nucleus (Figs 8 and 9), but it is generally not as clumped as in the rather poorly preserved material that is necessary for achieving useful labeling with the NG2 antibody (Fig. 7). In well-fixed material the cell bodies have somewhat irregular outlines, and a thin rim of cytoplasm surrounds the nucleus, although the cytoplasm may be more voluminous at one pole of the cell body. The cytoplasm is pale, contains a few short cisternae of rough endoplasmic reticulum, a number

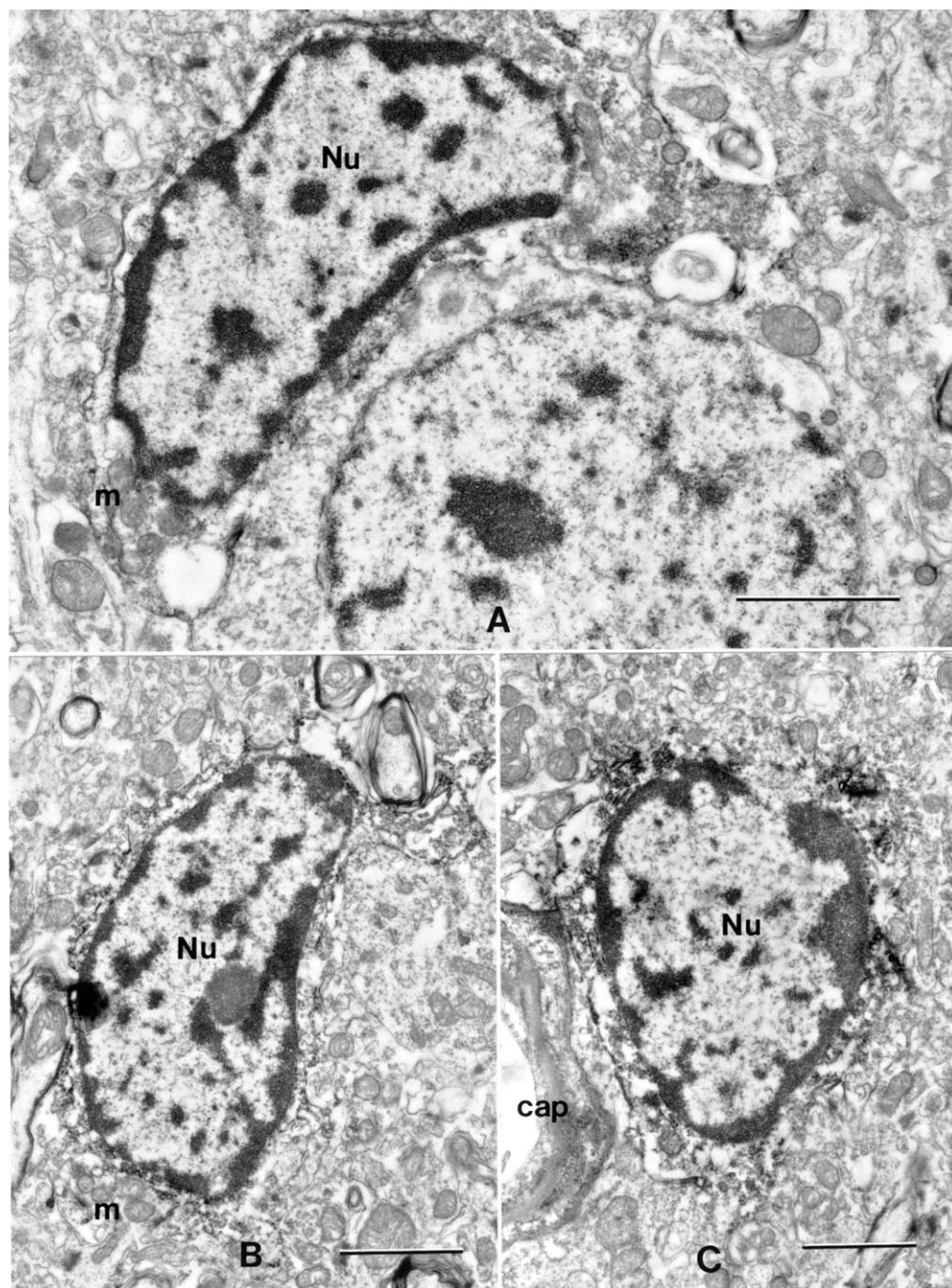


Figure 7. Electron micrographs of cells labeled by the NG2 antibody. The silver intensified DAB reaction product appears as dark granules on the surface and in the cytoplasm. All of the cells have bean-shaped or oval nuclei (Nu) in which there is a layer of heterochromatin beneath the nuclear envelope and patches of heterochromatin throughout the nucleoplasm. The nuclei have rather irregular outlines and much of the cytoplasm is disrupted. However, it can be seen that the mitochondria (m) are stubby and that the cisternae of rough ER are short. The cell in (A) is next to a neuron and the cell in (C) is adjacent to a capillary (cap). Scale bars = 2 μ m.

of free polyribosomes, and rather small and stubby mitochondria. No bundles of the filaments that characterize astrocytes are evident in the cytoplasm.

Having defined the features of the NG2⁺ oligodendroglial progenitor cells in electron microscopic preparations, semithick sections stained with toluidine blue were examined to ascertain if these same cells could be recognized by light microscopy. Some cells with lightly stained and irregular, elongate or bean-shaped nuclei, having a thin layer of dark chro-

matin beneath the nuclear envelope and a lightly stained cytoplasm, were found (Fig. 2; β). Such cells commonly occur next to neurons, oligodendrocytes and blood vessels, and their irregularly shaped nuclei distinguish them from astrocytes, which have round or oval shaped nuclei. In three young and three old monkeys examined, these nuclei accounted for only 1–2% of all neuroglial nuclear profiles. However, there are some nuclei that cannot be categorized with any degree of certainty, and so it is concluded that it is not possible to prop-

