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Effects of Age on the Thickness of Myelin Sheaths in Monkey Primary Visual Cortex

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ABSTRACT

The effect of age on myelin sheath thickness was determined by an electron microscopic examination of cross sections of the vertical bundles of nerve fibers that pass through primary visual cortex of the rhesus monkey. The tissue was taken from the cortices of young (4–9 years of age) and old (over 24 years of age) monkeys, and the sections were taken at the level of layer 4Cβ. From the electron photomicrographs, the diameters of axons and the numbers of lamellae in their myelin sheaths were determined. No change was found in the diameters of axons with age, although the mean numbers of myelin lamellae in the sheaths increased from 5.6 in the young monkeys to 7.0 in the old monkeys. Much of this increase in mean thickness was due to the fact that, in the old monkeys, thick myelin sheaths with more than ten lamellae are more common than in the young monkeys. While this increase in the thickness of myelin sheaths is occurring in old monkeys, there are also age-related changes in some of the sheaths. Consequently, it seems that, with age, there is some degeneration of myelin but, at the same time, a continued production of lamellae. J. Comp. Neurol. 435: 241–248, 2001.

Indexing terms: *Macaca mulatta*; myelin; aging; area 17; axons

In the primary visual cortex (area 17) of the rhesus monkey (*Macaca mulatta*), there are regularly spaced clusters of pyramidal cell apical dendrites that extend into layer 1, where they form their apical tufts. We have suggested that these clusters represent the axes of modules of pyramidal cells (Peters and Sethares, 1991), which are envisioned to be the basic functional neuronal units of the cerebral cortex, so that, in effect, they are equivalent to the minicolumns defined by Mountcastle (1978). Monkey primary visual cortex also contains prominent bundles of vertically oriented, myelinated nerve fibers. Like the dendritic clusters, these bundles of myelinated axons are arranged regularly, and they have a similar mean center-to-center spacing of 23 μm. For this and other reasons, it is proposed that each bundle of myelinated nerve fibers contains the efferent fibers that extend from the neurons within a single pyramidal cells module (Peters and Sethares, 1996).

In horizontal sections through monkey primary visual cortex, the myelinated fiber bundles are most discrete, and their nerve fibers are most tightly packed at the level of layer 4Cβ, making this a good location in which to examine the effects of normal aging on myelinated nerve fibers. Thus, in a recent study, we examined horizontal sections through layer 4Cβ of area 17 to determine whether there is a loss of intracortical nerve fibers with age (Nielsen and Peters, 2000). The results showed that, statistically, there is no difference in numbers of vertically oriented nerve fibers beneath 1 mm^2 of cortical surface between, young, middle-aged, and old rhesus monkeys. This is consistent with evidence of little axonal degeneration on electron photomicrographs and with the accumulating evidence that, during normal aging, there is no significant loss of neurons from the cerebral cortex (Morrison and Hof, 1997; Kim et al., 1997; Peters et al., 1998; Hof et al., 2000). However, even though there is no significant loss of the vertically oriented nerve fibers, there are age-related alterations in the structure of the myelin sheaths of many of the nerve fibers (Feldman and Peters, 1998; Peters et al., 2000). The most common change is that some of the my-

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TABLE 1. Data on Monkey Ages, Axonal Diameters, and Sheath Thickness

Monkey	Age (yrs)	Sex	Mean axonal diameter ± S.D. (μm)	Mean number of lamellae in sheath ± S.D.
Young monkeys				
AM 58	4	M	0.70 ± 0.21	4.8 ± 1.7
AM 16	5	M	0.88 ± 0.34	6.4 ± 2.9
AM 97	5	F	0.75 ± 0.27	5.5 ± 2.3
AM 76	6	F	0.78 ± 0.25	5.0 ± 1.8
AM 47	9	M	0.81 ± 0.31	6.3 ± 3.1
Mean	—	—	0.79 ± 0.27	5.6 ± 2.4
Old monkeys				
AM 19	24	F	0.90 ± 0.33	6.4 ± 3.9
AM 62	26	M	0.81 ± 0.24	6.4 ± 2.8
AM 15	28	F	0.77 ± 0.25	7.4 ± 4.1
AM 17	29	F	0.71 ± 0.24	7.4 ± 3.7
AM 91	30	M	0.81 ± 0.35	7.5 ± 5.2
AM 41	32	F	0.69 ± 0.24	6.6 ± 2.9
AM 13	35	M	0.87 ± 0.29	7.6 ± 5.0
Mean	—	—	0.79 ± 0.28	7.0 ± 4.0

elin sheaths of nerve fibers in old monkeys show focal splitting of the lamellae at the major dense line to accommodate dense cytoplasm. Other sheaths balloon out where there is a splitting of the intraperiod line to surround a fluid-filled space. Other age-related alterations are the formation redundant myelin, such that some sheaths are much too large for their enclosed axons, and the formation of what appear to be double sheaths, in which one compact sheath of myelin is surrounded by another. The purpose of the present study was to extend the investigation of the effects of age on the nerve fibers in the vertical bundles in monkey primary visual cortex by determining whether age has any effect on the diameters of axons and on the thickness of the myelin sheaths of the nerve fibers within the vertical bundles.

