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Increased microglial activation and protein nitration in white matter of the aging monkey☆

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Abstract

Activated microglia are important pathological features of a variety of neurological diseases, including the normal aging process of the brain. Here, we quantified the level of microglial activation in the aging rhesus monkey using antibodies to HLA-DR and inducible nitric oxide synthase (iNOS). We observed that 3 out of 5 white matter areas but only 1 of 4 cortical gray matter regions examined showed significant increases in two measures of activated microglia with age, indicating that diffuse white matter microglial activation without significant gray matter involvement occurs with age. Substantial levels of iNOS and 3-nitrotyrosine, a marker for peroxynitrite, increased diffusely throughout subcortical white matter with age, suggesting a potential role of nitric oxide in age-related white matter injury. In addition, we found that the density of activated microglia in the subcortical white matter of the cingulate gyrus and the corpus callosum was significantly elevated with cognitive impairment in elderly monkeys. This study suggests that microglial activation increases in white matter with age and that these increases may reflect the role of activated microglia in the general pathogenesis of normal brain aging.

Keywords: HLA-DR; Nitric oxide; iNOS; 3-Nitrotyrosine; Microglia; Aging; White matter; Rhesus monkey; Macaca mulatta

1. Introduction

Normal aging is associated with a decline in several aspects of cognitive function. In the normal aging rhesus monkey, deficits have been found in short term memory and executive system function [28]. Among the first impairments to appear are deficits in the acquisition and retention of visuospatial information [28,54]. Executive functions, such as cognitive flexibility and cognitive tracking, are also impaired with age [39,61]. These cognitive deficits accumulate with age, so that by age 30 all monkeys are significantly impaired.

The source of age-associated cognitive dysfunction remains unknown. Initially, nonautomated studies of neuronal counts of human cortices concluded that significant neuronal loss throughout the cortex occurs with age. A variety of investigators showed as much as 50% loss of neurons from a variety of neocortical sites [9,10,19,27]. When automated studies were performed to determine age-related neuronal losses more accurately, no extensive neuronal loss in human and monkey cortices was observed [3,14,61,74]. The only observed change using newer techniques was a shrinkage in large cortical neurons rather than a loss [25,26,40,41,61,74]. In addition, no neuronal loss has been detected in the hippocampal formation in the rhesus monkey [2,61], although losses have been reported in CA4 and the subiculum of the human brain [22,80].

Whether neurons are dysfunctional with age is a much harder question to address. Other than a thinning of layer I due to regressive changes to dendritic spines, limited morphological changes occur as a product of age in the monkey

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neocortex [61]. Although other studies have shown age-associated synaptic changes [43,44], several studies have shown that negligible synaptic loss occurs with age in primary motor cortex [31,75,86] and the hippocampal formation [61,76]. In addition, accumulation of amyloid plaques in cortical gray matter, while increasing with age, does not account for the age-associated cognitive loss seen in the rhesus monkey [69].

While mounting evidence suggests that age-related gray matter changes are subtle, substantial changes to cortical and subcortical myelin composition and content occur with age. Histological techniques have shown increased white matter pallor, suggesting myelin loss of the centrum semiovale and stratum sagittale interna [35]. Ubiquitin immunostaining identifies dense inclusions within glia and focal swellings within myelin lamellae in white matter of old humans [20] and dogs [78]. The levels of ubiquitin immunostaining and myelin swellings qualitatively increased in the human brain irrespective of the presence of dementia and, thus, is believed to represent a normal aging process [20].

In addition, the distribution of inflammatory glial cells throughout the brain further suggests that subcortical white matter rather than gray matter undergoes significant age-related changes. Microglial activation increases as a product of age in the neurologically normal human [45,47,68], the monkey [62,67], and the rat [52,59]. With advancing age, microglia activation occurs primarily in white matter [52, 59,67]. However, others suggest white matter microglia are constitutively activated [45]. In normal aging of the monkey CNS, microglia appear increasingly phagocytic with age as microglia in old individuals exhibit electron dense inclusions that have the appearance of myelin [62].

It is unclear whether the effect of activated microglia on white matter is beneficial, detrimental, or a combination of both. Several factors secreted by microglia, such as nitric oxide [48,49], TNF-α [29,42], and complement [82] are known oligodendrocyte toxins. In particular, nitric oxide preferentially promotes oligodendrocyte necrosis compared to other glial cell types [48–50]. The more toxic molecule peroxynitrite, a product of nitric oxide and superoxide, may also play a role in oligodendrocyte and myelin dysfunction as oligodendrocytes are differentially sensitive to peroxynitrite as well (J. Sloane, unpublished data).

