Memory Impairment in Aged Primates Is Associated with Focal Death of Cortical Neurons and Atrophy of Subcortical Neurons

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Mechanisms of cognitive decline with aging remain primarily unknown. We determined whether localized cell loss occurred in brain regions associated with age-related cognitive decline in primates. On a task requiring the prefrontal cortex, aged monkeys were impaired in maintaining representations in working memory. Stereological quantification in area 8A, a prefrontal region associated with working memory, demonstrated a significant $32 \pm 11\%$ reduction in the number of Nissl-stained neurons compared with young monkeys. Furthermore, the number of immunolabeled cholinergic neurons projecting to this region of cortex from the nucleus basalis was also reduced by $50 \pm 6\%$. In contrast, neuronal number was strikingly preserved in an adjoining prefrontal cortical region also associated with working memory, area 46, and in the component of the nucleus basalis projecting to this region. These findings demonstrate extensive but highly localized loss of neocortical neurons in aged, cognitively impaired monkeys that likely contributes to cognitive decline. Cell degeneration, when present, extends transneuronally.

Key words: aging; cell death; working memory; dorsolateral prefrontal cortex; stereology; cortex; cholinergic

Introduction

Normal aging is often accompanied by functional deficits in executive processes and declarative memory (Grady and Craik, 2000). The neural basis for age-related loss of cognitive and memory capacity, however, remains unclear. Analysis of working memory, the ability to retrieve and temporarily maintain a memory representation in support of cognitive functions such as learning, reasoning, and planning (Baddeley, 1998; Squire and Kandel, 2000), offers an opportunity to investigate possible causes for decrements in cognition associated with normal aging. Working memory commonly declines with age in both humans (Light and Anderson, 1985; Morris et al., 1988) and nonhuman primates (Walker et al., 1988; Rapp and Gallagher 1997), is subserved by a well defined neural substrate (Ungerleider et al., 1998), and is considered an integral component of the global cognitive decline that occurs in aging (Salthouse, 1992; Briggs et al., 1999). Thus, examining the basis for loss of working memory capacity may provide insights into general mechanisms that underlie age-related deficits in cognition and memory.

Among the potential causes for deficits in working memory are loss or dysfunction of neurons in either cortex or subcortical

nuclei that project to cortex and that modulate cortical activity. Whereas most studies reveal no significant loss in the total number of neurons in the aged human cortex (Pakkenberg and Gundersen, 1997; Peters, 2002), few studies have examined age-related changes in neuronal number in very focal regions of cortex (West, 1993; Simic et al., 1997). A single study that we are aware of investigated the effects of age on neurons of the dorsolateral prefrontal cortex and reported no loss (Peters et al., 1996); however, unbiased stereological methods were not used. Furthermore, no previous study has investigated age-related alterations in neuronal number using stereological methods in discrete neocortical regions in behaviorally characterized primates.

Thus, we behaviorally characterized young and aged rhesus monkeys on a task dependent on the functional integrity of the dorsolateral prefrontal cortex and then performed a detailed stereological analysis of neural circuitry in focal, functionally distinct components of this brain region. We find that cognitive decline with aging is associated with neuronal and transneuronal degeneration in highly selective brain regions, whereas distinct sparing of neural circuitry is present in adjoining cortical regions, indicating a highly selective regional vulnerability of the brain to age-related neural degeneration.

Materials and Methods

Behavioral methods

Rhesus monkeys (*Macaca mulatta*) were subjects of this study. One group consisted of young adult monkeys of mean $\pm$ SEM age of 11.0 ± 0.9 years ($n = 7$; four males and three females), and the other consisted of aged monkeys of mean age of 25.4 ± 1.2 years ($n = 6$; two males and four

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females). Monkeys were housed at the California Regional Primate Research Center, and animal care conformed to National Institutes of Health and institutional guidelines regarding health, safety, and comfort of experimental animals. A subset of these subjects (four young adult monkeys, mean age of 11.4 ± 1.5 years, three males and one female; three aged monkeys, mean age of 24.3 ± 2.0 years, one male and two females) was behaviorally characterized. Working memory was tested using a two-well delayed response (DR) task in a Wisconsin General Testing Apparatus at delays of 0, 1, 5, 10, 15, 30, 60, and 120 sec, as described previously (Eberling et al., 1997). Subjects were trained to a criterion of 90% correct without a delay and then were trained to 90% correct at a delay of 1 sec. Monkeys observed the baiting of one of two lateral wells with a food reward. During the training phase, both wells were covered in view of the subjects, and a clear Plexiglas screen was raised to permit a response. If the correct location was chosen, the subject was allowed to retrieve the reward. Delays were imposed by lowering an opaque screen after the baited and unbaited food wells were covered. Daily test sessions consisted of 30 trials with a 20 sec intertrial interval, with the left and right wells baited equally in pseudorandom order. After reaching performance criterion, testing proceeded using the same daily schedule as in the training phase. Choice accuracy was the percentage of correct responses across 90 total trials conducted at each delay. After completion of behavioral testing, subjects were deeply sedated with ketamine (10 mg/kg, i.m.) and deeply anesthetized with Nembutal (30 mg/kg, i.p.) and then transcardially perfused as described below for harvesting of brains. A detailed history of the behaviorally characterized monkeys is provided in Appendix.

