The effect of age on layer 1 of area 46 of prefrontal cortex was determined in the cerebral cortices of 15 rhesus monkeys, 13 of which had been behaviorally tested. Five of the monkeys were young (5–7 years of age), three were middle-aged (9–12 years) and seven were old (24–32 years). It was found that with age, layer 1 becomes significantly thinner and the glial limiting membrane becomes thicker. Counts of synapses in layer 1 of seven of these monkeys using the physical disector method on thin sections revealed that compared to young monkeys, there is a 30–60% reduction in the density of synapses per unit volume in old monkeys. This loss of synapses is accompanied by a reduction in the frequency of profiles of postsynaptic dendrites and their spines from the neuropil of layer 1, indicating that some spiny dendrites that belong to the apical dendritic tufts of pyramidal cells are degenerating and being lost with age. Correlation of these morphological changes with the behavioral data shows that there is a significant correlation between the thickness of layer 1 and memory function, as measured by the 2 min delay condition of the delayed non-matching to sample task. Also, there is significant correlation between the reduced number of synapses in layer 1 and three of the behavioral measures used, as well as the Cognitive Impairment Index. Thus, the changes that occur with age in layer 1 provide one possible basis for the age-related cognitive impairment evidenced in monkeys and humans alike.

Introduction
In our previous studies of the effect of age on the primary visual cortex (Vincent et al., 1989) and area 46 of prefrontal (Peters et al., 1994) cortex, only layer 1 was found to be substantially affected by age. This paper will provide an account of some of the alterations that occur with age in layer 1 of area 46 of prefrontal cortex, and it will be shown that some of these alterations can be correlated with the cognitive decline displayed by aging rhesus monkeys (see Peters et al., 1996). Aged monkeys as a group are impaired relative to young adults in spatial and reversal learning tasks, as well as in recognition memory tasks, and it is considered that in part, at least, this behavior is subserved by prefrontal cortex (Fuster, 1997; Kojima and Goldman-Rakic, 1982; Moss and Rosene, 1992).

All neocortical areas have an outer, cell-sparse layer, which is designated layer 1, or the molecular layer. This layer is derived from the primitive plexiform layer (see Marin-Padilla, 1971), which during development becomes split by the invading pyramidal cells of the cortical plate into two sublayers. These will become the outer, cell sparse layer 1, and layer 6b, which is represented by a layer of non-pyramidal cells that extend into the subcortical white matter (see Marin-Padilla, 1984).

The primitive neurons of layer 1 are the Cajal–Retzius cells. The fate of these neurons is not clear. Some authors (e.g. Bradford et al., 1977) consider that the Cajal–Retzius cells largely disappear during the course of maturation of the cortex (see also Hestrin and Armstrong, 1996; Zhou and Hablitz, 1996), but they may become sparse because of the expansion of the cortical mantle as the hemispheres enlarge (Marin-Padilla, 1990), or, as Parnavelas and Edmunds (1983) suggest, they may transform into other types of non-pyramidal cells. Zhou and Hablitz (1996) have shown that layer 1 does not just contain Cajal–Retzius cells. Other kinds of non-pyramidal cells are present early in postnatal development and, in addition, some small neurons are added to layer 1 during the course of later development (Marin-Padilla, 1984). The result is that in the mature brain, layer 1 contains a diverse population of neurons, and it is clear that most of them are inhibitory in function, for in monkey visual cortex at least 85% of the layer 1 neurons can be labeled by antibodies to GABA (Hendry et al., 1987; Beaulieu et al., 1992).

While the dendrites of these intrinsic neurons contribute to the neuropil of layer 1, the large majority of the dendrites belong to the apical dendritic tufts of pyramidal cells with perikarya in deeper layers of the cortex. Their apical dendrites branch profusely to form splays of thin and spiny branches (e.g. Marin-Padilla, 1984; Martin and Whitteridge, 1984) that seem to end just beneath the external glial limiting membrane. However, other dendrites in layer 1 are smooth (see Vaughan and Peters, 1973); these probably belong to both the intrinsic neurons of layer 1, and to neurons such as the non-pyramidal cells in layer 2 that label with antibodies to calbindin and calretinin and send their beaded dendrites into layer 1 (e.g. Gabbott and Bacon, 1996).

These dendrites in layer 1 form synapses with the very rich plexus of unmyelinated axons. The origins of these axons have not been fully investigated, but it is known that excitatory inputs are derived from such sources as the recurrent collaterals of pyramidal cells in deeper layers (e.g. Martin and Whitteridge, 1984; Blasdel et al., 1985); callosal afferents that terminate in the deeper half of the layer (e.g. Jones and Powell, 1970); thalamic afferents (e.g. Herkenham, 1986; Avendaño et al., 1990; Rausell et al., 1992); and feedback connections such as those that are derived from area 18 and terminate in layer 1 of primary visual cortex (see Rockland, 1994). Other inputs to layer 1 arise from the axons of the layer 1 neurons, and from such diverse sources as the basal nucleus of Meynert that provides a cholinergic input forming symmetric synapses (e.g. Mesulam et al., 1984; Campbell et al., 1987; Lewis 1991); from the substantia nigra that provides a dopaminergic input forming symmetric synapses (e.g. Levitt et al., 1984; Goldman-Rakic et al., 1989; Williams and Goldman-Rakic, 1993); and from the raphe nuclei that provide a serotonergic input forming asymmetric synapses (e.g. Campbell et al., 1987; Seguela et al., 1989).