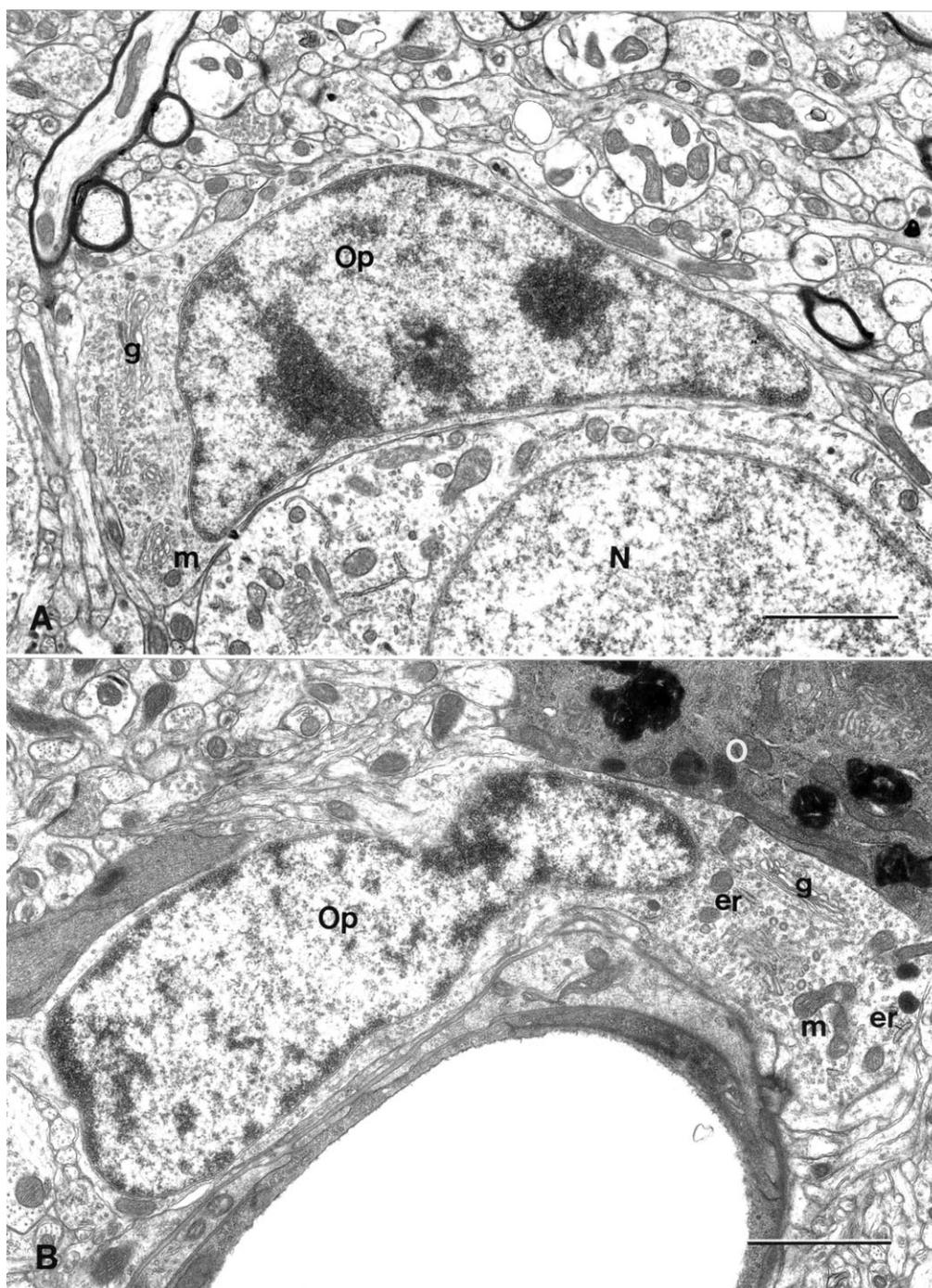


Figure 8. (A) This progenitor cell is from a 5-year-old monkey (AM 16) and is lying adjacent to a neuron (N). Like the NG2-labeled cells, this cell has a nucleus with an irregular outline and heterochromatin lying both beneath the nuclear envelope and dispersed throughout the nucleus. The cell has a somewhat irregular shape. The mitochondria (m) are small and stubby and the Golgi apparatus (g) is well developed. (B) An oligodendroglial progenitor (Op) from a 25-year-old monkey (AM 17) lying adjacent to a capillary. The cell has an irregular nucleus with heterochromatin beneath the nuclear envelope. Only a thin trim of pale cytoplasm surrounds the nucleus, but there is abundant cytoplasm at one pole of the cell, which shows the small stubby mitochondria (m), part of the Golgi apparatus (g), short cisternae of rough endoplasmic reticulum (er) and polyribosomes. Note the contrasting dark cytoplasm of the adjacent oligodendrocyte (O). Scale bars = 2 μ m.

erly determine the frequency of NG2+ cells in semi-thick sections of normally fixed material.

Discussion

Increase in Oligodendrocyte Frequency with Age

As far as we are aware, this is the first demonstration that the frequency of oligodendrocytes in primate cerebral cortex

increases with age. There was a suggestion of this increase in our earlier study (Peters *et al.*, 1991), but the number of monkeys examined at that time was too few to reach definite conclusions. The present study shows that there is a 50% increase in the number of oligodendrocytes in layer 4C β when young and old monkeys are compared. This increase in oligodendrocytes correlates with the increase in frequency of paranodal profiles (see Fig. 5) that we described in an earlier