Anatomical methods

Tissue preparation and histochemistry. Neuronal number and cortical volume were examined using unbiased stereological methods in thionin-stained coronal sections of prefrontal cortex. Subjects were transcardially perfused for 1 hr with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C, followed by infusion of 5% sucrose solution. Brains were stereotactically blocked in the coronal plane and sectioned on a freezing microtome set at 40 μ m. Sections were collected in cryoprotectant solution in six series, with each series consisting of every sixth section through the rostrocaudal extent of prefrontal cortex. These were maintained at -80°C until processed. A series of one-in-six sections was thionin-stained for quantification of cortical volume and neuron number.

Frontal cortex anatomical boundaries. Area 46, found within and on the banks of the principal sulcus, extends from a rostral boundary of the anterior extremity of the principal sulcus to a caudal boundary formed by the anterior extent of the superior limb of the arcuate sulcus, as judged on serial coronal sections (see Fig. 2). For area 8A, the rostral limit consists of the posterior extent of the principal sulcus, and the caudal boundary is formed by the posterior extent of the arcuate sulcus formed at the junction of its superior and inferior limbs, as judged on serial coronal sections (O'Donnell et al., 1999; Owen et al., 1999). This sulcal method of anatomical boundary determination was confirmed using clearly described architectonic characteristics of these cortical regions, as described by O'Donnell et al. (1999). Briefly, compared with surrounding cortical regions, area 46 contains the following: a thicker and denser layer II; darkly stained, medium-sized pyramidal neurons in deep layer III and superficial layer V; and a thin layer IV. Compared with surrounding cortical regions, area 8A contains the following: intensely stained and dense pyramidal neurons in layer III; no layer IV; and very large pyramidal neurons in layer V. Cortical layers I–IV were grouped together for quantification and analysis, as were layers V–VI. Corticocortical (Owen et al., 1999; Barbas, 2000) and thalamocortical (Krettek and Price, 1977; Giguere and Goldman-Rakic, 1988) afferents arrive in layers I–IV, which also perform operations native to a specific cortical area (Barbas, 2000; Ongur and Price, 2000). In contrast, deep layers V–VI provide efferent connectivity to other regions of the brain (Pandya et al., 1971; Pandya and Yeterian, 1990). Thus, layers I–IV share common patterns of neural connectivity and functions, as do layers V–VI.

Stereology. Stereology was conducted using Stereo Investigator software (MicroBrightField, Colchester, VT). Live video images were received from an Olympus Optical (Tokyo, Japan) OLY-200 video camera mounted on an Olympus Optical BX60 microscope fitted with a Ludl

99S000 XYZ motorized stage. Neuronal number and morphology and cortical volume were quantified in thionin-stained coronal sections of prefrontal cortex. Cell size was quantified using the nucleator method (Gundersen, 1988), and neuronal number was quantified using the optical fractionator method (West et al., 1996; Long et al., 1999) using the nucleolus as a reference object. This method also reports the volume within which neurons were contained (i.e., cortical volume). The optical fractionator relies on a three-dimensional probe (the optical disector) and a systematic uniform sampling scheme (the fractionator, set at 5% sampling area) to generate estimates of total cell number unbiased by tissue processing artifacts or assumptions regarding neuronal size and shape. For each frontal region quantified, the 5% sampling fraction was based on preliminary estimates of neuronal number; sampling was optimized to produce a coefficient of error lower than the biological variability. Within each optical disector, cells were counted only if the nucleolus reached focus within the disector sampling frame, which was set at the middle 75% of total tissue thickness for each section. Hence, a forbidden zone of at least 1–2 μ m was included at both the top and bottom of each disector, and objects were not counted within the forbidden zone. A motorized stage was used to focus through the full extent of the z-axis in each section to ensure that all neurons were counted through the inclusion zone. To correct for shrinkage attributable to dehydration of Nissl-stained sections, tissue thickness was measured at three points in each section using a 60 $\times$ objective, and the mean thickness was used to calculate the disector height for each section. The mean section thickness was 30 μm^2 .

Cells were included in counts if they met the following criteria: (1) cell diameter greater than 8 μ m; (2) polygonal or fusiform rather than round or ovoid soma; (3) clearly visible nucleus and nucleolus within the stereology inclusion boundaries; and (4) the nucleolus did not touch the exclusion boundaries. These parameters were chosen to exclude non-neuronal cells (Pakkenberg and Gundersen, 1997; Simic et al., 1997). In the present study, we confirmed that the 8 μ m cutoff size enriched neuronal counts without excluding shrunken neurons, as follows: stereological methods were used to measure cell number and size for all cells in area 46 and area 8A in all subjects. The results were used to construct histograms with a bin size of 1 μ m on the x-axis and the number of cells of that size on the y-axis. The histograms were all strongly bimodal, with a local peak <8 μ m, a second local peak >8 μ m, together with a local minimum at 8 μ m. Notably, all cells <8 μ m exhibited glial morphology (round soma, no distinguishable nucleolus), whereas cells larger than 8 μ m displayed typical neuronal morphology (pyramidal morphology, nucleolus visible). Total cell numbers >8 μ m in size, and smaller than 8 μ m in size, were then statistically compared between aged and young groups.

The distance between sections used for quantification and the size and spacing between counting frames were set to achieve a coefficient of error of <5% (Gundersen, 1987). In area 46 in young adult animals, every 24th section was used for quantification; the mean $\pm$ SEM number of sections was 9.00 ± 0.69 (minimum, 6; maximum, 11). In area 46 in aged monkeys, every 18th section was used for analysis; the mean $\pm$ SEM number of sections was 11.00 ± 0.63 (minimum, 9; maximum, 13). Every 12th section was used for quantification in area 8A. The mean number of sections analyzed in area 8A in young adult monkeys was 5.1 ± 0.5 (minimum, 4; maximum, 7) and in aged subjects was 5.0 ± 0.5 (minimum, 4; maximum, 7).