Layer 1 clearly has a very complex composition, and to assess how it is affected by aging, the cortices of young monkeys have been compared with those of old monkeys. Our data on the lifespan of the rhesus monkey (Tigges et al., 1988) indicate that the maximum lifespan of the rhesus monkey is ~35 years, that...
only ~25% of them survive to 25 years of age, and only some 6% attain an age >50 years. Consequently, monkeys that are >25 years of age can be considered to be old, and comparing their cortices with those from monkeys 5–7 years of age, and some of intermediate ages (9–12 years old), should reveal the effects of aging.

Materials and Methods
The ages of the 15 rhesus monkeys (Macaca mulatta) used in this study are given in Table 1. For 14 of the monkeys the exact ages are known, but for one of them (AM 26) the age has been estimated (est). In subsequent tables and throughout the text all of the ages will be given to the nearest year, as in our previous study in which the cortices of some of these same monkeys were used to determine the effects of age on the neurons and neuroglial cells in area 46 (Peters et al., 1994).

Fixation
The details of the fixation of the brains of the monkeys used in this study are given in Peters et al. (1994). Briefly, the monkeys were anesthetized and their brains fixed by intravascular perfusion through the ascending aorta with a warm fixing solution, containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate or 0.1 M phosphate buffer at pH 7.4. Immediately after perfusion the brains were removed and bisected. One half, which was to be used for this study, was immersed in a stronger fixing solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in the same strength buffer. These half brains remained in this solution, at 4°C, for a minimum of a week. Pieces of cortex were then removed from the floor of the principal sulcus at the level of the rostral end of the corpus callosum. This latter portion of prefrontal cortex is part of area 46 according to the designation given by Walker (1940), and it was chosen because it has a relatively even thickness (Peters et al., 1994). The pieces of area 46 were then osmicated, dehydrated in an ascending series of alcohols, stained en bloc with uranyl acetate and embedded in Araldite.

Two blocks of embedded cortex from area 46 of each monkey were sectioned in a plane vertical to the pial surface. The tissue blocks were adjusted on the microtome and carefully oriented until the plane of section passed parallel to the long axes of the apical dendrites of the pyramidal cells. When this orientation had been achieved a series of 1 µm thick sections extending through the entire thickness of the cortex were taken from each block and stained with toluidine blue. These semithick sections were used to determine the thickness of layer 1.

Thickness of Layer 1
The thickness of layer 1 was determined from camera lucida drawings of the semithick plastic sections. The drawings were made using an Olympus light microscope with a ×20 objective, to produce drawings with a magnification of ×250. For each monkey two sections spaced at least 20 µm apart were drawn from each of the two tissue blocks, to give a total of four drawings. Each drawing was of a 250 µm long strip of layer 1, whose thickness was taken to extend from the outside of the glial limiting membrane to the tops of the uppermost layer 2 pyramidal cells. The drawings were then scanned into a Power Macintosh computer, and NIH Image version 1.55 was used to determine the area of layer 1 included in the drawing. From the area, the thickness of layer 1 in each drawing was determined. The average thickness of layer 1 in area 46 for each monkey was then calculated from the measurements taken from the four drawings.

Some of these tissue blocks were thin sectioned for a general electron microscopic analysis of layer 1.

Neuropil Assessment and Synapse Types
To determine if there is a change in the neuropil and in the synaptic density in layer 1 with age, layer 1 of two young monkeys (AM 16, AM 76), one intermediate aged monkey (AM 47) and four old monkeys (AM 12, AM 62, AM 27 and AM 41) was examined by electron microscopy (Table 1 gives the ages of the individual animals). These animals were chosen for the quantitative analysis because their cortices were well fixed.

One block of cortex from the lower bank of the principal sulcus was selected from each of the seven monkeys, trimmed so that it included layers 1–3, and thin sectioned. The sections were mounted on formvar-coated slot grids (1 × 2 mm), stained with uranyl acetate and lead citrate, and a montage of electron micrographs passing through the entire depth of layer 1 was taken at a magnification of ×7500 and used to examine the distribution of dendrites and dendritic spines in layer 1.

An additional series of electron micrographs at one-third and two-thirds of the thickness through layer 1 were taken at a primary magnification of ×6000. These were taken to evaluate the frequency of synapses in outer and inner halves of the layer with respect to whether they were symmetric or asymmetric and whether the postsynaptic elements were dendritic shafts or spines, which can be readily distinguished from each other of their basis of their structure (Peters et al., 1991; Peters and Palay, 1996).

Densities of Synapses with Age
Serial thin sections were then taken from the same tissue blocks and sets of two or three serial sections, in a ribbon, were mounted on single-slot (1 × 2 mm), formvar-coated grids. These sections were stained with uranyl acetate and lead citrate, and used to determine synaptic densities by using the physical disector (e.g. Sterio, 1984; Calverley et al. 1988; Geinisman et al., 1996; Mayhew, 1996; Tiggges et al., 1996). Since the tissue blocks were all taken from the lower bank of the principal sulcus, the sampling used is selective and may not be representative of all of area 46.