MATERIALS AND METHODS

Blocks of tissue were removed from the opercular surface of the primary visual cortices of five young (4–9 years of age) and seven old (24–35 years of age) rhesus monkeys (*Macaca mulatta*). The ages of these monkeys and their code designations are given in Table 1. It is assumed that the five monkeys between 4–9 years of age have nerve fibers and sheaths with a normal structure. Consequently, these were used as controls against which to assess the effects of age, as exhibited by nerve fibers in the cortices of monkeys over 24 years of age. These ages were chosen because rhesus monkeys become sexually mature at about 5 years of age, and, in an analysis of the life span of the rhesus monkey, it has been shown that only approximately 25% of monkeys attain an age of 25 years (Tigges et al., 1988). These monkeys were from an aging colony maintained at Yerkes Regional Primate Research Center at Emory University. The colony is used to determine the effects of normal aging on behavior and on the brain. The colony is monitored to screen out individuals with known pathological conditions or treatment that may affect the normal aging process, and the animals are cared for by professional veterinary supervision in accordance with the National Institutes of Health guidelines for the use and care of laboratory animals.

Tissue preparation

The brains of the monkeys were fixed by vascular perfusion under deep anesthesia, as described by Peters et al. (1994) and in full accordance with approved Institutional Animal Care and Use Committee regulations. In brief, the monkeys were preanesthetized with ketamine, and sodium pentobarbital was administered intravenously until a state of areflexia was achieved. The anesthetized monkeys were then artificially respired with a mixture of 95% oxygen and 5% carbon dioxide and transcardially perfused with a warm solution of 1% paraformaldehyde and 1.25% glutaraldehyde in either a 0.1-M phosphate buffer or a 0.1-M cacodylate buffer, pH 7.4. At the end of the perfusion, the brains were removed, and one hemisphere was placed for additional fixation in a cold solution of 2% paraformaldehyde and 2.5% glutaraldehyde in the same buffer that was used for the perfusion.

After several days, eight to ten pieces of primary visual cortex were removed from the opercular surface of the occipital lobe (area 17) about 3 mm caudal to the lunate sulcus, where the center of the visual field is represented. The pieces of cortex, each about 2 mm thick, were osmicated, dehydrated in an ascending series of alcohols, and embedded in Araldite. Semithick, vertical sections were first taken through the depth of the cortex and stained with toluidine blue. The layers of the cortex were identified; then, the blocks were turned to obtain horizontal sections at the level of layer 4Cβ, where the bundles of vertically oriented nerve fibers aggregate into discrete bundles (Peters and Sethares, 1996). Thin sections taken for electron microscopic examination were stained with uranyl acetate and lead citrate.

Quantitative assessment

Electron photomicrographs of the cross-sectioned bundles of vertically oriented myelinated nerve fibers in layer 4Cβ were taken at a primary magnification of ×8,000 or ×10,000. At such magnifications, the lamellae of the myelin sheaths are visible. The electron microscopic negatives were then printed at an enlargement of ×2.5.

Another set of electron photomicrographs of myelin sheaths in each monkey was taken at a primary magnification of ×30,000 to determine whether the periodicity of myelin alters with age. The lamellar spacing was measured directly from the negatives using a measuring magnifier loupe with a 0.1-mm scale.

Number of lamellae in myelin sheaths. To determine the number of lamellae in the myelin sheaths of individual nerve fibers, the electron microscopic negatives were projected onto a screen. The number of lamellae in each sheath sectioned at the level of the internode was then counted and recorded on the corresponding image of the sheath on a matching photographic print. Profiles of sheaths sectioned at the levels of paranodes were not used, because the turning off of lamellae as the sheath terminates and approaches the node of Ranvier leads to a thinning of the sheath and a consequent reduction in the number of lamellae. In old monkeys in which some sheaths displayed age changes, such as splitting of the lamellae to enclose dark cytoplasm or ballooning, the number of lamellae was determined in a segment of the sheath where the myelin was compact and unaltered. It should be added that, because some sheaths were sectioned obliquely, the number of lamellae could not be determined for every sheath in a given vertical bundle. For each monkey, the number of lamellae was determined in the sheaths of at least 200 profiles of nerve fibers sectioned along their internodes.

