The Effects of Aging on Layer 1 of Primary Visual Cortex in the Rhesus Monkey

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The effect of age on layer 1 in primary visual cortex was determined in 19 rhesus monkeys of various ages. Twelve of the monkeys had been behaviorally tested. With age layer 1 becomes thinner and the glial limiting membrane becomes thicker. In the neuropil of layer 1 many of the dendrites in old monkeys appear to be degenerating and, as a consequence, electron micrographs from old monkeys display fewer dendritic and spine profiles per unit area than in young monkeys. As determined using both the dissector and size–frequency methods, there is also a concomitant decrease in the numerical density of synapses with age. Although there is a significant correlation between the thinning of layer 1 in area 17 and age, there is no significant correlation between either the thinning of layer 1 or its loss of synapses and any of the behavioral measures of memory function obtained from the 12 behaviorally tested monkeys. Similar morphological changes with age occur in layer 1 of prefrontal cortex of these same monkeys, but in area 46 both the thinning of layer 1 and the loss of synapses show a significant correlation with behavioral measures of memory function. These differences between layer 1 in these two cortical areas presumably relate to the fact that prefrontal cortex has a greater role in subserving cognition than does primary visual cortex.

Introduction

In our earlier studies of the effects of normal aging on the cerebral cortex of the rhesus monkey we found that with age there is no significant loss of neurons from area 17 (Vincent et al., 1989; Peters and Sethares, 1993), area 4 (Tigges et al., 1990) or area 46 (Peters et al., 1994). There is also strong evidence that the same holds true for the human brain, leading to the conclusion that the cognitive decline which occurs during normal aging in primates cannot be attributed to a substantial loss of cortical neurons (Peters et al., 1998a). However, cerebral cortex is not unaffected by age. For instance, in layer 1 there is a significant increase in the thickness of the glial limiting membrane and swelling of dendrites in the underlying neuropil (Peters, 1991). We investigated these age-related changes in more detail when we examined layer 1 of area 46 of prefrontal cortex (Peters et al., 1998b). This cortical area was chosen for study because aged monkeys as a group are impaired on spatial and reversal learning tasks, as well as recognition memory tasks, and it is considered that in part, at least, this type of behavior is subserved by prefrontal cortex (Kojima and Goldman-Rakic, 1982; Lai et al., 1995; Fuster, 1997; Moss et al., 1997; Moore et al., 1998). It was found that in prefrontal cortex layer 1 becomes significantly thinner with age and that this thinning is accompanied by a reduction in the frequency of profiles of dendrites and their spines, indicating that some of the branches of the spiny dendrites in the terminal tufts of underlying pyramidal cells are degenerating and being lost with age. Concomitantly, there is a 30–60% reduction in the numerical density of synapses per unit volume in the neuropil of layer 1 in prefrontal cortex of old monkeys.

When these morphological changes in area 46 were compared with behavioral data that had been derived from these monkeys, a significant correlation was found between impairment in cognition and both the decrease in thickness of layer 1 and the decrease in numerical density of synapses in layer 1. This led to the conclusion that the age-related changes occurring in layer 1 of area 46 provide at least one possible basis for the cognitive impairment evidenced in aged monkeys and, possibly, in aged humans.

The question then arises as to whether the age-related changes that occur in layer 1 of area 46 are ubiquitous and, if so, whether the same correlations between morphological changes and cognitive decline can be found in other areas of cortex not generally assumed to be involved in cognition. To at least partially answer this question, we have now examined layer 1 of area 17, or primary visual cortex. It will be shown that although there is an age-related thinning of layer 1 in visual cortex, proportionally it is not the same as that occurring in area 46. Further, neither the thinning of layer 1 in area 17 nor its age-related loss of synapses directly correlate with cognitive decline.

Materials and Methods

The ages of the 19 rhesus monkeys (Macaca mulatta) used in this study are given in Table 1. The exact ages of 16 of the monkeys are known, but for three of them (AM17, AM18 and AM13) the ages have been estimated (est.), since these monkeys were not born in captivity. In subsequent figures and in the text the exact ages of the monkeys will not be given; instead the ages will be given to the nearest whole year. It should be noted that for the 12 monkeys for which behavioral data are given in Table 1 the thickness of layer 1 in area 46 has also been determined [see table 1 in Peters et al. (Peters et al., 1998b)].

