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# Synapse loss from the rhesus monkey primary visual cortex does not correlate with cognitive decline during aging

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# SYNAPSE LOSS FROM THE RHESUS MONKEY PRIMARY VISUAL CORTEX DOES NOT CORRELATE WITH COGNITIVE DECLINE DURING AGING

by

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**SYNAPSE LOSS FROM THE RHESUS MONKEY PRIMARY VISUAL CORTEX DOES NOT CORRELATE WITH COGNITIVE DECLINE DURING AGING**

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## ABSTRACT

The effect of age on synapses in the neuropil of layers 2/3 in primary visual cortex was determined in 12 rhesus monkeys of various ages (6-33 years old). All of the monkeys had been behaviorally tested. As determined using the size–frequency method, there is a decrease in the numerical density of symmetric, but not asymmetric, synapses with age. There is no significant correlation between the loss of symmetric synapse frequency and the cognitive impairment indices (CII) of the 12 behaviorally tested monkeys. This lack of correlation between synapse frequency reduction and cognitive decline presumably relates to the fact that the primary visual cortex does not have a direct role in subserving cognition.

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## ABBREVIATIONS

| | |
|---|---|
| AI | Age-impaired |
| AU | Age unimpaired |
| CII | Cognitive Impairment Index |
| CSST | Conceptual set-shifting task |
| DNMS | delayed non-match to sample |
| dlPFC | Dorsolateral prefrontal cortex or area 46 |
| DRST | Delayed recognition span task |
| GABA | Gamma aminobutyric acid |
| MTL | Medial temporal lobe |
| PFC | Prefrontal cortex |
| PSC | Postsynaptic current |
| SD | Standard deviation |
| V1 | Primary visual cortex, striate cortex or Bodmann area 17 |

# BODY OF TEXT

## INTRODUCTION

Since all life eventually degrades with advancing chronological age, we accept as normal the non-pathological processes that lead to age-related degeneration. In the normal human brain, one such process is the cortical dysfunction exhibited by some elderly individuals whom, without obvious neuropathological findings, are cognitively impaired. The obvious question arises as to which structural changes underlie this cognitive decline. However, studies of normal aging in the human brain are difficult because of several factors. For example, it is usually not possible to preserve the human brain within the short postmortem interval that is required for optimal structural studies (Peters et al., 2002). Further limiting the study of the normal aging brain in humans is that the detailed cognitive status of individuals is usually unknown, making it impossible to correlate any structural changes with cognitive status. In light of these problems and limitations, it is appropriate to search for an alternative model in which to study the structural changes that occur during normal aging.

Fortunately, the rhesus monkey is an excellent model in which to examine normal aging. These monkeys live upwards of thirty-five years in captivity, so that they have a maximum lifespan of about 1/3 of humans and reach other milestones at this same fraction of human age (Tigges et al., 1988). We can, therefore, equate one rhesus monkey year with three human years and categorically define rhesus monkey age groups.

Furthermore, many of the problems accompanying human studies are avoided by using the rhesus monkey. For instance, rhesus monkeys display a diverse repertoire of complex behaviors similar to those of humans, making it possible to measure their cognitive status using tasks similar to those used to assess the cognitive status of humans. Consequently, it is possible to accurately record changes in cognitive abilities that occur during a rhesus monkey's lifetime, which has revealed that the primary cognitive changes in aged monkeys are similar to those exhibited by aged humans (Morrison et al., 2012; Herndon et al., 1997; Peters, 2002; Peters & Kemper, 2012). Although rhesus monkeys develop some beta amyloid plaques, there is no correlation between plaque load and cognitive decline, nor are there behavioral or structural indications of Alzheimer's disease in rhesus monkeys (Peters et al., 1996). Moreover, after cognitive tests have been carried out, it is possible to preserve the rhesus monkey brain within the short postmortem interval that is required for optimal structural studies (Peters 2002). Consequently, the use of rhesus monkeys allows for a determination of whether specific structural changes do, or do not, correlate with normal cognitive decline.

**Neurobiological basis for cognitive decline**

To explain the cognitive decline that occurs in normal aging, early investigation such as Brody (1955, 1977) and others (e.g. Colon, 1972) came to the conclusion that, in many cortical areas, there are significant losses of cortical neurons with age. However, subsequent studies by others (Cragg, 1975; Haug, 1985; Terry et al., 1987) reached the opposite conclusion. Haug (1985) suggested that the early reports of cortical neuron

losses were due to the fact that when cortical tissue is processed for neuronal count studies the brains of older individuals shrink less than those of younger individuals. The consequence of this is that neuronal densities are higher in younger cortices than older ones. Alternatively, Terry (1987) suggested the earlier reports of neuron loss could be due to the use of individuals with early stages of Alzheimer's disease. More recent investigations have confirmed that neuronal loss in the aged rhesus monkey does not occur in the hippocampus (Keuker et al., 2003; West et al., Gazzaley et al., 1997), cortical area 46 (Peters et al., 1994; Smith et al., 2004), or the primary visual cortex (Peters et al., 1997; Vincent et al., 1989), though neuronal loss has been reported to occur in cortical area 8A (Smith et al., 2004). From these few areas that have been investigated, conclusions regarding the cause of age-related cognitive decline have shifted away from neuronal loss towards other factors.

To determine what those other factors are, a series of structural studies have been completed in the primary visual cortex (also variously known as; V1, striate cortex and area 17) and dorsolateral prefrontal cortex (also known as dlPFC and prefrontal area 46) from cognitively tested rhesus monkeys (for reviews: Luebke et al., 2010; Peters & Kemper, 2012). Cortical area 46 has been chosen for the study of age-related cognitive decline because aged monkeys (as a group) are impaired on behaviors that are subserved by area 46. These area 46 dependent functions that decline with age include spatial and reversal learning tasks, as well as recognition memory tasks (Peters et al., 2001). In

contrast, cortical area 17 does not directly function in cognition, but functions in the initial processing of visual information.

In general, the outcomes of aging studies fall into three groups; (i) factors that do not change significantly with age; (ii) factors that change with age but do not correlate with cognitive decline; and (iii) factors that change with age and correlate with cognitive decline. In area 17, for example, the factors that do not change with age include: frequency of myelinated nerve fibers (Nielsen & Peters, 2000), axonal diameters (Peters et al., 2001), lengths of nodes of Ranvier and paranodes of myelinated fibers (Peters & Sethares, 2003), frequency of altered axons (Peters et al., 2000), frequency of microglia and astrocytes (Peters et al., 2008b), and the frequency of neurons in layer I (Peters & Sethares, 2002a). The area 17 factors, which have been shown to change with age, but show no correlation with cognitive decline include: increased myelin sheath thickness (Peters et al. 2001), increased paranode frequency (Peters & Sethares, 2003), and decreased layer I thickness and synapse density (Peters et al., 2001). The factors that alter with age, and correlate with cognitive decline include: increased frequency of altered myelin sheaths (Peters et al., 2000), and increased frequency of oligodendrocytes - the myelin producing cells (Peters et al., 2008b). In addition, other area specific structural studies identified alterations to myelin sheaths, extensive nerve fiber loss and decreased synaptic numerical density as factors that correlate with cognitive status (for review; Peters & Kemper, 2012).

**Changes to myelin**

As stated above, the frequency of structurally altered myelin sheaths surrounding axons have been shown to correlate with cognitive decline in area 17 (Peters et al., 2000), and the same is true in area 46 (Peters & Sethares, 2002b). In addition to myelin alterations occurring in these cortical areas, there is evidence suggesting myelin alterations also occur in white matter structures such as the splenium (Peters & Sethares, 2002b), fornix and cingulate bundle (Peters et al., 2010; Bowley et al., 2010). The alterations to myelin sheaths, in both the cortex and white matter, probably results from myelin degeneration, which include the local splitting of the sheath at the major dense line to accommodate oligodendrocyte derived dense cytoplasm, and fluid filled myelin balloons that are produced by the splitting of the intraperiod line (Peters, 2002). Other alterations to myelin sheaths may not be degenerative. For example, the increased frequency of nerve fibers with redundant myelin, and the increased frequency of thick myelin sheaths are not degenerative alterations that occur in the cortex (Peters et al. 2001). Regardless of the type of myelin alteration, it is hypothesized, that alterations to myelin sheaths cause a reduction in conduction velocities along the affected nerve fibers, which in turn diminish the integrity of the neuronal circuitry involved (Peters et al., 2000; Peters & Sethares, 2002).