Axon diameters. The diameters of the axons of those nerve fibers in which lamellae had been counted were determined from the photographic prints. The outlines of the axons were traced onto transparent sheets and scanned into a Macintosh computer (Apple Computers, Inc., Cupertino, CA). The software program N.I.H. Image (Bethesda, MD) was used to determine the areas of the cross-sectioned axons, and, from these areal measurements, the mean diameters of the axons were calculated.

Reliability. To determine whether the counts of the numbers of lamellae and the measurements of axon diameter were reliable, we conducted tests of the measurements. Some photomicrographs from which data had been collected previously were selected at random, and, on a total of 60 nerve fibers, the axon diameters were remeasured, and the numbers of lamellae in their sheaths were recounted. This second set of results was compared with the previous results using the Pearson correlation to determine whether there was qualitative agreement among the two sets of data and using a paired *t*-test to determine whether there was quantitative agreement. These tests revealed a high level of intrerater agreement in our data (r values > 0.95 and P values < 0.05; in all tests, P values < 0.10). This indicates that the methods used to collect data were reliable.

RESULTS

General description

An example of an electron photomicrograph of a cross-sectioned nerve fiber bundle taken at the level of layer 4Cβ from a young monkey is shown in Figure 1. In the bundles, the myelinated nerve fibers are intermixed with unmyelinated axons, and, typical of the central nervous system, the compact myelin sheaths are not very thick. Most of the nerve fibers profiles are of myelin sheaths that have been sectioned at levels between the paranodes, and, for descriptive purposes, such profiles are referred to herein as those of internodal myelin sheaths. In the nerve fiber bundle shown in Figure 1, the numbers of lamellae in the profiles of the internodal sheaths are indicated by the numerals on the ensheathed axons.

Although most of the profiles of the myelinated nerve fibers show internodal myelin, a few of the profiles are of paranodes. Cross sections of paranodes can be identified readily (Fig. 1, p), because the axon generally has a rather circular profile, whereas the axolemma and the membrane of the ensheathing oligodendrocyte are closely approximated to form a seven-layered complex (Peters et al., 1991). In addition, it is common to see the axon surrounded by a rim of cytoplasm from the helical tunnel of oligodendroglial cytoplasm that is between the compact myelin and the axon (Fig. 1, p).

Basically, the myelinated nerve fibers in the vertical bundles from the old monkeys are similar in appearance to those from the young monkeys (Fig. 2). The major difference is that, in old monkeys, some of the myelin sheaths show age-related alterations (Feldman and Peters, 1998; Peters et al., 2000). The most common of these alterations, as pointed out above, is a local splitting of the myelin at the major dense line to accommodate dark oligodendroglial cytoplasm that often contains vesicles (Fig. 2, asterisk). Other age-related defects are the ballooning of sheaths, an increased frequency of nerve fibers with redundant myelin such that sheaths are too large for the enclosed axons (Fig. 2, arrow), and the presence of apparently double sheaths in which one set of compact lamellae is separated by a space from an outer set. Another difference is that, in old monkeys, the sheaths generally seem to be somewhat thicker than in young monkeys. This can be seen by comparing the numbers of lamellae in the sheaths from young (Fig. 1) and old (Fig. 2) monkeys.