Fixation and Preparation of Material

The fixation of the brains used in this study was achieved by vascular perfusion under deep anesthesia, as previously described in detail (Peters et al., 1994), in full accordance with approved Institutional Animal Care and Use Committee regulations. In brief, the monkeys were preanesthetized with ketamine and a ketamine/Rompun mixture was administered i.v. to a state of areflexia. The anesthetized monkeys were artificially resired with a mixture of 95% oxygen and 5% carbon dioxide and their brains fixed by intravascular perfusion with a warm solution of 1% paraformaldehyde and 1.25% glutaraldehyde in either 0.1 M cacodylate or 0.1 M phosphate buffer, pH 7.4. After the brains had been removed, one cerebral hemisphere was immersed for further fixation in a stronger fixative containing twice the concentration of aldehydes in the same strength buffer at 4°C. After the hemispheres had fixed for 1 week or more, a strip of cortex ∼15 mm long and 2 mm wide was removed from the opercular lateral surface of area 17, from a region at least 3 mm caudal to the lunate sulcus. This is where the center of the visual field is represented and the cortex here has a relatively uniform thickness. The strip of visual cortex was then cut into seven or eight blocks which were osmicated, dehydrated in an ascending series of alcohols and embedded in Araldite.

Two blocks of cortex from area 17 of each monkey were then selected...
Density of Synapses with Age

The numerical density of synapses in layer 1 was determined in the six monkeys cited above using both the physical disector and the size–frequency methods.

Disector Method

Serial thin sections through layer 1 were taken from the same tissue blocks used for the neuropil assessment and ribbons of two or three serial sections were mounted on formvar-coated, single slot grids. The sections were stained with uranyl acetate and lead citrate and the numerical densities of synapses in layer 1 determined using the physical disector and applying the formula \( N_s = 2Q/a \times h \), where \( N_s \) is the number of synapses per unit volume of tissue, \( Q \) is the number of synaptic profiles that are present in the reference sections but do not appear in the experimental sections, \( a \) is the area sampled and \( h \) is the section thickness (Sterio, 1984; Calverley et al., 1988; Geinisman et al., 1996; Mayhew, 1996; Tigges et al., 1996; Peters et al., 1999b). The details of how the method was used have been given previously (Peters et al., 1999b) and so will not be repeated here.

Densities of the numbers of synapses \( (N_s) \) were made at one-third and two-thirds through the depth of layer 1 of each monkey to ascertain if the numerical density of synapses altered with depth. A profile was identified as belonging to a synapse if at least a portion of the density associated with a synaptic junction was visible and there was at least one synaptic vesicle nearby in the presynaptic axon terminal. For each determination of \( N_s \), a sufficient number of pairs of micrographs was examined to give a total \( Q \), the profiles of synapses that were present in one micrograph of the pair and not in the other, of at least 100. This ensured that for each separate determination of \( N_s \), the coefficient of error (CE), determined by standard statistical methods, was in the range 0.04–0.09 and in each instance the magnitude of the CE was at least half the value of the coefficient of variation (CV). Thus the estimates of \( N_s \) met the criterion suggested by West and Gundersen for obtaining meaningful values (West and Gundersen, 1990).

Size–Frequency Method

Recently, DeFelipe et al. made estimates of the number of synapses in cerebral cortex using both the physical disector method and the size–frequency method and found that both methods give similar results (DeFelipe et al., 1999). The size–frequency method uses the empirical formula suggested by Colonnier and Beaulieu: \( N_s = N_v/a \), where \( N_v \) is the number of synapses per unit volume, \( N_v \) is the number of synaptic junctions per unit area of an electron micrograph and \( d \) is the mean length of the synaptic junctions (Colonnier and Beaulieu, 1985). Since we already had electron micrographs available, we decided to compare the outcomes of using both methods. For the size–frequency analysis we examined each micrograph used for the disector analysis so that, in effect, the total area of neuropil examined was the same as that used for the disector analysis. Using these micrographs, which had a final magnification of \( \times 12500 \), the lengths of the profiles of at least 175 synaptic junctions were measured at the one-third and two-thirds depths through layer 1. If the synaptic junction profile was curved, the length of the junction was measured across the two ends of the junction and if it was en face the width of the synaptic density was measured. From these individual measures the mean synaptic junction length \( (d) \) was determined. Then the number of profiles of synaptic junctions in each micrograph was counted and the number per unit area \( (N_v) \) calculated. These values were subsequently inserted into the formula of Colonnier and Beaulieu to determine the value for \( N_v \).