**Nerve fiber degeneration**

In addition to containing axons with altered myelin sheaths, some white matter tracts in old monkeys lose nerve fibers because of degeneration. This occurs in the anterior

commissure (Sandell & Peters, 2003), splenium (Bowley et al., 2010), and fornix (Peters et al., 2010). In these tracts nerve fiber loss exceeds 30%, which correlates with cognitive decline. Conversely, when fiber loss is below 30% - as with the aging genu of the corpus callosum and cingulum bundle - then there is not a correlation with cognitive decline (Bowley et al., 2010). Since nerve fiber loss results from the degeneration of individual axons, nerve fiber loss should result in a loss of synaptic inputs to the target area(s). Since area 17 is of particular interest to this study, it is notable that there is a substantial (45%) age-related nerve fiber loss in the optic nerve, but whether this loss correlates with cognitive decline has never been investigated (Sandell & Peters, 2001). Accordingly, this nerve fiber loss suggests that there is a loss of synaptic input to the lateral geniculate nucleus (LGN), which in turn projects to area 17. Based on this, it is reasonable to hypothesize that if there is a loss synapses from area 17, in part it may be due to a loss of afferent inputs from the LGN.

**Age-related loss of synapses**

The study of the effects of age on area 46 synapses in the rhesus monkey, carried out by Peters and colleagues (2008a), represents the first in-depth analysis of the effects of age on the numerical density of synapses in the neocortex of cognitively tested rhesus monkeys. This study determined that in layers 2/3 and 5 of area 46 there is a significant decrease in asymmetric (excitatory) and symmetric (inhibitory) synapse frequency with age. Furthermore, it was determined that there is a significant correlation between the frequency of asymmetric synapses in layers 2/3 and the extent of cognitive impairment,

the same is true of symmetric synapses although the correlation is weaker. However, in layer 5 there is a decrease in synaptic frequency with age, but there is no correlation between loss of asymmetric or symmetric synapses and cognitive impairment (Peters et al, 2008).

Accompanying the loss of asymmetric and symmetric synapses in layers 2/3 of area 46, certain electrophysiological parameters have been shown to change during normal aging. For example, the layer 3 pyramidal neurons in this cortical area display increased action potential firing rates, decreased synaptic excitation and increased synaptic inhibition (Chang et al., 2005; Luebke et al., 2004). The significant age-related reduction of asymmetric (excitatory) synapses may explain the significantly decreased frequency of excitatory postsynaptic currents (PSCs) in layer 2/3 neurons. However, the significant age-related reduction in the frequency of symmetric (inhibitory) synapses observed in layers 2/3 seems inconsistent with the increased synaptic inhibition seen in these neurons. Other mechanisms must be considered to explain the increased inhibitory PSCs in layer 2/3 pyramidal cells. For example, an increase in action-potential dependent release of GABA from presynaptic interneurons may compensate for the loss of inhibitory synapses (Peters et al., 2008a).

To ascertain if the age-related structural and electrophysiological alterations in area 46 occur elsewhere in the cerebral cortex, we have examined the effects of age on the numerical density of synapses in the neuropil of area 17 of rhesus monkey. This

represents an area distinct in function from area 46, in that it is a primary sensory cortex not directly associated with cognition per se. Layer 2/3 was examined because physiology studies have shown that, in contrast to area 46, the response properties of its pyramidal neurons did not change with age or cognitive status (Luebke, unpublished results).

# BACKGROUND

## Location of the primary visual cortex

In the rhesus monkey (*Macaca mulatta*), the primary visual cortex, also known as area 17, striate cortex, or V1, is located bilaterally in the occipital lobe of the cerebral cortex. Area 17 occupies most of the domed operculum on the lateral surface of the hemisphere, where it is bounded rostrally by the lunate sulcus and inferiorly by the inferior occipital sulcus (Peters & Rockland, 1994). The total volume occupied by area 17 varies across individuals, but in the rhesus monkey it is estimated that area 17 occupies 12,000 square millimeters or about fifteen percent of the total cortical area (Peters & Rockland, 1994). This enormous area occupied by V1 mirrors the importance of vision to the largely arboreal rhesus monkeys.

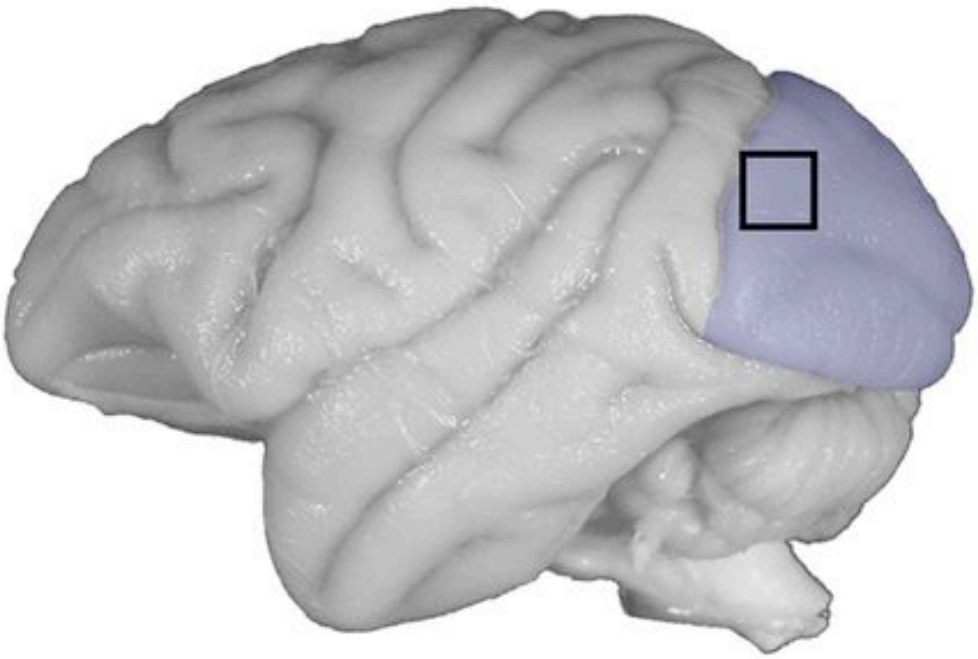

Figure 1. Location of the primary visual cortex.
Photomicrograph of the rhesus monkey brain indicating where the primary visual cortex (blue) is located. Figure amended from Amatrudo et al., 2012.

**V1 cytoarchitecture**

In terms of cytoarchitecture, area 17 is remarkable for its cellular density and lamination. According to stereological estimations, area 17 contains 120,000 neurons per $mm^3$. These neurons are organized into six horizontal layers of neurons that are most commonly labeled in the manner of Brodmann: 1, 2, 3, 4, 5, and 6. The layers are discernible with light microscopy in Nissl stained sections (Figure 1).

Layer 1 is sparsely populated with neurons and primarily contains the apical tufts of pyramidal cells, and the nerve fibers they synapse with. Layers 2 and 3 are often combined because discerning a border between the two is difficult in Nissl stained sections (Wandell, 1995). Layer 2/3 consists of a dense array of small to medium sized pyramidal cell bodies. In area 17, layer 4 is commonly subdivided into three parts. Layer 4A consists of a band of densely packed small and round cells, just below the pyramidal cells of layer 3. Layer 4B predominately contains pyramidal cells and a few large outer Meynert cells. When sections are stained for myelin, a striking band of myelination (known as the stripe of Gennari) is contained within layer 4B. Layer 4C has a heterogeneous cell population and is further subdivided into layers 4Ca and 4Cb, of which layer Cb is the more densely populated sublayer. Layer 5 is another cell-sparse layer, which contains medium-sized pyramidal cells. Layer 6 is subdivided into layer 6A and 6B. Layer 6A contains densely packed pyramidal cells and layer 6B is sparsely populated with neurons that are surrounded by myelinated axons, which are only observable when a myelin stain is employed.