The production of nitric oxide and peroxynitrite can be assessed by examining levels of inducible nitric oxide synthase (iNOS), the NOS isoform associated with microglial activation [56], and 3-nitrotyrosine (NTyr), an oxidation product of peroxynitrite. iNOS has been demonstrated in activated microglia [1,16,84] and astrocytes in vivo [16,37, 79]. Although NTyr levels are elevated in a number of neurological diseases [7,23,72], such as multiple sclerosis [5,15,60], it is unknown whether NTyr content changes with normal aging in brain regions exhibiting high levels of activated microglia.

In this study, we evaluated by immunohistochemistry age-related changes in activated microglia content in the rhesus monkey brain. Using monoclonal antibodies specific to HLA-DR, an MHC class II antigen, and iNOS, we used two different techniques to quantify activated microglia content in a variety of cortical and subcortical regions. With microglia activation, microglial surface expression of HLA-DR is upregulated and is a well established index of microglial activation [45,47,67]. The 12 monkeys used in this study were well characterized, as each was born in captivity and behaviorally assessed. We found that dramatic and significant increases in activated microglia density and percent stained area in subcortical white matter but not gray matter occurred with age. We also found dramatic, reproducible increases in NTyr immunostaining in subcortical white matter with age. These changes were associated with increased expression of iNOS. In addition, we used available behavioral data to assess the relationship between cognitive function and measurements of activated microglial content. Activated microglial content in several white matter areas correlated with cognitive status, suggesting a relationship exists between the two variables.

Table 1
Monkeys and their characteristics[a]

ID No.	Age	Z Score
AM61	30 years, 7 months	2.27
AM28	30 years, 1 month	2.47
AM29	29 years	1.02
AM30	25 years	0.53
AM31	23 years, 2 months	0.89
AM38	23 years, 2 months	2.26
AM49	15 years	0.19
AM56	10 years, 5 months	−0.01
AM22	10 years, 1 month	0.03
AM57	10 years, 1 month	0.58
AM32	8 years, 1 month	0.09
AM52	7 years, 6 months	0.46

[a] *Z* score refers to cognitive *Z* score. A higher *Z* score means more cognitively impaired. All monkeys were born in captivity, so precise ages were known for all individuals.

2. Methods

2.1. Subjects

Twelve rhesus monkeys, eight males and four females, were used in this study (Table 1). Of these, six were young to middle age adults (5–15 years old) and six were aged (>20 years old). Each of the monkeys in the present study had known birth dates and spent several years free ranging in social groups maintained at the Yerkes Regional Primate Center (YRPRC). All monkeys had MRI scans and underwent behavioral testing to assess cognitive function as described below [28,54].

All monkeys received medical examinations that included serum chemistry, hematology, urine analysis, and fecal analysis before entering the study. In addition, explicit

criteria were used to screen the monkeys for history of splenectomy or thymectomy, exposure to radiation, chronic illness including viral or parasitic infections, neurological disease, or chronic drug administration. All monkeys were visually inspected on a daily basis by both animal care personnel and research technicians and were given a medical exam on a semi-annual basis or more frequently if needed.

2.2. Cognitive assessment

The 12 monkeys in this study underwent extensive behavioral assessment of visual and spatial recognition memory, associative learning, and executive system function [33,39,54,55]. We obtained performance measures for these monkeys on six behavioral measures: [1] Delayed Non-Matching-to-Sample task (DNMS), [2] DNMS with a delay of 120 sec, [3] spatial condition of the Delayed Recognition Span Test (DRST), [4] non-spatial (color) condition of the DRST, [5] Spatial Reversal and [6] Object Reversal. A linear transformation of standardized scores on the six cognitive tests was derived by means of principal components analysis (PCA). The PCA included data from a group of 22 monkeys with available data on all six measures, and yielded a composite measure that declined linearly with increasing age ($r = -0.74$). The composite score was scaled as a Z score, with zero representing the sample mean and each increment of one representing a standard deviation. It is important to note that all monkeys in this study were not considered outliers in terms of their cognitive scores when compared to a much larger behaviorally tested monkey population.