Cytoarchitectonic boundaries for areas 46 and 8A were outlined at low power (4 $\times$ objective) using the criteria described above in the anatomical boundaries section, and neurons were then sampled in the indicated layers separately using the pseudorandom sampling scheme of the stereology program under a high-power, high-numerical aperture (1.4) oil (60 $\times$) objective. Careful inspection indicated that the Nissl stain penetrated the full thickness of each section used for stereological analysis.

Statistics. The total number of neurons and cortical volume were reported for each animal by the stereology program. Group means were computed and compared by two-tailed Student's *t* test. The null hypothesis in this and all other comparisons was rejected if $p > 0.05$.

Cholinergic neuron anatomical boundaries. Anatomical boundaries, as described previously (Mesulam et al., 1983; Smith et al., 1999), were used to delineate Ch4a and Ch4i. Ch4a and Ch4i are both ventral to the globus

pallidus. In addition, both Ch4a and Ch4i are bounded ventrally by the Ch3 cell group, which contains neurons that are fusiform or oval in morphology, with a long axis that is parallel to the ventral surface of the brain. The rostral extent of Ch4a is limited by the Ch2 cell group, in which neurons are also fusiform or oval in morphology, with the long axis of cells parallel to the ventral surface of the brain. Ch4a continues in a caudal direction until the ansa peduncularis begins to penetrate Ch4. This marks the caudal border of Ch4a and the rostral limit of Ch4i. The presence of the ansa peduncularis is the defining feature of Ch4i: Ch4i continues caudally until the ansa peduncularis completes its passage through Ch4 and the cholinergic neurons merge into a single group of neurons (Ch4p) that is more ventral and lateral than Ch4i.

Cholinergic neuron stereology. The number and size of cholinergic neurons in anterior and intermediate subdivisions of Ch4 (Ch4a and Ch4i, respectively) were quantified in sections containing basal forebrain that had been immunolabeled for p75, the low-affinity neurotrophin receptor. (Ch4p was not quantified because of the relative paucity of projections from this subregion to frontal cortex.) p75 colocalizes with 95% of neurons immunolabeled for choline acetyltransferase in the basal forebrain (Kordower et al., 1988) and does not label other cell types in this brain region. Subjects and tissue preparation are as described above. Every sixth section was processed for p75 immunoreactivity using a monoclonal antibody raised against p75 (monoclonal hybridoma cell line generated by M. Bothwell, University of Washington, Seattle, WA) at a dilution of 1:100. Labeled neurons were visualized using a biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:500, an avidin-biotin complex (Vector Laboratories, Burlingame, CA) and diaminobenzidine. Once again, the number of immunopositive neurons in Ch4a and Ch4i was quantified using the optical fractionator method (West et al., 1996; Long et al., 1999). Ch4a and Ch4i were outlined at low power (4 $\times$ objective), and quantification was performed at high power (60 $\times$ oil objective). Cells were included in neuronal counts in Ch4a and Ch4i if they met the following criteria: (1) they were immunopositive for p75; (2) the cell body was within the counting frame (or touched the inclusion boundary) but did not touch the exclusion boundary; and (3) the cell nucleus was best in focus within the inclusion volume. Careful inspection indicated that the p75 label penetrated the full thickness of each section used for stereological analysis. All other aspects of stereology and statistical analysis were as discussed above for quantification of the number of frontal cortical neurons. As noted above, the mean thickness of each section was measured, and cell objects (nuclei) were counted through the middle 75% of each section, leaving an exclusion zone of 1–2 μ m at the top and bottom of each frame (12.5% exclusion zone on the top and bottom of each section). Every 12th section was used for quantification through Ch4; the mean $\pm$ SEM number of sections quantified in young monkeys in Ch4a was 5.89 ± 0.25 (minimum, 5; maximum, 7) and in aged monkeys was 5.40 ± 0.56 (minimum, 5; maximum, 8). The mean $\pm$ SEM number of sections quantified in young monkeys in Ch4i was 8.05 ± 0.55 (minimum, 6; maximum, 9) and in aged monkeys was 7.75 ± 0.48 (minimum, 9; maximum, 7).

Staining and quantification of cholinergic innervation of cortex. Acetylcholinesterase (AChE) staining was performed using a modified Tago method (Di Patre et al., 1993) on a series of adjacent free-floating sections spaced 240 μ m apart. Sections were washed briefly in 0.05 M Tris-maleate buffer, pH 5.7, incubated for 10 min in Tris-maleate buffer containing 6 μ g/ml promethazine, and washed two additional times in Tris-maleate buffer. Sections were incubated for 30 min in a 32.5 mM Tris-maleate buffer solution containing 5 mM sodium citrate, 3 mM cupric sulfate, 0.5 mM potassium ferrocyanide, and 0.52 mg/ml acetylthiocholine iodide and then rinsed five times in 50 mM Tris-HCl, pH 7.6. Sections were incubated for 5 min in 50 mM Tris-HCl containing 0.25 mg/ml diaminobenzidine tetrahydrochloride and 3 mg/ml nickel ammonium sulfate. Hydrogen peroxide (0.006% final concentration) was added, and sections were allowed to incubate for 2–3 more minutes. The reaction was stopped by washing sections three to four times in 50 mM Tris-HCl buffer.