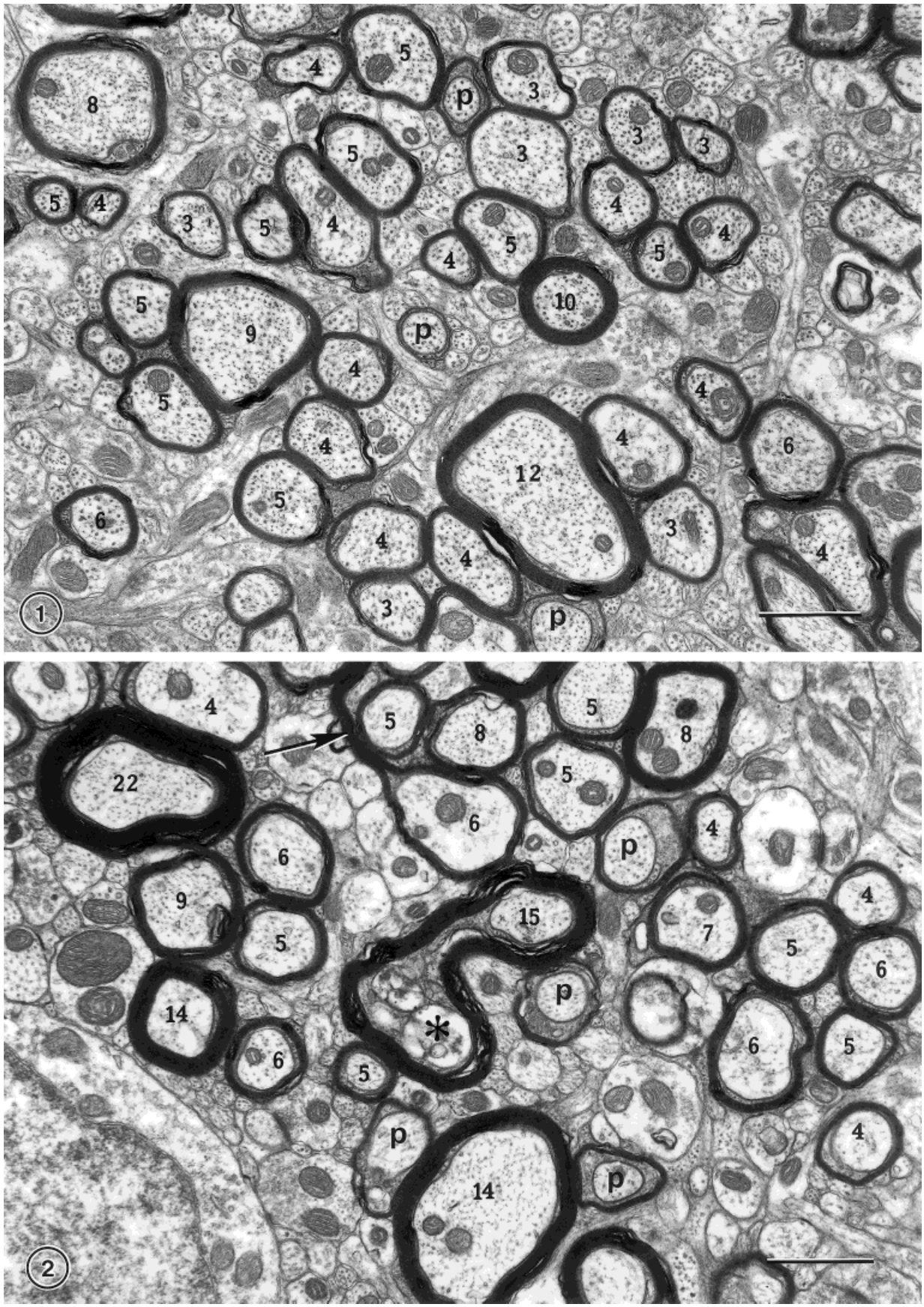
Quantitative analyses

Axonal diameter. Figure 3 shows that axons range from as small as 0.1 μm to as large as 2.2 μm, and, in young and old monkeys, the mean diameters of axons are similar (Table 1). In the five young monkeys, the mean diameter is 0.79 μm ± 0.27 μm, whereas, for the seven old monkeys, it is 0.79 μm ± 0.28 μm. A Student's *t*-test shows that there was no significant difference in the diameters of axons between the young and old groups, and there was no correlation between axon diameters and the sexes of the monkeys, even though the mean weight of the brains of male monkeys is about 10% greater than that of female monkeys (Herndon et al., 1998).

Sheath thickness. The numbers of lamellae in the sheaths of nerve fibers in visual cortex range from as few as 2 to as many as 36. The distribution of sheath thickness in young and old monkeys in terms of the numbers of lamellae in their sheaths is shown in Figure 4. A comparison of the numbers of lamellae using a Student's *t*-test shows that there was a significant difference in the distribution of sheath thickness between the young and old groups ($t = 11.36$; $P = 0.0001$), confirming that sheaths are thicker in old monkeys compared with young monkeys. The mean number of lamellae in the myelin sheaths of the five young monkeys was 5.6, whereas, in the seven old monkeys, the mean number was 7.0 (Table 1).

However, in both young monkeys and old monkeys, there was a correlation between axonal diameter and the thickness of the sheath. Thus, Pearson r correlations revealed that, in young monkeys, there was a significant ($r = 0.59$; $P = 0.001$; one-tailed test), positive, linear correlation between axonal diameter and the number of lamellae in sheaths, so that, as axons increase in diameter, there is a correlated increase in sheath thickness. A similar relationship between axon diameter and sheath thickness also exists for old monkeys ($r = 0.56$; $P = 0.001$; one-tailed test).

