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Conservation of Neuron Number and Size in Entorhinal Cortex Layers II, III, and V/VI of Aged Primates

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ABSTRACT

Past dogma asserted that extensive loss of cortical neurons accompanies normal aging. However, recent stereologic studies in humans, monkeys, and rodents have found little evidence of age-related neuronal loss in several cortical regions, including the neocortex and hippocampus. Yet to date, a complete investigation of age-related neuronal loss or size change has not been undertaken in the entorhinal cortex, a retrohippocampal structure essential for learning and memory. The aged rhesus macaque monkey (*Macaca mulatta*), a species that develops β-amyloid plaques and exhibits cognitive deficits with age, is considered the best commonly available model of aging in humans. In the present study, we examined changes in total neuron number and size in layers II, III, and V/VI of the intermediate division of the entorhinal cortex in aged vs. nonaged rhesus monkeys by using unbiased stereologic methods. Total neuron number was conserved in aged primates when compared with nonaged adults in entorhinal cortex layer II (aged = 56,500 ± 12,100, nonaged adult = 48,500 ± 10,900; P = 0.37), layer III (aged = 205,600 ± 50,700, nonaged adult = 187,600 ± 60,300; P = 0.66), and layers V/VI (aged = 246,400 ± 76,700, nonaged adult = 236,800 ± 69,600; P = 0.87). In each of the layers examined, neuronal area and volume were also conserved with aging. This lack of morphologically evident neurodegeneration in primate entorhinal cortex with aging further supports the concept that fundamental differences exist between the processes of normal "healthy" aging and pathologic age-related neurodegenerative disorders such as Alzheimer's disease. J. Comp. Neurol. 422:396–401, 2000.

Indexing terms: aging; rhesus monkey; stereology; memory; neurobiology

Aging in primates clearly results in declines in brain size, weight and function (Chrzanowska and Beben, 1973; Dekaban, 1978; Bachevalier et al., 1991; Murphy et al., 1992; Cabeza et al., 1997; Herndon et al., 1997; Andersen et al., 1999; Small et al., 1999). Yet the cellular and morphologic substrates underlying these changes remain poorly characterized. Although long-held dogma suggested that aging is associated with a frank loss of neurons in the primate brain, careful recent studies have revealed modest if any change in cell number and size in a variety of brain regions with aging, including the neocortex and hippocampus (West, 1993; Wickelgren, 1996; Morrison and Hof, 1997; Simic et al., 1997; Peters et al., 1998).

Among the clear functional changes that occur with aging in primates are declines in some aspects of cognition and memory. In particular, aging affects short-term memory (Bartus et al., 1978), memory acquisition and early retrieval (Small et al., 1999), working memory (Grady et al., 1998), recognition memory (Rapp and Amaral, 1991; Grady et al., 1995; Moss et al., 1997), reasoning (Gilinsky and Judd, 1994), and processing speed (Kail and Salthouse, 1994; Robbins et al., 1994). Some of these functions are mediated through the temporal lobe memory system

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(TLMS), which includes the hippocampus proper, subiculum, presubiculum, parasubiculum, and entorhinal cortex (Squire and Zola, 1996). The entorhinal cortex in particular serves as a gateway structure that functionally interconnects broad areas of cerebral cortex with the hippocampus. The entorhinal cortex receives widespread cortical inputs from polymodal association areas and projects from layers II and III by means of the perforant path to the hippocampal dentate gyrus and Ammon's horn, respectively (Bjorklund et al., 1987). After extensive hippocampal processing of this information, the entorhinal cortex receives projections back from the hippocampus and subiculum. These inputs are then sent to widespread cortical regions by neurons in layers V/VI. Thus, each layer of entorhinal cortex is a key component in memory formation. Age-related loss of neurons in just one of these entorhinal layers could have a profound impact on function of the hippocampus and the diverse regions of cortex to which entorhinal cortex projects, potentially disrupting some of the same memory systems that become dysfunctional with aging.