Behavioral Testing

Twelve of the monkeys used in this study had been trained in a battery of behavioral tasks to assess learning and memory. The others had been used in pilot studies at the beginning of the project and so comparable data on

<table>
<thead>
<tr>
<th>Monkey code</th>
<th>Age (yr, est.)</th>
<th>Sex</th>
<th>Thickness of layer 1 (mm)</th>
<th>DMNS (errors)</th>
<th>DRST (2 min delay)</th>
<th>CII</th>
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<td>–0.01</td>
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<td>–0.01</td>
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<td>–0.03</td>
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<td>F</td>
<td>0.14</td>
<td>0.01</td>
<td>0.46</td>
<td>0.72</td>
</tr>
<tr>
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<td>0.16</td>
<td>1.85</td>
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<td>1.37</td>
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<td>0.11</td>
<td>–1.28</td>
<td>–1.33</td>
<td>1</td>
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<td>0.19</td>
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<td>M</td>
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The individual Z scores shown in this table are based upon a population of young adult and aged monkeys. The higher the Z score, the worse the performance of the monkey (see Herndon et al., 1997). The 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 (Herndon et al., 1997).
their behavior was not available. The details of the behavioral testing are given in an earlier article (Peters et al., 1998b) and so need not be repeated here. Suffice it to state that the battery of tests included three visual recognition memory tasks: (i) a delayed non-matching to sample (DNMS) task with a 10 s delay that has been used by several laboratories to assess memory in aged monkeys; (ii) a variation of this task which is more difficult due to an increase in the delay between presentation of a sample and the recognition trials to 2 min (DNMS 2 min delay); (iii) a spatial delayed recognition span task (DRST) to assess ‘memory load’.

Although it is important to assess performance for each of these tasks individually, it is also helpful to characterize an animal’s overall performance. This is done by generating a Cognitive Impairment Index (CII), which is derived from the normalized scores, i.e. the Z-scores. This is done by generating a Cognitive Impairment Index (CII), individually, it is also helpful to characterize an animal’s overall performance. Specifically, the individual scores on the three behavioral measures (errors on DNMS acquisition; percent correct DNMS 2 min delay; mean span length on spatial DRST) for each of the 12 monkeys behaviorally tested were transformed to scores normalized to a population of 53 adult rhesus monkeys, as described by Herndon et al. (Herndon et al., 1997), to generate a Cognitive Performance Index of global ability. The inverse of this Cognitive Performance Index is the CII, which is a measure of how much the cognition of the monkeys is impaired.

Results

Morphology of Layer 1

In area 17 layer 1 is ~0.1 mm thick. Even in young monkeys the dendrites just beneath the outer surface of the layer tend to appear swollen, but with increasing depth the swelling gradually decreases so that in the deeper part of layer 1 the dendrites appear more normal. The reason for this frequent swelling of the dendrites in the outer portion of layer 1 is not apparent and it occurs even though the astrocytic processes of the glial limiting membrane do not exhibit a similar swelling. This is odd, since, as pointed out in a description of the effects of age on layer 1 in area 46 (Peters et al., 1998b), it is usually assumed that astrocytes are the best barometers of fixation: when the overall fixation is poor the astrocytic processes are invariably swollen (Peters et al., 1991).

Young Monkeys

The glial limiting membrane is largely formed by the processes of astrocytes, with the interposition of an occasional astrocytic cell body, and the cytoplasm of both the processes and the cell bodies is filled with the intermediate filaments that characterize these cells. In the visual cortex of young monkeys the glial limiting membrane is relatively thin (Fig. 1, G), but of variable thickness, so that in some places it may be only one astrocytic process thick, while elsewhere several processes are layered. However, in addition to these regional variations, there are also differences among monkeys, so that the glial limiting membrane can be somewhat thicker in some young monkeys than in others.

Below the glial limiting membrane layer 1 is almost entirely composed of neuropil, for there are few neuronal cell bodies and a scattering of the cell bodies of neuroglia. In electron micrographs of the neuropil (Fig. 2) the most common profiles are those of thin unmyelinated axons, which are between 0.2 and 0.4 µm in diameter. At intervals these axons give rise to terminals that synapse with the shafts of dendrites and with the spines which originate from many of the dendrites. These spiny dendrites are largely from pyramidal cells of layers 2/3 and 5, whose apical dendrites ascend to upper layer 2, where they begin to form the profuse apical dendritic tufts that enter layer 1 (Peters and Sethares, 1991). The profiles of these pyramidal cell dendrites generally have rather irregular outlines because of the spines that extend from them and they receive few synapsing axon terminals on their shafts. Other dendrites, which are less common, have smooth outlines and have frequent axon terminals synapsing with their shafts. These smooth dendrites originate either from non-pyramidal cells in deeper layers or from neurons with cell bodies in layer 1.

The other common component of the neuropil of layer 1 is the processes of astrocytes (Fig. 2, As). Although the profiles of the thicker astrocytic processes have irregular outlines and can be recognized by their cores of intermediate filaments, the profiles of the majority of astrocytic processes have few visible cytoplasmic inclusions beyond small cisternae of rough endoplasmic reticulum and glycogen granules. Their shapes are also very irregular, because, like pieces of a jigsaw puzzle, they fit themselves to the contours of the surrounding neuronal elements of the neuropil.