In Figure 5, the axonal diameter and the number of lamellae in the sheaths of individual nerve fibers are plotted against each other. This graph contains all of the data obtained from both young monkeys and old monkeys. The mean number of 5.6 lamellae in the sheaths of axons from young monkeys also is indicated. Visual inspection of Figure 5 suggests that, with age, there is a relative increase in the number of axons with thick sheaths. To test this directly, we operationally defined thick sheaths as those containing more lamellae than the mean of the young group ± 2 S.D. These fibers are in the shaded area of Figure 5, and, essentially, they have more than ten lamellae in their sheaths. We directly compared the percentage of fibers with thick sheaths in the young and old groups using a Student's *t*-test, and, as suggested by Figure 5, the percentage of axons with thick sheaths was significantly greater in older monkeys ($t = 3.2$; $P = 0.002$) than in young monkeys. In young monkeys, 5.4% of the nerve fibers have sheaths with more than ten lamellae;



Figures 1 & 2

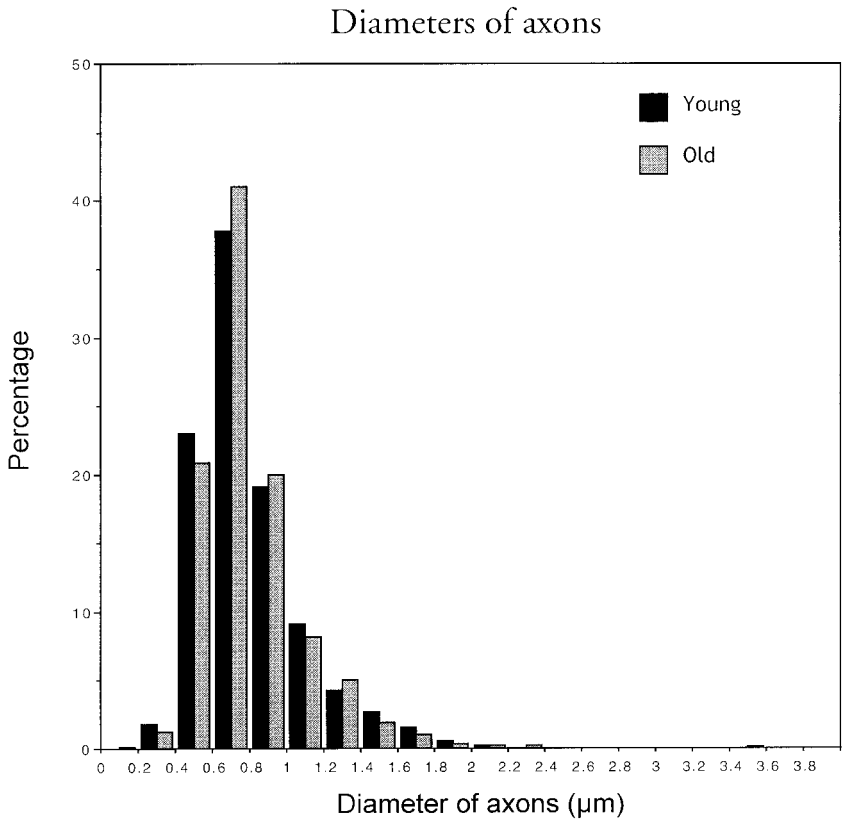


Fig. 3. A comparison of the percentage distribution of the diameters of axons in young monkeys and old monkeys.

however, in old monkeys, the proportion increases to 15.2%.

Lamellar thickness. No differences were found in the thickness of the lamellae in the myelin sheaths from young and old monkeys. In both young and old, the mean thickness of the lamellae, measured from center to center of adjacent major dense lines, was 11 nm.

DISCUSSION

Our data on the myelinated nerve fibers in visual cortex show, that in both young monkeys and old monkeys, there is a correlation between axon size and myelin sheath thickness. Such a correlation has been documented previously for peripheral nerves, which have been studied by both light microscopy (Duncan, 1934) and electron microscopy (Friede and Samorajski, 1967; Matthews, 1968; Friede and Bischhausen, 1982), and for nerve fibers in the central nervous system that have been examined by electron microscopy (Samorajski and Friede, 1968; Bishop et al., 1971; Waxman and Swadlow, 1976). However, from these studies, it is obvious that, for similar sized axons, the myelin sheaths in the peripheral nervous system that are formed by Schwann cells are significantly thicker than those formed by oligodendrocytes in the central nervous system. In the study by Friede and Samorajski (1967), for example, it was found that, in the sciatic nerve, the number of lamellae in the sheaths ranged from 5 to as many as 80. In contrast, in their study of the pyramidal tract of the rat, the same authors (Samorajski and Friede, 1968) found that the thickest sheaths had only 25 lamellae, similar to the thickest sheaths we encountered in monkey visual cortex.