Electron micrographs were taken of the identical part of layer 1 as it appeared in two or three adjacent sections. The micrographs were taken at ×5000 magnification and printed to a final magnification of ×12 500. This magnification was determined to be sufficient to recognize synapses in micrographs that are in focus. Initially 10 pairs of electron micrographs were taken at one-third and two-thirds of the way through the thickness of layer 1 from each monkey, to determine if there is a difference in synaptic density in the inner and outer halves of the depth through layer 1. These depths were determined by ascertaining the thickness of layer 1 from the glial limiting membrane to the level of the tops of the uppermost neuronal cell bodies of layer 2. In taking the micrographs of the synapse-containing neuropil, cell bodies of neurons and neuroglial cells, as well as blood vessels were avoided. Micrographs were also taken of folds present in the section, so that the thicknesses of the sections in the pair could be determined using the minimal fold method proposed by Small (1968), in which the width of the narrowest folds is equivalent the twice the thickness of the section (Calverley et al., 1988; de Groot, 1988; Hunter and Stewart, 1989). This method provides a reasonably accurate estimate of the average thickness of the section, as shown by Tiggges et al. (1996), who used the minimal fold method to determine the thickness of some sections and then embedded those same sections so that they could be resectioned perpendicular to the original plane of sectioning to measure their thickness.

The pairs of electron micrographs that had been taken from consecutive thin sections were then marked with disector frames that encompassed identical fields. For the most part the overlap between the images in the pairs of micrographs was good, and the areas of overlap were usually of the order of 250–300 µm². The pairs of micrographs were then examined in detail; those synapses which displayed profiles of their junctions in both micrographs were marked in one color, and those synapses which had a profile of the synaptic junction in one micrograph and not the other were marked in a different color. In the cases of synapses sectioned parallel to their junctions, the presence of a synapse was marked only if the section passed through the junction itself and not through the presynaptic grid, which is in the presynaptic element (Peters et al., 1991; Peters and Palay, 1996). Having marked the synapses, in turn each micrograph in the pair was used as the reference and the look-up member of the pair, and the total number of synaptic profiles (Q) within this micrograph frame was counted as well as the number of profiles with profiles in only the reference micrograph and not the look-up micrograph. For each micrograph the number of synapses per unit volume (Nv) was then calculated using the formula Nv = (Q/A) × T, where A is the area of the disector frame and T is the thickness of the section as determined by the minimal fold method. Since the images in serial thin sections are dependent upon each other, the mean Nv was determined for each pair of micrographs, and from these means the overall mean and coefficient of error was calculated. For each level in layer 1 of each monkey a sufficient number of pairs of micrographs was examined to
The individual behavioral Z scores shown in this table are based on a population of young adult and aged monkeys (see Hendon et al., 1997). The higher the Z score, the worse the performance of the monkey.

Cognitive Impairment Index (CII) is expressed as the number of standard deviation units from the mean performance of a cohort of young adult rhesus monkeys (Hendon et al., 1997).

Results

The Morphology of Layer 1

Even in young animals, it is difficult to obtain good fixation of layer 1, because although other components of the neuropil appear to be well fixed, dendrites are frequently swollen. This is especially true of those in the outer portion of layer 1 in which the dendrites frequently have enlarged profiles with pale cytoplasm and dispersed organelles. This swelling of the dendrites is curious, because it can occur even though there is no obvious swelling of the astrocytes and their processes, and the presence of swollen astrocytes in the neuropil is usually regarded as the best indicator that the fixation is poor (see Peters et al., 1991). By a careful selection of material it is possible to obtain specimens in which the dendrites in layer 1 in young monkeys are not swollen excessively, but in a study of the present type, in which each monkey is a crucial member of a series, such selection is not possible or desirable. Indeed, as will become apparent, it turns out that much of the dendritic swelling is a consequence of aging.

Young Monkeys

In young monkeys the most prominent profiles in layer 1 are those of dendrites belonging to the apical tufts of pyramidal cells (Fig. 1). In the bottom of layer 1, where the main trunks of the apical tufts are present, some of these dendritic profiles have diameters of as much as 3 μm and are frequently sectioned along or oblique to their long axes. But toward the outer half of layer 1 the overall sizes of the pyramidal cell dendritic profiles become smaller; most of them are 1–1.5 μm thick and tend to be sectioned more or less transversely. As Golgi-impregnated material shows, this is because as they ascend, the dendrites in layer 1 are so big that for individual dendrites it is not possible to see their apical tufts. However, it is possible to see the terminal branches of the apical tufts (Fig. 1). When they are not swollen the profiles of the apical dendrites of the pyramidal cells have irregular outlines because of the spines that extend from them, and they contain an array of microtubules, as well as mitochondria and a few small profiles of granular endoplasmic reticulum. However, as pointed out above, even in young animals a number of the terminal branches of the apical tufts show a degree of swelling and a concomitant disruption of their microtubules (di in Fig. 1), and some of the dendritic profiles contain irregular vacuoles (ds in Fig. 1).

As well as the apical dendrites of pyramidal cells, layer 1 also contains smooth dendrites. Such dendrites lack spines and...
Figure 1. In young monkeys the neuropil of layer 1 contains dendrites (d) that give rise to spines (sp), and both of these components synapse with axon terminals (at). The neuropil also contains numerous unmyelinated axons (ax) as well as the irregular processes of astrocytes (As). Even in young monkeys some of the dendrites (d1) have vacuoles in their cytoplasm. From AM 16; 5 years of age. ×11 000.

