Published in final edited form as:

J Comp Neurol. 2012 October 15; 520(15): 3492–3508. doi:10.1002/cne.23101.

A stereological study of the numbers of neurons and glia in the primary visual cortex across the lifespan of male and female rhesus monkeys

Eustathia Lela Giannaris¹ and Douglas L. Rosene^{1,2}

¹Department of Anatomy and Neurobiology, Boston University School of Medicine, Boston, MA 02118

²Yerkes National Primate Research Center, Emory University, Atlanta, GA 30322

Abstract

Mild age-related declines in visual function occur in humans and monkeys, independent of ocular pathology, suggesting involvement of central visual pathways (Spear, 1993). While many factors might account for this decline, a loss of neurons in primary visual cortex (V1) could be a contributing factor. Previous studies of neuron numbers in V1 reported stability across age, but were limited in the ages and genders studied and sampled only limited parts of V1 or limited cell types, allowing for the possibility of a subtle loss of neurons. We pursued this question in 26 behaviorally tested adult male and female rhesus monkeys ranging from 7.4 to 31.0 years of age using design-based stereology to estimate numbers of NeuN-labeled neurons and thionin-stained glia within three laminar zones, supragranular (layers II–IVB), granular (IVC), and infragranular (V–VI), across the entirety of V1. There were no significant differences between males and females on any measures, except for total brain weight ($p=0.0038$). There was an average of 416,000,000 neurons in V1, but no effect of age on this total or numbers within any laminar zone. Similarly, there was an average of 184,000,000 glia in V1 (44% the number of neurons), but no effect of age on this total. However, there was a significant age-related increase in numbers of glia in the infragranular zone, perhaps reflecting a glial response to pathology in myelinated projection fibers. This study provides further evidence that in normal aging neurons are not lost and hence cannot account for age-related dysfunction.

Keywords

Aging; Area 17; NeuN; immunohistochemistry; optical fractionator; Cavalieri method

Correspondence: Eustathia Lela Giannaris, Ph.D., Department of Anatomy and Neurobiology, Boston University School of Medicine, 700 Albany Street, W-701, Boston, MA 02118, 617-638-4060 (office), 617-638-4922 (fax), elgianna@bu.edu.

Conflict of interest statement

The authors state there is no conflict of interest.

Role of Authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: ELG, DLR. Acquisition of data: ELG. Analysis and interpretation of data: ELG, DLR. Drafting of the manuscript: ELG. Critical revision of the manuscript for important intellectual content: DLR. Statistical analysis: ELG, DLR. Obtained funding: DLR. Administrative, technical, and material support: DLR. Study supervision: DLR.

INTRODUCTION

In normal, non-pathological aging, even in the absence of ocular pathology, humans and monkeys exhibit deficits in visual function (Spear, 1993). Since these changes cannot be explained by ocular deficits, they have been hypothesized to be due to functional and/or degenerative changes in central visual areas such as the visual cortices (Spear, 1993; Schmolesky et al., 2000). It has been shown that relative to young monkeys, V1 neurons of old monkeys lose orientation and direction selectivity while exhibiting an increased excitability (Schmolesky et al., 2000). It has also been reported that these deficits are ameliorated by treatment with a GABAergic agonist (Leventhal et al., 2003). These functional changes could result from subtle anatomical or physiological changes in the cortex including age-related degeneration of cortical neurons.

Several published studies have reported the absence of neuronal loss in primary visual cortex, but this conclusion is limited in a number of ways. For example, these studies sampled a limited number of subjects (e.g. from 5 to 14 subjects total – O’Kusky and Colonnier, 1982; Vincent et al., 1989; Peters and Sethares, 1993; Suner and Rakic, 1996; Peters et al., 1997; Hof et al., 2000), did not fully examine gender differences in their measures (only females - O’Kusky and Colonnier, 1982; only males – Suner and Rakic, 1996), did not sample the entire adult lifespan (only young - O’Kusky and Colonnier, 1982; Suner and Rakic, 1996; only young and old but not middle aged - Vincent et al., 1989; Peters et al., 1997; Hof et al., 2000) and only sampled very limited parts of the primary visual cortex and/or limited cell types (e.g. a 250 micron strip of the lateral operculum - Vincent et al., 1989; Peters et al., 1997; Layer IVB and Meynert cells - Hof et al., 2000; Meynert cells - Peters and Sethares, 1993).

Nevertheless, a variety of studies in other parts of monkey cortex including area 4 (Tigges et al., 1990), area 46 (Peters et al., 1994), hippocampus (Keuker et al., 2003), and entorhinal cortex (Merrill et al., 2000) also failed to identify age-related loss of neurons, findings that lend plausibility to the more restricted studies of V1. However, a recent stereological study by Smith et al. (2004) reported a significant loss of neurons (32%) in area 8A of prefrontal cortex despite preservation of neurons in adjacent area 46, suggesting that loss of neurons may indeed occur but be quite limited in location. This leaves open the possibility that there could be a significant but modest loss of neurons in the primary visual cortex underlying visual dysfunction that has gone undetected because of these limitations.

In order to address this question thoroughly, we undertook a stereological study of total numbers of neurons and glia covering the entire primary visual cortex of 26 monkeys of both sexes ranging in age from young adult (7.4 yrs old) to extreme old age (31.0 yrs old). Misclassification of neurons and glia is a particular problem in the primary visual cortex because of the high packing density of small neurons that are easily confused with glia. Therefore, we utilized NeuN immunohistochemistry to positively identify neurons along with a Nissl counterstain to reveal the NeuN-negative glia, allowing for unambiguous identification of both neurons and glia.

MATERIALS AND METHODS

Subjects

This study utilized tissue from twenty-six rhesus monkeys (*Macaca mulatta*), 11 male and 15 female, between the ages of 7.4 and 31.0 years of age. The subject data is summarized in Table 1. Monkeys were obtained from national primate research centers and all had known birth dates and complete medical records. Upon entering the study, monkeys were housed at the Laboratory Animal Science Center at Boston University Medical Center, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal procedures were performed according to the National Institutes of Health guidelines and the Institute of Laboratory Animal Resources Commission on Life Sciences' *Guide for the care and use of laboratory animals* (1996). The Boston University Institutional Animal Care and Use Committee approved all animal procedures. Upon entering the study, all monkeys underwent a battery of behavioral tests to assess behavioral function (Herndon et al., 1997; Moss et al., 1997; Moore et al., 2005). Once completed and prior to perfusion, all monkeys received MRI scans to ensure that there was no occult brain damage.

Tissue Preparation

Monkeys were tranquilized with ketamine (10 mg/kg, intramuscular), deeply anesthetized with sodium pentobarbital (15 mg/kg, intravenous to effect) and then euthanized by exsanguination during transcardial perfusion of the brain. Perfusion consisted of either Krebs-Henseleit buffer at 4°C, followed by 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4) at 37°C or the paraformaldehyde solution alone (Table 1). Brains were blocked, in situ, in the coronal plane, rostral to the lunate sulcus and the inferior occipital sulcus to yield an occipital lobe block containing the entire primary visual cortex (area 17). All tissue blocks were cryoprotected in graded glycerol solutions and flash frozen in 2-methylbutane at -75°C (Rosene et al., 1986), and then stored at -80°C until cut into serial sections.