There are also myelinated fibers in layer 1 (Fig. 2, M) and in light microscopic preparations it is seen that in the deeper part of layer 1 these are concentrated to form a horizontal band (Peters and Sethares, 1996).

Old Monkeys

In old monkeys there is a thinning of layer 1 and a thickening of the glial limiting membrane. The glial limiting membrane becomes many astrocytic processes thick (Fig. 3) and much of the cytoplasm of these processes is occupied by filaments. In old monkeys filamentous astrocytes are also common in the astrocytic processes that permeate the neuropil of the underlying layer 1 (Fig. 4, As) and the bundles of filaments that form the cores of the larger astrocytic processes are stouter and more prominent than in young monkeys. The cell bodies of most of the astrocytes in layer 1 of older monkeys also have increased numbers of filaments in their perikaryal cytoplasm usually contains dense inclusions. The other obvious change is in the dendritic profiles (Fig. 4, D). In some of the dendrites in old monkeys the cytoplasmic components, such as microtubules and mitochondria, are not evenly distributed, but tend to be clumped to one side, while in other dendritic profiles the cytoplasm appears to be largely devoid of organelles. Also, the cytoplasm of many dendrites in some of the older monkeys contains vacuoles or membranous whorls of the type first described by Feldman (Feldman, 1976). These whorls are generally assumed to be a sign of degeneration.

Another age-related change is that some of the profiles of myelinated axons in layer 1 of the older monkeys have altered myelin sheaths (Fig. 4, M). Some of the myelin sheaths display ballooning, such that the sheath is herniated on one side (Feldman and Peters, 1998), but the most common age-related change is a splitting of the lamellae on one side of the sheath to accommodate dark cytoplasm. Since such splitting of the sheath occurs at the major dense line, it is assumed that the dark cytoplasm in derived from the oligodendrocyte forming the sheath (Peters et al., 2000).

The other components of the neuropil of layer 1 appear to be less affected by age, so that it is rare to observe any structural changes in the profiles of either the numerous small unmyelinated axons or the axon terminals. Nevertheless, as will be shown in a subsequent section of these results, there is a decrease in the frequency of synapses in layer 1 of older monkeys.

Depth of Layer 1

An overall thinning of layer 1 accompanies these age-related alterations in morphology. The depth or thickness of layer 1 was measured in 19 monkeys; six young monkeys (5–7 years of age), four intermediate aged monkeys (7–12 years of age) and nine old
monkeys (25–35 years of age). The thickest layer 1 was encountered in a 6-year-old monkey, AM10, in which it measured 0.158 mm. However, this is abnormal, because in the other 18 monkeys the thickness of layer 1 only varied between 0.122 and 0.089 mm. The lowest values occur in old monkeys and, as shown in Figure 5, the decrease in thickness of layer 1 correlates significantly with increasing age ($P < 0.01$). It should be mentioned that even if AM10 is regarded as an outlier and so removed from the data set, the relationship between thickness of layer 1 and age remains statistically significant (Fig. 5).

In order to assess group differences in layer 1, the mean thickness of layer 1 in the young (4–12 years of age) was also compared with that of old (>24 years of age) monkeys. Discounting the data for AM10, a non-parametric Mann-Whitney test shows that there is a significant difference in the thickness of layer 1 between the young and old monkeys ($U = 9, P < 0.02$).

Density of Synapses

To determine if there is a loss of synapses from layer 1 with age, both the physical disector and the size–frequency formulae were used to estimate the number of synapses per unit volume of neuropil. Estimates were made in two young monkeys, 5 (AM16) and 6 (AM76) years of age, one 9-year-old monkey (AM47) and four old monkeys, between 27 and 32 years of age (AM12, AM62, AM27 and AM41). To ascertain if any loss of synapses varied with depth, in each monkey determinations of the frequency of synapses were made at one-third and two-thirds of the depth from the pial surface.

The results of the analysis of the numerical density of synapses in layer 1 of the primary visual cortices of these seven monkeys using the two methods are given in Table 2. In the case of AM16, for example, at one-third of the depth through layer 1 the total area of thin sections examined by electron microscopy was 4034 $\mu$m$^2$ and the total number of profiles of synapses that were present in one thin section of the disector pair and not in the other, i.e. $Q'$, was 108. Using the minimal fold method (Small, 1968) to determine the thickness of the thin sections examined, it was estimated that the number of synapses per unit volume ($N_v$) is $4.02 \times 10^8$ synapses/mm$^3$ tissue. In applying the size–frequency method to the micrographs used for the physical disector analysis, it was determined that the mean lengths of the profiles of synaptic junctions in those micrographs was 0.28 $\mu$m, resulting in an estimated $N_v$ of $5.13 \times 10^8$ synapses/mm$^3$.