The data from the present study show that, although there is no change with age in the dimensions of the axons, there is an overall increase in the thickness of their myelin sheaths. This is due largely to the presence of a three-fold increase in the population of nerve fibers with thick sheaths comprised of between 10 and 36 lamellae in the old monkeys. It is interesting to note that it is not only fibers with large diameter axons that have these thick

Fig. 1 A transversely sectioned bundle of myelinated fibers from a 4-year-old monkey (AM 58). The myelin sheaths of nerve fibers sectioned at their internodes are compact, and the numbers of lamellae in these sheaths are given. Profiles of paranodes (p) can be recognized by the close approximation of the axolemma with the plasma membrane of the myelin-forming cell and the presence of cytoplasm of the terminal helix. Scale bar = 1 μm.

Fig. 2. A transversely sectioned nerve fiber bundle from a 29-year-old monkey (AM 17). Like in Figure 1, paranodes (p) are labeled, and the numbers of lamellae in the sheaths of nerve fibers sectioned at their internodes are given. A comparison with Figure 1 shows the generally increased number of lamellae in the older monkey, with one sheath having 22 lamellae. One nerve fiber with 15 lamellae in the center of the field has dark, vacuolated cytoplasm in its sheath (asterisk), whereas the nerve fiber adjacent to it, one with six lamellae, has a redundant sheath (arrow). Scale bar = 1 μm.

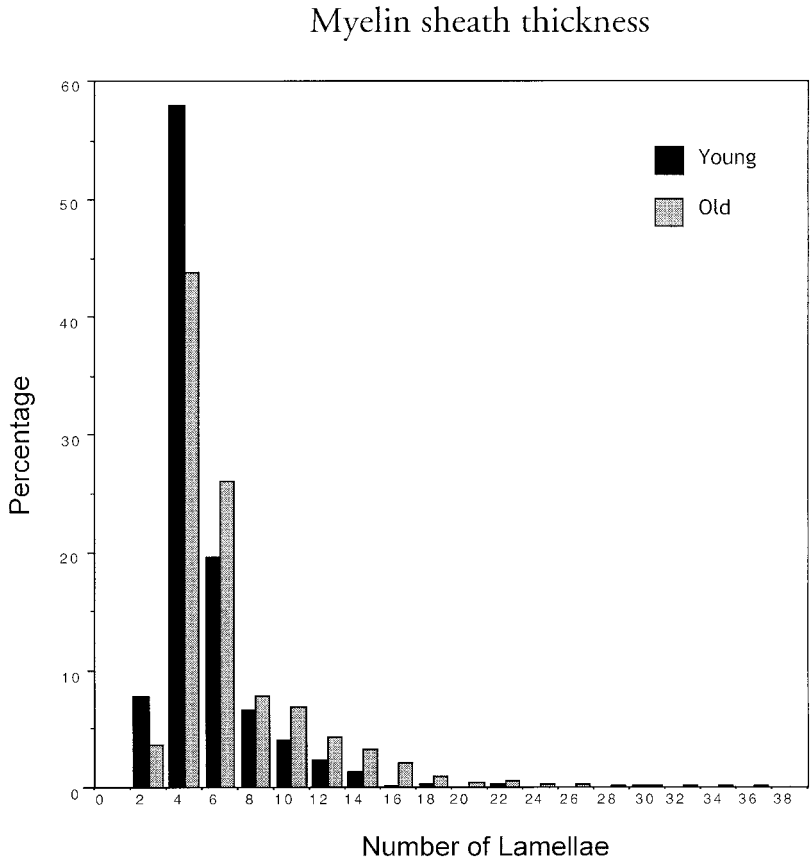


Fig. 4. A comparison of the percentage distribution of the numbers of lamellae in the myelin sheaths of young monkeys and old monkeys.

sheaths, because some of the thick sheaths surround axons with diameters of less than 0.5 μm (see Fig. 5).