Tissue Processing for Immunohistochemistry

Frozen sections were cut with a sliding microtome in the coronal plane. Blocks were from the right hemisphere in all but two subjects. All blocks were sectioned at 30µm into ten interrupted series. Sections were collected in vials containing 15% glycerol in phosphate buffer (0.1M, pH 7.4) and stored at -80°C until tissue from all subjects could be processed together as a single batch.

For NeuN immunohistochemistry, vials from all subjects containing one eighth of a series of an occipital block that included 6 to 8 equally spaced sections from each of the 26 subjects were removed from the freezer, warmed to room temperature, and processed together at the same time in the same batch of reagents to minimize the possibility of procedural variance. All steps were performed with agitation and at room temperature unless otherwise indicated. Free-floating sections were washed in tris-buffered saline (TBS, 0.05M, pH 6.8) to remove the cryoprotectant. Then sections were incubated in 1% hydrogen peroxide for 30 minutes to quench endogenous peroxidases. Sections were rinsed in TBS and then blocked for one hour

in a solution of 10% normal goat serum and 0.4% Triton-X100 in TBS. Sections were then incubated for 48 hours at 4°C in a stock solution (1% normal goat serum, 0.2% Triton-X100 in TBS) containing the mouse monoclonal anti-NeuN antibody (1:1,000, MAB377, Chemicon). After primary antibody incubation, sections were washed 3 times in stock solution and treated with biotinylated goat anti-mouse secondary antibody diluted in stock solution (1:200, BA-2000, Vector Laboratories) for 2 hours. Sections were rinsed in stock solution 3 times and then incubated with an avidin-biotinylated horseradish peroxidase enzyme complex (1:200, Vectastain Standard ABC kit, PK-4000, Vector Laboratories) in stock solution for 1 hour. After washing in TBS, sections were developed for 5 minutes in a solution of TBS with 3,3-diaminobenzidine tetrahydrochloride (DAB, 0.05%, Sigma) and hydrogen peroxide (0.005%). The immunostained sections were mounted on gelatin-coated slides and allowed to air dry. Sections were then defatted in chloroform: ethanol (1:1), rehydrated, counterstained for Nissl substance with thionin (0.05% thionin at pH 4.8), dehydrated in a graded series of ethanols, cleared in xylenes and coverslipped with Permount (Fisher Scientific).

Antibody Characterization

The specifications of the antibody used in this study are summarized in Table 2. The NeuN antibody is generated against purified mouse brain cell nuclei (Mullen et al., 1992) and recognizes both the 46-kDa and 48-kDa isoforms of the NeuN (*Neuronal nuclei*) protein (Lind et al., 2005). It has been shown to be a sensitive and specific marker for neurons (Wolf et al., 1996). DAB reaction product on NeuN-immunostained sections was observed in the nucleus, as well as perinuclear cytoplasm of neurons. When sections were counterstained, no Nissl-stained neurons were found that were unreacted with the NeuN antibody. Also, the antibody selectively labeled neurons, leaving glial and endothelial cells unlabeled from the immunostaining. Negative controls lacking primary antibody were run in parallel with experimental sections and no staining was observed.

Regions of Interest

The entire primary visual cortex, or area 17, (Figure 1) was sampled using design-based stereological methods. In the rhesus monkey, the primary visual cortex is the largest cortical area and covers approximately 17% of the total brain surface area (Van Essen et al., 1981). It extends across most of the lateral surface of the occipital lobe where it is separated from the parietal and temporal lobe by the lunate sulcus. Medially, the primary visual cortex is found along the banks and within the depths of the calcarine sulcus, which begins caudal to the splenium of the corpus callosum and runs along the occipital lobe. This large neocortical region is readily delineated from adjacent cortical regions, even at low magnification, because of its distinct cytoarchitecture and the myelinated band of axons that comprise the stria of Gennari. Though there has been some controversy in the literature concerning the layering scheme of area 17 (reviewed by Billings-Gagliardi et al., 1974), Brodmann's scheme was used in this study. It divides striate cortex into six layers, with three zones of layer IV (A, B, and C) and two zones of layer VI (A and B).

To obtain stereological estimates of total number in area 17, the rostral boundary was identified as the first section rostral to the ventral tip of the lunate sulcus so that the entire

foveal representation in the operculum of the occipital lobe could be sampled (Figure 1). The caudal boundary was the last section of the occipital pole in which all laminae were distinguishable from the pial surface to the white matter. This was necessary as sections through the occipital pole are tangential to the cortex and do not contain all layers or underlying white matter. In order to obtain localized estimates of total numbers of cells, the cortex was further subdivided into 3 laminar zones: supragranular, granular, and infragranular. The supragranular zone included layers II, III, IVA, and IVB; granular included layer IVC; and infragranular included layers V and VI (Figure 2). Layer I was outlined only to obtain volume estimates.

Design-based Stereology

The optical fractionator was used to estimate the total number of neurons and glia in each laminar zone. This method allows for an accurate estimation of the total number of objects within a 3-dimensional space (West et al., 1991). It is unbiased to the shape of the object and does not depend on tissue volume. In its most basic form, the method analyzes a known fraction of sections throughout the region of interest, a known fraction of the area of each section, as well as a known fraction of the thickness of each section, to obtain an estimate of total number of objects (West et al., 1991). All sections were blind coded to eliminate any bias by the experimenter. StereoInvestigator software (version 8.21.7, MBF Bioscience, Williston, VT) was used to outline regions of interest using a 2x Nikon Plan objective on a Nikon Eclipse E600 microscope and stereological counts were made using a 100x Nikon Plan Fluor oil objective.

Estimation of Volume

The Cavalieri estimator was used to estimate the volumes of the laminar zones as well as the volume of Layer I (Gundersen and Jensen, 1987). A $750\mu\text{m} \times 750\mu\text{m}$ sampling grid was superimposed on sections viewed at low magnification using a 2x objective and the number of intersection points that fell within each of the three laminar zones, as well as layer I, were counted using the StereoInvestigator software. One in every 80 sections, each $30\mu\text{m}$ thick, was analyzed, yielding a distance of 2.4mm between sections. There were 6 to 8 sections per subject. The volume estimates for layer I and the three laminar zones were combined to yield an estimate of the total volume for area 17.

Estimation of Total Number of Neurons and Glia

The total number of neurons and glia in each laminar zone was obtained using the formula by West et al. (1991) for the optical fractionator. Table 2 shows the stereological parameters used for each of the laminar zones. Counting sites were spaced every $120\mu\text{m}$ in both the x and y planes. At each grid intersection, a $20\mu\text{m} \times 20\mu\text{m}$ counting frame was placed and counts were made within a $5\mu\text{m}$ deep disector. An example of one counting frame including the two lateral exclusion planes is shown in Figure 3. Thickness measurements were made at every counting site and ranged from 7 to 9 microns. A guard zone of one micron above the disector box was used leaving a variable guard volume below the $5\mu\text{m}$ deep disector box of 1 to 3 microns. The bottom z-axis plane was used as the third exclusion plane. As shown in Figure 3, at high magnification, the appropriate immunostaining pattern was verified and cells that were very closely packed were readily distinguished. The criterion for counting

neurons was the appearance of the brown DAB immuno-product in the nucleus and the presence of perinuclear cytoplasm (with or without reaction product). Glial cells were unlabeled by DAB, were easily visualized by their thionin-stained nucleus. Thionin-stained endothelial cells and pericytes were easily identified and excluded for all counts.

