The Effects of Age on the Cells in Layer 1 of Primate Cerebral Cortex

Although there is significant thinning of layer 1 with age in both occipital area 17 and prefrontal area 46 of the rhesus monkey, there are no significant age-related changes in the numbers of neurons, astrocytes, or microglia and oligodendrocytes in this layer. A few profiles of degenerating neurons have been encountered in old monkeys, but they are uncommon. Some astrocytes undergo hypertrophy with age, as evidenced by the increased thickness of the glial limiting membrane, and throughout layer 1 the amount of filaments in the cytoplasm of both their cell bodies and processes increases. The astrocytes also come to contain phagocytic material in the old monkeys, as do the microglial cells. We have previously shown that in both areas 17 and 46 there is an age-related loss of synapses from layer 1 and a concomitant loss of dendritic branches from the apical tufts of pyramidal cells from layer 1. These may be the sources of the material phagocyted by the astrocytes and microglial cells.

Materials and Methods
Twenty-one rhesus monkeys (Macaca mulatta) with ages ranging from 5 to 35 years were used in this study. For 18 of these monkeys their exact ages were known, but for three of the older monkeys, which were not born in captivity, their ages had to be estimated. The details of the fixation protocol for the brains used in this study have been given in detail in earlier publications (Peters et al., 1994). The perfusions were carried out in full accordance with the approved Institutional Animal Care and Use Committee regulations. In brief, the monkeys were anesthetized with ketamine, and sodium pentobarbital (35 mg/kg) was administered i.v. until a state of areflexia was attained. The monkeys were then artificially resired using a mixture of 95% O2 and 5% CO2 and their brains fixed by vascular perfusion with a warm solution of 2% paraformaldehyde and 2.5% glutaraldehyde in either 0.1 M cacodylate or 0.1 M phosphate buffer at pH 7.4. Following this initial fixation, the brain was removed and one hemisphere placed for several days in a cold solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate or phosphate buffer at pH 7.4.

Small blocks of cerebral cortex were then taken from two cortical areas: (1) from the primary visual cortex on the lateral surface of the occipital lobe, ∼3 mm caudal to the lunate sulcus, where central vision is represented and (2) from the floor of the principal sulcus of the frontal lobe at the rostral end of the corpus callosum, (Peters et al., 1998b), where area 46 (Walker, 1940) is located. The pieces of cortex were osmicated, dehydrated in an ascending series of alcohols and embedded in Araldite. At least two blocks of cortex from each area were sectioned in a plane vertical to the pial surface. The tissue blocks were adjusted on the microtome until the plane of section passed exactly parallel to the lengths of the apical dendrites of the pyramidal cells. This ensured that the true thickness of the cortex would be displayed. When this had been achieved, a series of 1 µm thick sections was taken and stained with toluidine blue for light microscopic examination. Thin sections were also taken from these blocks for electron microscopy. Such thin sections were stained with uranyl acetate and lead citrate, for examination in a JEOL 100 S electron microscope.

Thickerness of Layer 1
For those monkeys in which the thickness of layer 1 in area 17 and area 46 had not already been ascertained in our previous studies (Peters et al., 1998b, 2001), the thickness was determined by making camera lucida drawings of the 1 µm thick sections using an Olympus microscope. A ×20 objective lens was used and drawings of 250 µm long strips of layer 1 were scanned into a computer. NIH Image v.1.55 was used to determine the area of layer 1 contained in the strips and from this, the mean thickness of layer 1 was ascertained. The locations of the cell bodies of

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the pyramidal cells in upper layer 2 were taken to mark the boundary between layers 1 and 2 (Peters et al., 1998b).

In terms of the measurements of the thickness of layer 1, it is pertinent to point out that there is no indication that the overall depth of the cerebral cortex changes appreciably with age (Vincent et al., 1989) and although it is not known if there is a change in the volume of cortex when it is fixed by perfusion, it is known that when fixed cortex is prepared and embedded for electron microscopy there is a linear shrinkage that is fixed by perfusion, it is known that when fixed cortex is prepared and embedded for electron microscopy there is a linear shrinkage that amounts to only –0.7% (Peters et al., 1985).