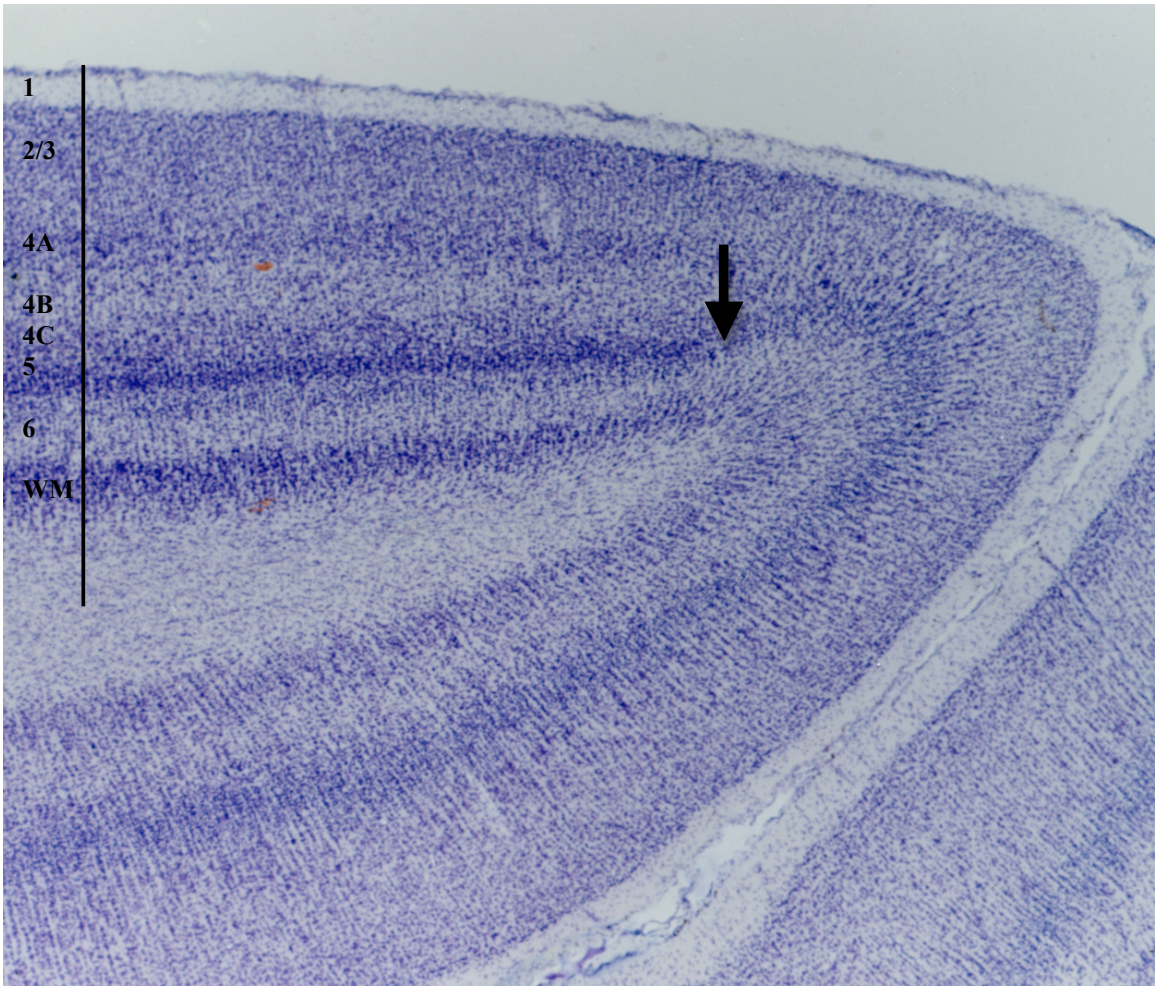


Figure 2. Lamination of the primary visual cortex.
Nissl stained 60 μm-thick section of the primary visual cortex. Note the difference in layering pattern between the primary visual cortex to the left of arrow, and the visual association area 18 to the right of arrow.

## V1 Connectivity

Area 17 is a primary sensory cortex and is the principal destination of visual sensory (afferent) projections and the source of (efferent) projections to the visual association cortices. The visual pathway begins with the retinal photoreceptors, which initiate

electrical signals that travel along the optic nerve to the optic tract and synapse principally in the dorsolateral geniculate nucleus (dLGN) of the thalamus. Afferent fibers from the LGN then project to area 17 thorough three parallel but anatomically and physiologically distinct pathways. The M-pathway consists of magnocellular neurons in the LGN that provide inputs to area 17 and are concerned with motion perception. The P-pathway consists of parvocellular neurons in the LGN and these neurons provide inputs to area 17 that are concerned with detailed vision and colour perception. The K-pathway consists of koniocellular, or interlaminar, neurons and its function(s) remain ambiguous (Peters & Rockland, 1994). This visual circuit, also known as the feed-forward stream of information, then passes directly or indirectly to the visual association areas that include: visual area two (V2), visual area three (V3), visual area four (V4), and visual area five (V5) or middle temporal visual area (MT).

**Morphology of synapses**

At the most basic level, a chemical synapse is the site where one neuron communicates with a second neuron via neurotransmitters. In the cerebral cortex, each chemical synapse consists of a presynaptic axon terminal containing neurotransmitter laden synaptic vesicles and a postsynaptic element (soma, dendrite or dendritic spine) with neurotransmitter receptors. In electron micrographs, the characteristic feature of the presynaptic plasma membrane is the presence of a cytoplasmic density known as the active zone, where vesicles are released. Whereas the characteristic feature of the postsynaptic plasma membrane is the presence of a cytoplasmic density, where synaptic

vesicles bind to receptors (Peters & Palay, 1991). Separating the plasma membranes of the pre- and postsynaptic elements is a gap known as the synaptic cleft, which measures 10 to 20 nm in width (Peters et al., 1991).

In the cerebral cortex there are two morphologically distinct types of chemical synapses, which are described as asymmetric and symmetric synapses (Colonnier, 1968). Morphological parameters used to distinguish between these two types of synapses are: the width of synaptic cleft, the thicknesses of the postsynaptic densities, and the shape of presynaptic vesicles (Peters et al., 2008a). Asymmetric synapses have a wide synaptic cleft, a thick postsynaptic density and uniformly spherical vesicles (Fig. 3. A). In contrast, symmetric synapses have narrower synaptic clefts, thinner and less prominent postsynaptic densities, and smaller and pleomorphic vesicles (Fig. 3 S).

Synapses may also be defined on the basis of their electrophysiological properties. In the cerebral cortex, asymmetric synapses are excitatory and symmetric synapses are inhibitory (Peters & Palay, 1991). This correlation between morphology and electrophysiology gives rise to a synaptic lexicon, in which, “asymmetric” is synonymous with excitatory and “symmetric” is synonymous with inhibitory. The vesicles of axon terminals forming symmetric synapses contain gamma aminobutyric acid (GABA) or glycine (Peters & Palay, 1991). Asymmetric excitatory synapses use the neurotransmitters glutamate and aspartate, which excite postsynaptic elements (Peters

and Palay, 1996). However, the identity of the synapse also relies on the identities of neurotransmitter receptors found on the postsynaptic membrane.

Neurotransmitter receptors change the permeability (conductance) of the postsynaptic membrane, which may increase or decrease the probability of generating an action potential. In the cortex, the vast majority of inhibitory chemical synapses release GABA and exhibit three types of receptors, called $GABA_A$, $GABA_B$ and $GABA_C$. GABA receptors are inhibitory because their associated ion channels become more permeable to chloride ions ($GABA_A$ and $GABA_C$). Alternatively, GABA receptors may activate potassium channels or block calcium channels ($GABA_B$). In the cortex, excitatory chemical synapses chiefly release glutamate, and exhibit three types of receptors, called NMDA receptors, AMPA receptors, and kainite receptors. All glutamate receptors increase the permeability of sodium and potassium across the postsynaptic membrane, and therefore, the glutamate receptors increase the probability of generating an action potential.