Data Analysis

Student's t-tests were first performed to determine if there was a difference in any measure due to perfusion method or between males and females. Pearson's correlations with age as a continuous variable were used to determine if there were significant linear relationships between age and estimates of volume, numbers of neurons and number glia both for area 17 as a whole and in each laminar zone.

Photomicrographs

Photographs of medial and lateral views of a whole brain hemisphere (Figure 1, A and B) were taken with a digital camera (Fujifilm FinePix S602 Zoom) and adjusted to a common brightness using Adobe Photoshop CS5 Extended (version 12.0 x64). Photomicrographs of occipital lobe sections (Figure 1, C and D) were captured with a digital camera (Qimaging Retiga OEM Fast Color 12-bit) mounted on a Nikon Eclipse E600 microscope and montaged using the Surveyor software (Objective Imaging, version 6.0.1.3). Photomicrographs of stained sections in area 17 in Figure 2 and Figure 3 were taken with a digital camera (MBF-CX9000) mounted on a Nikon Eclipse E600 microscope using the StereoInvestigator software (version 8.21.7, MBF Bioscience, Williston, VT).

RESULTS

Perfusion Method and Gender

There was no significant difference between subjects perfused with Krebs-4%PFA, or those with 4%PFA alone on any measure. Likewise, there were no significant differences between males and females on any measure with the exception of total brain weight ($p=0.0038$) with the average male brain weight of 85.1g and female brain weight of 77.4g. Although brain weight and total area 17 volume were correlated, there was no significant difference between the sexes in total area 17 volume.

Volume Measures

Volume estimates for each region of interest were obtained using the Cavalieri Method (Table 4). As shown in Figure 4, there was no significant correlation between age and volume of area 17. Likewise, when the volumes of individual laminar zones were examined separately, there was no significant change with age. Furthermore, there was no change with age in the volume of layer 1 ($p=0.827$). Of note, while the average volume of area 17 was $1,478 \text{ mm}^3$, it ranged from a low of $1,034 \text{ mm}^3$ to a high of $1,743 \text{ mm}^3$, a variation of ± 18 to 24% from the mean. Looking at the total range, there is a 1.7 fold difference across subjects. Similar variation was present in the individual laminar zones with variation in the supragranular, granular and infragranular zones of 1.8, 1.6, and 1.8 fold, respectively. In layer I there was a 2.4 fold difference across subjects. The individual laminar zone volumes each correlated significantly with the total volume of area 17 ($p<0.0001$, two-tailed).

Total Numbers of Neurons

Estimates of total numbers of neurons made using the optical fractionator are presented in Table 5. The mean coefficient of error (CE) value was less than 0.05 for all regions assessed. As shown in Figure 5 the total number of neurons for area 17 was unchanged with age. Additionally there was no age-related change in any laminar zone. Like volume, this measure also displayed a large amount of variation among subjects. Estimates of total numbers of neurons for area 17 ranged from 268,440,790 to 480,695,150 with a mean of 415,794,415. There was a 1.8 fold difference across all subjects in area 17. When the individual laminar zones were analyzed, there was a 1.8, 1.8, and 1.9 fold difference in the supragranular, granular and infragranular zones, respectively.

Total Numbers of Glia

Estimates of total numbers of glia are shown in Table 6. The mean CE values were less than 0.073 for all regions assessed. As shown in Figure 6, the total number of glia is unchanged with age for area 17 as a whole. However, when analyzing individual laminar zones, while there was no change in the supragranular or granular zones separately, there was a modest (21.9%), but significant increase in the total number of glia cells in the infragranular layers with age (Figure 6D, $R=0.459$, $p=0.018$). The average number of glia was 46,800,000, comprising 25% of the total glia in area 17. This significant difference was observed, even in light of a high degree of individual variability. Total glial estimates in the infragranular zone ranged from 31,531,500 to 56,036,250, which is a 1.8 fold difference. A similar degree of variability was observed overall for area 17, the supragranular zone, and the granular zone, with fold variations of 1.9, 2.3, and 1.8, respectively, however there was no significant correlation with age.

Statistical Outlier

Of note, there was one subject that appeared to be an outlier in all measures. AM161 had the minimum values in all estimates of total numbers of neurons and glia, as well as all volume estimates, differing from the mean of all subjects by approximately 2.5 to 3 times the standard deviation. This subject was a 19.2 year-old female that was obtained from the Yerkes National Primate Research Center, as were the majority of the subjects. This subject also had the smallest brain weight of the entire cohort. Removing this subject had no effect on any of the statistical analyses and has therefore been included in all the foregoing analyses.

Relationship of Brain Weight to Measured Parameters

In order to determine if total brain size contributed to the variability or if it obscured the effects of aging, brain weight was used to normalize data on total numbers of neurons and glia. This normalization narrowed the range of variation on all measures. However, it did not reveal any additional significant linear relationships between age and any measures of total number neurons, glia or volume, nor was the significant increase in glia in the infragranular zone lost. Most importantly, the statistical outlier, AM161 fell within the data range of other subjects with this normalization. Figure 7 shows the total numbers of neurons (A) and total numbers of glia (B) in area 17 normalized to brain weight.

Correlations between brain weight and measures of volume and total numbers of neurons and glia were performed. There was a significant positive linear relationship between brain weight and volumes of area 17 ($R=0.506$, $p=0.008$), the supragranular zone ($R=0.509$, $p=0.008$), and the infragranular zone ($R=0.394$, $p=0.046$). Likewise, there was a significant positive linear relationship between brain weight and numbers of neurons and numbers of glia in area 17 overall (neurons: $R=0.435$, $p=0.026$; glia: $R=0.471$, $p=0.015$), as well as the supragranular (neurons: $R=0.467$, $p=0.016$; glia: $R=0.509$, $p=0.008$) and infragranular zones (neurons: $R=0.399$, $p=0.043$; glia: $R=0.388$, $p=0.0502$). Interestingly, brain weight did not correlate with either the volume ($R=0.315$, $p=0.117$) or the total number of neurons ($R=0.287$, $p=0.155$) or glia ($R=0.219$, $p=0.282$) in the granular zone.

Relationship Between Neurons and Glia

Numbers of neurons versus numbers of glia were analyzed for area 17 and each of the laminar zones. The relationship between total numbers of neurons and total numbers of glia in area 17 is shown in Figure 8. A highly significant linear relationship existed in all laminar zones and in area 17 overall, indicating that subjects with more neurons also have more glia (area 17: $R=0.676$, $p=0.0001$; supragranular: $R=0.628$, $p=0.0006$; granular: $R=0.533$, $p=0.005$; infragranular: $R=0.675$, $p=0.0002$; two-tailed). Likewise, when comparing volume estimates and total number estimates of neurons and glia, there was a strong significant linear relationship (area 17 - neurons: $R=0.780$, $p<0.0001$; glia: $R=0.654$, $p=0.003$, two-tailed) as shown in Figure 9. A larger area 17 correlated with a greater number of neurons and a greater number of glia. Similarly, a larger laminar zone was correlated with more neurons and more glia (supragranular – neurons: $R=0.778$, $p<0.0001$, glia: $R=0.645$, $p=0.0004$; granular- neurons: $R=0.740$, $p<0.0001$, glia: $R=0.503$, $p=0.009$; infragranular – neurons: $R=0.561$, $p=0.003$, glia: $R=0.548$, $p=0.004$).