Figure 2. In young monkeys the glial limiting membrane (G) is thin and is usually formed by only one or two layers of astrocytic processes (As). Above the glial limiting membrane is the pia mater (P). The profiles of both dendrites (d) and of unmyelinated axons (ax) are obvious in the neuropil of layer 1 beneath the glial limiting membrane. AM 46; 5 years of age. ×7500.
Figure 3. In the neuropil of layer 1 in old monkeys the profiles of dendrites (d) and of spines (sp) are less frequent than in young monkeys, and many of the dendrites contain membranous whorls (w). Also, in the neuropil the processes of astrocytes (As) are generally thicker and contain filaments. AM 27, 28 years of age. ×11 000.

Figure 4. In older monkeys the glial limiting membrane (G) is much thicker than in young monkeys, and on its outside projections (arrows) protrude into the subpial space. In the field are the nuclei (nuc) of two astrocytes, and the lower cell body can be seen to give rise to thick processes (p) filled with filaments. Note the profiles of dendrites that contain membranous whorls. AM 27, 28 years of age. ×6000.
Figure 5. This micrograph shows the thickened glial limiting membrane (G) in an old monkey, in which the astrocytic processes are filled with filaments. Beneath the glial limiting membrane is a neuron (N) that has an irregular outline and has vacuoles and membranous whorls (arrows) in its cytoplasm. AM 27; 28 years of age. ×7500.

Figure 6. A dark neuron in the neuropil of an old monkey. The nucleus (nuc) and the cytoplasm (cyt) are much darker than is normal. The nuclear envelope (arrows) and the cisternae of the endoplasmic reticulum are swollen and vacuoles (v) are present in the cytoplasm, suggesting that the neuron is dying. AM 12; 27 years of age. ×10000.
membrane, have thickenings similar to hemidesmosomes (Peters et al., 1991). These axons give rise to vesicle-filled terminals (at in Fig. 1) that form synapses with the spines (sp in Fig. 1) extending from the pyramidal cells dendrites and with the shafts of both these dendrites and the smooth ones. A few myelinated axons are also dispersed throughout layer 1, but they are most frequent in its deeper half, sometimes designated as layer 1B.

The only other common component of the neuropil are the processes of astrocytes (As in Fig. 1). The profiles of the thicker processes can be identified by the bundles of intermediate filaments in their cytoplasm, together with glycogen particles. However, most of the profiles of astrocytic processes appear as thin, irregular sheets that often lie next to dendritic spines and synapses. Some of these processes arise from the astrocytes that occur throughout layer 1, but others extend down from astrocytes with cell bodies within, or just beneath the glial limiting membrane on the outside of the cortex. The glial limiting membrane is formed by astrocytes whose processes overlap and interlock with each other to produce a continuous sheet that is usually between one and three processes thick (Fig. 2). Unlike those in the neuropil, the cytoplasm of the astrocytic processes forming the glial limiting membrane is filled with intermediate filaments, and the plasma membranes on their outer faces, adjacent to the basal lamina the glial limiting membrane, have thickening similar to hemidesmosomes (Peters et al., 1991).

As pointed out, there are few neuronal cell bodies in layer 1 and the profiles of the intrinsic neurons that have been encountered have nuclei with a slightly folded envelope and a cytoplasm in which the Nissl substance is not very prominent. Their cell bodies have few synapses and the dendrites have smooth contours.

Aged Monkeys
In old monkeys the frequency of dendritic profiles in layer 1 is less than in the young monkeys, and a greater proportion of the profiles are swollen (Fig. 3). It is also common for the cytoplasm of the dendrites to be almost devoid of organelles, and for more of them to contain vacuoles, as well as membranous whorls of the type first described by Feldman (1976). These membranous whorls (w in Fig. 3), which are taken to be an indication of degeneration, are composed of several layers of loosely wrapped, irregular membranes. They appear to be most common in dendrites in the outer portion of layer 1 and to become somewhat less frequent with depth. Not only do the pyramidal cells dendrites contain these inclusions, but they also occur in the bulbous enlargements of the smooth dendrites.

Despite these alterations in the dendrites, morphologically the unmyelinated axons and the axon terminals in layer 1 show no obvious effects of age. The same is true of the myelinated axons, but it is common for their sheaths to show splits between successive turns of the lamellae and for the sheaths to have blebs protruding from them. Moreover the splits in the sheaths sometimes contain a dark, vacuolated cytoplasm, so that it would appear that the sheaths are breaking down.

The other obvious change is in the astrocytes. In the older animals the astrocytic cell bodies usually contain inclusions, and their processes in the neuropil are more frequent and prominent, and more of them contain bundles of filaments (As in Fig. 3). This proliferation of astrocytic processes is especially evident in the glial limiting membrane, which is much thicker in the older monkeys and consists of many more layers of astrocytic processes (Fig. 4). In addition, the outer face of the glial limiting membrane is more irregular in older monkeys, since protrusions stick out into the space below the pia mater (Peters, 1991), and thick strands of astrocytic processes extend down into the underlying neuropil. Furthermore, microglial cells containing inclusions are often associated with, and are sometimes embedded within, the thickened glial limiting membrane.