The data given in Table 2 have been plotted in Figure 6, to show the means and standard deviations (68% confidence intervals) of the $N_v$ determinations. The values for $N_v$ estimated using the disector method are all slightly lower and have greater standard deviations than those obtained using the size–frequency formula. However, in each case there is overlap between the standard deviations of the $N_v$ values obtained using both methods on the same set of micrographs. It can also be noted that both methods show the numerical density of synapses at the one-third and two-thirds depths through layer 1 to be very similar.

It is also evident from the data that irrespective of whether the $N_v$ values were determined using either the disector method of the size–frequency formula, the highest values for the numerical densities of synapses occur in the two younger monkeys. The $N_v$ values for the 9-year-old monkey (AM47) are somewhat lower than those for the two younger monkeys, but significantly higher than any of the $N_v$ values for any of the four old monkeys. In broad terms the $N_v$ values for the four old monkeys were between 30 and 50% lower than those for the two young monkeys.

The sets of data derived by the disector method and the empirical formula both show that there is a significant correlation ($P < 0.01$) between the numerical density of synapses in layer 1 and age. It is interesting to note that as this age-related decrease in the numerical density of synapses occurs, there is some suggestion that the mean lengths of the synaptic junctions tend to increase (Table 2).

As shown in Figure 5, with age there is a decrease in the thickness of layer 1, and this occurs as the numerical density of synapses decreases. When these two factors are taken together and the $N_v$ data from the disector method used, the number of synapses beneath 1 mm$^2$ of cortical surface in the two young monkeys is 0.52 and 0.55 $\times 10^8$, but between 0.24 and 0.34 $\times 10^8$ for the four old monkeys. This amounts to a substantial overall age-related loss of 40–55% in the number of synapses beneath a unit area of cortical surface.

The Effects of Age on Dendrites

An analysis of the distribution of synaptic profiles in the layer 1 neuropil of the young monkeys, AM76 and AM47, reveals that 64 and 62%, respectively, of the synapses are axosomatic, with the remaining 36 and 38% being axodendritic. The loss of synapses from layer 1 with age would imply either that the post-synaptic elements, the dendrites and their spines, have been lost with age or that the post-synaptic elements are intact although the numbers of synapses upon their surfaces has been reduced. To determine if there is a loss of dendrites from layer 1 with age it would normally be possible to carry out a volume fraction analysis by point counting, but such an analysis would have little meaning in this material because of the variable swelling of the layer 1 dendrites among animals. In those monkeys in which there was swelling of dendrites, the swelling would have the effect of artificially increasing the volume fraction attributed to dendrites. This would mask any loss of dendrites that might occur. Consequently, as in our earlier examination of layer 1 in area 46 (Peters et al., 1998b), we attempted to get some measure of dendrite and spine loss by counting the numbers of profiles of these elements in montages of electron micrographs passing through the depth of layer 1. This was done in the seven monkeys in which synaptic densities were estimated.

Determination of the numbers of profiles of dendrites and of dendritic spines per 1000 $\mu$m$^2$ at successive 20 $\mu$m depths through layer 1. Since we were only trying to obtain a broad picture, the data for the three 5- to 9-year-old monkeys (AM16, AM76 and AM47) were pooled to obtain a mean value and the data for the four old monkeys >27 years of age (AM12, AM62, AM27 and AM41) were similarly pooled. The results are given in graphic form in Figures 7 and 8. Figure 7 shows that there is a decrease in the numbers of dendritic profiles with age.

Figure 1. In young monkeys the glial limiting membrane (G) is thin and only 1–3 astrocytic processes thick. Beneath the glial limiting membrane the neuropil largely consists of dendrites (D), some of which may be swollen, dendritic spines, myelinated axons (M) and numerous unmyelinated axons (Ax). From AM16, 5 years of age. ×14 000.

Figure 2. In layer 1 from a young monkey the neuropil is rich in profiles of dendrites (D) and their spines (s), axon terminals (At), unmyelinated axons (Ax), the processes of astrocytes (As) and a few myelinated axons (M). From AM16, 5 years of age. ×14 000.
with the decrease being most severe in the superficial portion of layer 1. Since many of the dendrites in the older monkeys have lost organelles and have membranous bodies in their cytoplasm, it is suggested that some of the branches of the apical tufts of the pyramidal cell dendrites in layer 1 are degenerating and being lost with age. Presumably, this leads to the loss of dendritic spines (Fig. 8), which are the main post-synaptic targets of synapsing axon terminals. It is interesting that overall the number of dendritic spine profiles per unit area of electron micrographs in old monkeys is only ~66% of that in young monkeys, which is in the same range as the loss of synapses from layer 1 of the old monkeys (Table 2).