2.3. Perfusion and tissue preparation

At the conclusion of behavioral testing, all monkeys were deeply anesthetized and killed by exsanguination during transcardial perfusion of the brain with isotonic Krebs buffer (pH 7.4, 4°C). The brains were blocked in situ in the coronal stereotactic plane and then flash frozen in −60 degree C isopentane. They were stored at −80 degrees C until cut on a motorized cryostat into interrupted series of 15 micrometer thick sections spaced approximately 750 microns apart. These sections were thaw mounted onto subbed slides, dried rapidly under a cool stream of air, and stored at −20 degrees C until removed for processing. For this study a partial series of sections from the rostrum to the splenium of the corpus callosum was selected. This included the posterior part of the frontal lobe from the precentral gyrus caudally, as well as most of the temporal and parietal lobes.

2.4. Immunohistochemistry

For processing the selected series were thawed rapidly on 37°C warming plate and then fixed with 10% buffered formaldehyde or acetone for 1 h or 20 min, respectively. Sections were then rinsed in distilled water for 3 min followed by a 3-min rinse in Tris buffer-saline-Tween 20 (TBST) buffer (10 mM Tris, pH8, 0.15 M NaCl, 0.05% Tween 20). Sections were permeabilized for 5 min in 0.3% Triton X-100 and then incubated in 3% H_2O_2 in phosphate-buffered saline (PBS) for 30 min to inhibit endogenous peroxidase activity. Sections were incubated in a blocking solution of 5% Carnation dried milk in TBST buffer followed by overnight incubation at 4°C with primary monoclonal antibodies directed at HLA-DR (1:100; Boehringer-Mannheim, Germany) or iNOS (1:100; Transduction Labs). We detected antibody/antigen complexes using biotinylated horse anti-mouse antibody and the ABC system (Vector).

A different technique was used for NTyr immunostaining. After fixation in 10% buffered formaldehyde for 1 h, cryostat sections were permeabilized with 0.3% Triton X-100, microwaved in 0.1 M aqueous sodium citrate buffer pH 6.5 [23], and treated with 3% H_2O_2 for 30 min. The sections were then treated with a mixture of chloroform: methanol (2:1) to delipidate the sections. After blocking with 5% goat serum, NTyr immunostaining was performed using polyclonal antibodies directed at NTyr (1:100; Upstate), biotinylated goat anti-rabbit antibody, and the ABC system (Vector). Nondelipidated and delipidated sections showed no detectable immunostaining when reacted against anti-NTyr polyclonal antibodies preabsorbed with 10 mM NTyr. In addition, nondelipidated sections displayed insignificant immunostaining after reacted with anti-NTyr antibodies.

To control for day-to-day variability in detection by immunocytochemistry, sections from all monkey brains were reacted in parallel in the identical incubation solutions so that valid comparisons in immunostaining could be made. Each set of slides stained with each antibody was fixed with the same fixative. Controls also were run in parallel and consisted of sections for known human Alzheimer disease brain or old monkey brain sections known to contain immunoreactive material. Sections were stained with or without primary antibody to verify the specificity of the secondary antibody. After immunostaining, sections from old monkeys were stained with Thioflavin S to identify amyloid deposits.

2.5. Quantitative microscopy

HLA-DR$^+$ microglia were identified in nine different cytoarchitectonic areas of cortex using standard cytoarchitectonic criteria [69]. Adjacent subcortical white matter no deeper than 2 mm underneath select cytoarchitectonic gray matter areas was used for white matter measurements. We used 10 different monkeys for density calculations of HLA-DR immunostaining. We identified activated microglia based on their characteristic morphology and excluded immunoreactive blood vessels and artifactual staining. The boundary of each area or subfield was demarcated directly on the slide and stained activated microglia were counted

within each area at 100 × total magnification on an Olympus BH2 microscope.

For each measurement, we counted activated microglia in three nonoverlapping randomly chosen areas within each of the four defined cytoarchitectonic regions, the four adjacent white matter fields, and the corpus callosum. For each, we calculated an average density from the three measurements. Activated microglial densities were calculated by averaging HLA-DR$^+$ microglia numbers counted in separate microscopic fields that were each 1 mm^2 [83].