Thus, each of the layers of the entorhinal cortex is a logical target for investigation of effects of aging on memory, because each layer comprises a major input or output from entorhinal cortex. Only one study to date has examined change in neuron number in entorhinal cortex as a function of age; this study was restricted to layer II and reported intact numbers of neurons in aged rhesus monkeys relative to nonaged monkeys (Gazzaley et al., 1997). To date, a comprehensive examination of all layers of the primate entorhinal cortex as a function of age has not been conducted, however. Furthermore, no studies of age-related change in cell size have been conducted in the entorhinal cortex, despite the possibility that reduction in neuronal size could comprise an important component of age-related atrophy. The goal of this study was to comprehensively investigate the layers of primate entorhinal cortex by using unbiased stereologic methods to determine whether changes in either cell number or size occur as a potential mechanism correlating with age-related cognitive decline.

MATERIALS AND METHODS

Subjects

Two groups of rhesus monkeys were studied: four aged monkeys (mean age, 24.3 ± 1.9 years; all males) and four nonaged adult monkeys (mean age, 11.3 ± 2.1 years; 3 males, 1 female). All subjects were born at the California Regional Primate Center and spent the majority of their lives in 0.5-acre field enclosures containing social groups of 80–100 animals. All animal care procedures adhered to American Association for the Accreditation of Laboratory Animal Care and institutional guidelines.

Tissue processing and histochemistry

Animals were sedated with 25 mg/kg i.m. ketamine and were then deeply anesthetized with Nembutal (30 mg/kg i.p.). After verifying that all reflex responses to cutaneous stimulation were absent, subjects were perfused transcardially for 1 hr with a 4% solution of paraformaldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C followed by 5% sucrose solution. Brains were stereotaxically blocked in the coronal plane, and serial coronal sections through the brain were cut on a freezing microtome set at 40μm. Every 12th section was Nissl stained with thionin for stereologic quantification.

Anatomic boundaries

The intermediate subdivision of the primate entorhinal cortex, the largest of the three major components of entorhinal cortex, was examined because of the well-defined laminar and anatomic boundaries of this region. Specifically, the intermediate division of the entorhinal cortex is the only region that contains layer II "cell islands" and a clearly definable cell-sparse layer IV "lamina dessicans" (Fig. 1B). In contrast, the rostral entorhinal cortex contains cell islands but no lamina dessicans (Fig. 1A), and layer II in the caudal entorhinal cortex contains a continuous cell layer (rather than islands) and lacks a lamina dessicans (Fig. 1C). Furthermore, the anatomic boundaries between the caudal entorhinal cortex and the adjacent parahippocampal gyrus are indistinct, rendering a stereologic quantification of the caudal entorhinal cortex problematic. Thus, the intermediate division of the entorhinal cortex is most distinct, constitutes the largest contribution of primary entorhinal inputs and outputs to hippocampus and cortex, and is, therefore, a logical and valid target for stereologic quantification.

The following anatomic features were used to define borders of the intermediate entorhinal cortex for stereologic quantification (Van Hoesen and Pandya, 1975; Amaral et al., 1987). *Rostral:* the *first* coronal section moving from rostral-to-caudal that contained cell islands in layer II and a complete layer IV; *Caudal:* the *last* coronal section moving from rostral-to-caudal that contained cell islands in layer II and a complete layer IV; *Medial:* the appearance of layer IV and cell islands in layer II just ventral to the parasubiculum; and *Lateral:* the disappearance of a clear layer IV in lateral entorhinal, or prorhinal, cortex. The prorhinal cortex was not quantified because of the absence of a clear layer IV, which made the border between prorhinal and perirhinal cortex at the rhinal sulcus difficult to define precisely.