**Behavioral Correlations**

As shown in Figure 9, there is no significant correlation between the thickness of layer 1 in area 17 and the CII. Neither is there any correlation between the thickness of layer 1 and the performance of the monkeys on any of the three behavioral tasks (see Table 1). There is also no correlation between CII and the density of synapses, regardless of whether \( N_v \) is determined using the disector or the size–frequency method.

**Discussion**

The age-related changes that occur in area 17 are very similar to those that we previously encountered in layer 1 of area 46 (Peters et al., 1998b). In both cortical areas there is a decrease in the thickness of layer 1 and a decrease in the frequency of synapses with age. These changes are accompanied by a decrease in the number of dendritic and spine profiles, suggesting that the apical tufts of pyramidal cells are pruned with age. In addition to the effect of age on neuronal processes, there is an obvious thickening of the glial limiting membrane in old monkeys, as well as an increase in the frequency of intermediate filaments in the perikarya and processes of the astrocytes throughout layer 1 and the thickness of layer 1 in area 17 and the CII. Neither is there any correlation between the thickness of layer 1 and the performance of the monkeys on any of the three behavioral tasks (see Table 1). There is also no correlation between CII and the density of synapses, regardless of whether \( N_v \) is determined using the disector or the size–frequency method.

**Table 2**

<table>
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<th>Monkey code</th>
<th>Age (years)</th>
<th>Depth</th>
<th>Total area examined (µm²)</th>
<th>Disector method</th>
<th>Size–frequency method</th>
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<td>AM 16</td>
<td>5</td>
<td>1/3rd</td>
<td>4034</td>
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<td>0.28 5.13 (0.62)</td>
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<td>3724</td>
<td>5.01 (1.82)</td>
<td>0.28 5.50 (0.68)</td>
</tr>
<tr>
<td>AM 76</td>
<td>6</td>
<td>1/3rd</td>
<td>3832</td>
<td>4.83 (1.52)</td>
<td>0.34 5.41 (1.25)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>4.92 (1.55)</td>
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<tr>
<td>AM 47</td>
<td>9</td>
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<td>6192</td>
<td>3.86 (1.13)</td>
<td>0.29 4.58 (1.01)</td>
</tr>
<tr>
<td></td>
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<td>4994</td>
<td>4.01 (1.20)</td>
<td>0.30 4.20 (1.68)</td>
</tr>
<tr>
<td>AM 12</td>
<td>27</td>
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<td>6746</td>
<td>2.53 (0.99)</td>
<td>0.33 3.63 (0.57)</td>
</tr>
<tr>
<td></td>
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<td>2.46 (0.82)</td>
<td>0.33 3.49 (0.63)</td>
</tr>
<tr>
<td>AM 62</td>
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<td>4336</td>
<td>3.53 (1.14)</td>
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<tr>
<td></td>
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<td>3.06 (0.69)</td>
<td>0.35 3.63 (0.55)</td>
</tr>
<tr>
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<td>28</td>
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<td>8424</td>
<td>2.22 (0.82)</td>
<td>0.33 3.20 (0.66)</td>
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<td></td>
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<td>AM 41</td>
<td>32</td>
<td>1/3rd</td>
<td>5546</td>
<td>3.27 (0.81)</td>
<td>0.32 4.23 (0.50)</td>
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<tr>
<td></td>
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<td>2/3rd</td>
<td>5354</td>
<td>3.66 (1.02)</td>
<td>0.34 3.94 (0.33)</td>
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**Figure 5.** A plot of the thickness of layer 1 against the ages of the monkeys.

**Figure 6.** A comparison of the means and the standard deviations of the \( N_v \) values obtained using the size–frequency method (squares) and the physical disector (diamonds).

**Figure 3.** In old monkeys the glial limiting membrane (G) is thickened and consists of several layers of astrocytic processes, as well as the cell bodies of astrocytes (As). In the neuropil beneath the glial limiting membrane the dendritic profiles (D) are usually swollen. From AM62, a 28-year-old monkey. ×7000.

**Figure 4.** In the neuropil of layer 1 in old monkeys the dendrites (D) are often swollen and contain dense inclusion bodies. The dendritic spines (s), axon terminals (At) and unmyelinated axons (Ax) are usually unaltered, but the sheaths of the myelinated axons (M) often show defects. The astrocytic processes (As) have more filaments in their cytoplasm than in young monkeys. From AM62, a 28-year-old monkey. ×14 000.
an increase in the frequency of phagocytosed material in the astrocytes. However, despite these similarities, there are important differences between the effects of age on layer 1 in areas 46 and 17. These differences will be considered after a brief discussion of the methods used to determine $N_v$.