Frequency of Neurons and Neuroglial Cells

In Nissl-stained material prepared for light microscopy it is frequently difficult to distinguish between the profiles of the various cell types in layer 1. For this reason a stereological analysis of the effects of age on the populations of the individual cell types in layer 1 cannot be carried out using such material. However, it is easy to distinguish between neurons and astrocytes in semithick, plastic-embedded and osmicated tissue stained for light microscopy with toluidine blue, although even in this material it is not always possible to distinguish between oligodendrocytes and microglial cells. Consequently, it was decided to group the profiles of oligodendrocytes and microglial cells together and to put them into a category referred to as ‘dark cells’.

To determine if there is a change in the frequency of neurons, astrocytes and ‘dark cells’ in layer 1 with age, using a ×40 objective lens drawings were made of 500 µm long strips of layer 1 to show the locations of the profiles of every neuron and neuroglial cell that contained a nucleus. For each monkey at least six separate drawings were made of randomly selected nuclei of both cell types from each monkey. The drawings were then scanned into a computer and the areas of the profiles of the nuclei measured employing the program NIH Image. From the measured areas, the mean diameters of the nuclear profiles were calculated.

Glial filament antibody labeling of astrocytes

To visualize the filament-containing processes of astrocytes in layer 1, an antibody to glial filament protein (GFAP) was used. Vibratomed sections 50 µm thick were taken from area 17 of four monkeys: AM 76 (5 years old), AM 47 (9 years old), AM 62 (27 years old) and AM 41 (32 years old). The sections were treated with 1% sodium borohydride and incubated overnight in a monoclonal mouse anti-human GFAP antibody (Dako). The binding sites were then visualized with a fluorescein (FITC) conjugated AffiniPure F(ab’)'2 fragment, goat anti-mouse IgG (Jackson Laboratories, Burlingame, CA) and the sections examined by confocal fluorescent microscopy.

Results

The following account of the morphology of the cells in layer 1 is equally applicable to both areas 17 and 46. The main difference between these two areas is that layer 1 in area 46 is almost twice as thick as layer 1 in area 17 (see Table 1).

Morphology of Cells in Layer 1 of Young Monkeys

In semithick plastic sections taken from layer 1 of young monkeys and stained with toluidine blue, one of the most common elements in the neuropil of layer 1 are the dendrites (Fig. 1). These are largely derived from the apical tufts of the pyramidal cells in layers 2/3 and 5, and they appear as pale profiles of various sizes. Scattered through the neuropil, profiles of myelinated axons are also evident.

There are few cells in layer 1, but of these the neurons are the largest and they can be recognized by their pale nuclei, which have mottled chromatin and usually contain a large nucleolus together with several smaller heterochromatic bodies. In addition,
Figure 1. A semithick section through layer 1 of area 17 in a young monkey (AM 10) to show the glial limiting membrane (g) and the various cell types. Neurons (N) occur throughout the layer and are sometimes located just beneath the glial limiting membrane (N₁). Neurons have large pale nuclei, usually with a folded nuclear envelope. The astrocytes (A) have smaller nuclei with smooth contours, which are readily distinguished from the darker nuclei (D) of microglia and oligodendrocytes. At the bottom of the micrograph are the pyramidal cells (N₂) of upper layer 2. Scale bar, 10 µm.

Figure 2. A semithick section through the glial limiting membrane of layer 1 in area 17 of a 9-year-old monkey (AM 47). In this example astrocytes (A) are common just beneath the thick glial limiting membrane (g). Scale bar, 10 µm.
the nuclear envelopes of the neurons are usually folded and surrounded by a pale perikaryon with smooth contours. Neurons can occur at any depth of layer 1, even just beneath the glial limiting membrane (Fig. 1, N).