DISCUSSION

Summary

The combination of NeuN immunohistochemistry and Nissl staining facilitated the definitive identification of neurons and glia and stereological estimation of total numbers of neurons and glia, as well as total volume of area 17. Results demonstrated that the overall volume of area 17 (mean = 1478 mm³), as well as the volume of all its major laminar zones, is stable over the lifespan in both males and females. This study also found that the total number of neurons throughout area 17 (mean = 416,000,000), as well as each of its laminar zones, is preserved with age. In addition, the current study demonstrated that the total number of glia in area 17 (mean = 184,000,000) is stable with age and that number of glia is about 44.3% of the number of neurons. The one exception to stability across age is a significant age-related increase of 21.9% in the total number of glia in the infragranular zone. This study also showed that there is no significant difference between the sexes on measures of volume or neuron and glial numbers overall in area 17 or in any of the laminar zones. Furthermore, it was shown that glia comprise a little less than half the cells in area 17. Overall these results provide further evidence that in the normal aging rhesus monkey, there is no age-related loss of neurons. Similarly there is no major change in glial cells with the exception of an increase in the infragranular layers. Therefore, age-related changes in

visual function are not due to neuron loss, but likely reflect sublethal changes in neuron functions.

Technical Considerations

Small neurons, as found in great abundance in the granular zone of area 17 (layer IVC), can be difficult to distinguish from glial cells in standard Nissl preparations of 30 micron or thicker frozen sections as many small neurons are round, with little cytoplasm surrounding the nucleus and hence very difficult to distinguish from glia. To avoid this problem, we utilized a combination of immunohistochemistry for NeuN to label neurons combined with a thionin counterstain to reveal any NeuN negative neurons as well as glia. As no NeuN-negative neurons were detected, this method enabled an unambiguous distinction between the two populations.

Supporting this approach is a study of the human anterior cingulate cortex by Gittins and Harrison (2004). These authors compared the neuronal density obtained from sections of area 24b stained with NeuN to values of neuron density in adjacent sections stained with cresyl violet for Nissl substance. They reported a significant correlation between these numbers, but the absolute values were 10–20% higher for the NeuN-positive neurons compared with the Nissl-positive neurons. The authors suggested that this is likely due to misclassification of small neurons as glia in the Nissl preparation, leading to an underestimate of neuron numbers and an overestimate of glial numbers. However, they did not counterstain the NeuN tissue, nor count the glia to get estimates of that population.

Volume of Area 17 Across Gender and Lifespan

Though previous studies of visual cortex in aging monkeys have included both male and female subjects, they have not had a sufficient sample to analyze gender differences. In the rhesus monkeys analyzed here, the variation in total volume of area 17 was the same order of magnitude in both male and female subjects across all ages (7.4 to 31 years), where males showed a range of 1350 mm³ to 1643 mm³ for 1.2 fold difference, while females showed a range of 1034 mm³ to 1743 mm³ for 1.7 fold difference.

Numbers of Neurons Across the Lifespan

A loss of neurons has been hypothesized to account for both cognitive and sensory declines with age. Early investigations concluded that neurons are lost with age and contribute to the cognitive declines observed in the elderly. Using density measures, Brody (1955; 1970) reported a 50% loss of neurons with age in human frontal and temporal cortices and approximately a 25% loss in visual cortex. Pakkenberg and Gundersen (1997) estimated a 9.5% overall loss of neocortical neurons with age. In addition, West (1993) reported for human hippocampus a loss of about 52% in the subiculum and 31% in the hilus of the dentate gyrus with no loss in the other subfields. However, in an analysis of 120 human brains, Haug (1985) reported that there was no significant decrease in cortical neuron density with age when differential shrinkage was taken into account. Terry et al. (1987) also concluded that overall neurons are not lost in the cerebral cortex with age. Similarly, Gomez-Isla et al. (1997) reported that there is no loss of cortical neurons in humans who are free of Alzheimer's disease.

In non-human primates, Brizzee et al. (1975; 1980) reported an age-related decrease in density, supporting the notion that neuronal cell loss was part of normal aging. In contrast, Vincent et al. (1989, area 17), Tigges et al. (1990, area 4); Peters et al. (1994, area 46; 1997, area 17); Merrill et al. (2000, entorhinal cortex); Keuker et al. (2003, hippocampus), all concluded that neurons are not lost with age in the monkey brain. However, Smith et al. (2004) reported neuron loss of about 32% in area 8A of monkey prefrontal cortex while confirming in the same subjects that neurons were not lost in adjacent area 46. This report of neuron loss in 8A suggests that there could be focal losses of neurons with age that may have been missed when sampling large cortical regions. It also supports the classic hypothesis of Coleman and Flood (1987) that any loss of neurons is likely to be localized, raising the possibility that areas of neuron loss may have been overlooked.

The possibility of localized loss is especially important for area 17 since previous studies sampled it in limited ways. Peters et al. (1997) sampled only 250-micron strips of the opercular part of area 17, while Hof et al. (2000) sampled only the Meynert cells in layer IVB and at the border of layers V and VI. In contrast, the current study sampled the entirety of area 17 in 26 monkeys of both sexes whose ages spanned the entire adult lifespan, separated neurons from glia immunohistochemically for identification, and confirmed that cortical neurons are not lost in normal aging rhesus monkeys.

V1 Neuron Numbers

Given the stability of neuron numbers with age, it is of interest to consider the quantitative aspects of these results in comparison with other studies of visual cortex. In the current study, the mean number of neurons for area 17 was approximately 416,000,000 per hemisphere, a number that differs from previous reports in monkeys for a number of reasons. For example, O'Kusky and Colonnier (1982) assessed neuron numbers per unit volume in rhesus and cynomolgus monkeys (*Macaca mulatta* and *Macaca fascicularis*) and then used correction factors based on neuron shape (Weibel and Gomez, 1962) and shrinkage due to tissue processing. The authors then extrapolated to a total of 160,000,000 neurons per hemisphere from a density of 128,500 neurons per mm³ in layers II – VI of area 17. Both of these measures are less than 45% of numbers obtained in the current study (416,000,000 total and 281,000 neurons per mm³ in layers II – VI). Overall this discrepancy in estimates of neuronal number and density suggests that the non-stereological methods employed by O'Kusky and Colonnier (1982) introduced bias into their counts as documented by Mouton (2002).

In another study, Suner and Rakic (1996) found that the neuron numbers in area 17 are stable between ages 1 month postnatal and 10 years. They counted neurons in cresyl violet stained sections from both hemispheres of five male rhesus macaques. The average number of neurons in area 17 per hemisphere was approximately 341,000,000. This slightly smaller estimate of neuron number, as compared to the current study, is likely due to the fact that they did not use immunohistochemistry for identification of neurons.

In a more recent study, Christensen et al. (2007) used the optical fractionator to estimate total numbers of neurons and glia across several brain regions, including the occipital lobe, in adolescent monkeys only 2.8 (+0.46) years of age. They reported approximately

334,000,000 neurons in the occipital lobe, which appears to have included all of area 17 plus some visual association cortex. This estimate of neuron number is about 80% of the approximately 416,000,000 neurons found in area 17 alone in the current study. This lower estimate might be attributed to misclassification of small neurons as glia in their Nissl preparations, and sampling of visual association cortex in addition area 17.