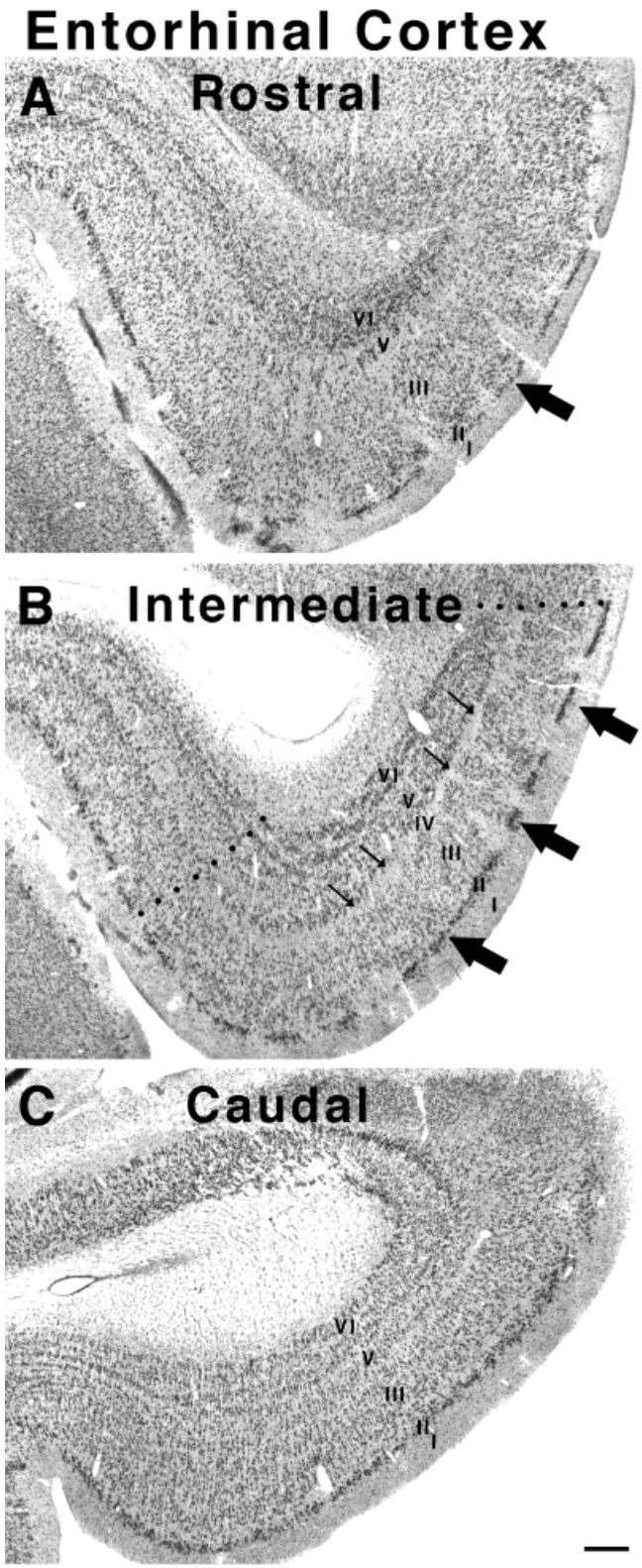
Borders between individual laminae in the entorhinal cortex were distinguished as follows (see Fig. 1B): Laminar boundaries between layers II and III were easily distinguishable due to the increased size and staining intensity of layer II cell islands. Cell-sparse layer IV clearly separated layer III from layer V, providing a distinct boundary between III and V. Layers V and VI were quantified together, because they are difficult to resolve into individual laminae within a single section, and their projection patterns are similar (Amaral et al., 1987; Bjorklund et al., 1987).

Stereology

A 1-in-12 series of Nissl-stained sections through the entire extent of the intermediate entorhinal cortex was used to quantify cell number and size in layers II, III, and V/VI of each subject. To correct for shrinkage during processing, tissue thickness was measured in each section at 60× in five random locations, and a mean thickness from these measurements was used in stereologic equations.

The unbiased optical fractionator and nucleator stereologic methods were used to estimate total number and size of principal neurons, respectively. Briefly, the optical fractionator relies on a three-dimensional probe, the optical disector, and a systematic uniform sampling scheme (the

fractionator) to provide unbiased estimates of total neuron number (West et al., 1991). Optical fractionator estimates are free of assumptions about neuron size and shape, and are unaffected by tissue processing artifacts. Within each optical disector, cells were counted only if the nucleolus came into focus within the disector height, which was set at the middle 75% of total tissue thickness for each section. The optical disector dimensions were set at 50 μm^2. For each entorhinal cortical layer, a specific fraction of the total area was sampled based on preliminary estimates of cell number. Sampling was optimized to produce a coefficient of error (CE) under the observed biological variability. The nucleator was used to provide estimates of the profile area of cells and absolute volume (Gundersen, 1988). The nucleator identifies a point associated with a cell, in this case the nucleolus, from which five rays are extended. The intersections of the rays with the boundary of the cell are located and marked. It is assumed that the tissue has been sectioned in parallel planes and that the cells will be observed in random three-dimensional positions.