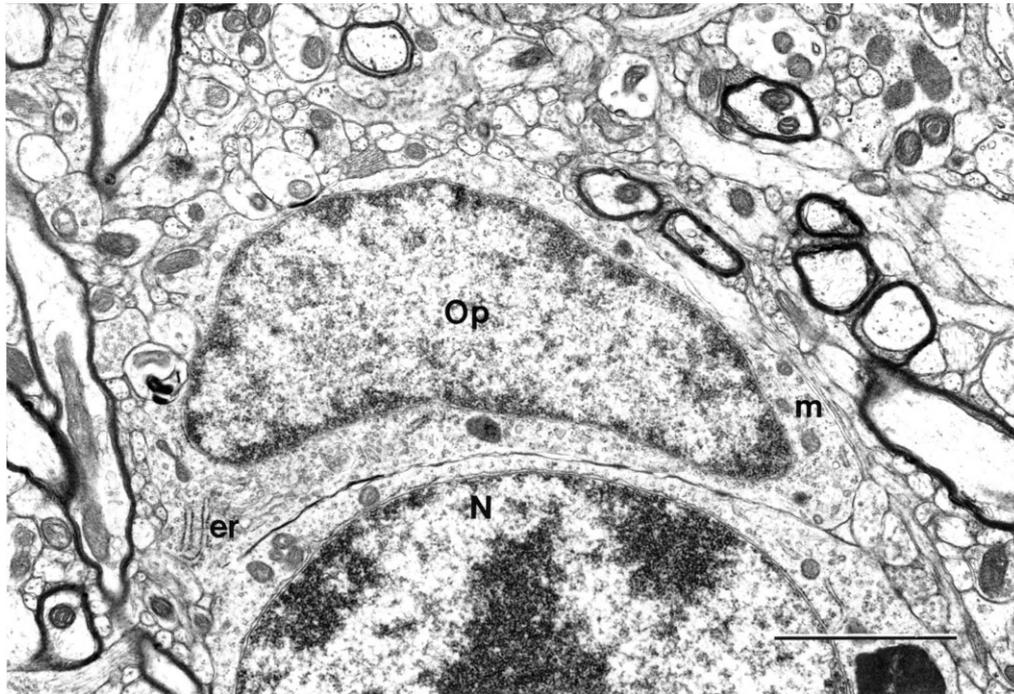


Figure 9. An oligodendroglial progenitor cell (Op) from a 35-year-old monkey (AM 13). The cell is lying adjacent to a neuron (N). The nucleus has a rim of heterochromatin beneath the irregular nuclear envelope and dark patches of chromatin throughout the nucleus. The cell has a somewhat irregular outline and the thin rim of cytoplasm contains a few short cisternae of rough endoplasmic reticulum (er) and small mitochondria (m). Scale bar = 2 μ m.

publication (Peters and Sethares, 2003). To account for this increase in the frequency of paranodal profiles it was proposed that in the aging cerebral cortex there is some breakdown of internodes of myelin with subsequent remyelination, resulting in the formation of some new, short segments of myelin. Such short internodal lengths of myelin are present in the cerebral cortices of old monkeys, as are some usually thin myelin sheaths, and both of these features are generally accepted to be the hallmarks of remyelination (Peters and Sethares, 2003). It seems likely that the additional oligodendrocytes are necessary to generate the increasing number of myelin segments, and this may explain the necessity for the persistence of oligodendroglial progenitors in the mature brain.

Age Changes in the Neuroglial Population

In young monkeys, profiles of oligodendroglial cells that display nuclei account for 63% of the neuroglial cells, astrocytes 30% and microglial cells 7%. However, with age, the 50% increase in oligodendrocytes alters the proportion of oligodendrocytes to ~70% of the total population of neuroglia, even though the numbers of myelinated nerve fibers appear not to change (Nielsen and Peters, 2000). In an earlier study involving all layers of area 17 (Peters *et al.*, 1991) we found the proportions to be: astrocytes 57%; oligodendroglia 35%; and microglia 7%. These are similar to the overall proportions reported for monkey visual cortex by O'Kusky and Colonnier (1982), who also found the greatest frequency of oligodendrocytes to be in layer 4C, where the highest concentration of myelinated nerve fibers occurs.

Although it is a white matter tract, the proportions of neuroglial cells in the young optic nerve closely resemble those in layer 4C β . In optic nerve of young monkeys oligodendrocytes account for 59% of the neuroglial cell population, astrocytes

35% and microglial cells 6% (Sandell and Peters, 2002). It might be assumed that the percentage of oligodendroglial cells would be greater in the optic nerve because it is composed largely of nerve fibers, but in the optic nerve there is a large percentage of astrocytes, which are involved in the fasciculation of the nerve fibers and the formation of the glial limiting membrane around the fascicles. In optic nerve there is a 40% increase in the number of oligodendrocytes with age, and microglial cells also increase in number as they are activated to phagocytose the large number of degenerating nerve fibers. However, in another white matter tract, the anterior commissure, oligodendrocytes account for 86% of all neuroglial nuclear profiles, because in the anterior commissure there is little fasciculation of the nerve fibers by astrocytes. As in optic nerve, the anterior commissure also shows a reduction in the total number of myelinated nerve fibers with age, but at any one time few myelinated nerve fibers are seen to be degenerating, and the frequency of neuroglial cells does not change with age (Sandell and Peters, 2003).