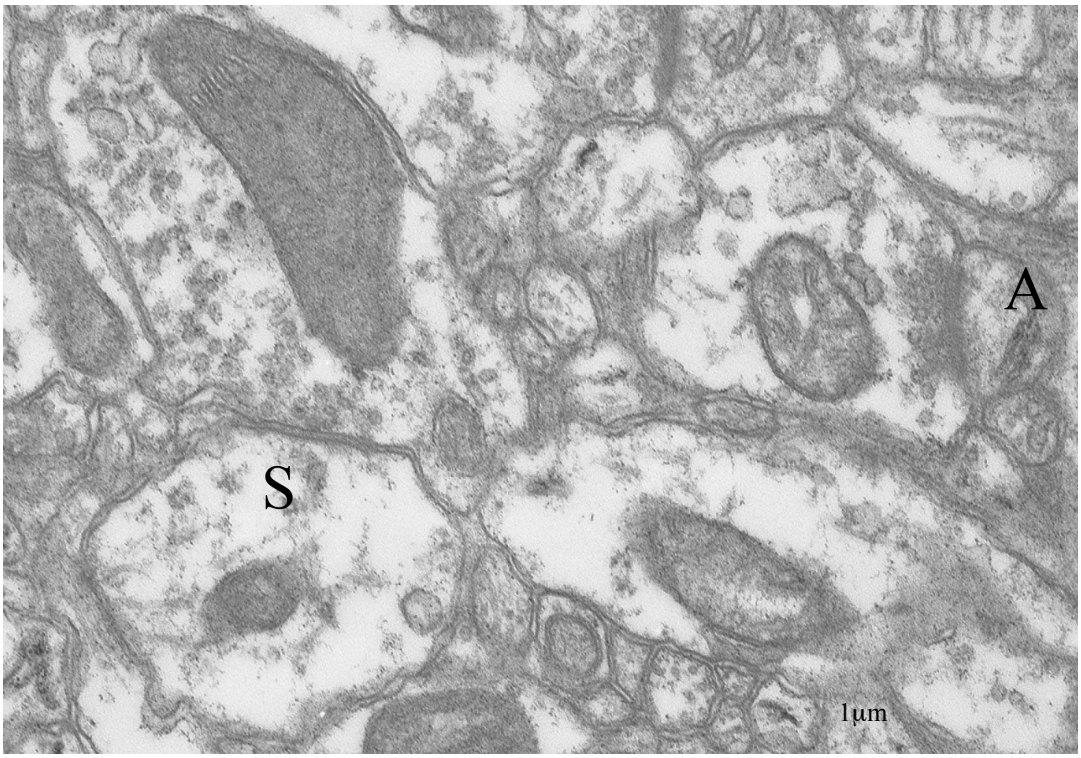


Figure 3. Asymmetric and symmetric synapses.
The electron micrograph from an aged monkey shows a symmetric axodendritic synapse (S), with a narrow synaptic cleft, pleomorphic vesicles, and an inconspicuous postsynaptic density. The postsynaptic element (dendrite) is defined by a relatively large size, the presence of a mitochondria and microtubules. The micrograph also shows an asymmetric axospinous synapse (A), with a thick postsynaptic density and larger spherical vesicles (compared to S). The postsynaptic element is defined as a spine because it contains a spine apparatus.

## OBJECTIVES

The objectives of this study are to ascertain the effects of age on the frequency and ultrastructural characteristics of synapses in the neuropil of layers 2/3 in the rhesus monkey visual cortex.

# METHODS

## Experimental subjects

Monkeys were obtained from Yerkes National Primate Research Center at Emory University (Atlanta, GA, USA), and were subsequently housed individually, under a 12 hour light-dark cycle, in a colony room at the Boston University School of Medicine (BUSM) Laboratory Animal Science Center (LASC). Animals were maintained in strict accordance with the guidelines outlined in the NIH *Guide for the Care and Use of Laboratory Animals* and the *U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals*. Both the Yerkes National Primate Research Center and BUSM LASC are fully accredited by the Association of Assessment and Accreditation of Laboratory Animals and approved all procedures outlined in the following studies.

## Cognitive testing

All of the monkeys used in this study had been behaviorally tested. Rhesus monkeys were trained on, and examined using a number of cognitive tests that are similar to those used for the testing human cognition. These include three visual recognition tests; the delayed non-match to sample (DNMS), the DNMS-2 minute delay, and the delayed recognition span task (DRST). Each of these tests is designed to determine whether specific modalities of memory are affected by aging, and were carried out according to procedures used by the Boston University group (Moss et al., 1988; Moss et al., 1997).

To measure overall cognitive ability in aging monkeys, the Boston University group has employed the cognitive impairment index (CII). The CII was derived based on the principal components analysis, which indicated that overall impairments were predicted by a weighted average of each subject's scores on the DNMS, the DNMS-2 minute delay, and DRST-spatial (see test descriptions above). To simplify this, the scores on each of the three tasks for each individual were converted to a z-score relative to the mean performance of young adults, or the baseline reference group. The CII was then computed as a simple average of the three standardized scores, with positive numbers indicating increasing impairments in z-units from the mean of the baseline reference group of young adult monkeys. With this index, the cutoff for impairment is a CII that is 2.5 standard deviations above the mean for young adult monkeys.

**Tissue specimens and processing**

Each monkey was tranquilized by an intramuscular injection of ketamine (0.1ml/kg body weight), artificially respired, and then anesthetized by an intravenous administration of sodium pentobarbital (Nembutal). Each animal also received an intravenous administration of heparin. The thoracic cavity was opened and an initial warm perfusion solution was injected into the left ventricle of the heart, consisting of 400 ml of 6% dextran and 1% sodium nitrite. This was followed by a second warm perfusion solution consisting of 4 liters of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate or phosphate buffer at 7.4 pH. Immediately following perfusion the brain was removed from the skull. Upon removal one-half of the brain was then immersed in a

stronger aldehyde solution, containing 2% paraformaldehyde and 2.5% glutaraldehyde in the same buffer used for the fixation. Immersion storage lasted for at least one week at 4°C. Small blocks of tissue were taken from the primary visual cortex of four young (6 - 8 years old) and eight aged (25 - 33 years old) rhesus monkeys (Table 2), osmicated and embedded in plastic.

Table 1. Animal tissue preparation.

| Animal AM number | Fixative | Time between anesthetization and first perfusion (minutes) |
|---|---|---|
| Young | | |
| 76 | Karnovsky | 32 |
| 77 | Karnovsky | 20 |
| 129 | Karnovsky | 6 |
| 130 | Karnovsky | 104 |
| Aged | | |
| 19 | Karnovsky | Unknown |
| 100 | Karnovsky | 8 |
| 15 | Karnovsky | Unknown |
| 11 | Karnovsky | 8 |
| 26 | Karnovsky | Unknown |
| 12 | Karnovsky | Unknown |
| 17 | Karnovsky | Unknown |
| 65 | Karnovsky | 54 |

Semithick (1μm) sections were cut from the plastic embedded blocks and orientated so the plane of section passed parallel to the lengths of the apical dendrites, that is, the vertical plane at a right angle to the pial surface. Thick sections were mounted on glass slides and stained with toluidine blue for light microscopic examination. Using a light microscope with a camera lucida, a drawing, was made to identify layers 2/3. Thin

sections (0.1μm) were then taken from the same blocks used to produce thick sections and cut in the same vertical plan. These thin sections were mounted on copper mesh grids and stained with lead citrate.

The thin sections were examined with a JEOL 100S transmission electron microscope. At first, low magnification was used to make a drawing of the thin section to determine the location of layer 3. This was done by comparing the drawings of the section from the light microscope and electron microscope. In a systematic fashion, between ten and fifteen micrographs were taken of layers 2/3 neuropil, of each monkey, at a magnification of X 6000. In taking the electron micrographs of layers 2/3, areas containing a zone of neurons or neuroglial cells were avoided. The result was that the postsynaptic elements examined were either dendrites or dendritic spines, but not neuronal soma. The negatives were then digitally scanned and analysed using RECONSTRUCT$^{TM}$ software.

**Counting Synapses**

The digital images of the electron microscope micrographs were imported into the RECONSTRUCT program (Fiala, 2001). RECONSTRUCT is an application designed for montaging, counting, aligning, tracing, measuring, and reconstructing objects from images of sections. Profiles of synaptic junctions were counted, the lengths of the postsynaptic densities measured, and the synaptic profiles were identified as one of the following types of junction; asymmetric axospinous, asymmetric axodendritic, symmetric axospinous, symmetric axodendritic or uncharacterized. A synaptic junction was counted

only if at least two vesicles were observable, pre- and post-synaptic elements were present and a postsynaptic density was apparent (Peters et al., 2008). More than one investigator made the counts of synapses.

While the majority of synaptic profiles can be easily identified as symmetric or asymmetric, some problems are encountered when synapses are sectioned obliquely. In rare cases the plane of section passes parallel to the synaptic junctions revealing *en face* synapses, and such profiles were not included into synaptic counts. When it was not possible to define the postsynaptic element of a given synapse, the synaptic profile was defined as uncharacterized.

The lengths of synaptic densities of the synaptic junctions were measured in RECONSTRUCT, but only if there was a discernable cleft separating the pre- and postsynaptic elements. If a synaptic junction was curved or perforated, the length of the postsynaptic density was measured linearly between its two end points. For the set of micrographs, for each animal, the lengths of at least 100 asymmetric and at least 50 symmetric synaptic densities were measured and the mean lengths determined.

**Estimating numerical density of synapses**

To determine the numerical density of synapses, the empirical formula suggested by Colonnier and Beaulieu (1985), known as the size-frequency method, was used. The formula is $N_V=N_A/d$, where $N_V$ is the frequency of synapses per unit volume, $N_A$ is the

number of synaptic profiles per unit area of electron micrographs, and *d* is the mean length of synaptic densities. The alternative method used to estimate the synaptic density, is the disector method, which involves preparing serial thin sections and examining identical fields in adjacent sections of a series of thin sections (e.g. Sterio, 1984; Calverley et al., 1988). In comparing the data generated by the disector and size frequency method it has been shown that the two methods give comparable results (Peters et al, 2001; DeFelipe et al., 1999). Since the two methods reveal comparable data and the disector method is more time intensive, it was decided to use the size-frequency method.