The nuclei of astrocytes have smooth contours, and their nuclear envelopes rarely show folds (Fig. 1, A). Their nuclei stain slightly darker and more evenly than those of neurons and, in addition to the dark nucleolus, the chromatin usually has several condensations located just beneath the nuclear envelope. The cytoplasm of the astrocytes is very pale, often forms only a thin rim around the nucleus and may contain inclusions. These cells have irregular shapes and sometimes thick pale processes can be seen to emerge from the perikaryon. Astrocytes occur throughout layer 1 and their processes form the glial limiting membrane at the surface of the cortex. Interestingly, in some monkeys there are only a few astrocytic perikarya associated with the glial limiting membrane, while in others the cell bodies of astrocytes are regularly spaced just beneath it (Fig. 2).

Microglia and oligodendroglia can be recognized because their smaller nuclei are more darkly stained than those of neurons and astrocytes and they contain large clumps of heterochromatin. The microglia and oligodendroglia also have dark staining cytoplasm, but their profiles can be sufficiently alike that it is not always possible to distinguish between them in semithick sections. However, this is not a problem in thin sections, in which the features of these cells can be examined in more detail (Peters et al., 1991b).

**Morphology of Cells in Layer 1 of Old Monkeys**

As pointed out in earlier publications (Peters, 1991, 1999; Peters et al., 1991a), each of the cell types in layer 1 undergoes some alterations with age. In semithick sections most neurons appear to be unchanged beyond the appearance of some granules of lipofuscin in their cytoplasm. However, infrequent neuronal profiles appear to have a watery cytoplasm with irregularly dispersed organelles in their perikarya, while other rare neurons appear dense, suggesting that they have become pyknotic. Neurons displaying these kinds of changes are shown in Figures 3 and 4, which are from layer 1 in area 46 of a 27-year-old monkey. The neuron in Figure 3 has a nucleus that appears normal, but a broken-down perikaryal cytoplasm that contains few organelles, is watery at the periphery and has membranous inclusions. It is assumed that neurons with such features are dying, but by a process that is different from that shown by the pyknotic neuron in Figure 4. This neuron has become electron-dense and has an irregular outline, suggesting that it has become shrunken. In its dense cytoplasm the cisternae have become swollen and some of them contain membranous inclusions.

In young monkeys the glial limiting membrane is only a few astrocytic processes thick and, while the cytoplasm of these processes is filled with astrocytic filaments, the cell bodies and processes of astrocytes deeper in layer 1 have only a few thin bundles of filaments in their cytoplasm. In most old monkeys the glial limiting membrane is much thicker than in younger ones (Fig. 5), and it is formed from sheets of long, parallel astrocytic processes. The cell bodies of some astrocytes may be either em-bedded within the glial limiting membrane, or lie just beneath it. As in young monkeys, these astrocytic processes at the surface of the cortex are packed with filaments, but in contrast to young monkeys, the processes and cell bodies of astrocytes throughout layer 1 of old monkeys also contain prominent bundles of filaments (Figs 5 and 6). In addition, the cell bodies of astrocytes in old monkeys contain frequent cytoplasmic inclusions, which resemble the lipofuscin in neurons, in that the inclusions consist of a granular dense material with a pale component (Figs 5 and 6). It is presumed that these inclusions are derived from material that astrocytes have phagocytosed.

This increase in the frequency of filaments in the cell bodies and processes of astrocytes in layer 1 of old monkeys is readily evident in sections of cortex that have been labeled with antibodies to glial fibrillary acidic protein (GFAP; Fig. 7). In young monkeys there is labeling of the glial limiting membrane and of processes just beneath the glial surface, but deeper in the cortex only a few bundles of filaments are evident. In contrast, in the cortices of older monkeys, not only are the thickened glial limiting membrane and the processes just beneath it labeled, but there is intense labeling of many more astrocytic processes throughout the outer layers of the cortex. In addition, the cell bodies of many astrocytes in these outer cortical layers are now visible because of the thick bundles of filaments in their perikarya (Fig. 7, arrows).