Comments on the Determinations of $N_v$

As will be seen from the results (Table 2), in our hands determinations of the numerical densities of synapses using the physical dissector resulted in lower $N_v$ values than when the size–frequency formula was used. Nevertheless, both methods show that there is a significant age-related loss of synapses from layer 1. One reason for the lower $N_v$ values obtained using the dissector could be the application of the criteria we used to identify synaptic junctions. Thus, to define a structure as a synaptic junction some of the density associated with the junction must be visible and at least one synaptic vesicle must be present in the vicinity of the junction. This means that more peripheral portions of synaptic junction profiles, which can be difficult to identify, will necessarily be omitted from the synapse count, and the assessment of $Q$.

Figure 7. The frequency of dendritic profiles as a function of depth, as visualized in montages of electron micrographs extending through the depth of layer 1. The number of dendritic profiles per 1000 $\mu$m$^2$ of neuropil was determined at successive 20 $\mu$m depths, so the outer surface of layer 1 is represented at the left. The data for the three young (AM16, AM76 and AM47) and the four old (AM12, AM62, AM27 and AM41) monkeys analyzed were pooled to generate these plots.

Figure 8. The frequency of dendritic spine profiles as a function of depth. The number of spine profiles per 1000 $\mu$m$^2$ was determined at successive 20 $\mu$m depths. As explained in the legend to Figure 7, the data for the three young monkeys and for the four old monkeys were pooled.

Figure 9. A plot of the depth of layer 1 against the CI for the 12 monkeys for which both measures were available (see Table 1).

Figure 10. A plot of the depth of layer 1 in area 17 against the depth of layer 1 in area 46 in the same individual monkeys.
obtained with the size–frequency method (see Fig. 6) is that there can be wide differences in the values of \(Q\), even between pairs of electron micrographs as they are used in turn as the experimental or reference member of the pair. There is much less variation in the number of synaptic junction profiles identified in each member of the pair of micrographs when the size–frequency method is used, so that the standard deviations are lower.

To determine if there is a significant difference in the direction of the synaptic density values obtained using the disector and size–frequency methods for the two sets of the 14 values for \(N_v\) (Table 2) and to determine if the magnitude of those differences varies significantly across all values, a non-parametric Sign Test and Wilcoxon Matched Pairs Signed Rank (Siegel, 1956) were used. Secondly, in order to ascertain the extent to which the values of \(N_v\) for the disector method correlated with those obtained with the size–frequency method, the Spearman Ranked Correlation was determined for the entire data set. The results of these analyses revealed that the sign and magnitude of the 14 values obtained with the size–frequency method as compared with the values obtained using the disector method were significantly equivalent (\(\chi = 0\), \(P < 0.001\), two tailed test; \(T = 0\), \(P < 0.001\), two tailed test). Further, the ranking of the values using the size–frequency method as compared with the disector method yielded a positive and significant correlation (\(r = 0.964\), \(P = 0.01\), two tailed test).

The analyses suggest that although the size–frequency method values are, in absolute terms, different from those obtained using the disector method, the size and sign of the differences are consistent for all values obtained. Moreover, the values obtained with one method correlate very highly with those obtained with the other. This leads to the conclusion that in our hands there is no significant difference between the two methods in determining relative measures of synaptic density.

Unfortunately, it cannot be determined whether the results obtained with the physical disector are more valid than those obtained using the size–frequency method. Both methods have inherent problems. A number of other investigators have also compared data obtained using both methods on the same pre- and post-central gyri (Adams, 1987). These data raise the question as to whether age-related changes in layer 1 might be area specific.

Comparison of Layer 1 in Area 17 with Layer 1 in Area 46

The mean overall thickness of the cortex in area 17 is \(-1.5\) mm (Vincent et al., 1989), while in area 46 it is \(-1.65\) mm (Peters et al., 1994). However, the thickness of layer 1 is quite different. In area 46 of young monkeys the thickness of layer 1 is between 0.178 and 0.211 mm, while in area 17 layer 1 is only between 0.111 and 0.158 mm thick. Thus, on average layer 1 in area 17 is only \(-60\%\) of the thickness of layer 1 in area 46. Also, interestingly, in the two young monkeys we have examined (AM16 and AM76) the synaptic density in layer 1 in area 46 is \(-6 \times 10^9/mm^3\) (Peters et al., 1998b), slightly higher than that in area 17, in which it is \(-5 \times 10^9/mm^3\) (Table 2). When the thicknesses of layer 1 and the synaptic densities are both taken into account it can be determined that the number of synapses beneath 1 mm\(^2\) of cortical surface in area 46 is \(-1.1 \times 10^8\), while in area 17 it is only \(0.5 \times 10^8\). In other words the tufts of the apical dendrites of pyramidal cells in layer 1 of area 46 receive about twice the number of synapses than those in layer 1 of area 17. In the neocortex the majority of dendrites that occupy layer 1 are derived from the apical tufts of pyramidal cells in layers 2, 3 and 5 (Cauller et al., 1998) and Cauller and Connors have shown that the excitatory inputs to these apical tufts in layer 1 have a powerful excitatory effect on the pyramidal neurons (Cauller and Connors, 1994). Since there are more synapses in layer 1 of area 46 of prefrontal cortex than in layer 1 of area 17, it is to be expected that the layer 1 input to the area 46 pyramidal cells will play a more important role in functioning of the cortex than in area 17.