Unfortunately, no previous studies on the effects of age on sheaths of central nerve fibers in primates could be found. There are reports on the effects of age on nerve fibers in rodents, but they have reached differing conclusions. For example, Godlewski (1991) noted that the myelin sheaths in the optic nerve and corpus callosum of 2.0- and 2.5-year-old rats were thicker than those of 4-month-old rats. In contrast, Sturrock (1976) reported that, in his study of the anterior and posterior limbs of the anterior commissure in the brains of 5- and 18-month-old mice, there was no significant change with age either in the diameters of axons or in the mean numbers of myelin lamellae. In the peripheral nervous system, Cebellos et al. (1999) found that the myelin sheaths in the tibial nerves of mice became thicker between 6 and 33 months of age, with some sheaths becoming extremely thick, as they do in monkey visual cortex. However, Caselli et al. (1999) found no change in the numbers of lamellae in the sciatic nerves of rats between 12 and 30 months of age. Why these studies on rodents reach such differing conclusions is not apparent, although the two studies that reported increases in sheath thickness used animals that were somewhat older than the studies that reported no change in thickness.

Even though myelin sheaths in monkey visual cortex become thicker with age, there is no indication of a change in the thickness of the myelin lamellae. At all ages, the thickness of the myelin lamellae averaged 11 nm. This is similar to the thickness measured for fixed central myelin by Raine (1984) and others (see Peters et al., 1991). One reason for measuring the thickness of the myelin lamellae is that, in an earlier study of central myelin, Hildebrand (1972) reported that, in cat spinal cord, the thickness of the myelin lamellae is related inversely to the number of lamellae. Thus, Hildebrand (1972) reported that, in sheaths with over 60 lamellae, the myelin period was about 9.6 nm, whereas, in sheaths with 10 lamellae, the spacing was 11 nm, suggesting that one reason for the difference may lie in the composition of the myelin in thin and thick sheaths. However, to date, there have been few investigations into how the composition and properties of myelin in the central nervous system may alter with age (see, e.g., Malone and Szoke, 1982; Chia et al., 1983). Godlewski (1991) also reported an increase in interlamellar distances in the optic nerves and corpus callosum of old rats compared with young rats. However, the interlamellar distances were not measured directly but were derived from a ratio of the overall sheath thickness to the number of lamellae in the sheaths.

From our observations, it is apparent that, although the sizes of the axons are not changing with age in the monkey, there is an increase in the mean number of lamellae of myelin, with the sheaths of some fibers of all sizes becoming markedly thicker. This must mean that the