The microglial cells are activated by the age changes that take place in layer 1, so that it is common to encounter microglial cells whose perikarya are filled with debris. Such debris-laden microglia are particularly evident either embedded in, or just beneath the glial limiting membrane (Fig. 8), but they can occur throughout the depth of layer 1.

The oligodendrocytes also respond to age-related changes in the sheaths of the myelinated axons that pass through layer 1. As in other layers of the cortex the myelin sheaths commonly split to accommodate dense cytoplasm or vesicles (Fig. 3, m), and some sheaths balloon out (Peters et al., 2000), while the oligodendrocytes come to contain dense inclusions, both in their perikarya and in swellings of their processes (Peters, 1996).

**The Frequency of Cell Types in Young and Old Monkeys**

To ascertain if there is a change in the frequency of occurrence of neurons, astrocytes and dark cells (microglia plus oligodendrocytes) in layer 1 with age, 1 μm thick sections stained with toluidine blue were used (see Fig. 1). A comparison was made between the number of profiles of cell bodies of each cell type that display nuclei in 500 μm long strips of layer 1 in young (5–12 years of age) and old (>25 years of age) monkeys. Both area 17 and area 46 were examined and the results are given in Table 1.

In comparing the cell counts obtained from areas 17 and 46, it is evident that there are about twice as many neurons and astrocytes in layer 1 of area 46 than in area 17. Presumably this is related to the fact that layer 1 in area 46 is about twice as thick as that in area 17 (Table 1). But, interestingly, the frequencies of ‘dark cells’ in areas 46 and 17 are similar.

Within area 17 and within area 46, there is not a great deal of

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**Figure 3.** A neuron (N) in layer 1 of area 46 in a 27-year-old monkey (AM 12). This neuron appears to be degenerating since it has a watery cytoplasm and at the periphery of the perikaryon there are membranous inclusions (arrows). In the neuropil, note the dendrites (D) with membranous inclusions and the degenerating myelin sheath (m). Scale bar, 2 μm.

**Figure 4.** A degenerating, pyknotic neuron. The nucleus and perikaryon of the neuron are electron-dense. The cisternae of the endoplasmic reticulum and the nuclear envelope are swollen (asterisks). Also note the membranous inclusions (arrows) at the periphery of the cell body. Above the cell body are processes of astrocytes that are filled with filaments (f). From area 46 of a 27-year-old monkey (AM 12). Scale bar, 2 μm.
variation in the numbers of profiles of neurons and of dark glial cells among individual monkeys, but there are large variations in the numbers of profiles of astrocytic cell bodies. In general, the highest numbers of astrocytic cell body profiles occur in layer 1 of those monkeys with a large number of these cells just beneath the glial limiting membrane (Fig. 2). However, it is evident from the data shown in Table 1 that there are no significant differences in the mean numbers of profiles of either neurons, astrocytes, or dark cells in layer 1 between young and old monkeys. There are also no differences in the mean sizes of the nuclear profiles of neurons and astrocytes in layer 1 of young and old monkeys, in either area 17 or area 46. Consequently, our data indicate that with age there are no significant losses of neurons from layer 1 and no significant increases in the numbers of astrocytes.