# RESULTS

## Age groups

For the purposes of description, the monkeys used in this analysis are divided into two groups: young (5-8 years old) and aged (25 years old and above).

## Lengths of postsynaptic densities

In layers 2/3 the lengths of measurable postsynaptic densities are shown in Tables 3 and 4. As shown in Figure 4, the mean lengths of asymmetric synaptic junctions do not change with age ($p = 0.464$), and neither does the mean length of symmetric synaptic junctions ($p = 0.432$).

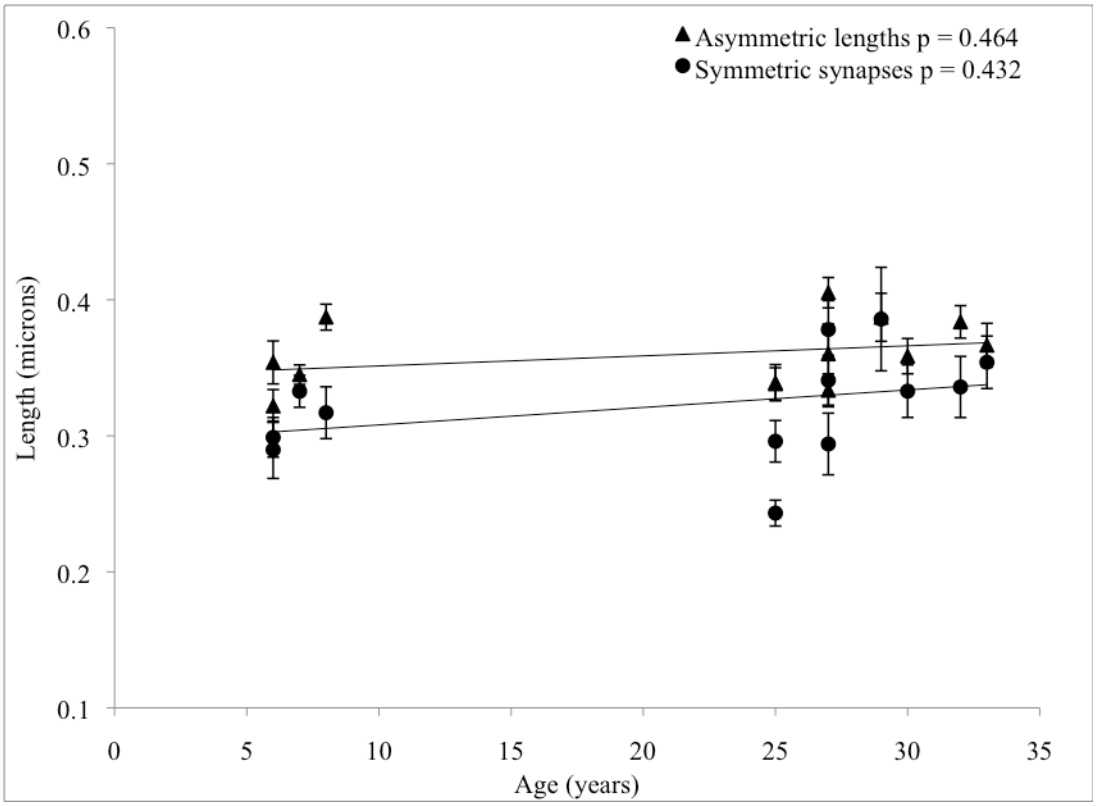


Figure 4. Postsynaptic density lengths versus age.
A plot of the lengths of asymmetric and symmetric postsynaptic densities in the neuropil of layers 2/3 against age.

**Distribution of synapses**

The distribution of asymmetric and symmetric synapses in layers 2/3 of the primary visual cortex is similar for young and aged monkeys, as shown in Table 1 and Figures 4 and 5. In young monkeys 71.8% of asymmetric synapses are axospinous and only 28.2% are axodendritic; and of the symmetric synapses, 40.5% are axospinous and 59.5% are axodendritic. Virtually the same percentage distributions of synapses are present in aged monkeys (Fig. 5 and 6). If a two-tailed t-test is employed, it is evident that the distribution of asymmetric axospinous synapses ($p = 0.806$) and axodendritic synapses ($p = 0.806$), as well as, symmetric axospinous synapses ($p = 0.095$) and symmetric axodendritic synapses ($p = 0.02$) do not change significantly with age in area 17.

Table 2. Percentage distribution of synaptic profiles.

| Group | Asymmetric synapses (%) | | Symmetric synapses (%) | |
|---|---|---|---|---|
| | Axospinous | Axodendritic | Axospinous | Axodendritic |
| Young | 71.8 $\pm$ 0.83 | 28.2 $\pm$ 0.83 | 40.5 $\pm$ 2.51 | 54.0 $\pm$ 2.51 |
| Aged | 71.2 $\pm$ 2.13 | 28.8 $\pm$ 2.13 | 59.5 $\pm$ 4.23 | 46.0 $\pm$ 3.58 |

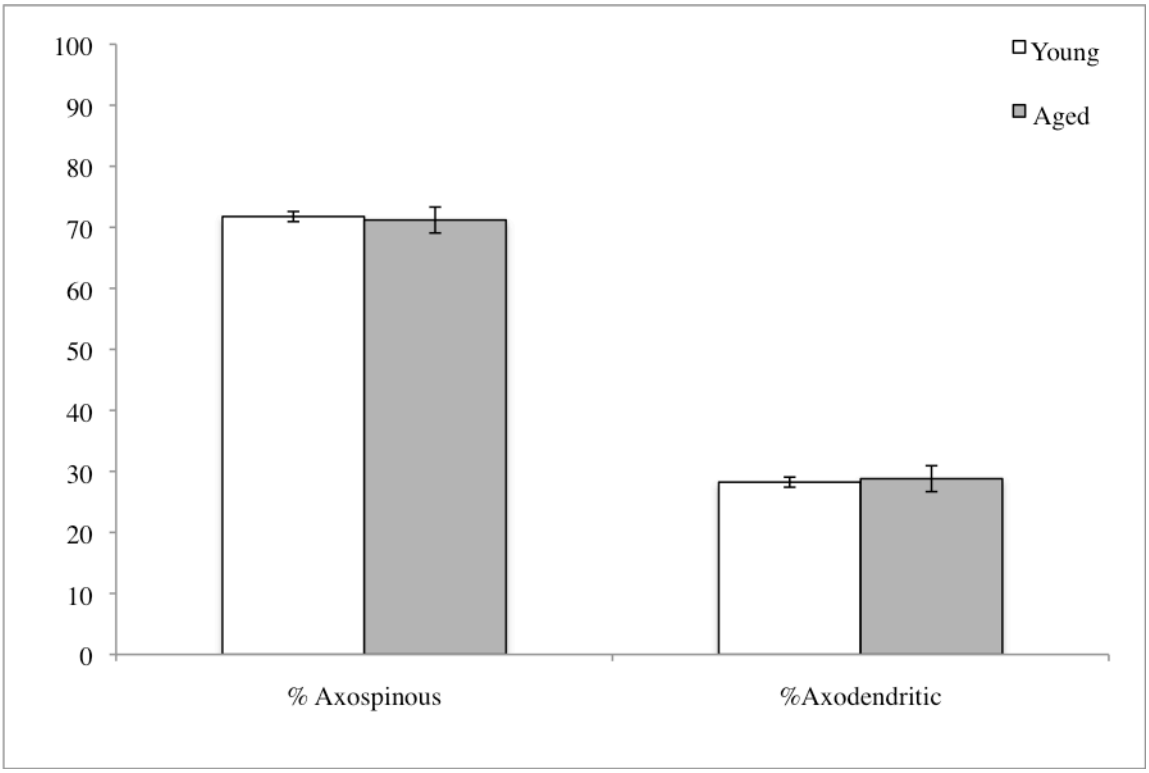


Figure 5. Distribution of asymmetric synapses.
Bar graphs showing the percent distribution of axospinous and axodendritic synaptic profiles in young and aged animals.