The stereology set-up consisted of an Olympus AX-70 microscope fitted with a Ludl-2002 motorized stage and an Olympus video camera. Stereology was completed by using Stereo Investigator software (Microbrightfield, Inc.). Each entorhinal cortical layer was outlined at low-power (4×) and then cells were quantified by using the stereology program's pseudorandom sampling scheme under a high-power oil objective (60×, numerical aperture = 1.4). All analyses were performed with the examiner blinded to the identity of the subject. Neurons were distinguished from other objects such as astrocytes and microglia based on the presence of a readily distinguishable nucleolus within the cell in question, in accordance with criteria previously used in stereologic studies to identify neurons (West, 1993; West and Slomianka, 1998).

Statistical analysis

Comparisons between the aged and nonaged groups were made by using Student's two-tailed, unpaired *t* test. Criteria for significant differences were set at the 95% probability level. Data are reported as mean ± standard deviation.

RESULTS

On the basis of the anatomic boundaries described above, the mean number of Nissl-stained histologic sections quantified for stereologic analysis in the aged and nonaged groups was identical: 5.75 ± 1.50 sections were examined in the aged group vs. 5.75 ± 1.50 sections in the nonaged group. Similarly, mean tissue section thickness was similar in the two groups: mean thickness was 20.7 ±

Fig. 1. Coronal sections of nonaged adult primate entorhinal cortex illustrating the anatomic boundaries (black dots) used to demarcate the intermediate subdivision of entorhinal cortex. **A:** Rostral entorhinal cortex contains layer II "cell islands" (large arrow) but no layer IV "lamina dessicans." **B:** Intermediate entorhinal cortex includes both layer II "cell islands" (large arrows) and a clearly definable layer IV "lamina dessicans" (small arrows), which is a cell-sparse layer only visible in intermediate entorhinal cortex with Nissl stains. **C:** In caudal entorhinal cortex, layer II becomes continuous and lacks "cell islands," whereas layer IV is no longer present. The figure was produced by using Adobe Photoshop (Adobe Systems, Mountain View, CA). Scale bar = 0.5 mm (applies to A–C).

TABLE 1. Number of Neurons in the Intermediate Entorhinal Cortex

Layer	Group	Animal	Age (yr)	Neuron number	Group mean	SD	*P* value
II	Non-aged	Y1	8.2	64,209			
		Y2	8.7	45,264			
		Y3	10.4	45,629			
		Y4	15.3	39,043	48,500	±10,900	
	Aged	A1	20.4	64,445			0.37
		A2	21.7	41,160			
		A3	26.3	67,737			
		A4	28.7	52,449	56,400	±12,100	
III	Non-aged	Y1	8.2	276,800			
		Y2	8.7	147,252			
		Y3	10.4	155,150			
		Y4	15.3	171,226	187,600	±60,300	
	Aged	A1	20.4	225,529			0.66
		A2	21.7	174,400			
		A3	26.3	267,156			
		A4	28.7	155,176	205,600	±50,700	
V/VI	Non-aged	Y1	8.2	336,000			
		Y2	8.7	182,465			
		Y3	10.4	195,138			
		Y4	15.3	233,636	236,800	±69,600	
	Aged	A1	20.4	348,690			0.87
		A2	21.7	179,200			
		A3	26.3	260,757			
		A4	28.7	196,769	246,400	±76,700	