An intersect analysis was used to obtain a quantitative estimate of cholinergic axon density in areas 8A and 46, using standardized methods developed by Geula and Mesulam (1996). This method was chosen be-

cause it is relatively insensitive to intersubject variability in AChE staining intensity and is thought to reliably reflect axon density (Geula and Mesulam, 1996; Conner et al., 2001, 2003). Briefly, very high resolution digital images of AChE-stained, 40- μ m-thick sections were captured from an Olympus Optical AX-70 microscope at 360 $\times$ magnification (600 $\times$ for hippocampus) with high numerical aperture optics using a digital Spot camera (Diagnostics Instruments, Sterling Heights, MI) with a computer interface. Images were displayed on a high-resolution Sony (Tokyo, Japan) monitor, and a $6 \times 6 \mu$ m grid ($175 \times 175 \mu$ m for 360 $\times$) was superimposed on each quantification frame using Adobe Photoshop (Adobe Systems, San Jose, CA). The number of AChE-stained axons intersecting all gridlines in the field was quantified and summed in each image. This method of analysis was chosen because it is capable of quantifying cholinergic fiber numbers, independent of the intensity of the AChE staining intensity (Geula and Mesulam, 1996; Conner et al., 2003). Four fields per animal were quantified in this manner from each region of area 8A and 46 that was sampled for neuron counts (see above). Comparable sections were chosen from each experimental subject using fiducial landmarks by an investigator blinded to group identity. The AChE stain penetrated the full thickness of each cortical section.

Results

Neuronal number and morphology were examined using stereological methods in prefrontal cortex in aged ($n = 6$) and young ($n = 7$) rhesus monkeys. A subset of these monkeys (aged, $n = 3$; young, $n = 4$) had been behaviorally characterized on a DR test of working memory that is sensitive to the functional integrity of prefrontal cortex (Bauer and Fuster, 1976; Levy and Goldman-Rakic, 1999). Accurate performance in this task required subjects to remember which location in a manual test apparatus was baited with a food reward before a retention interval (Eberling et al., 1997). A repeated measures ANOVA, using performance across memory delays of 5–120 sec as a within-subject factor revealed significant main effects of age ($F_{(1,6)} = 23.1$; $p < 0.005$) and delay ($F_{(5,30)} = 5.2$; $p < 0.005$) on task accuracy. The age $\times$ delay interaction was also significant ($F_{(5,30)} = 2.7$; $p < 0.05$), consistent with previous reports (O'Donnell et al., 1999). Thus, aged monkeys in this study were significantly impaired on choice accuracy relative to the young monkeys and exhibited a larger behavioral deficit at longer delay periods. Notably, however, aged subjects scored normally at short delays of 0–5 sec compared with young monkeys, suggesting that aged monkeys retained the ability to select appropriate responses (Fig. 1). Specifically, there was no group difference in the mean number of trials required to reach criterion performance at the 0 and 1 sec delays (90% correct; $p > 0.05$). Similarly, at a short delay of 5 sec, aged and young monkeys scored comparably: aged monkeys, $86.3 \pm 3.5\%$ correct, and young monkeys, $91.9 \pm 0.5\%$ correct ($p > 0.05$). However, aged monkeys exhibited significant impairment compared with young adult monkeys at delays of 10 sec and longer (p values < 0.05) (Fig. 1), suggesting that the ability to maintain representations in working memory across longer retention intervals declines with age.

Neuronal number was then quantified using stereological methods in thionin-stained sections from two cytoarchitectonically defined regions of prefrontal cortex implicated previously in working memory, areas 8A and 46 (Fig. 2) (Funahashi et al., 1993; Fiez et al., 1996; Owen et al., 1999; Quintana and Fuster, 1999; Levy and Goldman-Rakic, 2000; Rowe et al., 2000; Fuster, 2002). In each region, quantification was performed separately for superficial (layers I–IV) and deep layers (V and VI) on the basis of similarities in the projection patterns and operations of these respective layers. Stereological quantification revealed that neuronal number was preserved in area 46 in aged monkeys. The mean $\pm$ SD total neuronal number across all layers of area 46 was

Figure 1. Working memory performance on delayed response task. Performance of aged monkeys did not differ significantly from young adult monkeys at delays of 5 sec and less, but significant impairments were present at each delay period of 10 sec and longer.

$30.09 \pm 2.75 \times 10^6$ in young adult monkeys and $28.92 \pm 2.77 \times 10^6$ in aged monkeys ($p = 0.46$). Subdivided by layer groupings, young adult and aged monkeys displayed similar neuronal numbers in layers I–IV and in layers V–VI of area 46. In layers I–IV, the mean $\pm$ SD neuronal number in area 46 in young adult monkeys was $19.48 \pm 1.73 \times 10^6$ and was $18.90 \pm 1.40 \times 10^6$ in aged monkeys ($p = 0.53$) (Fig. 2). In layers V–VI of area 46, young adult monkeys exhibited a mean neuronal number of $10.61 \pm 1.51 \times 10^6$, and aged monkeys had a mean of $10.02 \pm 1.41 \times 10^6$ neurons ($p = 0.48$) (Fig. 2).