Numbers of Glia Across the Lifespan

There was no significant age-related change in the total numbers of glia when area 17 was examined as a whole. However, there was a modest and significant age-related increase in the total number of glia in the infragranular zone alone. This increase in glia is compatible with reports by Peters et al. (2008) that there is an age-related increase in the density of the oligodendroglial cells in all layers of area 17. Given that the oligodendrocyte population accounts for approximately 55% of all glia in layers V–VI and that Peters et al. (2008) reported a 50% increase in oligodendroglia with age, one would predict a 27.5% increase in the total number of glia in the infragranular zone assuming stability of other glia. In fact, in the current study, we observed a 21.2% increase in the total numbers of glia in the infragranular zone when comparing the oldest and youngest subjects. In layers I to IV, equivalent to the supragranular and granular zones in this study, oligodendrocytes account for 57–62% of all glia (Peters et al., 2008), so an increase of 28.5–31% increase would have been predicted. A significant increase in glia was not observed in these laminar zones, likely due to high variability in these layers as fold changes of 2.3 and 1.8 were observed in the supragranular and granular zones, respectively.

Variability of Volume, Neurons, and Glia Across the Lifespan

A major difficulty in studying the brain with age is that it is not possible to get estimates of quantitative anatomical parameters such as total number of cells from the same subject over time, requiring a cross-sectional design using post-mortem samples from subjects of different ages. One problem with any cross-sectional design is that variation due to age can be obscured by interindividual variation. The primary visual cortex has been reported to manifest perhaps the largest variation across individuals of any cortical area. For the human brain, Stensaas et al. (1974) reported a three-fold variation in the total area of primary visual cortex while Leuba and Kraftsik (1994) reported a two-fold variation in volume. Andrews et al. (1997) found a two to three fold variation in both the surface area and volume of primary visual cortex. In monkeys, Van Essen et al. (1984) found a two-fold difference in the total surface area of rhesus monkey primary visual cortex. Similarly, Peters et al. (1997) examined six young (4–12 years) and 8 old (25–35 years) male and female rhesus monkeys and noted a 2.1 fold variation in volume of V1. In the current study, there was a 1.7 fold variation in the estimates of total volume of area 17. Although the volume estimates of V1 in rhesus monkeys vary less than those of V1 in humans, the overall implication is that relatively large sample sizes ($n = 26$ in the current study) are necessary to use cross-sectional methods to study aging.

Quantitative Relationship of Neurons to Glia

A further metric of interest is the relationship of neurons and glia within brain regions. As highlighted by Hilgetag and Barbas (2009), there is some controversy over whether there

are, as some have asserted, “ten times” more glia than neurons in the brain (Kandel et al., 2000). While little data exists for the human brain, in monkey prefrontal cortex Dombrowski and colleagues (2001) found that the glia to neuron ratio was approximately 1:1. Similarly, Christensen et al. (2007) examined a number of cortical brain regions in young monkeys and found glia to neuron ratios ranging from 0.47:1 to approximately 1:1, with a ratio of 0.56:1 over the entire cerebral cortex and a ratio of 0.58:1 for the occipital cortex as a whole. O’Kusky and Colonnier (1982) found the ratio to be 0.49:1 in primary visual cortex. Therefore, our finding of a glia to neuron ratio for area 17 of 0.44:1 is comparable to the results of O’Kusky and Colonnier (1982) and Christiansen et al. (2007) and provides further evidence that the number of glia does not exceed the number of neurons.

Statistical Considerations

While it is not possible to “prove” the null statistical hypothesis that there is no loss of neurons across age, it is important to point out that with our sample size of 26 animals across the full adult life span we could have detected at $p=0.05$ two-tailed, an age-related loss reflected in a correlation as small as 0.388 which would account for as little as 15% of the variance. Nevertheless, we were unable to detect any effect of age on the number of neurons for area 17 as a whole or for any of the laminar zones.

Conclusions

The results of this study demonstrate that age-related impairments in visual function cannot be explained by even a modest age-related loss of neurons in area 17 either globally or in any of the three laminar zones examined. In fact, in contrast to age-related neurodegenerative diseases like Alzheimer’s where massive neuron death occurs, this study further supports the view that cortical neurons are preserved in normal aging though likely evidence sublethal functional changes as proposed by Morrison and Hof (1997). From this perspective there seem to be two major plausible explanations for the age-related impairment in visual function. On the one hand, the abundant evidence of age-related damage to myelinated fibers in the rhesus monkey brain (see Peters 2002 for review) might be manifested in alterations of input to primary visual cortex or in output from primary visual cortex or both. Alternatively, given the reports of Leventhal and colleagues (2003) suggesting that age-related decrements in visual function result from diminished inhibitory processing, it is possible that there might be changes in presynaptic or postsynaptic inhibitory processes or even a loss of GABAergic neurons, which constitute only 15 to 20% of the total neurons in primary visual cortex (Fitzpatrick et al., 1987; Hendry et al., 1987). The latter would be lost in the total numbers assessed here as even a 25% loss of these neurons would only result in a 3.5 to 5.0% overall change.

In terms of the estimates of glial cell numbers, the absence of any overall increase in glia as reported here is compatible with the absence of any major loss of neurons since the latter would be expected to produce significant gliosis. On the other hand, the modest but significant increase in glia detected in the infragranular layers (V–VI) is compatible with the hypothesized damage to myelinated fibers and resultant proliferation of oligodendroglia as myelinated fibers are abundant in layers V and VI as they enter and leave the cortex. While

a global loss of neurons is unlikely to occur and certainly does not appear to account for impairments in visual function, a number of more subtle changes remain to be explored.

Acknowledgments

Funded by:

National Institute of Health Grants: P01-AG00001, P51-RR00165, R01-AG021133

LITERATURE CITED

- Andrews TJ, Halpern SD, Purves D. Correlated size variations in human visual cortex, lateral geniculate nucleus, and optic tract. *J Neurosci.* 1997; 17:2859–2868. [PubMed: 9092607]
- Billings-Gagliardi S, Chan-Palay V, Palay SL. A review of lamination in area 17 of the visual cortex of *Macaca mulatta*. *J Neurocytol.* 1974; 3:619–629. [PubMed: 4142638]
- Brizzee, KR.; Klara, P.; Johnson, J. Changes in microanatomy, neurocytology, and fine structure with aging. In: Ord, JM.; Brizzee, KR., editors. *Neurobiology of Aging*. New York: Plenum Press; 1975. p. 574-594.
- Brizzee KR, Ord JM, Bartus RT. Localization of cellular changes within multimodal sensory regions in aged monkey brain: possible implications for age-related cognitive loss. *Neurobiol Aging.* 1980; 1:45–52. [PubMed: 7266734]
- Brody H. Organization of the cerebral cortex. III. A study of aging in the human cerebral cortex. *J Comp Neurol.* 1955; 102:511–516. [PubMed: 14381544]
- Brody H. Structural changes in the aging nervous system. *Interdiscip Top Gerontol.* 1970; 7:9–21.
- Coleman PD, Flood DG. Neuron numbers and dendritic extent in normal aging and Alzheimer's disease. *Neurobiol Aging.* 1987; 8:521–545. [PubMed: 3323927]
- Christensen JR, Larsen KB, Lisanby SH, Scalia J, Arango V, Dwork AJ, Pakkenberg B. Neocortical and hippocampal neuron and glial cell numbers in the rhesus monkey. *Anat Rec.* 2007; 290:330–340.
- Dombrowski S, Hilgetag CC, Barbas H. Quantitative architecture distinguishes prefrontal cortical systems in the rhesus monkey. *Cereb Cortex.* 2001; 11:975–988. [PubMed: 11549620]
- Fitzpatrick D, Lund JS, Schemmehel DE, Towles AC. Distribution of GABAergic neurons and axon terminals in the macaque striate cortex. *J Comp Neurol.* 1987; 264:73–91. [PubMed: 3680625]
- Gittins R, Harrison PJ. Neuronal density, size and shape in the human anterior cingulate cortex: a comparison of Nissl and NeuN staining. *Brain Res Bull.* 2004; 63:155–160. [PubMed: 15130705]
- Gómez-Isla T, Hollister R, West H, Mui S, Growdon JH, Petersen RC, Parisi JE, Hyman BT. Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Ann Neurol.* 1997; 41:17–24. [PubMed: 9005861]
- Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J Microsc.* 1987; 147:229–263. [PubMed: 3430576]
- Haug, H. Are neurons of the human cerebral cortex really lost during aging? A morphometric evaluation. In: Traber, J.; Gispén, WH., editors. *Senile dementia of the Alzheimer type*. Berlin: Springer; 1985. p. 150-163.
- Hendry SH, Schwark HD, Jones EG, Yan J. Numbers and proportions of GABA-immunoreactive neurons in different areas of monkey cerebral cortex. *J Neurosci.* 1987; 7:1503–1519. [PubMed: 3033170]
- Herndon JG, Moss MB, Rosene DL, Killiany RJ. Patterns of cognitive decline in aged rhesus monkeys. *Behav Brain Res.* 1997; 87:25–34. [PubMed: 9331471]
- Hilgetag CC, Barbas H. Are there ten times more glia than neurons in the brain? *Brain Struct Funct.* 2009; 213:365–366. [PubMed: 19198876]
- Hof PR, Nimchinsky EA, Young WG, Morrison JH. Numbers of meynert and layer IVB cells in area VI: a stereologic analysis in young and aged macaque monkeys. *J Comp Neurol.* 2000; 420:113–126. [PubMed: 10745223]