In both area 46 and area 17 there is a significant correlation between the thickness of layer 1 and age, but, as shown in Figure 10, there is no correlation between the thickness of layer 1 in area 46 and layer 1 in area 17 of the same monkeys. In other words, with age layer 1 in area 46 can decrease in thickness at a different rate than layer 1 in area 17. This suggests that there is no common factor that brings about a proportionally similar thinning of layer 1 with age in the two cortical areas.

It should be pointed out that not all studies have concluded that there is a decrease in the thickness or volume of layer 1 with age. Thus, in contrast to our observation that layer 1 in area 46 becomes thinner with age (Peters et al., 1998b), O’Donnell and co-workers have concluded that in the rhesus monkey there is no decrease in the volume of layer 1 with age (O’Donnell et al., 1999). O’Donnell et al. used Nissl stained frozen sections and applied stereological methods to estimate the total volume of area 46 and of layer 1. The data generated by both O’Donnell and co-workers (O’Donnell et al., 1999) and ourselves (Peters et al., 1998b) both agree that layer 1 occupies some 12% of the total volume of area 46. Since the mean thickness of layer 1 in area 46 is 0.191 mm in young monkeys and 0.164 mm in old monkeys (Peters et al., 1998b) and the overall thickness of area 46 cortex is \(-1.64\) mm (Peters et al., 1994), our reported thinning of layer 1 with age would produce a decrease of only 1.6% in the total thickness or volume of area 46. So there is a question of whether such a small volume change would be detected by the stereological methods that O’Donnell et al. applied.

Also, in studies of the dentate gyrus in monkeys Tigges and co-workers found no change in the thickness of the molecular layer with age and concluded that no age-related loss of synapses occurs (Tigges et al., 1995, 1996). Adams examined layer 1 in the pre- and post-central gyri of human brains from subjects aged 45–84 years and concluded that there is no significant decrease in the thickness of layer 1 with age, although there is a loss of synapses from layer 1 of the pre-central gyrus with age, but not from the post-central gyrus (Adams, 1987). These data raise the question as to whether age-related changes in layer 1 might be area specific.

Correlations with Behavior

While there is a significant correlation between the thickness of layer 1 in area 46 and the performance of monkeys on the 2 min delay condition of the delayed non-matching to sample (DNMS) task (Peters et al., 1998b), there is no correlation between the thickness of layer 1 in area 17 and any of the behavioral...
measures. This difference between the two areas also extends to synaptic loss with age. When the scores for the behavioral tasks were compared with the mean number of synapses per unit volume in layer 1 of area 46, a significant correlation was found with CII and with each of the three individual behavioral measures (Peters et al., 1998b). However, the synaptic frequency in layer 1 of area 17 in these same monkeys bears no correlation with any of the behavioral measures.

Presumably this difference in correlation between the behavioral data and the age-related morphological findings in layer 1 of area 46 and the lack of correlation between these same parameters for layer 1 of area 17 has to do with the functions of these cortical areas. Age-related impairments in spatial and reversal learning, as well as in recognition memory tasks, are considered, in part, to be subserved by prefrontal cortex (Kojima and Goldman-Rakic, 1982; Lai et al., 1985; Fuster 1997; Moss et al., 1997; Moore et al., 1998). Indeed, evidence from lesion studies in monkeys suggest that the integrity of area 46 is essential to complex network functions that subserve working memory (Goldman-Rakic, 1996; Petrides, 1996), as well as other higher order executive functions (Goldman-Rakic et al., 1971). In contrast, area 17 is primarily involved in vision and is not considered to play a role in learning and recognition memory tasks, so that a lack of correlation between the age-related decline in cognitive behavioral tasks and age-related morphological changes should not come as a surprise. If anything, the data on the effect of age on layer 1 serve to emphasize the functional differences between prefrontal and visual cortices and the important role prefrontal cortex plays in learning and other higher cortical functions.

Notes
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References