TABLE 2. Area of Neurons in the Intermediate Entorhinal Cortex

Layer	Group	Animal	Age (yr)	Area estimate	Group mean	SD	*P* value
II	Non-aged	Y1	8.2	312			
		Y2	8.7	302			
		Y3	10.4	306			
		Y4	15.3	305	306	±4	
	Aged	A1	20.4	272			0.67
		A2	21.7	298			
		A3	26.3	280			
		A4	28.7	345	299	±33	
III	Non-aged	Y1	8.2	252			
		Y2	8.7	284			
		Y3	10.4	264			
		Y4	15.3	252	263	±15	
	Aged	A1	20.4	257			0.21
		A2	21.7	250			
		A3	26.3	247			
		A4	28.7	253	252	±4	
V/VI	Non-aged	Y1	8.2	205			
		Y2	8.7	258			
		Y3	10.4	215			
		Y4	15.3	220	225	±23	
	Aged	A1	20.4	207			0.25
		A2	21.7	196			
		A3	26.3	216			
		A4	28.7	216	209	±9	

TABLE 3. Volume of Neurons in the Intermediate Entorhinal Cortex

Layer	Group	Animal	Age (yr)	Volume estimate	Group mean	SD	*P* value
II	Non-aged	Y1	8.2	4,667			
		Y2	8.7	4,399			
		Y3	10.4	4,509			
		Y4	15.3	4,558	4,533	±112	
	Aged	A1	20.4	3,751			0.65
		A2	21.7	4,291			
		A3	26.3	3,901			
		A4	28.7	5,452	4,349	±770	
III	Non-aged	Y1	8.2	3,399			
		Y2	8.7	4,076			
		Y3	10.4	3,691			
		Y4	15.3	3,502	3,667	±298	
	Aged	A1	20.4	3,499			0.13
		A2	21.7	3,353			
		A3	26.3	3,257			
		A4	28.7	3,457	3,391	±109	
V/VI	Non-aged	Y1	8.2	2,506			
		Y2	8.7	3,549			
		Y3	10.4	2,772			
		Y4	15.3	2,795	2,906	±449	
	Aged	A1	20.4	2,456			0.22
		A2	21.7	2,415			
		A3	26.3	2,699			
		A4	28.7	2,753	2,581	±170	

2.6 μm in the aged group vs. 21.2 ± 3.2 μm in nonaged subjects ($P = 0.50$).

Significant differences between aged and nonaged primates in neuron number or size were not detected in any layer of the intermediate division of the entorhinal cortex (Tables 1–3). Neuron numbers were preserved in layers II, III, and V/VI of the entorhinal cortex (Table 1). Thus, the total number of neurons in the intermediate component of the entorhinal cortex of adult, nonaged monkeys is 473,000 ± 139,000 neurons (summing counts in layers II–VI); this value increases slightly (but nonsignificantly) to 508,400 ± 126,900 neurons in aged monkeys.

Similarly, overall neuronal area and volume were preserved in the entorhinal cortex of primates with aging (Tables 2, 3). Mean neuronal area in the entorhinal cortex was 264 ± 11 μm^2 in nonaged adult monkeys and was 253 ± 12 μm^2 in aged monkeys ($P = 0.22$). Mean neuronal volume in the entorhinal cortex was 3,700 ± 212 μm^3 in nonaged monkeys, and this value decreased nonsignificantly to 3,440 ± 302 μm^3 in aged monkeys ($P = 0.21$). Thus, aging is not associated with alteration in neuronal survival or morphology in the entorhinal cortex.

To determine whether valid and efficient optical fractionator parameters are used and whether adequate object sampling is performed in a stereologic study, *variation in the estimate of neuron number within an individual* (CE^2) is compared with *variation in the estimate of neuron number among all individuals* (CV^2). Overall variation in an estimate is composed of biological variation between subjects and several other causes of variation that are produced by measurement error. To prove that biological variability between subjects rather than measurement error is the main component of variability, the observed variation of the estimate of N (neuron number) for an individual (CE^2) should be less than half that (0.5) of the observed variance among individuals (CV^2). In the present experiment, CEs were similar within and between groups. CE values fell within a range from 0.06 to 0.15, with a mean of 0.10. The coefficients of variation (CV = standard deviation/mean) ranged from 0.22 to 0.32, with a mean of 0.27. This resulted in a CE^2/CV^2 ratio of 0.14 (West and Gundersen, 1990). Because the mean CE^2 of the individual estimates was less than half of the mean CV^2, the group estimates primarily reflected true biological variation between groups rather than measurement error (West and Gundersen, 1990).