- Institute of Laboratory Animal Resources Commission of Life Sciences NRC. Guide for the care and use of laboratory animals. Washington, DC: National Academy Press; 1996 p.
- Kandel, ER.; Schwartz, JH.; Jessel, TM., editors. Principles of neural science. 4. New York: McGraw-Hill; 2000. p. 19-20.
- Keuker JJ, Luiten PG, Fuchs E. Preservation of hippocampal neuron numbers in aged rhesus monkeys. *Neurobiol Aging*. 2003; 24:157–165. [PubMed: 12493561]
- Leuba G, Kraftsik R. Changes in volume, surface estimate, three-dimensional shape and total number of neurons of the human primary visual cortex from midgestation until old age. *Anat Embryol*. 1994; 190:351–366. [PubMed: 7840422]
- Leventhal AG, Wang Y, Pu M, Zhou Y, Ma Y. GABA and its agonists improved visual cortical function in senescent monkeys. *Science*. 2003; 300:812–815. [PubMed: 12730605]
- Lind D, Franken S, Kappler J, Jankowski J, Schilling K. Characterization of the neuronal marker NeuN as a multiply phosphorylated antigen with discrete subcellular localization. *J Neurosci Res*. 2005; 79:295–302. [PubMed: 15605376]
- Merrill DA, Roberts JA, Tuszyński MH. Conservation of neuron number and size in entorhinal cortex layers II, III, and V/VI of aged primates. *J Comp Neurol*. 2000; 422:396–401. [PubMed: 10861515]
- Moore TL, Killiany RJ, Herndon JG, Rosene DL, Moss MB. A non-human primate test of abstraction and set-shifting: an automated adaptation of the Wisconsin Card Sorting Test. *J Neurosci Methods*. 2005; 146:165–173. [PubMed: 16054506]
- Morrison JH, Hof PR. Life and death of neurons in the aging brain. *Science*. 1997; 278:412–419. [PubMed: 9334292]
- Moss MB, Killiany RJ, Lai ZC, Rosene DL, Herndon JG. Recognition memory span in rhesus monkeys of advanced age. *Neurobiol Aging*. 1997; 18:13–19. [PubMed: 8983028]
- Mouton, PR. Principles and practices of unbiased stereology: an introduction for bioscientists. Baltimore: Johns Hopkins University Press; 2002.
- Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. *Development*. 1992; 116:201–211. [PubMed: 1483388]
- O’Kusky J, Colonnier M. A laminar analysis of the number of neurons, glia, and synapses in the adult cortex (area 17) of adult macaque monkeys. *J Comp Neurol*. 1982; 210:278–290. [PubMed: 7142443]
- Pakkenberg B, Gundersen HJ. Neocortical neuron number in humans: effect of sex and age. *J Comp Neurol*. 1997; 384:312–320. [PubMed: 9215725]
- Peters A. The effects of normal aging on myelin and nerve fibers: a review. *J Neurocytol*. 2002; 31:581–593. [PubMed: 14501200]
- Peters A, Leahu D, Moss MB, McNally KJ. The effects of aging on area 46 of the frontal cortex of the rhesus monkey. *Cereb Cortex*. 1994; 4:621–635. [PubMed: 7703688]
- Peters A, Nigro NJ, McNally KJ. A further evaluation of the effect of age on striate cortex of the rhesus monkey. *Neurobiol Aging*. 1997; 18:29–36. [PubMed: 8983030]
- Peters A, Sethares C. Aging and the Meynert cells in rhesus monkey primary visual cortex. *Anat Rec*. 1993; 236:721–729. [PubMed: 8379594]
- Peters A, Verderosa A, Sethares C. The neuroglial population in the primary visual cortex of the aging rhesus monkey. *Glia*. 2008; 56:1151–1161. [PubMed: 18449941]
- Rosene DL, Roy NJ, Davis BJ. A cryoprotection method that facilitates cutting frozen sections of whole monkey brains for histological and histochemical processing without freezing artifact. *J Histochem Cytochem*. 1986; 34:1301–1315. [PubMed: 3745909]
- Schmolesky MT, Wang Y, Pu M, Leventhal AG. Degradation of stimulus selectivity of visual cortical cells in senescent rhesus monkeys. *Nat Neurosci*. 2000; 3:384–390. [PubMed: 10725929]
- Smith DE, Rapp PR, McKay HM, Roberts JA, Tuszyński MH. Memory impairment in aged primates is associated with focal death of cortical neurons and atrophy of subcortical neurons. *J Neurosci*. 2004; 24:4373–4381. [PubMed: 15128851]
- Spear PD. Neural bases of visual deficits during aging. *Vision Res*. 1993; 33:2589–2609. [PubMed: 8296455]