A number of the neuronal cell bodies encountered in layer 1 appear to be affected by age. The cytoplasm of some of the neurons contains vacuoles (Fig. 5), and other neurons even appear to be dying, since their cytoplasm and nucleoplasm is much darker than normal and the cells are shrunken. Such a neuron is shown in Figure 6. Its nucleoplasm is dark and granular, the nuclear envelope is swollen, while in the dark cytoplasm there are vacuoles and the cisternae of the rough endoplasmic reticulum are swollen.

Thickness of Layer 1
The measurements of the thickness of layer 1 were made in the 15 monkeys and the results are given in Table 1. This group consists of five young (5–7 years of age), three intermediate (9–12 years of age) and seven old (25–35 years of age) monkeys.

The thickness of layer 1 varies from 0.21 to 0.16 mm, and, as shown in Figure 7, with age its thickness decreases significantly ($P < 0.01$). As will be discussed later, the decrease in the thickness of layer 1 correlates not only with age, but with some of the cognitive deficits displayed by those monkeys.

Density of Synapses
To ascertain if there is a loss of synapses from layer 1 with age, the frequency of synapses in layer 1 was determined in two

**Figure 7.** A plot of the thickness of layer 1 in area 46 against the age of the monkey.
young, one intermediate and four old monkeys. And to ascertain if there is any difference in synaptic density with depth, determinations were made at two levels, at one-third and two-thirds of the depth through layer 1. The results are presented in Table 2. In AM 16, for example, at one-third of the depth through layer 1 the total number of synaptic profiles present in one section and not the other of the pairs of micrographs analyzed, i.e. Q−, was 146, and the total area containing these synapses was 4512 µm². Taking into account the thickness of the thin sections examined, as determined using the minimal fold method, this leads to a estimated $N_v$ of 5.82 × 10⁸ (SEM 0.39) synapses/µm³. In this monkey, and in the other young monkey (AM76), $N_v$ has a similar value at both one-third and two-thirds of the depth through the layer 1, suggesting that there is little difference in the synaptic density through layer 1. However, for the 9 year old monkey, AM 47, the synaptic density at one-third of the way through the depth of layer 1 is lower than at the two-thirds depth. Perhaps this indicates the beginning of a synaptic loss that is evident in the four old monkeys the synaptic density is only 40–70% of the value at both one-third and two-thirds of the depth through the layer 1, suggesting that there is little difference in the synaptic density through layer 1. However, for the 9 year old monkey, AM 47, the synaptic density at one-third of the way through the depth of layer 1 is lower than at the two-thirds depth. Perhaps this indicates the beginning of a synaptic loss that is evident in the four old monkeys, in which there is also no change with age in the overall distribution of synapses. Consequently, the loss with age seems to affect all types of synapses equally.

**Distribution of Synapses**

To ascertain if there is a change in the distribution of synapses with age, the neuropil was examined in electron micrographs of layer 1 taken from the same monkeys in which the densities of synapses were determined. The analysis of the synapses was done at at one-third and two-thirds of the way through the depth of layer 1 in micrographs at a final magnification of ×15 000. This was sufficient magnification to characterize those profiles of synapses displaying enough of their pre- and postsynaptic elements and their synaptic density, as being either asymmetric or symmetric and axospinous or axodendritic. For each monkey and for each of the two levels of layer 1 a sufficient number of micrographs was examined so that for each analysis 100 profiles of synapses were characterized. Axosomatic synapses were not taken into account since cell bodies are sparse and they have few synapses. The results of this analysis are given in Table 3.

In both the young and old monkeys there appears to be little difference in the frequency with which the profiles of the four different types of synapses occur at the two depths in layer 1, and there is no obvious change with age in the proportion of the synaptic profiles that are axospinous. In both the young and the old monkeys, between 69 and 72% of the synaptic profiles are axospinous: between 57 and 62% of them are asymmetric, and between 9 and 11% are symmetric. In both young and old monkeys the percentage of profiles of synapses involving dendritic shafts is between 28 and 32% of the total, and even though there are some individual differences there is no obvious trend to suggest that with age there is a change in the proportion of profiles of symmetric to asymmetric axodendritic synapses.

The results of this analysis suggest that although there is a decrease in the numerical density of synapses with age there is no change in the distribution of the different types of synapses involving dendrites and their spines. Consequently, the loss seems to affect all types of synapses equally.

**The Effect of Age on Dendrites**

Since there is no change with age in the overall distribution of profiles of synapses involving dendritic shafts and spines, even though there is a reduction in the density of synapses, it seems probable that with age there is a general loss of postsynaptic sites, namely dendrites and their spines. The problem is how to quantify such a change in these monkeys in which there is a variable amount of swelling of dendrites among specimens. If there is a loss of dendritic branches and spines with age, it could...
normally be detected by carrying out a volume fraction analysis. However, such an analysis would not generate meaningful data in this material, because in specimens in which the dendrites are swollen the dendritic profiles occupy an artifactually large amount of space. It was therefore decided to carry out an analysis by visualizing the distribution of the profiles of dendrites and spines in electron micrographic montages of strips of layer 1 passing from the pial surface towards the tops of the pyramidal cells of layer 2, in each of the seven monkeys in which synaptic densities and synaptic distributions were determined. The montages were taken at a primary magnification of ×3000 and printed at ×7500. Xerox copies were then made of the photographic prints, and the profiles of dendrites and of dendritic spines were colored. The colored Xerox copies were then glued together in a strip.