We also used an automated image analysis system (Inquiry; Loats Associates, Westminster, MD) to analyze HLA-DR$^+$ microglia to obtain an estimate of the percent of tissue area occupied by microglial processes and cell bodies in each of the nine cytoarchitectonic areas assessed by profile counts. For each region, background was excluded by manually adjusting the computerized imaging sensitivity until only HLA-DR$^+$ tissue was selected. Then immunoreactive blood vessels, nonspecific staining, and artifacts were edited out before quantitation. When a subfield was too large to be analyzed at one time, several measurements of nonoverlapping areas were made and then averaged. For each region the area that was HLA-DR$^+$ was divided by the total area analyzed to obtain an estimate of the percent area occupied by activated microglial cells and processes.

We also used a mathematically derived measure of immunostained individual cell area using percent area and density calculations. Individual cell area was derived by the following formula: (percent area)/100*density. This measure represents all immunostaining as described above for percent area measurements and therefore all cellular elements of the microglia such as cell soma and processes.

2.6. Statistical analysis

Statistical analysis was performed using Statview SE+ (Abacus Concepts). Spearman rank correlations coefficients were calculated to determine if dependent data sets with non-Gaussian distribution were correlated with each other. The one-way ANOVA with post hoc Bonferroni correction for multiple comparisons was used for tests of significance between two or more groups. To examine the relationship between cognitive status and microglial content, only monkeys older than 20 years of age were used. Old monkeys with cognitive Z score less than 2.0 were considered "non-impaired" in comparison to young monkeys. Old monkeys with cognitive scores greater than or equal to 2.0 were considered cognitively impaired. Statistical analyses were conducted with and without monkeys with high levels of gray matter amyloid plaques. Statistical significance was accepted with $p \leq 0.05$.

3. Results

Although regions of cortical gray matter showed minimal evidence of HLA-DR immunoreactivity for activated microglia in either old or young monkeys, there was considerable immunoreactivity in the white matter of the aged monkeys. Thus, HLA-DR immunohistochemistry demonstrated diffuse activation of microglia in the corpus callosum and subcortical white matter in old monkeys (Fig. 1d and f), whereas young monkeys exhibited limited white matter microglial activation (Figs. 1c and 1e). In contrast, cortical gray matter showed minimal increases in activated microglia as a function of age (Figs. 1a and 1b).

In all examined areas of white matter, the density of activated microglia was elevated in old compared to young monkeys (Fig. 2a). All subcortical white matter regions with the exception of subcortical white matter beneath motor cortex exhibited a significant correlation between activated microglia density and age (Table 2).

Using percent area stained as a measure of HLA-DR$^+$ microglia levels, we observed qualitative increases in percent area in all white matter regions in old compared to young monkeys (Fig. 2c). We detected increases in percent stain that correlated significantly with age in all white matter areas except the subcortical white matter underlying somatosensory cortex (Table 2). Using a mathematically derived measure of immunostained individual cell area ((percent area)/100*density), no significant correlation was detected between age and immunostained individual cell area for any area examined (Table 2).

Among the gray matter areas measured for activated microglia density (Fig. 2b), only the cingulate gyrus and motor cortex gray matter showed a significant correlation between age and activated microglial density (Table 2). When amyloid bearing monkeys were omitted from statistical analysis, density in only the cingulate gyrus was significantly correlated with age by Spearman correlation (Table 2).

In terms of percent area occupied by HLA-DR$^+$ stain, slight qualitative increases in percent area were seen in old compared to young monkeys with age (Fig. 2d). The inferior temporal gyrus and cingulate gyrus were found to have significant correlations between percent area and age (Table 2). When monkeys that evidenced amyloid deposition in the cortex were omitted from statistical analysis, only the inferior temporal gyrus exhibited significant correlation between age and percent area stain (Table 2). No change in significance differences between age groups was seen with one way ANOVA when amyloid plaque bearing monkeys were deleted from analysis of gray matter regions for either percent area or density calculations.

We also compared activated microglial content data with behavioral data generated before the monkeys were sacrificed. Among the six old monkeys that were cognitively assessed, we found a significant increase in the percent stain in the subcortical white matter of the cingulate gyrus in old cognitively impaired monkeys (13.71 ± 0.98% (n = 3)) compared to old cognitively normal monkeys (9.44 ± 1.02 (n = 3)) (ANOVA with Bonferroni correction, $p < 0.05$) (Fig. 3a). Using all the old monkeys (AM31, AM30, AM29,