**Discussion**

The necessity to differentiate between cell types, and especially between neurons and astrocytes in layer 1, meant that this analysis had to be carried out using semithick sections, so that it was not practical to carry out a formal stereological study. Nevertheless, the data indicate that there are no significant changes in the numbers of neurons and neuroglia in layer 1 with age, in either area 17 or area 46. In the only other study of the effects of age on the neuroglial cells of the cerebral cortex of the rhesus monkey, Peters et al. (Peters et al., 1991a) report that throughout the entire depth of area 17 there are no increases in the numbers of astrocytes and oligodendrocytes, but a 44% increase in the number of microglial cells, which account for ~7.6% of the total population of neuroglial cells in young monkeys and 9.4% in old monkeys. In a study of the auditory cortex of the rat, Vaughan

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**Figure 5.** Glial limiting membrane from area 46 of a 27-year-old monkey (AM 12). In old monkeys the glial limiting membrane is formed from several layers of astrocytic processes that contain thick bundles of filaments (f). On the undersurface of the glial limiting membrane is the cell body of an astrocyte (As) with part of its nucleus (N). The cell body also contains filaments as well as several inclusions bodies (I). Scale bar, 2 μm.

**Figure 6.** An astrocyte (As) in layer 1 of a 27-year-old monkey (AM 12). In old monkeys both the cell bodies and processes of astrocytes contain thick bundles of filaments (f) and the cell bodies usually contain several dense inclusions (I). Note the dendrites (D) with membranous inclusions in the surrounding neuropil. Scale bar, 2 μm.

**Figure 7.** Sections of area 17 from a 9-year-old monkey (AM 47) on the left and from a 32-year-old monkey (AM 41) on the right. The sections have been reacted with an antibody to GFAP in order to visualize the astrocytic filaments. The numbers between the micrographs show the locations of the cortical layers. In these confocal, fluorescent-labeled images the antibody shows the thickening of the glial limiting membrane (g) with age and the remarkable age-related increase in astrocytic filaments, with the result that many more processes and cell bodies (arrows) of astrocytes are visible in the outer cortical layers of old monkeys. Scale bar, 100 μm.
and Peters (Vaughan and Peters, 1974) also report little change in the frequency of astrocytes and oligodendrocytes, but a 65% increase in the number of microglia with age. However, Long et al. (Long et al., 1998) found no change in the numbers of astrocytes and microglia in the hippocampus of the normally aging mouse, while Pilegaard and Ladefoged (Pilegaard and Ladefoged, 1996) reported only a slight age-related increase in the total numbers of astrocytes in the molecular layer of the dentate gyrus of rats. In another study of layer 1, Peinado et al. (Peinado et al., 1993) found a decrease in the neuronal density in the frontal cortex of rats with age, but in a more recent study of the effects of age on rat parietal cortex they found no change in the neuronal density in any layer (Peinado et al., 1997). Overall, then, it would seem that with age there are only small changes in the numbers of neurons, astrocytes and oligodendrocytes in the cortex, although there may be increases in the numbers of microglia.

However, with age each of the cell types undergoes some morphological alterations. In the case of the neurons, this involves the formation of lipofuscin in the cell body and degenerative changes in a small number of neurons. But even though we have found a few examples of dying neurons, any loss of layer 1 neurons as a result of degeneration cannot be extensive, as indicated by the fact that there is no significant change in the numbers of neuronal profiles in either area 17 or area 46 with age (see Table 1). Indeed, the existing evidence suggests that neurons are not lost in significant numbers from any portion of the cerebral cortex of primates (Leuba and Kräftrik, 1994; Morrison and Hof, 1997; Peters et al., 1998a) and O’Donnell et al. (O’Donnell et al., 1999) have pointed out that the volume of area 46 is totally preserved in aged monkeys, while Peters et al. (Peters et al., 1997) have shown that there is no change in the volume of area 17 with age in rhesus monkeys.