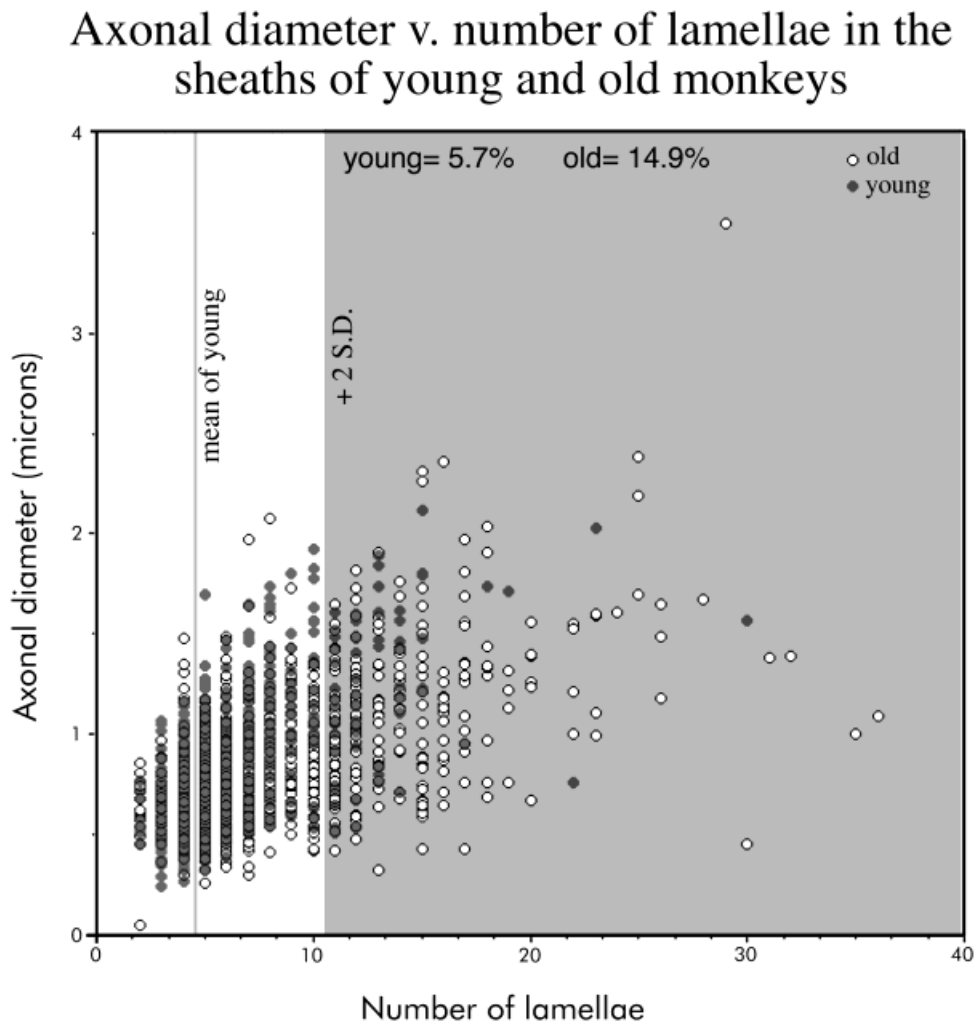


Fig. 5. Plots of the diameters of axons against the numbers of lamellae in their myelin sheaths in young monkeys and old monkeys. The mean number of lamellae in the sheaths of young monkeys is shown as well as those nerve fibers with sheaths that are thicker than 2 S.D. from the mean number of lamellae in the sheaths of young monkeys, that is, ten lamellae. Whereas 5.4% of the sheaths in the young monkeys have ten lamellae (i.e., are thicker than the mean of the young plus 2 S.D.), 15.2% of the sheaths in the old monkeys have more than ten lamellae.

oligodendrocytes not only are actively producing myelin to maintain their sheaths but that they also are adding lamellae during the normal aging process. The continued, active production of myelin also is indicated by the fact that, with age, there is an increase in the frequency of occurrence of sheaths with redundant myelin (Sturrock, 1976; Peters et al., 2000). One question that arises is whether the increased thickness of sheaths with age is due to the addition of lamellae to existing sheaths or whether a second sheath is being formed around an existing one, a possibility that is suggested by the presence of so-called double sheaths. The double sheaths that become more common in aging monkeys, as far as we can determine, are due to extensive splits in thick sheaths. There is no evidence that they are produced by one complete sheath becoming surrounded by another. For example, we have never seen cytoplasm-containing inner and outer tongue processes within the space separating the inner and outer sets of compact lamellae, and, in longitudinal sections, we have never encountered paranodes or nodes covered by compact lamellae or one paranode covering another. Such images would be expected, because the connection between the parent oligodendrocyte and its sheath occurs about in the middle of an internodal length of myelin, and the existence of this connection likely would preclude the formation of a second internodal length of myelin completely covering and coextensive with a previous length. It seems then, that the so-called double sheaths probably are produced by the formation of extensive splits between lamellae in thick sheaths, and, because thick sheaths are more common in old monkeys than in young monkeys, the apparently double sheaths become more common with age.

Concomitant with the increase in the mean thickness of the myelin sheaths, some sheaths show signs of degeneration. This is revealed by the occurrence of significant numbers of nerve fiber profiles that show splitting of the major dense line to accommodate dense cytoplasm that

originates from the oligodendroglial glial cells and ballooning of sheaths at splits of the intraperiod line (Peters et al., 2000).

These data suggest that, during aging, oligodendroglia continue to actively produce myelin, even though some sheaths are degenerating. With age, as shown in an earlier study (Peters, 1996), some oligodendrocytes develop bulbous swellings of their processes, and such swellings are filled by inclusions resembling age pigment. Similar inclusions also occur in the perikarya of some oligodendrocytes in old monkeys. It may be that oligodendrocytes with such inclusions are those that are connected to sheaths containing dense cytoplasm and that the oligodendrocytes are resorbing their own sheaths. In addition, although oligodendrocytes usually occur singly in the cortices of young monkeys, it is not uncommon in old monkeys to encounter oligodendrocytes in pairs and groups (Peters, 1996). Such groups of oligodendrocytes may have been produced by cell division, because Levison et al. (1999) provided evidence that, in rat neocortex, oligodendrocytes continue to be generated throughout life from resident progenitor cells (see also Levine et al., 2001). It also is known that demyelinated axons can be remyelinated (Ludwin, 1995, 1997; Rosenbluth et al., 1999; Woodruff and Franklin, 1999), and this process probably would require additional numbers of oligodendrocytes. Together, these observations on myelin sheaths and oligodendrocytes may suggest that, with age, there is a breakdown of some myelin sheaths concomitant with the continued formation of myelin to produce increased sheath thickness and even remyelination if some sheaths degenerate. The next phase of this study will be to determine whether demyelination and remyelination do occur with increasing age.

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