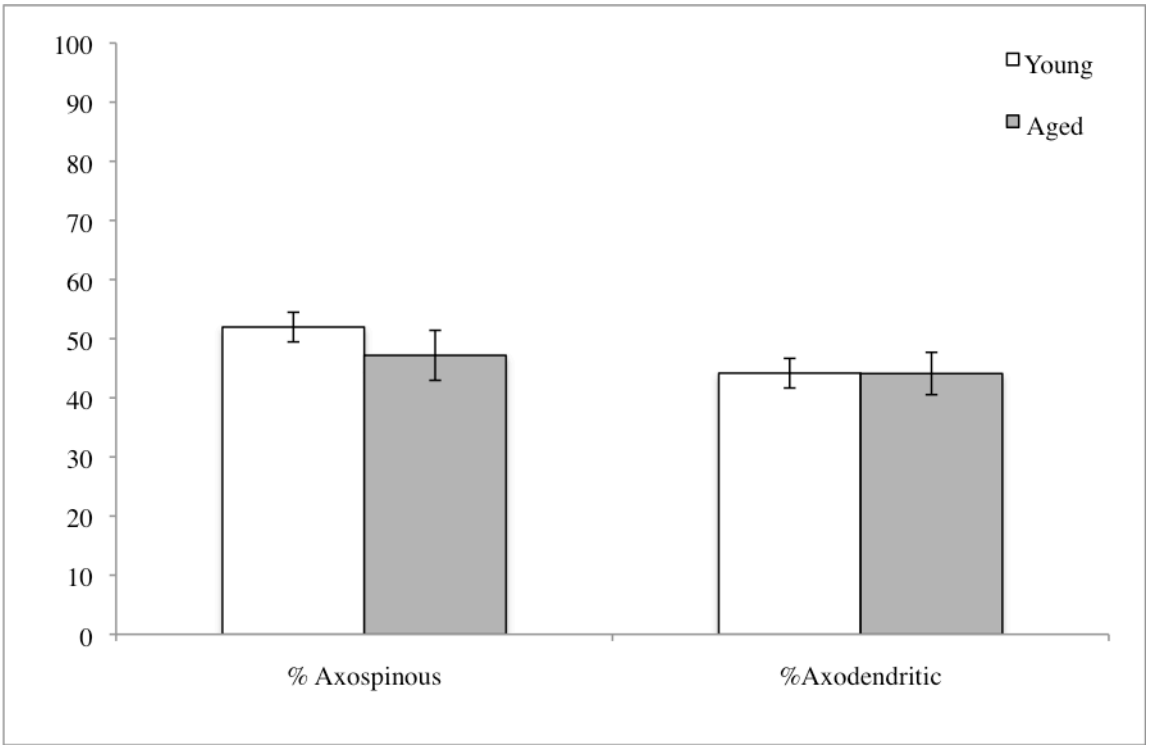


Figure 6. Distribution of symmetric synapses.
A bar graph showing the percent distribution of axospinous and axodendritic synaptic profiles in young and aged animals.

**Numerical density of synapses**

As shown in Table 2, in layers 2/3 of the primary visual cortex of young monkeys the mean numerical density of asymmetric synapses is 436± 61 $x10^6$ per $mm^3$, and for symmetric synapses the mean numerical density is 157±13 $x10^6$ per $mm^3$. Among aged monkeys the mean numerical density of asymmetric synapses is 374± 21 $x10^6$ per $mm^3$, and for symmetric synapses the mean numerical density is 105± 6 $x10^6$ per $mm^3$. Thus, when young and aged monkeys are compared there seems to be a loss of synapses with age (Fig. 7). There is a 62 $x10^6$ per $mm^3$ reduction in mean asymmetric synapse frequency ($p = 0.162$), and a 52 $x10^6$ per $mm^3$ reduction in mean symmetric synapse frequency ($p = 0.001$).

Table 3. Mean numerical densities of asymmetric and symmetric synapses in young and aged groups.

| | Mean numerical synaptic density ($N_v$ $x10^6$ per $mm^3$) | |
|---|---|---|
| Synapses | Young | Aged |
| Asymmetric | 436 ± 61 | 374 ± 21 |
| Symmetric | 157±13 | 105 ± 6 |

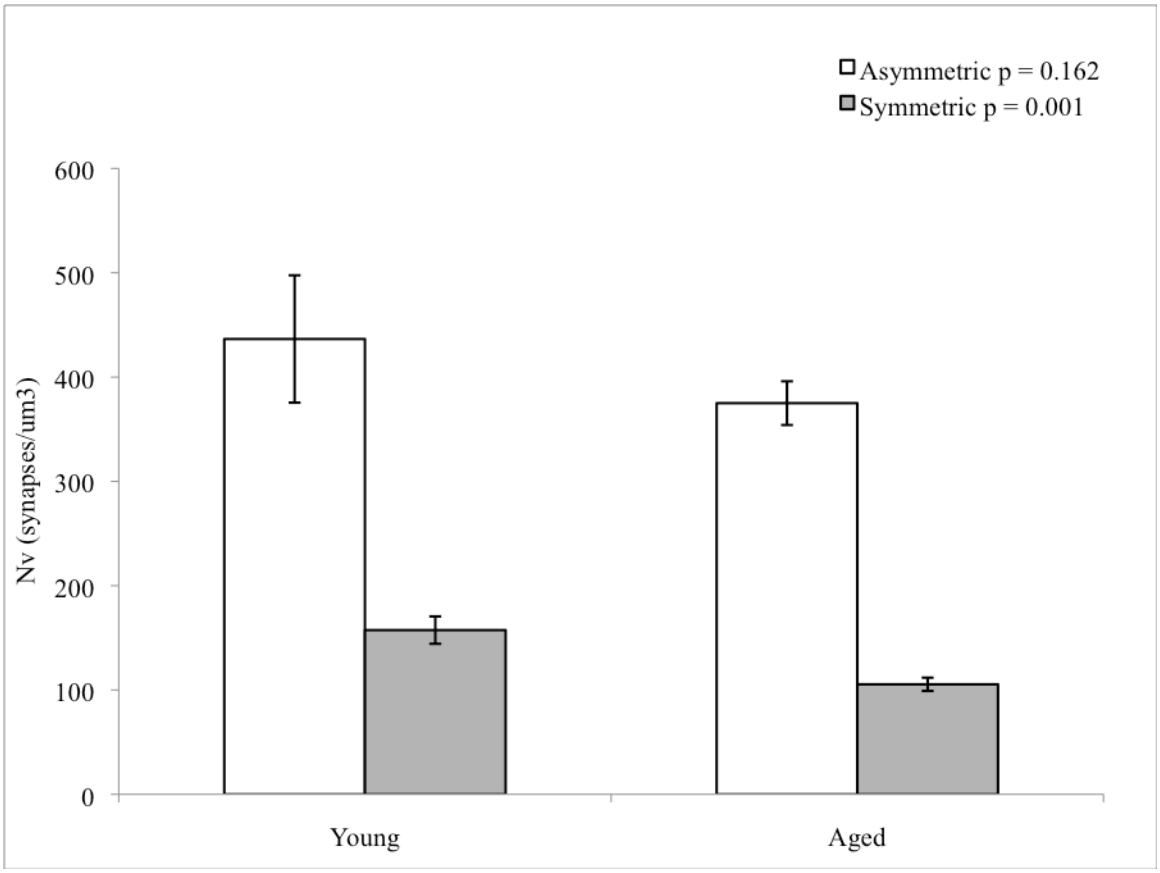


Figure 7. Mean numerical densities of synapses.
Bar graphs showing the mean numerical asymmetric and symmetric synaptic densities for young and aged monkeys.

**Correlation between numerical density and age**

As shown in Figure 8, when all subjects (young and aged) are considered, a linear correlation (Pearson Product Moment) of the date, shows the loss of asymmetric synapses with age is not significant ($p = 0.154$); and when the correlation is done within the aged group only (Fig. 9) the asymmetric synapse frequency loss after 25 years of age is not significant ($p = 0.247$). However, a linear analysis (Fig. 8) of symmetric synapses density data shows that there is a correlation between loss of symmetric synapses and age ($p = 0.003$). Again, when the correlation is done within the aged group only (Fig. 9) it appears that the loss of symmetric synapses after 25 years of age is not significant ($p = 0.222$).