In contrast, a significant $32 \pm 11\%$ reduction in neuronal number was observed in aged monkeys across all layers of area 8A compared with young monkeys, and this decline correlated significantly with impaired working memory performance in the respective groups of animals ($R^2 = 0.81$; $p < 0.01$). The mean $\pm$ SD total number of neurons in area 8A in young adult monkeys was $14.09 \pm 1.63 \times 10^6$, whereas aged monkeys exhibited a mean of $9.64 \pm 1.02 \times 10^6$ neurons ($p < 0.0001$). Neuronal loss in area 8A was evident in aged subjects in both layers I–IV ($38 \pm 10\%$, from a mean of $8.91 \pm 0.80 \times 10^6$ neurons in young adult monkeys to a mean of $5.60 \pm 0.73 \times 10^6$ neurons in aged monkeys; $p < 0.0001$) (Fig. 2) and layers V–VI ($22 \pm 15\%$, from a mean of $5.19 \pm 0.98 \times 10^6$ neurons in young adult monkeys to a mean of $4.06 \pm 0.53 \times 10^6$ neurons in aged monkeys; $p < 0.05$) (Fig. 2). Neuronal number, therefore, was significantly reduced in one subregion of prefrontal cortex associated with working memory, area 8A, but was maintained in an adjoining prefrontal region contributing to working memory, area 46.

To determine whether the observed reduction in cell number in area 8A represented true cell death rather than cell shrinkage (atrophy), the number of thionin-stained cells in area 8A was quantified as a function of size. There was no significant increase in the percentage of small cells ($<8 \mu\text{m}$ diameter) in aged subjects. In fact, the proportion of small neurons ($<8 \mu\text{m}$ diameter) was slightly greater in young monkeys ($44 \pm 6\%$) than in aged subjects ($34 \pm 5\%$; $p > 0.05$). Furthermore, there was no significant difference in mean overall size across all cells when comparing young adult and aged monkeys ($p = 0.77$). Thus, cell shrinkage below the limits of detection did not account for reductions in cell number in area 8A.

Memory deficits in normal aging have also been attributed to disruption of cholinergic function (Kadar et al., 1990), and degeneration of cholinergic neurons that project to cortex has been reported previously in aged humans (de Lacalle et al., 1991) and nonhuman primates (Smith et al., 1999). Cholinergic innervation of dorsolateral prefrontal cortex was examined by measuring the density of cholinergic axons in sections histochemically stained for AChE using methods established by Mesulam and colleagues (Mesulam et al., 1983; Conner et al., 2001, 2003). Alterations in cholinergic axon density with age exhibited a pattern consistent with changes in neuronal number in prefrontal areas 46 and 8A (Fig. 3). In area 46, in which neuronal number was preserved with aging, cholinergic axon density was normal (Fig. 3). However, in area 8A, aged subjects exhibited a significant $23 \pm 5\%$ reduction in the density of cholinergic axons in layers I–IV, from a mean $\pm$ SD of $0.53 \pm 0.10 \times 10^6$ fibers/ mm^3 in young adult subjects to a mean of $0.40 \pm 0.03 \times 10^6$ in aged monkeys ($p < 0.05$) (Fig. 3). Layers I–IV of neocortex contain the primary target of corticocortical and thalamocortical projections. No significant reduction in cholinergic inputs to layers V and VI of area 8A was detected: cholinergic fiber density was $0.40 \pm 0.09 \times 10^6$ fibers/ mm^3 in young adult monkeys and $0.38 \pm 0.07 \times 10^6$ fibers/ mm^3 in aged subjects ($p = 0.60$). Layers V–VI of cortex provide the primary output projections to other brain regions. Changes in cortical volume were not artifactually responsible for reductions in cholinergic axon density in any region, because the volume of prefrontal areas, measured stereologically, did not significantly differ between young adult and aged monkeys ($p = 0.97$ in area 46; $p = 0.79$ in area 8A). Thus, cholinergic innervation was significantly reduced only where the most pronounced loss of cortical neurons was observed, in layers I–IV of area 8A.

Reduced cholinergic innervation in area 8A may reflect degeneration of cholinergic neurons that project to cortex. The number of cholinergic neurons that innervate prefrontal areas 46 and 8A was therefore examined using stereological methods in sections immunolabeled for the low-affinity neurotrophin receptor (p75), a specific marker of basal forebrain cholinergic neurons in this brain region (Kordower et al., 1988). The largest portion of cholinergic innervation of area 46 arises from the anterior division of the nucleus basalis of Meynert (designated Ch4a), whereas the intermediate division of the nucleus basalis of Meynert (designated Ch4i) provides the majority of cholinergic innervation to area 8A (Mesulam et al., 1983). In Ch4a, there was a trend toward a slight increase in number of immunolabeled neurons in aged monkeys: the mean $\pm$ SD number of immunolabeled neurons in Ch4a in young adult monkeys was $5.98 \pm 0.90 \times 10^4$ versus $7.24 \pm 1.35 \times 10^4$ in aged monkeys ($p = 0.07$) (Fig. 4). There was no significant difference in the size of Ch4a neurons between young ($460 \pm 41 \mu\text{m}^2$) and aged ($436 \pm 17 \mu\text{m}^2$; $p = 0.21$) monkeys. In contrast, the intermediate component of Ch4, which originates inputs to area 8A, exhibited reductions in the number of p75-immunolabeled cells, consistent with patterns of cell loss in area 8A. There was a significant $50 \pm 6\%$ reduction in the number of immunolabeled cholinergic neuronal somata within Ch4i in aged subjects, from a mean $\pm$ SD of $5.23 \pm 0.97 \times 10^4$ in young adult monkeys to a mean of $2.59 \pm 0.39 \times 10^4$ in aged subjects ($p < 0.0001$) (Fig. 4). Immunolabeled neurons within Ch4i also exhibited a significant $12 \pm 3\%$ decrease in cell size in aged subjects. The mean $\pm$ SD cross-sectional area in young adult monkeys was $513 \pm 44 \mu\text{m}^2$, whereas in aged monkeys the mean size was $451 \pm 33 \mu\text{m}^2$ ($p < 0.05$) (Fig. 4). Previous results (Smith et al., 1999) have demonstrated that a reduction in the number of immunolabeled neurons in Ch4 in the aged