- Stensaas SS, Eddington DK, Dobelle WH. The topography and variability of the primary visual cortex in man. *J Neurosurg.* 1974; 40:747–755. [PubMed: 4826600]
- Suner I, Rakic P. Numerical relationship between neurons in the lateral geniculate nucleus and primary visual cortex in macaque monkeys. *Vis Neurosci.* 1996; 13:585–590. [PubMed: 8782386]
- Terry RD, DeTeresa R, Hansen LA. Neocortical cell counts in normal human adult aging. *Ann Neurol.* 1987; 21:530–539. [PubMed: 3606042]
- Tigges J, Herndon JG, Peters A. Neuronal population of area 4 during the life span of the rhesus monkey. *Neurobiol Aging.* 1990; 11:201–208. [PubMed: 2362653]
- Van Essen, DC.; Maunsell, JHR.; Bixby, JL. Organization of extrastriate visual areas in the macaque monkey. Woolsey, CN., editor. Vol. 2. Cortical Sensory Organization; Clifton: Humana Press; 1981. p. 157-170.
- Van Essen DC, Newsome WT, Maunsell HR. The visual field representation in striate cortex of the macaque monkey: asymmetries, anisotropies, and individual variability. *Vision Res.* 1984; 24:429–448. [PubMed: 6740964]
- Vincent SL, Peters A, Tigges J. Effects of aging on the neurons within area 17 of rhesus monkey cerebral cortex. *Anat Rec.* 1989; 223:329–341. [PubMed: 2923284]
- Weibel ER, Gomez DM. A principle for counting tissue structures on random sections. *J Appl Physiol.* 1962; 17:343–348. [PubMed: 14005589]
- West MG, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec.* 1991; 231:482–497. [PubMed: 1793176]
- Wolf HK, Buslei R, Schmidt-Kastner R, Schmidt-Kastner PK, Pietsch T, Wiestler OD, Blümcke I. NeuN: a useful neuronal marker for diagnostic histopathology. *J Histochem Cytochem.* 1996; 44:1167–1171. [PubMed: 8813082]

Figure 1. Region of Interest

A) Lateral and B) medial views of monkey brain with the primary visual cortex highlighted. The vertical lines indicate the levels sampled for stereological counts. The asterisk (*) indicates the level of the sections shown in C and D. C) Nissl staining. D) NeuN immunostain with Nissl counterstain. Arrows in C and D indicate boundary between Area 17 and Area 18. Note that in both preparations, Area 17 is easy to identify based on the cytoarchitecture.

Figure 2. Laminar zones of area 17 and comparison of staining procedures

A) Area 17 was divided into three laminar zones for all counting procedures. The supragranular zone included layers II–IVB, the granular zone included layer IVC, and the infragranular zone included layers V–VI. Layer I was only outlined to obtain volume estimates. Scale bar = 200 microns. **B)** Nissl staining in the granular zone (layer IVC) of area 17. It is difficult to distinguish small neurons from glial cells. **C)** NeuN immunohistochemistry with Nissl counterstain in granular zone of area 17. Neurons and glia can easily be distinguished. Neurons are labeled brown with the NeuN antibody and DAB chromagen. Glial cells, as well as endothelial cells, are stained blue with Nissl. Scale bar = 50 microns.

Figure 3. Counting frame

A photomicrograph of a counting frame used for stereological procedures viewed using a 100x oil objective. The x–y dimensions were 20µm by 20µm. Brown NeuN+ neurons and blue Nissl-stained glia were unambiguously identified.

Figure 4. Estimated volume of area 17 and laminar zones vs. age

(A) Area 17, (B) supragranular zone, (C) granular zone (D) infragranular zone. There is no significant linear relationship between the volume of area 17 and age or volume of any laminar zones and age. There is no change in the volume of layer 1 with age (data not shown).

Figure 5. Estimated total number of neurons in area 17 and laminar zones vs. age
 (A) Area 17, (B) supragranular zone, (C) granular zone (D) infragranular zone. There is no significant linear relationship between age and total number of neurons in area 17 or any of the laminar zones.

Figure 6. Estimated total number of glia in area 17 and laminar zones vs. age
 (A) Area 17, (B) supragranular zone, (C) granular zone (D) infragranular zone. There is a significant linear relationship between age and total number of glia in the infragranular zone of area 17 with age.

Figure 7. Estimated total number of neurons and glia in Area 17 vs. age normalized to brain weight

Even when counts of neurons (**A**) and glia (**B**) are normalized using the brain weight, there is no significant change with age.

Figure 8. Estimated total number of glia vs. estimated total number of neurons in area 17
Scatter plot showing the estimated total number of glia versus the estimated total number of neurons in area 17. There is a highly significant linear relationship between these two measures ($R=0.676$, $p=0.0001$ two-tailed).

Figure 9. Estimated total number of neurons and glia vs. volume of area 17

The total number of neurons (A) and total number of glia (B) are strongly correlated with the volume of area 17.

Table 1

Subject data, brain weight, and supplier

Subject	Sex	Age	Perfusate	Hemi-sphere	Supplier	Brain Weight (g)
AM222	M	7.4	Krebs + 4% PFA	Right	YNRPC	87.2
AM132	M	7.5	4% PFA	Right	YNRPC	97.8
AM255	F	9.5	Krebs + 4% PFA	Right	YNRPC	76.7
AM199	F	10.6	4% PFA	Right	YNRPC	80.4
AM254	F	11.4	Krebs + 4% PFA	Right	YNRPC	73.9
AM194	F	11.9	4% PFA	Right	YNRPC	76.6
AM138x	M	12.0	4% PFA	Right	VA	86.0
AM195	F	12.1	4% PFA	Right	YNRPC	80.2
AM215H	M	16.6	4% PFA	Right	TNPRC	78.5
AM158H	M	16.8	4% PFA	Right	VA	82.3
AM257	F	17.6	Krebs + 4% PFA	Right	YNRPC	67.5
AM250	F	18.0	Krebs + 4% PFA	Right	YNRPC	83.7
AM190	F	18.0	4% PFA	Right	YNRPC	73.1
AM253	F	18.4	Krebs + 4% PFA	Right	YNRPC	75.7
AM161	F	19.2	4% PFA	Right	YNRPC	67.2
AM153H	M	19.4	4% PFA	Right	VA	84.2
AM216H	M	19.7	Krebs + 4% PFA	Right	TNPRC	94.2
AM256	F	20.7	Krebs + 4% PFA	Right	YNRPC	79.6
AM242	M	23.5	Krebs + 4% PFA	Right	YNRPC	77.1
AM179	F	23.6	4% PFA	Left	UPSM	79.6
AM243	M	24.4	Krebs + 4% PFA	Right	YNRPC	77.9
AM189	M	24.5	4% PFA	Right	YNRPC	79.9
AM235	F	24.5	Krebs + 4% PFA	Right	YNRPC	80.7
AM110	M	25.8	4% PFA	Right	YNRPC	90.4
AM104	F	28.9	4% PFA	Left	YNRPC	78.8
AM119	F	31.0	4% PFA	Right	YNRPC	86.8

Abbreviations: Krebs = Krebs-Henseleit Buffer; 4% PFA = 4% paraformaldehyde; YNPRC = Yerkes National Primate Research Center, Emory University, Atlanta, Georgia; VA = Labs of Virginia Inc., Yemassee, South Carolina; Tulane = Tulane National Primate Research Center, Tulane University, Covington, Louisiana; UPSM = University of Pittsburgh School of Medicine, Primate Research Laboratory, Pittsburgh, Pennsylvania; n/a = not available

Table 2

Characteristics of the Antibody Used in This Study

Antigen	Immunogen	Manufacturer, Species, Clonality, Catalog Number, Lot Number
Neuronal Nuclei (NeuN, clone A60)	Purified cell nuclei from mouse brain	Chemicon, mouse monoclonal, MAB377, Lot LV1388597

Table 3

Stereological parameters for optical fractionator

Region of Interest	Section Interval	Grid spacing (μm)	Counting frame area (μm ²)	Disector height (μm)	Guard Zone (μm) Above
Supragranular	1/80	1150 × 1150	400	5	1
Granular	1/80	750 × 750	400	5	1
Infragranular	1/80	750 × 750	400	5	1