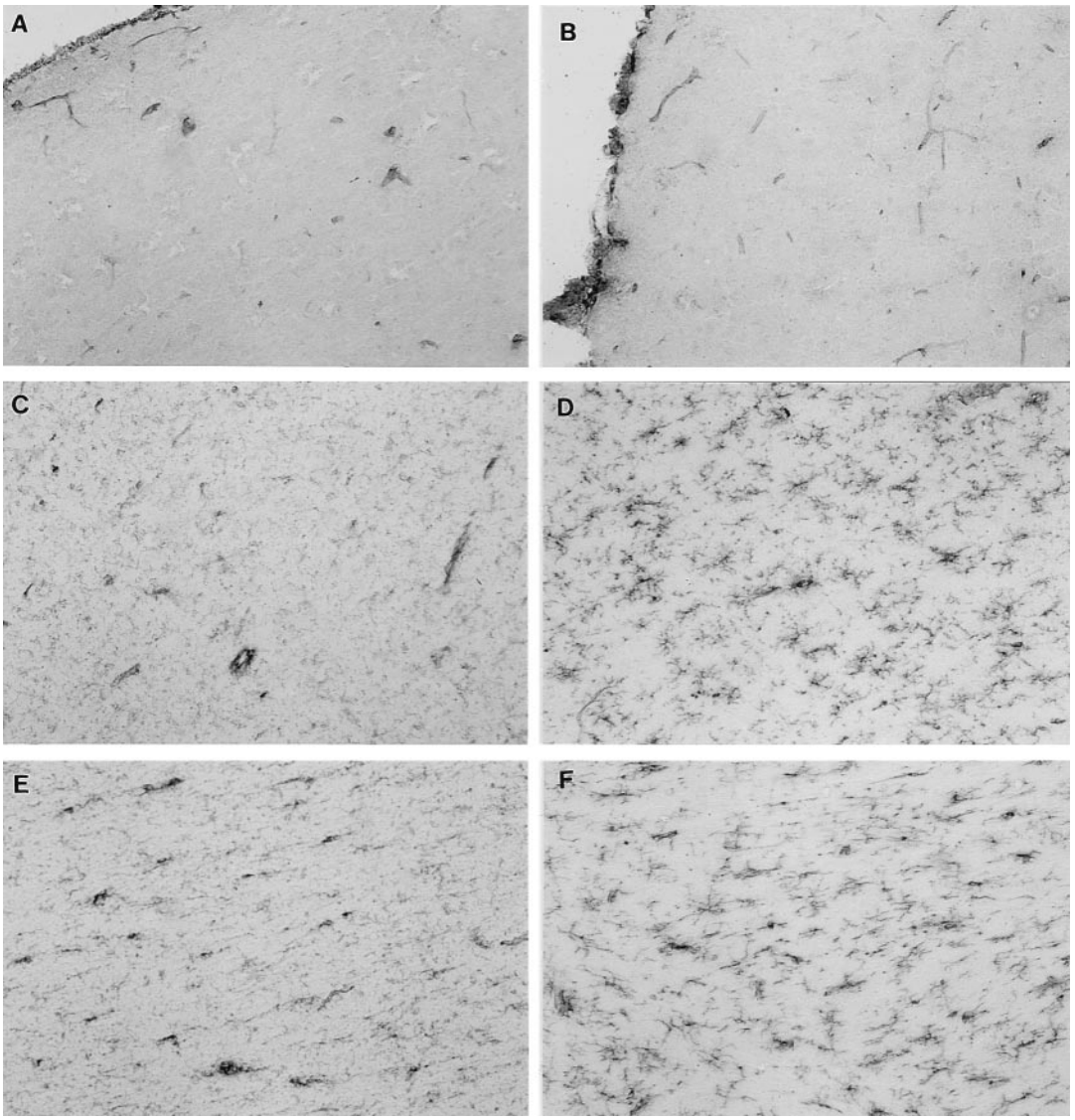

Fig. 1. Diffuse activation of microglia in white matter. Formaldehyde-fixed coronal cryostat sections from a 7-year-old monkey (a, c, e) and a 30-year-old monkey (b, d, f) were stained with HLA-DR monoclonal antibody. Representative staining of frontal lobe gray matter (a, b), frontal lobe subcortical white matter (c, d), and corpus callosum (e, f) is shown (100×).

AM61, and AM28) assayed for activated microglia density, we also found a significant increase in the activated microglia density in the corpus callosum in old cognitively impaired monkeys (4.73 ± 0.74 cells/mm^2 ($n = 3$)) compared to old cognitively normal monkeys (9.76 ± 0.32 ($n = 2$)) (ANOVA with Bonferroni correction, $p < 0.05$) (Fig. 3b). No significant difference in age was detected between the old cognitively impaired monkey group (30.5 ± 0.42 yrs ($n = 3$)) and the old cognitively nonimpaired monkey group (25.72 ± 1.72 yrs ($n = 3$)) (ANOVA with Bonferroni correction, $p < 0.05$).