In these colored images it is obvious that dendritic and spine profiles are less frequent in the neuropil of the four old monkeys than in that of the three young ones. An example of this is shown in Figure 8, which shows tracings of the disposasions of both dendritic profiles (open outlines) and of dendritic spine profiles (black) in the neuropil of a young monkey (AM 76) compared with that from an old monkey (AM 27). These two monkeys were chosen since their dendrites showed the least amount of swelling.

To put this data into a more quantitative frame, determinations have been made of the number of profiles of dendrites and of spines per 1000 µm² at four successive 40 µm segments of depth through layer 1, beginning at the pial surface and extending towards the top of layer 2. The results are plotted in Figures 9 and 10.

In Figure 9 it can be seen that the number of dendritic profiles per 1000 µm² of neuropil in the first and second 40 µm segments of depth is greater in the three younger monkeys than in the four old monkeys. With increasing depth, the number of profiles in the young and old monkeys become more similar to each other, and the frequency of dendritic profiles in each monkey becomes less because the apical dendritic tufts have fewer branches as the parent apical dendrites are approached.

The obvious interpretation to be put onto these data on profile frequency is that with age, dendritic branches are lost from the apical tufts of the pyramidal cells, and this loss is most severe in the terminal portions of the tufts, in the outer part of layer 1. The loss may be even more severe than the data suggest, because the swollen dendrites in the older monkeys would generate more profiles than those of young monkeys. The loss of profiles is less marked with increasing depth. This interpretation fits with the general appearance of the dendrites in electron micrographs, in which the dendrites in the outer portion of layer 1

Figure 8. A tracing of the disposition of dendrites (open profiles) and of dendritic spines (black) in the outer portion of the neuropil of layer 1 in a young monkey (AM 76) and an old monkey (AM27) taken from montages of electron micrographs. The horizontal tracing at the top is the outer face of the glial limiting membrane. It is obvious that the neuropil of the young monkey contains more dendrites and spines than that of the old monkey.

Figure 9. The frequency of the profiles of dendrites as a function of depth, as visualized in a montage of electron micrographs extending through the depth of layer 1. The number of profiles of dendrites per 1000 µm² of neuropil was determined at successive 40 µm depths, so that the outer surface of layer 1 is represented at the left.

Figure 10. The frequency of the profiles of dendritic spines as a function of depth, as visualized in a montage of electron micrographs extending through the depth of layer 1. The number of profiles of dendritic spines per 1000 µm² of neuropil was determined at successive 40 µm depths through layer 1.
1 in old monkeys exhibit more signs of degeneration than the deeper ones. The distribution of the profiles of dendritic spines at successive depths through layer 1 is shown in Figure 10. Electron micrographs from each of the three younger monkeys have almost twice as many profiles of dendritic spines as ones from the four older monkeys, and since the sizes of the spines do not appear to alter with age, the differences in the spine profile frequencies between the young and old monkeys are a reflection of the extent of spine loss, which amounts to an average of some 50%. As with the dendritic profiles, the differences between the young and old monkeys becomes less as layer 2 is approached. Presumably the decreased frequency of spine profiles in the older monkeys is largely due to the loss of dendritic branches from the apical tufts of the pyramidal cells and decreased spine frequency in the older monkeys must correlate with the diminished frequency of synapses encountered in the old monkeys, since some 70% of the synapses in layer 1 are axospinous.

Behavioral Correlations
As shown in Figure 11, comparison of the behavioral findings with the thickness of layer 1 revealed a significant correlation between the thickness of layer 1 and the performance of the monkeys on the 2 min delay condition of the DNMS ($r = 0.602, P < 0.25$).

When the scores for each behavioral task were compared with the mean number of synapses per unit volume for each monkey, a significant correlation was found with the CII (see Table 1, Figs 12 and 13), as well as with each of the three individual memory measures given in Table 1 ($r = 0.833–0.980, P < 0.05$ to < 0.025).

Discussion
This study shows that there are significant age-related changes in layer 1 of area 46. There is a loss of dendritic profiles from the neuropil, and this is accompanied by a reduction in the numbers of dendritic spine profiles. Concomitantly there is a decrease in synaptic density in the neuropil, although the relative proportions of axodendritic and axospinous synapses appear not to alter. In addition, there are indications that some of the neurons in layer 1 are dying, and it is presumed that these alterations in the neuronal components of layer 1 are the underlying cause of the decrease in the thickness that occurs with age, and lead to the astrocytosis that is most obvious in the increase in the thickness of the glial limiting membrane.

Dendritic Changes
Since most of dendrites in layer 1 are derived from the apical tufts of pyramidal cells it can be presumed that it is branches of these apical tufts that are the dendritic components most affected by age. These dendrites are unusual, because even in young monkeys the terminal branches are difficult to preserve, so that they readily swell and show vacuoles. This effect is even more pronounced in old monkeys. In old monkeys the dendrites are also lose their organelles and contain whorls of membranes that have been taken to be a sign of degeneration (Feldman, 1977). That degeneration of dendrites does occur with aging in area 46 is evidenced by the fact that the number of dendritic profiles in the outer portion of layer 1 of old animals is markedly reduced as compared to young monkeys. As pointed out, in reality this loss is even greater than the raw counts of dendritic
profiles reveal, since the greatly swollen dendrites of the older cortices would generate more oblique and longitudinal profiles than those in layer 1 of the younger monkeys.