Figure 2. Quantification of neuronal number and morphology in dorsolateral prefrontal cortex. *A*, Lateral surface of prefrontal cortex indicating area 46 (dark shading) and area 8A (light shading), in which neuronal number was quantified. Vertical lines indicate stereological boundaries. Lines marked r46 and c46 delineate rostral and caudal limits of area 46, and lines r8A and c8A denote rostral and caudal boundaries of area 8A. *B*, Thionin-stained coronal section of prefrontal cortex, showing grouping of layers II–IV and V–VI for quantification, on the basis of projection patterns. Scale bar, 150 μ m. *C*, Higher magnification of thionin-stained sections in layers III–IV of area 8A in young adult; *D*, aged monkey, suggesting reduced cell density in aged monkey. Scale bar: *C*, *D*, 70 μ m. *E*, Stereological quantification of neuronal number in area 46 and area 8A. In area 46, neuronal number was preserved in aged monkeys. In contrast, highly significant reduction in neuronal number was observed in layers II–IV ($p < 0.0001$) and V–VI in area 8A of aged monkeys ($p < 0.05$).

Figure 3. Reduction of AChE-stained axon density in dorsolateral prefrontal cortex in aged monkeys. AChE-staining of axons in area 8A of young (*A*) and aged (*B*) monkeys. Scale bar: *A*, *B*, 55 μ m. *C*, Quantification of AChE-stained axon density shows significant reductions in layers I–IV of area 8A ($p < 0.05$), in which Nissl-stained neurons are also lost. In contrast, AChE-stained axons are preserved in area 46, in which the number of Nissl-stained neurons was also preserved. Reductions in cortical volume do not account for these changes (see Results).

primate reflects cellular atrophy rather than death. Thus, atrophy of basal forebrain cholinergic neurons was observed only in a division of the basal forebrain that projects to an area of prefrontal cortex in which substantial neuronal loss occurred. Furthermore, the number of immunolabeled neurons in Ch4i correlated significantly with the number of cortical neurons in layers I–IV of area 8A ($R^2 = 0.76$; $p < 0.0001$).

Discussion

Findings of this study demonstrate significant reductions of cell number in cortical regions involved in age-related memory decline, together with extensive transneuronal degeneration of subcortical neurons that project to these cortical regions. This loss is highly focal and selective: adjacent cortical regions that contribute to similar memory functions are preserved, together with their subcortical inputs. Thus, compared with young adult mon-

keys, area 8A of aged primates exhibited a 38% loss of thionin-stained neurons, a 23% reduction in cholinergic axon terminals in superficial cortical layers, and a 50% atrophy of cholinergic neurons in a subcortical nucleus that innervates this cortical area. Area 8A has been implicated in the working memory task on which the aged monkeys were impaired (Funahashi et al., 1993; Fiez et al., 1996; Quintana and Fuster, 1999; Levy and Goldman-Rakic, 2000; Rowe et al., 2000; Fuster, 2002). However, an adjoining region of cortex that also contributes to working memory, area 46, exhibited anatomical sparing with no reduction in cortical cell number, size, or inputs from cholinergic systems. The reduction of cortical neuron number in area 8A is not an artifact of cell shrinkage: the proportion of small cells did not increase in aged compared with young monkeys, nor did mean cortical neuron size differ among young and aged subjects. Thus, highly focal cell loss occurs in the cortex with aging and is accompanied by

transneuronal degeneration that can substantially extend the region of age-related atrophy beyond the immediate region of cortical involvement.

Aged monkeys in this study showed no impairment in selecting correct responses at short delays on a working memory task but did exhibit significant impairment in recalling those responses at longer delays. Does the observed anatomical pattern of cell sparing in area 46 and cell loss in area 8A correlate with the behavioral sparing at short delays and impairment at longer delays? A previous functional magnetic resonance imaging study in humans reported that selection, but not maintenance, of working memory was associated with activation of prefrontal cortical area 46, whereas activation of prefrontal area 8 and the inferior parietal cortex was associated with memory maintenance (Rowe et al., 2000). This pattern of behavioral performance corresponds with the observed patterns of cell loss and sparing in our study. However, several lesion and electrophysiological studies in non-human primates have not demonstrated this pattern of functional segregation in the prefrontal cortex (Funahashi et al., 1993; Quintana and Fuster, 1999; Levy and Goldman-Rakic, 2000; Fuster, 2002), suggesting instead that area 46 has an active role in the delayed component of working memory on which our subjects were impaired. Because this study was not prospectively designed to examine the functional parcellation of prefrontal cortex in working memory tasks, further conclusions in this respect are not warranted. Nonetheless, because areas 8A and 46 are required to perform the working memory task of this study, one can conclude that loss of cells and their inputs in area 8A likely contribute to the decline of working memory with age.