DISCUSSION

Data from this study further challenge the concept that aging is associated with a loss of neuronal number or size. Aged rhesus monkeys develop mnemonic deficits with age and have been reported to exhibit β-amyloid plaque formation in the entorhinal cortex (Struble et al., 1985; Mufson et al., 1994), yet the present results demonstrate conservation of neuron number and size in this region. Cognitively intact aged humans can also exhibit a sub-

stantial β-amyloid plaque load yet fail to exhibit substantial cell loss with aging in such regions as the hippocampus (West, 1993; Simic et al., 1997) and entorhinal cortex (Gómez-Isla et al., 1996). These findings differ from observations in Alzheimer's disease, for which extensive cell loss occurs in the entorhinal cortex early in the disease (Gómez-Isla et al., 1996). Cell loss in the brain in Alzheimer's disease becomes yet more severe over time. Thus, biological processes underlying functional decline with aging seem to differ fundamentally from degenerative processes active in Alzheimer's disease.

The precise mechanism(s) contributing to decline in neuronal function with age remain to be identified, although several recent studies provide some insights. Gazzaley et al. (1996) recently reported a decrease in NMDA-subunit 1 receptor expression in the outer molecular layer of the dentate gyrus, a region that receives inputs from the perforant path (Gazzaley et al., 1996). This loss of excitatory neurotransmitter signaling systems, rather than frank cell death, could account for some age-related disturbances in neuronal and, therefore, cognitive function. Emborg et al. (1998) also recently reported a 40% decline in the number of tyrosine hydroxylase immunolabeled neurons in the substantia nigra of aged rhesus monkeys; furthermore, this decline in immunolabeling correlated with functional motor deficits in the animals (Emborg et al., 1998). Thus, loss of functional markers in neurons has been defined in at least two brain regions. In addition, we recently reported a 40% reduction in p75 low-affinity neurotrophin receptor labeling and choline acetyltransferase labeling in the basal forebrain of the same aged monkeys examined in the present study, but no loss of Nissl staining in the cholinergic basal forebrain region (Smith et al., 1999). These findings indicate that although aged neurons have not died, they fail to express functional proteins as efficiently as younger neurons. Interestingly, these age-related functional declines in cholinergic neurons were substantially reversed by nerve growth factor delivery (Smith et al., 1999), raising the possibility that age-related declines in neuronal function may be remediable if cells are atrophic but still alive. Thus, recent careful studies have failed to demonstrate that extensive cell loss occurs as a consequence of aging in the primate brain but have identified extensive down-regulation of key enzymes and receptor functions. Future investigations will aim to identify molecular and cellular processes that render aged neurons vulnerable to alterations in neuronal protein and receptor expression. The correlation of molecular and cellular alterations with declines in performance on functional tasks (Rapp and Amaral, 1991; Morrison and Hof, 1997; Emborg et al., 1998) may also yield valuable insights into mechanisms of age-related dysfunction of the nervous system.

This study also establishes that the normal intermediate component of the nonhuman primate entorhinal cortex contains a total of 491,000 neurons per hemisphere. Based on previous studies that the intermediate entorhinal cortex occupies roughly 30% of total entorhinal volume in monkey (Amaral et al., 1987), it is estimated that the entire monkey entorhinal cortex contains roughly 1.5 million neurons per side. Thus, complex interactions between temporal lobe memory systems and diverse cortical regions are supported by relatively few neurons. The stereologic method of analysis of neuron number in this study is consistent with the single previous report of quantification of cell number in layer II of the rhesus entorhinal cortex (Gazzaley et al., 1997). Notably, the proportion of neurons in each layer of the entorhinal cortex is highly conserved between species, possibly reflecting functional conservation in entorhinal cortical processing and channeling of neurotransmission through the hippocampus and neocortex. Rat entorhinal cortex was previously estimated to contain approximately 700,000 neurons (Mulders et al., 1997), whereas two estimations of human entorhinal cortex cell number have found an average of 7 million (Gómez-Isla et al., 1996) and 8 million neurons (West and Slomianka, 1998a,b). Differences in absolute cell numbers between rodent, nonhuman primate, and human entorhinal cortex likely reflect the progressively greater volume of information flow that this system supports in more highly evolved species.

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