Whether entire branches of the terminal dendritic tufts are lost with age, or whether branches become shorter is not evident from the present study. However, some insight into the events that are taking place comes from the study by Cupp and Uemura (1980), who examined Golgi-impregnated preparations from the frontal gyrus of rhesus monkeys aged from 7 to 28 years old and concluded that in old monkeys, i.e. 27–28 years of age, entire branches or segments are lost from the apical tufts of dendrites of pyramidal cells. In another study Uemura (1980) counted visible dendritic spines on Golgi-impregnated pyramidal cells in prefrontal cortex and concluded that compared with younger monkeys, monkeys aged 27–28 years old show a 25% loss of dendritic spines from both the apical dendritic shafts and from the apical tufts of these dendrites. Coleman and Flood (1987) have reviewed the literature dealing with changes in dendritic extent in normal aging; though they note a number of descriptions of dendritic regression and of spine loss with age, most of the descriptions relate to rodents.

Synaptic Changes
The results obtained by Uemura (1980) on changes in synaptic frequency, or density, in prefrontal cortex of rhesus monkeys with age are in agreement with our data. Uemura (1980) determined synaptic density in material stained with ethanolic phosphotungstic acid, which stains the electron-dense material associated with synaptic junctions. Uemura (1980) calculated that the synaptic density in cortex is of the order of $8-9 \times 10^8$ synapses/mm$^3$, which is somewhat higher than the value we obtained, and concluded that with age there is a 27% loss of synapses from the upper third of the cortex and a losses of 21% and 18% from the middle and lower thirds respectively of the cortical depth.

Uemura (1980) did not address changes that might be specific to layer 1, but synaptic changes in layer 1 with age have been examined by Adams (1987) in a study of human pre- and post-central gyrus. The material was from patients aged 45–84 years; it was obtained from biopsies and had been immersion fixed. Adams (1987) concluded that there is no decrease in the thickness of layer 1 with age in either cortical area, but that there is a significant decrease in the number of synapses from layer 1 of precentral gyrus, a decrease that is largely attributable to a loss of asymmetric axospinous synapses. In contrast, in postcentral gyrus no significant change in synapse number was detected. This result raises the question of whether alterations with age in layer 1 are area specific, as results obtained by other members of our group also suggest.

In studies of the dentate gyrus, Tigges et al. (1995, 1996) found no change in the thickness of the molecular layer with age and concluded that overall the number of synapses in the outer third of the molecular layer is unchanged with age (Tigges et al., 1995). In the molecular layer of the dentate gyrus, 87% of the synapses involve dendritic spines, as compared with 69–72% in layer 1 of area 46, and 13% of synapses in dentate gyrus are axodendritic. If these axodendritic synapses are considered as a separate population, then Tigges et al. (1995) found that with age there is a significant loss of axodendritic synapses, amounting to ~3% of the total. Tigges et al. (1996) then went on to examine the population of asymmetric synapses in the inner one-third of the molecular or supragranular layer of the dentate gyrus, using the empirical formula derived by Colonnier and Beaulieu (1985) as well as the physical dissector. They determined that in the inner third of the molecular layer of the dentate gyrus the density of synapses is $13-14 \times 10^8$/mm$^3$, which is about two times greater than in layer 1 of area 46 in young monkeys; however, as in the outer third of this layer, Tigges et al. (1996) found no change in the overall numerical density of synapses with age.

Another indication that layer 1 in different parts of the cortex might react differently in some conditions comes from the study of Brun et al. (1995). They compared the density of synapses in layer 1 of the frontal (area 10) and parietal (area 39) cortex in human material from control subjects (mean age 70 years), subjects with frontal lobe degeneration of non-Alzheimer type (mean age 65 years) and subjects with Alzheimer's disease (mean age 73 years), by using antibodies to synaptophysin to label axon terminals, whose frequency was then measured by optical densitometry. Brun et al. (1995) found that compared to the controls, in the frontal pole the density of synapses in the two disease groups had declined by 40%, whereas in the parietal lobe the decrease was greater, and showed a 50% loss in the Alzheimer brains, but no significant change in the brains with frontal pole degeneration. At the same time the density of astrocytes in the molecular layer in both diseased groups of brains had increased, similar to what has been observed in our electron micrographs of layer 1 in area 46 of the monkey.

Clearly, the question of whether layer 1 in some portions of the cortical mantle are more susceptible to aging and to disease requires a fuller investigation, especially in the context of any cognitive changes that are recorded.

Neurons
In the neocortex as a whole there is no evidence of significant losses of neurons during normal aging, except in layer 1 (see Peters et al., 1998), in which most of the neurons are inhibitory. As indicated, while most of the cell bodies of layer 1 neurons appear to be unaffected by age, some of them show a vacuolation of their cytoplasm, and in other neurons both the nucleoplasm and the cytoplasm exhibit an increased electron density, together with a swelling of the cisternae of the nuclear envelope and endoplasmic reticulum, indicating that these latter neurons are dying. What proportion of neurons may be lost from layer 1 during normal aging in the monkey is not known, and could be difficult to determine because of the sparse distribution of layer 1 neurons, but it is of interest that in their study of the effects of aging on neurons in the frontal lobe of the rat, Peinado et al. (1993) found that the only significant age-related decrease in neuronal density was in layer 1, from which ~18% of the neurons were lost.