Table 4. Asymmetric synapses in layers 2/3.

| Animal AM number | Age (years) | Mean postsynaptic density length (μm) | $N_v$ x $10^6$/mm$^2$ | Standard error |
|---|---|---|---|---|
| Young | | | | |
| 76 | 6 | 0.32 | 579 | 34 |
| 77 | 6 | 0.35 | 411 | 23 |
| 129 | 7 | 0.34 | 431 | 22 |
| 130 | 8 | 0.39 | 325 | 17 |
| Aged | | | | |
| 19 | 25 | 0.34 | 448 | 21 |
| 100 | 25 | 0.34 | 444 | 29 |
| 15 | 27 | 0.41 | 401 | 31 |
| 11 | 27 | 0.33 | 298 | 25 |
| 26 | 29 | 0.39 | 415 | 27 |
| 12 | 27 | 0.36 | 303 | 27 |
| 17 | 30 | 0.36 | 327 | 30 |
| 65 | 33 | 0.37 | 362 | 20 |

Table 5. Symmetric synapses in layers 2/3.

| Animal AM number | Age (years) | Mean postsynaptic density length (μm) | $N_v$ x $10^6$/mm$^2$ | Standard Error |
|---|---|---|---|---|
| Young | | | | |
| 76 | 6 | 0.29 | 137 | 14 |
| 77 | 6 | 0.30 | 139 | 14 |
| 129 | 7 | 0.33 | 181 | 15 |
| 130 | 8 | 0.32 | 172 | 17 |
| Aged | | | | |
| 19 | 25 | 0.24 | 104 | 10 |
| 100 | 25 | 0.34 | 125 | 11 |
| 15 | 27 | 0.38 | 108 | 11 |
| 11 | 27 | 0.29 | 137 | 10 |
| 26 | 29 | 0.39 | 77 | 7 |
| 12 | 27 | 0.34 | 113 | 12 |
| 17 | 30 | 0.33 | 112 | 10 |
| 65 | 33 | 0.35 | 110 | 7 |

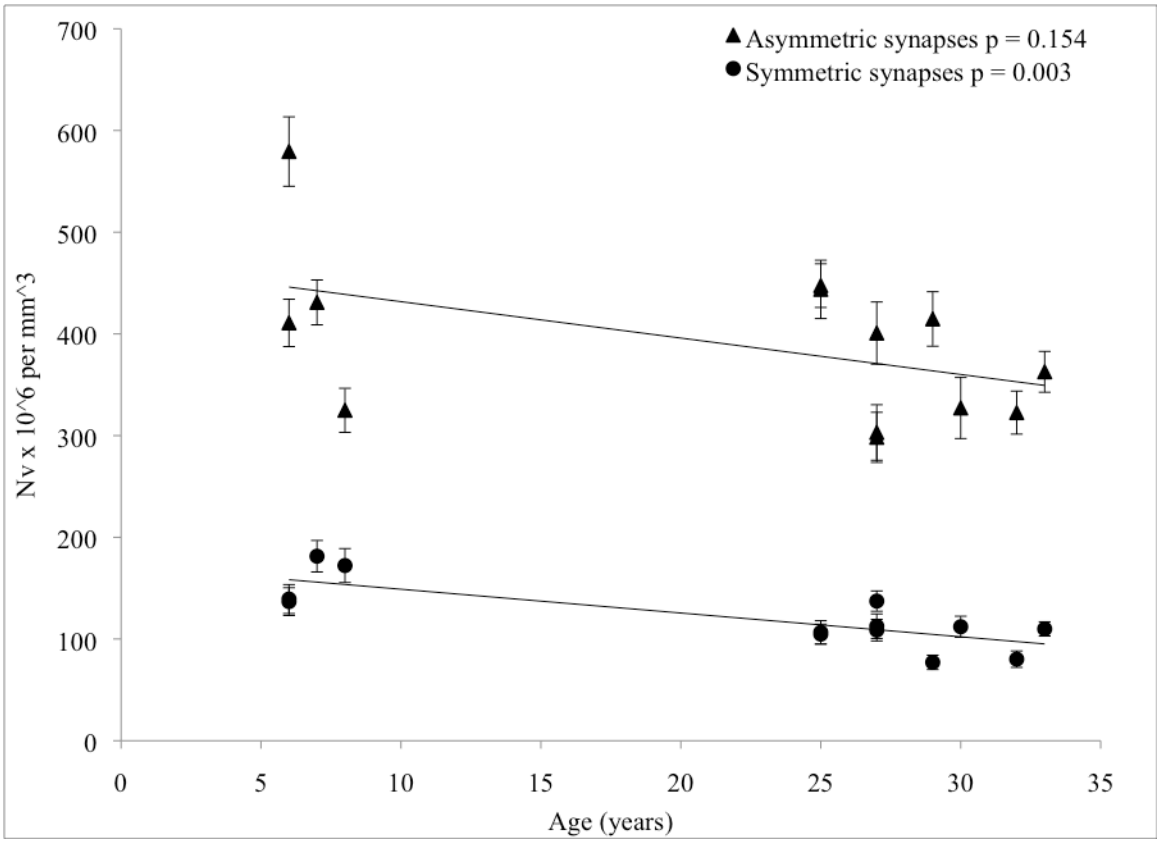


Figure 8. Numerical density of synapses versus age.
A plot of the numerical density of asymmetric and symmetric synapses per $mm^3$ of the neuropil of layers 2/3 against age.

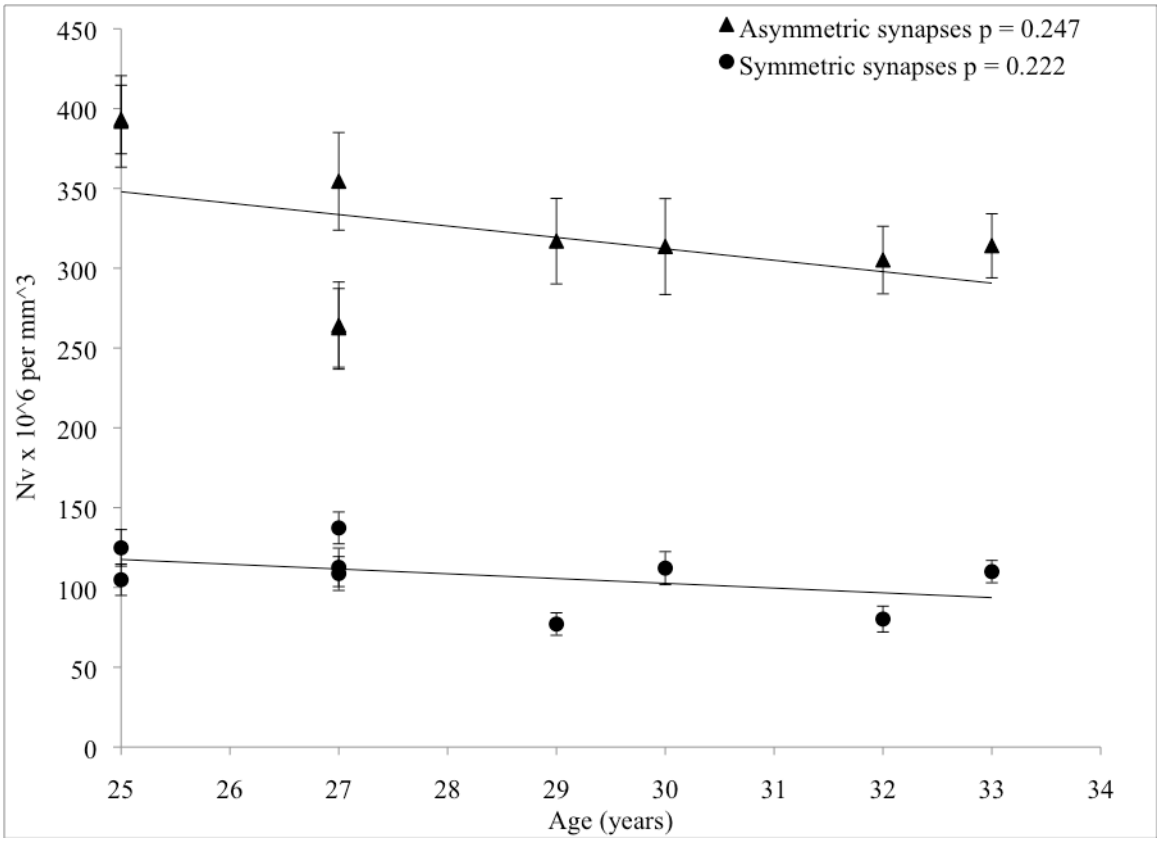


Figure 9. Numerical density of synapses versus age, aged individuals only.
A plot of the numerical density of aged monkey asymmetric and symmetric synapses per $mm^3$ of the neuropil of layers 2/3 against age.

**Behavioral correlates**

The scores that the thirteen monkeys in this study achieved on the behavioral tasks are given in Table 5. As shown in Figure 10, there is no correlation between the numerical density of asymmetric synapses and cognitive impairment index (CII) (P = 0.136), nor is there a correlation between symmetric synapses and CII (P = 0.552).