The highly focal nature of this cellular and transcellular degeneration probably accounts for the failure of previous studies to identify significant cell loss as a substrate of age-related cognitive decline. Previous investigations of cell number in the cortex and hippocampus of aged primates concluded that the brain is essentially spared of neuronal loss with aging (Walker et al., 1988; Vincent et al., 1989; Ahmad and Spear, 1993; Peters et al., 1994; Gazzaley et al., 1997; Kemper et al., 1997; Kim et al., 1997; Hof et al., 2000; Peters, 2002; Keuter et al., 2003). However, the lack of stereological methodology in some studies may have led to erroneous conclusions because of age-related changes in brain size, volume, or neuronal packing density that have been described in several reports (Walker et al., 1988; Vincent et al., 1989; Peters et al., 1994; Kemper et al., 1997; Kim et al., 1997; Peters, 2002). Other studies used stereological methods but sampled broader cortical regions rather than focal functional subdivisions (Kemper et al., 1997; Hof et al., 2000; Keuter et al., 2003).

Figure 4. p75-labeled cholinergic neuronal numbers are selectively reduced in aged monkeys. Low-magnification views of representative sections from a young adult (*A*) and an aged (*B*) monkey demonstrate distribution of immunolabeled neurons in the intermediate division of the basal forebrain (Ch4i). ap, Ansa pdenularis; Ch4id, intermediodorsal component of Ch4 region; Ch4iv, intermedioventral component of Ch4 region. Scale bar: *A*, *B*, 270 μ m. Higher-magnification views from a young adult (*C*) and an aged (*D*) monkey show a reduction in neurite density in old monkeys. Scale bar: *C*, *D*, 45 μ m. *E*, Stereological quantification of the number of p75-labeled neurons revealed significant reductions in Ch4i ($p < 0.0001$), the division of the cholinergic basal forebrain that projects to the area of prefrontal cortex in which neuronal loss occurred (area 8A). Ch4a, which innervates the area of prefrontal cortex in which neuronal number was preserved, displayed no reduction ($p > 0.05$).

Indeed, the examination of overlapping, broader regions of cortex may lead to a loss of sensitivity in identifying focal regions of cell loss during the course of age-related decline in cognition. Although some brain regions do in fact exhibit cell sparing with aging, including thalamic nuclei (Ahmad and Spear, 1993) or area 46 as shown in the present study, other adjoining regions (e.g., area 8A) clearly exhibit cell loss. Thus, future studies of age-related brain dysfunction would benefit from examination of very focal and well defined functional subdivisions of neural circuitry, when possible.

It is notable that both cortical neurons and their inputs from subcortical regions degenerated in aged, cognitively impaired monkeys. Cholinergic innervation of prefrontal cortex primarily

arises from the Ch4 group of neurons examined in this study, although a small component also arises from the hypothalamus, a region that we did not examine. Basal forebrain cholinergic neurons exert an important role in modulating cortical plasticity (Kilgard and Merzenich, 1998; Conner et al., 2003). Cholinergic neurons degenerate if deprived of nerve growth factor (NGF) (Tuszynski et al., 1990; Liberini et al., 1994) and remain sensitive to NGF even in the aged primate (Kordower et al., 1994; Smith et al., 1999; Conner et al., 2001). Therefore, declining trophic support from target cells in superficial layers of prefrontal area 8A could cause degeneration of cholinergic neurons in Ch4i. Alternatively, degeneration of cholinergic basal forebrain neurons could cause trans-synaptic changes that ultimately result in loss of cortical neurons (Unger and Schmidt, 1994; Zhang et al., 1998).

This study was not prospectively designed or powered to examine gender-related effects on age-related changes in cognition or cellular parameters, and estrogen levels were not measured in the experimental subjects. Thus, conclusions regarding gender-related effects cannot be made. However, the performance of the two aged female monkeys was not worse than that of the two aged monkeys on the delayed response task, nor were cell numbers suggestive of a gender difference in the cortex or Ch4 region (data not shown) in these subjects.

Together with alterations in neuronal gene expression (Colombo et al., 1997; Guarente and Kenyon, 2000; Murphy et al., 2003), intracellular transport, neurotransmitter systems–receptors (Arnsten et al., 1995; Gazzaley et al., 1997; Hof et al., 2002), synapses (Tigges et al., 1995; Smith et al., 2000), dendrites–spines (Uemura, 1985a,b; Peters et al., 1994; Hayashi et al., 2001; Page et al., 2002), electrophysiological mechanisms (Tanila et al., 1997), astroglial (Sloane et al., 2000), and white matter changes (Peters, 2002), a more comprehensive picture of mechanisms underlying age-related neural degeneration emerges. The mechanism of this degeneration is likely multifactorial and varies, depending on the brain region affected.

Appendix

As noted above, three aged monkeys in this study were behaviorally tested. Twelve months after completion of behavioral testing, these three subjects received injections of primary autologous fibroblasts genetically modified to express the inert reporter gene β -gal into five evenly spaced sites covering the rostral-to-caudal extent of the Ch4i region, thereby serving as control subjects in another study examining growth factor delivery to other sets of monkeys (Smith et al., 1999). Control fibroblasts ($10 \mu\text{l}$) were injected into each site. Aged subjects included in the present study were not exposed to growth factors. Previously, we reported that these control fibroblast grafts to the Ch4i region had no effect on either the number or morphology of immunolabeled cholinergic basal forebrain neurons (Smith et al., 1999) or the density of their projections to cortex (Conner et al., 2001) when compared with aged-matched non-operated monkeys. To extend and confirm these results in the present study, we compared the following: (1) the number of immunolabeled cholinergic basal forebrain neurons, (2) the density of cholinergic projections to prefrontal cortex, and (3) the number of neurons in prefrontal cortex in groups of aged control fibroblast-grafted ($n = 3$) versus age-matched non-operated ($n = 3$) monkeys. There were no differences in any measured parameter between these groups. In aged monkeys (graft recipient mean age, 24.3 ± 2.0 years; unoperated mean age, 26.1 ± 1.7 years), control grafts did not alter any of the following: (1) the mean $\pm$ SD number of immunolabeled