It is evident, then, that the proportions of the three principal types of neuroglial cells varies with location, and in general the greater the concentration of myelinated nerve fibers the greater the frequency and the proportion of oligodendrocytes. What happens to the neuroglial cell population with age seems to depend upon whether there is only breakdown of some myelin sheaths, as in cerebral cortex, a low level of degeneration as in anterior commissure, or an extensive degeneration of nerve fibers as occurs in optic nerve.

The Origins of the Increased Numbers of Oligodendroglial Cells

We have encountered no dividing neuroglial cells in the monkey material, but an indication that cell division is taking

place comes from the observation that pairs, groups and rows on oligodendrocytes become more common with age (Fig. 4), while at the same time the frequency of occurrence of oligodendrocytes increases (Fig. 3). It is possible that division of mature oligodendrocytes generates these pairs, groups and rows, but there appears to be little evidence that mature oligodendrocytes divide (see Ludwin, 1995; Norton, 1996; Keirstead and Blakemore, 1997). The prevailing view is that in the mature brain increased numbers of oligodendrocytes are derived from oligodendroglial progenitor, or precursor cells (see Norton, 1996; Levine *et al.*, 2001; Chen *et al.*, 2002; Watanabe *et al.*, 2002) that can be visualized by staining with antibodies to NG2 chondroitin sulfate proteoglycan and to platelet-derived growth factor α receptor (e.g. Levine *et al.*, 1993; Nishiyama *et al.*, 1997; Stallcup, 2002). The appearance of these NG2+ multipolar cells has been described in light microscopic preparations of various structures, including rat cerebellum (Levine and Card, 1987), rat hippocampus (Ong and Levine, 1999; Nishiyama *et al.*, 2002), rat neocortex (Nishiyama *et al.*, 1997, 1999; Reynolds and Hardy, 1997; Levison *et al.*, 1999); rat brain stem (Cenci di Bello *et al.*, 1999); rat optic nerve (Butt *et al.*, 1999; Jennings *et al.*, 2002), and human brain and spinal cord (Scolding *et al.*, 1998; Chang *et al.*, 2000).

The role of glial progenitors in the normal, mature cerebral cortex is not certain. It is clear that gliogenesis does not cease (e.g. Kornack and Rakic, 1999) and it has been found that initially both astrocytes and oligodendrocytes are produced, but that with increasing age oligodendrocytes are preferentially generated (Levison *et al.*, 1999; Parnavelas, 1999). Our counts of the frequency of neuroglial cell profiles in monkey visual cortex would support this contention, since although we found an increase in the frequency of oligodendrocytes in layer 4C β , astrocytes did not increase in number (Fig. 3).

NG2-positive Cells and Classical Neuroglia

Although it is generally agreed that they cannot be equated with any of the three classical types of neuroglial cells encountered in the mature central nervous system, the identity of the NG2+ cells has been uncertain and a variety of views have been expressed. Thus, in a light and electron microscopic study of rat cerebellum, Levine and Card (1987) suggested that although the progenitor cells do not label with intracellular markers for astrocytes, they have a morphology similar to smooth protoplasmic astrocytes. Fulton *et al.* (1992), on the other hand, concluded that in rat optic nerve the progenitors are neither astrocytes nor oligodendrocytes, but bear a resemblance to both β astrocytes and to the small glioblasts described in rat optic nerve by Vaughn (1969). Nishiyama *et al.* (1997) showed that the NG2+ cells are distinct from both resting and activated microglia, while after examining the NG2+ cells in rat hippocampus Ong and Levine (1999) concluded that they do not have the features of either astrocytes, oligodendrocytes, or microglial cells, and Ye *et al.* (2003) have shown that NG2+ cells in mouse cerebral cortex do not possess cell-type specific proteins typical of astrocytes, neurons, or microglia. Interestingly Butt *et al.* (1999) found NG2+ cells in white matter to have processes that contact nodes of Ranvier, suggesting that they are specialized perinodal glia (also see Butt *et al.*, 2002). However, we have encountered no similar processes in cerebral cortex, where the nodes of Ranvier do not show perinodal sheaths, so that there might

be a difference in the role that NG2+ cells play in gray and in white matter.