Table 4

Estimated volume of area 17 and laminar zones

Subject	Sex	Age	Layer I	Estimated Volume (mm ³)				Total: Area 17
				Supra-granular	Granular	Infra-granular		
AM222	M	7.4	126	732	328	293		1478
AM132	M	7.5	97	791	375	354		1617
AM255	F	9.5	92	694	275	323		1384
AM199	F	10.6	107	757	354	381		1598
AM254	F	11.4	122	752	346	351		1570
AM194	F	11.9	97	794	389	338		1617
AM138x	M	12.0	97	753	377	375		1602
AM195	F	12.1	88	726	257	302		1373
AM215H	M	16.6	78	674	277	347		1376
AM158H	M	16.8	86	622	325	316		1350
AM257	F	17.6	78	730	331	333		1473
AM250	F	18.0	140	844	360	398		1743
AM190	F	18.0	112	637	286	293		1328
AM253	F	18.4	107	635	297	329		1368
AM161	F	19.2	74	479	239	242		1034
AM153H	M	19.4	105	662	348	331		1446
AM216H	M	19.7	122	748	290	382		1542
AM256	F	20.7	93	819	374	387		1674
AM242	M	23.5	107	684	342	424		1557
AM179	F	23.6	107	686	327	328		1447
AM243	M	24.4	84	667	313	321		1385
AM189	M	24.5	104	763	348	315		1530
AM235	F	24.5	58	624	320	329		1331
AM110	M	25.8	109	828	335	371		1643
AM104	F	28.9	108	697	308	354		1466
AM119	F	31.0	115	749	293	348		1505

Estimated Volume (mm ³)						
Subject	Sex	Age	Layer I	Supra-granular	Granular	Infra-granular
Total: Area 17						
		Mean	100	713	324	341
		SD	18	79	39	38
						1478
						147

SD = standard deviation

Table 5

Estimated total number of neurons in area 17 and laminar zones

Estimated Total Number of Neurons						
Subject	Sex	Age	Supra-granular	Granular	Infra-granular	Total: Area 17
AM222	M	7.4	221,333,600	125,460,000	80,190,000	426,983,600
AM132	M	7.5	236,658,730	114,426,000	84,942,000	436,026,730
AM255	F	9.5	201,866,400	113,724,000	78,781,500	394,371,900
AM199	F	10.6	223,026,400	117,526,500	75,816,000	416,368,900
AM254	F	11.4	230,099,130	126,382,500	83,652,750	440,134,380
AM194	F	11.9	241,181,680	144,463,500	87,885,000	473,530,180
AM138x	M	12.0	233,611,690	137,416,500	84,609,000	455,637,190
AM195	F	12.1	228,834,820	102,377,250	72,369,000	403,581,070
AM215H	M	16.6	157,636,710	93,035,250	60,705,000	311,376,960
AM158H	M	16.8	208,484,190	118,383,750	85,338,000	412,205,940
AM257	F	17.6	220,809,890	125,955,000	87,075,000	433,839,890
AM250	F	18.0	218,217,790	127,363,500	82,046,250	427,627,540
AM190	F	18.0	222,608,490	117,261,000	73,822,500	413,691,990
AM253	F	18.4	179,151,140	100,845,000	72,877,500	352,873,640
AM161	F	19.2	137,677,540	79,481,250	51,282,000	268,440,790
AM153H	M	19.4	221,661,580	115,740,000	86,204,250	423,605,830
AM216H	M	19.7	230,368,920	126,126,000	80,907,750	437,402,670
AM256	F	20.7	247,757,150	136,170,000	96,768,000	480,695,150
AM242	M	23.5	204,236,320	133,920,000	84,017,250	422,173,570
AM179	F	23.6	230,696,900	128,560,500	84,150,000	443,407,400
AM243	M	24.4	218,625,120	124,929,000	76,819,500	420,373,620
AM189	M	24.5	228,602,060	127,563,750	70,479,000	426,644,810
AM235	F	24.5	184,409,400	135,702,000	81,983,250	402,094,650
AM110	M	25.8	242,366,640	138,546,000	89,122,500	470,035,140
AM104	F	28.9	188,154,720	107,581,500	77,962,500	373,698,720
AM119	F	31.0	234,749,040	119,196,000	89,887,500	443,832,540
Mean			215,108,694	120,697,529	79,988,192	415,794,415

Estimated Total Number of Neurons						
Subject	Sex	Age	Supra-granular	Granular	Infra-granular	Total: Area 17
SD			26,537,082	14,957,916	9,403,748	46,940,037
Mean CE			0.046	0.041	0.050	0.045
SD			0.003	0.003	0.003	0.003

SD = standard deviation
CE = coefficient of error

Table 6

Estimated total number of glia in area 17 and laminar zones

Subject	Sex	Age	Estimated Total Number of Glia			Total: Area 17
			Supra-granular	Granular	Infra-granular	
AM222	M	7.4	88,448,800	56,272,500	44,651,250	189,372,550
AM132	M	7.5	103,461,820	54,756,000	47,736,000	205,953,820
AM255	F	9.5	65,172,800	44,469,000	36,531,000	146,172,800
AM199	F	10.6	71,097,600	56,088,000	47,567,250	174,752,850
AM254	F	11.4	102,409,110	54,427,500	49,207,500	206,044,110
AM194	F	11.9	71,573,700	49,999,500	45,738,000	167,311,200
AM138x	M	12.0	98,208,850	51,246,000	46,926,000	196,380,850
AM195	F	12.1	90,533,060	44,043,750	47,020,500	181,597,310
AM215H	M	16.6	71,690,080	40,540,500	36,936,000	149,166,580
AM158H	M	16.8	83,761,860	43,796,250	42,570,000	170,128,110
AM257	F	17.6	69,679,880	59,535,000	47,587,500	176,802,380
AM250	F	18.0	105,376,800	56,772,000	49,916,250	212,065,050
AM190	F	18.0	78,593,530	50,890,500	44,178,750	173,662,780
AM253	F	18.4	70,272,360	46,314,000	43,911,000	160,497,360
AM161	F	19.2	48,472,270	38,981,250	31,531,500	118,985,020
AM153H	M	19.4	100,636,960	65,880,000	47,931,750	214,448,710
AM216H	M	19.7	75,583,520	40,713,750	46,431,000	162,728,270
AM256	F	20.7	103,419,500	56,418,750	50,463,000	210,301,250
AM242	M	23.5	67,140,680	47,880,000	49,572,000	164,592,680
AM179	F	23.6	91,337,140	52,065,000	44,946,000	188,348,140
AM243	M	24.4	74,208,120	61,236,000	50,890,500	186,334,620
AM189	M	24.5	113,650,360	67,511,250	46,309,500	227,471,110
AM235	F	24.5	76,398,180	63,504,000	53,597,250	193,499,430
AM110	M	25.8	100,547,030	69,273,000	56,036,250	225,856,280
AM104	F	28.9	87,475,440	52,474,500	54,400,500	194,350,440
AM119	F	31.0	82,344,140	60,984,000	54,123,750	197,451,890
Mean			84,288,215	53,310,462	46,796,538	184,395,215

Estimated Total Number of Glia						
Subject	Sex	Age	Supra-granular	Granular	Infra-granular	Total: Area 17
		SD	15,916,007	8,493,970	5,579,368	25,745,293
		Mean CE	0.073	0.060	0.064	0.066
		SD	0.007	0.004	0.004	0.004

SD = standard deviation
CE = coefficient of error