cholinergic basal forebrain neurons in either Ch4i (aged grafted animals, $2.58 \pm 0.50 \times 10^4$ neurons; aged unoperated animals, $2.60 \pm 0.36 \times 10^4$ neurons; $p = 0.95$) or Ch4a (aged grafted animals, $6.80 \pm 1.41 \times 10^4$ neurons; aged unoperated animals, $7.68 \pm 1.42 \times 10^4$ neurons; $p = 0.49$); (2) the mean $\pm$ SD density of cholinergic innervation in either area 8A (aged grafted animals, $3.94 \pm 0.43 \times 10^5$ fibers/ mm^3 ; aged unoperated animals, $4.1 \pm 0.17 \times 10^5$ fibers/ mm^3 ; $p = 0.57$) or area 46 (aged grafted animals, $2.91 \pm 1.16 \times 10^5$ fibers/ mm^3 ; aged unoperated animals, $2.47 \pm 0.36 \times 10^5$ fibers/ mm^3 ; $p = 0.58$); or (3) the mean $\pm$ SD number of cortical neurons in either area 8A (aged grafted animals, $10.12 \pm 0.32 \times 10^6$ neurons; aged unoperated animals, $9.16 \pm 1.33 \times 10^6$ neurons; $p = 0.29$) or area 46 (aged grafted animals, $29.26 \pm 4.31 \times 10^6$ neurons; aged unoperated animals, $28.59 \pm 0.54 \times 10^6$ neurons; $p = 0.80$). Similarly, of the seven young adult monkeys that were subjects in this study, the four that were behaviorally characterized received transduced fibroblast grafts to Ch4i 12 months after completion of behavioral testing. Behavioral testing was conducted as part of a previous study that examined cognitive decline associated with normal aging. After completion of that behavioral study, these young adult monkeys received $10 \mu\text{l}$ suspension grafts of autologous fibroblasts that were genetically modified to produce NGF into five sites of Ch4i, as part of an NGF dosing study in young monkeys. NGF delivery to the intact brains of young animals has been reported previously to have no effect on the number or morphology of immunolabeled cholinergic basal forebrain neurons (Chen and Gage, 1995). To determine whether NGF grafts altered any of the parameters measured in this study, we compared the following: (1) the number of immunolabeled cholinergic basal forebrain neurons, (2) the density of cholinergic projections to prefrontal cortex, and (3) the number of neurons in prefrontal cortex of these four young adult NGF-grafted to three age-matched young adult non-operated monkeys. No differences in cell number or morphology were found between these groups. NGF-secreting grafts in young adult monkeys (mean age, 11.4 ± 1.5 years), compared with young nongrafted monkeys (mean age, 10.6 ± 1.2 years), did not change any of the following: (1) the mean $\pm$ SD number of immunolabeled cholinergic basal forebrain neurons in either Ch4i (young NGF-grafted animals, $5.29 \pm 1.27 \times 10^4$ neurons; young unoperated animals, $4.57 \pm 1.24 \times 10^4$ neurons; $p = 0.45$) or Ch4a (young NGF-grafted animals, $5.88 \pm 0.74 \times 10^4$ neurons; young unoperated animals, $6.12 \pm 1.25 \times 10^4$ neurons; $p = 0.77$); (2) the density of cholinergic innervation ($\pm$ SD) in either area 8A (young NGF-grafted animals, $5.19 \pm 0.95 \times 10^4$ fibers/ mm^3 ; young unoperated animals, $5.33 \pm 1.36 \times 10^4$ fibers/ mm^3 ; $p = 0.88$) or area 46 (young NGF-grafted animals, $5.19 \pm 0.95 \times 10^4$ fibers/ mm^3 ; young unoperated animals, $5.33 \pm 1.36 \times 10^4$ fibers/ mm^3 ; $p = 0.88$); or (3) the mean $\pm$ SD number of cortical neurons in either area 8A (young NGF-grafted animals, $14.33 \pm 1.80 \times 10^6$ neurons; young unoperated animals, $13.77 \pm 1.71 \times 10^6$ neurons; $p = 0.70$) or area 46 (young NGF-grafted animals, $30.10 \pm 3.68 \times 10^6$ neurons; young unoperated animals, $30.07 \pm 1.53 \times 10^6$ neurons; $p = 0.99$). To summarize, three of six aged monkeys and four of seven young adult monkeys used in this study were behaviorally characterized. All animals were unoperated and intact throughout behavioral testing. Twelve months after completion of behavioral testing, aged monkeys received autologous grafts of primary β -gal-producing fibroblasts. These grafts had no effect on any cellular parameter measured. Four of seven young adult monkeys received grafts of autologous NGF-secreting fibroblasts after completion of behavioral testing, and, consistent with pre-

vious reports, NGF delivery in the young intact brain had no effect on any cellular parameter measured. Thus, grafts placed in monkeys used in this study (1) did not influence behavioral results because behavioral testing was completed before grafts were placed and (2) did not alter any cellular parameter, as noted above.

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