The Morphological Characteristics of NG2-positive Cells

Upon examination of the NG2-labeled cells by electron microscopy, Ong and Levine (1999) found them to have round or elongated outlines and their nuclei to have dense heterochromatin on the inner aspect of the nuclear envelope. More recently Nishiyama *et al.* (2002) have studied the cytological features of NG2+ cells in rat hippocampus and cerebral cortex. They describe two kinds of cells. One has clumped chromatin and scant cytoplasm, while the other has a pale, irregular shaped nucleus with pale cytoplasm. The cytological features of the NG2+ oligodendroglial progenitor cells described by Ong and Levine (1999) and the pale cells of Nishiyama *et al.* (2002) are similar to those of the cells that we have encountered in the present study of monkey cerebral cortex and there seems to be little doubt that the cells fit the description of β astrocytes as given by Reyners *et al.* (1982, 1986) in 3-month-old rat cerebral cortex. Reyners *et al.* (1982) found these cells to be radiosensitive, so that they disappear shortly after the cortex is irradiated with high doses of X-rays, even though other cell types seem to be unaffected. In a later study Reyners *et al.* (1986) showed the β astrocytes to be mitotically active, and suggested that they are glial progenitor cells, which they estimate to account for ~13% of the profiles of all neuroglial cells in the cerebral cortex of the adult rat.

Differences between NG2-labeled Progenitor Cells and other Neuroglia

The β astrocytes, which are synonymous with the NG2-labeled oligodendrocyte progenitor cells as identified in thin sections of normally prepared tissue, are clearly distinct from oligodendrocytes, which have a characteristic dark cytoplasm and generally have round nuclei (Fig. 1). They are also different from astrocytes, which have pale and usually round or oval nuclei and a pale cytoplasm that often shows bundles of filaments. Moreover, the cell bodies of astrocytes have irregular shapes that closely follow the contours of the surrounding elements of the neuropil, whereas the progenitor cells have more regular outlines (see Figs 8 and 9). In addition, the mitochondria of the progenitor cells are stubby and generally smaller than those of typical astrocytes, being ~0.2 μ m in diameter compared to the mitochondria of astrocytes, which are ~0.25–0.3 μ m in diameter. In some respects the progenitor cells resemble microglia, which also often lie next to neurons, but they differ in that the nuclei of microglial cells are darker and their cytoplasm contains long cisternae of rough endoplasmic reticulum, while in old monkeys it is common for microglial cells to have large inclusions in their cell bodies. In short, the cells that label with the NG2 antibody have distinct ultrastructural features, and they are different from the three classically defined neuroglial cell types.

One has to wonder why the cells that label with the NG2 antibody, and have the ultrastructural features of β astrocytes, have been largely overlooked in electron microscopic studies of the cerebral cortex. The probable reason is that these cells are essentially nondescript in appearance and have no outstanding features that would draw attention to them. However, when one is conscious that β astrocytes exist, and looks for them in routinely prepared material, it becomes apparent that they are not uncommon in cerebral cortex.

Conclusions

In summary, it is proposed that the increased number of oligodendrocytes present in the aging monkey cerebral cortex is necessary to generate the increased number of internodal segments produced during normal aging. Evidence for the generation of new internodal segments of myelin is the increase in the frequency of paranodal profiles, as well as the presence of short internodes and some unusually thin myelin sheaths. Both of the latter are the hallmarks of remyelination. The new oligodendrocytes are most likely produced by cell division, and this is supported by the fact that as the number of oligodendrocytes increases with age, there is an increase in the frequency with which oligodendrocytes occur in pairs, rows and groups. Since there is no evidence that mature oligodendrocytes can undergo mitosis, it is likely that the additional oligodendrocytes are generated from oligodendroglial progenitor cells that exist in the mature brain. It is generally agreed that these progenitor cells label with the NG2 antibody, and such cells have the features of neuroglial cells previously been described as β astrocytes. However, these cells are clearly not astrocytes and have features that also distinguish them from oligodendrocytes and microglia.

Notes

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