Afferents in Layer 1
Whether the loss of synapses with age is brought about solely as a consequence of a loss of postsynaptic sites, the dendrites and their spines, or whether synaptic sites are lost due to a loss of afferent axons to layer 1, is not yet evident. There is no obvious morphological evidence of a widespread degeneration of axons or their terminals in layer 1, and whether there is a significant loss of axons cannot be determined stereologically by carrying out a volume fraction analysis, because of the inconstant swelling of the dendrites.

However, it can be assumed that there is a loss of afferent axons from some sources such as the pars compacta of the substantia nigra, because in our rhesus monkeys Siddiqi et al. (1994, 1995) have found that as many as 27% of the total number
of neurons are lost with age, and Goldman-Rakic and Brown (1981) and Wenk et al. (1989) have shown that with age there is a decrease of as much as 50% of the endogenous dopamine from the frontal cortex of monkeys. The substantia nigra provides a strong dopamine input to layer 1 of prefrontal cortex (Williams and Goldman-Rakic, 1993) and gives rise to terminals forming symmetric synapses (Smiley and Goldman-Rakic, 1993).

There is also a reduction in the level of choline acetyltransferase with age in monkey cortex (Beal et al., 1991), and presumably this reflects a reduction in the strong cholinergic input, which also provides symmetric synapses to layer 1 of prefrontal cortex (e.g. Campbell et al., 1987; Lewis, 1991). In contrast, Beal et al. (1991) found no differences in the levels of glutamate with age in the monkey cerebral cortex. Glutamate is the prime excitatory neurotransmitter in the cortex, and a decrease in glutamate levels might be expected because the majority of axon terminals in layer 1 are excitatory and form the asymmetric synapses, which are certainly reduced in frequency with age.

As pointed out earlier, it is assumed that the axons forming most of the asymmetric synapses in layer 1 are derived from ascending branches of the plexuses of the pyramidal cells, and it has to be considered that the morphological changes seen in the apical dendritic tufts of layer 1, and in the layer 1 neuronal cell bodies, might be due to excitotoxicity. Olney (1978) first reported that systemically injected glutamate can bring about a pattern of dendritic and neuronal cell body swelling with a sparing of axons, which, superficially, seems to describe the changes that occur in layer 1. N-Methyl-D-aspartate (NMDA), kainate and other agonists that act on glutamate receptors also bring about neuronal damage when injected into the brain, and Dietrich et al. (1992) have shown that when NMDA is injected into the lateral ventricles of adult rats, there are severe changes near to the ependymal layer of the ventricles. But in addition, brain regions adjacent to the CSF spaces are affected; this includes the superficial layers of the cortex (Dietrich et al., 1992), just as in layer 1 of aged monkeys. The dendrites become swollen and the most affected ones contain membranous profiles, while the axons and terminals appear to be unaffected. Obviously, the possibility that the age changes in layer 1 are brought about by excitotoxicity requires further investigation, as does the question whether layer 1 in other neocortical areas display similar changes to those encountered in prefrontal cortex.

**Relationship to Behavioral Findings**

As shown, there is a significant correlation between the thickness of layer 1 and memory function (Fig. 11). Specifically, the degree of impairment in the delay condition of the DNMS can be related to the degree of thinning of layer 1 in area 46, and further, there is a significant correlation between the numerical density of synapses in layer 1 and several behavioral measures, including the extent of impairment on the acquisition and performance of the DNMS task, the spatial condition of the DRST task and the CII, which is an overall measure of cognitive dysfunction (see Table 1, Figs 12 and 13).

These age changes in layer 1 are of particular interest in light of the notion advanced by Vogt (1991) that one of the essential functions of layer 1 circuits is ‘event holding’, i.e. briefly holding the memory of an event until it can be integrated with the memory of other events. In this hypothesis, layer 1 plays a pivotal role in regulating information processing within cerebral cortex.

Evidence from lesion studies in monkeys has accumulated to suggest that the integrity of area 46 is crucial to a complex network of functions that subserve working memory (Goldman-Rakic, 1996; Petrides, 1996) as well as other higher order ‘executive functions’ (Goldman et al., 1971). In humans, damage to the dorsolateral prefrontal cortex, which includes area 46, produces impairments in executive system function similar to that seen in non-human primates (Goldman-Rakic, 1996; Petrides, 1996). It is also of interest to note that executive system function is one of the first cognitive domains to evidence decline with age both in humans (see Albert and Moss, 1996) and in non-human primates (Moss et al., 1997). Taken together, the findings from behavioral studies place area 46 in a pivotal role in ‘higher cortical’ information processing.

Thus, the relationship identified in the present study between the morphological alterations in layer 1 of area 46 and behavioral impairment, together with our earlier observation of white matter alterations in area 46 of aged monkeys (Peters et al., 1994), provide a possible basis for the age-related decline in executive system function evidenced in monkeys and humans alike.

**Notes**

We are grateful to Dr Ron Killiany for helping with the correlations between the behavioral and morphological data and for Dr Jim Herndon for his careful reading of the manuscript. Supported by ational Institute on Aging grant 2PO1-AG 00001.

Address correspondence to Dr Alan Peters, Department of Anatomy and Neurobiology, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118, USA. Email. apeters@cajal-1.bu.edu.

**References**