Although the numbers of astrocytes in layer 1 do not appear to increase with age, those contributing to the glial limiting membrane hypertrophy. This is evidenced by the marked thickening of the glial limiting membrane, which becomes thicker because of an increase in the numbers of processes of astrocytes that contribute to it. This thickening may occur in response to the loss of dendrites from the apical dendritic tufts of pyramidal cells and the consequent loss of synapses. There is also an obvious increase in the numbers of intermediate filaments in the cell bodies and processes of astrocytes throughout layer 1. This can be seen in both electron micrographs and in material labeled with antibody to GFAP, since the latter preparations show a marked increase in the thickness and frequency of staining of filament bundles in the long astrocytic processes that extend from layers 1 and 2 into the upper half of the cortex. This strong labeling of astrocytes with GFAP antibody has been remarked upon earlier by Hanson et al. (Hanson et al., 1987), who examined the labeling in human cerebral cortex and commented upon the fact that the numbers of labeled astrocytes in layer 1 vary widely among individuals, but show no correlation with advancing age. Other studies have recorded an overall increase in GFAP with age, both in terms of the increase in the intensity of labeling of GFAP with antibodies (O’Callaghan and Miller,
As well as the marked increase in the content of intermediate filaments, the perikarya of astrocytes come to contain inclusions. Inclusions are also usually present within microglia in layer 1 of old monkeys and presumably they are derived from materials that have been phagocytosed by the astrocytes and microglia. The source of these phagocytosed materials cannot be determined from the morphology of the inclusions. However, degenerating dendrites may be one source of these inclusions, since it is known from our earlier studies that some dendrites from the apical tufts of pyramidal cells in both area 46 (Peters et al., 1998b) and area 17 (Peters et al., 2001) are lost with age.

Sheffield and Berman (Sheffield and Berman, 1998) have shown that in the brains of Macaca nemestrina the microglial expression of major histocompatibility complex (MHC) class II antigens increases with age, indicating that these cells are more activated. However, the effect is more pronounced in white matter, in which Sheffield and Berman suggest the microglial cells may influence myelin loss with age (Sloane et al., 1999). Nevertheless, most of the microglial cells in layer 1 accumulate phagocytosed material as they age, and this is particularly evident in microglial cells associated with the glial limiting membrane.

Whether myelinated axons are lost from layer 1 with age is not known, but it is clear that many of the myelin sheaths in the axonal plexus of layer 1 undergo age-related changes of the kinds that occur elsewhere (Feldman and Peters, 1998; Peters et al., 2000). Also, the oligodendrocytes in old monkeys show accumulations of dense bodies in both their perikarya and in swellings of their processes, as they do in other cortical layers (Peters, 1996). At present, the nature and source of these inclusions is not known.

In summary, age has little affect on the frequency of cells in layer 1. However, each cell type is affected by age and some of the intrinsic neurons may degenerate. All of the cell types accumulate some inclusions in their cell bodies. In the case of the neurons and the oligodendrocytes it is essentially age pigment, but with astrocytes and microglial cells the inclusions are formed through phagocytosis. There is also some hypertrophy of the astrocytes, so that the glial limiting membrane becomes thickened, perhaps forming a scar in response to the age-related loss of some dendrites and synapses from layer 1 and, in addition, the proliferates and cell bodies of astrocytes throughout layer 1 come to contain increased amounts of fibrillary protein.

In terms of what effect aging has on the functioning of layer 1, its inputs need to be considered. The inputs come from a wide variety of sources. These include: backward projecting axons; the axons of the local intrinsic neurons; axons of pyramidal cells that ascend into layer 1; subpopulations of neurons in both specific and unspecific thalamic nuclei; and cholinergic and monaminergic inputs from brain stem nuclei (Vogt, 1991; Cauller and Connors, 1994). Cauller and Connors have shown that in the somatosensory cortex of the rat the horizontally projecting glutaminergic axons of layer 1 give a powerful excitation to pyramidal cells with apical tufts in layer 1. Since aging results in a pruning of the apical tufts of pyramidal cells and a loss of synapses (Peters et al., 1998b, 2001), inputs to the apical dendritic tufts will be reduced, so that this excitation of pyramidal cells through their apical dendritic tufts can be expected to be diminished with age. But so far, this has not been examined and there is no information available about whether only some, or all of the axonal types entering layer 1 are affected by the normal aging process.