Table 6. Behavioral task scores.

| Animal AM Number | Age (years) | CII | DNMS (errors) | DNMS, 120-minute delay | DRST (spatial) |
|---|---|---|---|---|---|
| 76 | 6 | 0.08 | 58 | 0.91 | 2.35 |
| 77 | 6 | 2.27 | 85 | 0.66 | 2.07 |
| 129 | 7 | 1.87 | 114 | 0.75 | 2.24 |
| 130 | 8 | 1.28 | 121 | 0.84 | 2.32 |
| 19 | 25 | 1.98 | 111 | 0.72 | 2.31 |
| 100 | 25 | 3.59 | 241 | 0.73 | 2.04 |
| 15 | 27 | 1.76 | 166 | 0.90 | 1.84 |
| 11 | 27 | 1.51 | 50 | 0.72 | 2.03 |
| 15 | 27 | 1.76 | 166 | 0.90 | 1.84 |
| 26 | 29 | 1.05 | 83 | 0.85 | 1.98 |
| 12 | 27 | 3.31 | 235 | 0.77 | 1.97 |
| 17 | 30 | 2.85 | 195 | 0.78 | 1.89 |
| 65 | 33 | 3.24 | 265 | 0.85 | 1.81 |

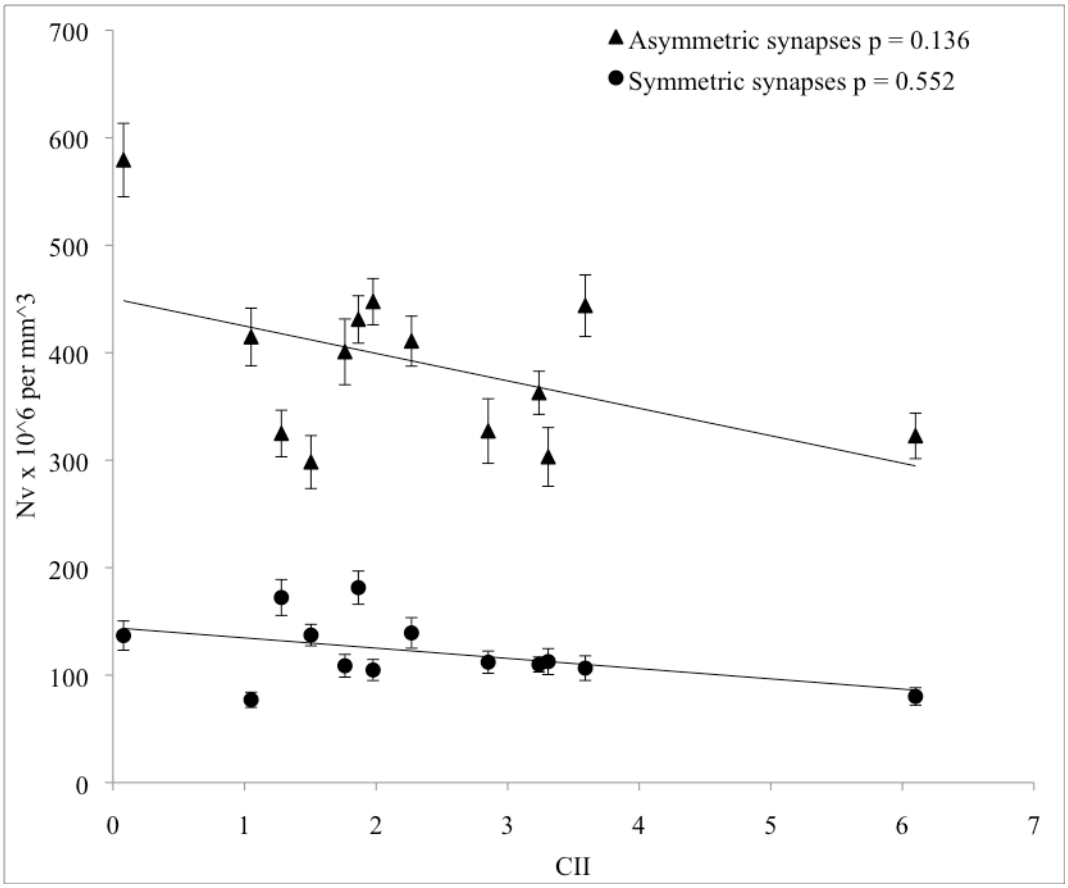


Figure 10. Synapse density versus CII.
A plot of the number of asymmetric and symmetric synapses present per $mm^3$ in the neuropil of layers 2/3 against CII scores.

## DISCUSSION

In summary, this study shows that while there is a significant age-related reduction in symmetric synapse frequency in layers 2/3 of the rhesus monkey primary visual cortex. However, there is not a concomitant age-related loss of asymmetric synapse frequency. Additionally, there is no correlation between symmetric, or asymmetric, synapse frequency and cognitive status (CII). At which age in the monkey lifespan symmetric synapse frequency loss begins remains unknown. However, since there is no correlation between numerical synaptic density and age when only the aged group is considered (Fig. 8), the decline in symmetric synapse frequency must occur before the age of twenty-five, which is the age of the youngest monkey in the aged cohort.

Similar to area 17, in cortical area 46 the numerical symmetric synapse density decreases in layers 2/3 (Peters et al., 2008). However, unlike area 17, the age-related decrease in symmetric numerical synaptic density correlates with cognitive impairment index (CII) (Peters et al., 2008). By comparing the results from area 17 to those from area 46, there is a difference in the effect caused by symmetric synapse loss. Presumably, area 46 symmetric synapse frequency correlates with CII because the area participates in functions related to cognition; and area 17 symmetric synapse frequency does not correlate with CII because the area does not participate in functions related to cognitive status. Similarly, the same hypothesis is supported the fact that numerical synaptic density of layer 1 decreases with age in both area 17 and 46, but only in area 46 does the loss of synapses correlate with CII (Peters et al., 1998; Peters et al., Peters et al., 2001).

The fact that symmetric synapse loss in layers 2/3 of area 17 does not correspond with cognitive decline is interesting because the electrophysiological parameters of the pyramidal cells residing in layers 2/3 do not change significantly with age (Luebke; unpublished). In area 46, by comparison, the electrophysiology of layer 2/3 pyramidal neurons does alter with age (Luebke et al., 2010 review). These pyramidal neurons show an increase in the frequency of spontaneous inhibitory postsynaptic currents (IPSCs) (Luebke et al., 2004), which suggests there is a compensatory increase of activity in the remaining inhibitory axon terminals, and thereby affecting cognitive status. A similar compensatory mechanism may occur in layers 2/3 of area 17, but since IPSC frequency does not increase in area 47, the compensation maintains rather than alters the function of these pyramidal neurons.

Even though the symmetric synapse frequency decreases with age in layers 2/3 of area 17, the loss does not correlate with cognitive decline, as measured by the cognitive impairment index (CII). However, it is not yet known if the decrease in synaptic frequency correlates with other behavioral changes. For example, the age-related symmetric synapse frequency decrease may lead to the reported age-related alterations to receptive field properties in area 17, especially since these properties are thought to largely depend on inhibitory mechanisms (Schmolesky et al., 2000; Leventhal et al. 2003; Wang et al., 2005). It would be interesting to determine whether symmetric synapse loss corresponds with the visual functions that contribute to the age-related deficits in these

receptive field properties, such as decreased orientation and direction selectivity, increased visual responsiveness, increased spontaneous activity, and a decreased ability to signal visual stimuli above background activity (Schmolesky et al., 2000).

In contrast to the area 17 symmetric (inhibitory) synaptic density - and what has been reported in the prefrontal cortex area 46 - the asymmetric synapse density is not affected by age, on the basis of linear plots (Fig. 8). However, when the mean asymmetric synapse frequencies are compared between young and aged groups a trend towards age-related loss of synapse frequency is observed (Fig. 7). This discrepancy between the two analyses is likely due to the unusually high asymmetric numerical synaptic density of one individual (AM 76) in the young group of monkeys. This skews the mean numerical synapse density of this group. If the data from AM 76 is omitted, then the decline in mean numerical synaptic density between young and aged groups disappears. Supporting this interpretation is that when AM 76 is included, the standard error for the young mean numerical synaptic density is above 10%, but when AM 76 is omitted the standard error drops below 10%. Further analyses of using additional young animals are needed to confidently determine the relationship of asymmetric numerical synapse density with age.

Interestingly, earlier studies show that there is no loss of dendritic spines from the basal apical dendrite of pyramidal neurons residing in layers 2/3 of area 17 (Amatrudo, 2012). These dendritic spines represent the postsynaptic elements in the majority of asymmetric

synapses residing in layers 2/3. Therefore, the absence of loss of basal dendritic spines supports the finding that there is no loss of asymmetric synapses in layers 2/3 of area 17.

In conclusion, in layers 2/3 of area 17 there is a significant loss of symmetric synapses with age, but this decline does not correlate with cognitive status (CII). However, the loss of symmetric synapses may be involved in the age-related decline in visual receptive field properties. In contrast, the population of asymmetric synapses examined in layers 2/3 of area 17, is not affected by age. To determine if declining synapse frequency is only found in areas of the brain directly involved in cognition, more association areas of the brain need to be examined.

## LIST OF JOURNAL ABBREVIATIONS

| | |
|---|---|
| ILAR | Journal of the National Research Council, Institute of Laboratory Animal Resources |

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