We next examined the relationship between gray and subcortical white matter activated microglia content as a function of age. When the ratio between the activated microglial content of subcortical white matter versus adjacent gray matter was used, there was a consistent, yet insignificant, increase in the ratio of activated microglia in white matter versus gray matter that occurred with age for all regions examined, suggesting that young monkeys exhibit relatively less activated microglia in white matter compared to old monkeys.

Using antibodies to iNOS, we further confirmed that microglial activation increases with age. Expression of iNOS occurs principally in activated microglia in a variety of neurological diseases [1,16,84]. In Alzheimer's disease (AD) brain, we have been able to localize iNOS to white matter HLA-DR$^+$ microglia but not astrocytes, suggesting that iNOS expression occurs chiefly in activated microglia [70]. Here, we have shown that iNOS$^+$ cells showed characteristic microglial morphology and therefore may represent activated microglia (Fig. 4d and f). By immunohistochemistry, iNOS expression appears to increase with age in

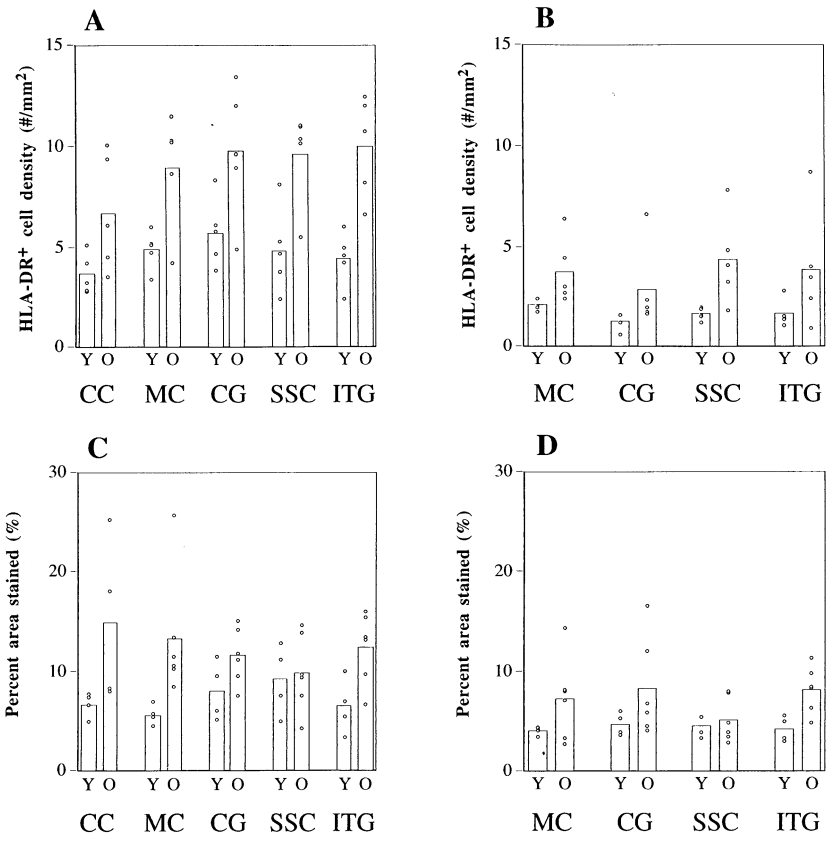

Fig. 2. Activated microglial content as a function of age in gray and white matter. Activated microglial content was measured by two different methods. HLA-DR$^+$ cell body density (#/mm^2) was assessed in four subcortical white matter areas and the corpus callosum (a) and four gray matter areas (b) of 11 different monkeys. The area of HLA-DR$^+$ cellular stain as a percentage of total area was assessed in four subcortical white matter areas and the corpus callosum (c) and four gray matter areas (d) of 11 different monkeys. Data from only 10 monkeys are displayed because one monkey was between 10 and 20 years of age. Circles refer to measurements of individual monkeys and each bar graph represents the mean for each region. (Y = young (4 to 10 years old), O = old ≥20 years old), CC = corpus callosum, MC = motor cortex, CG = cingulate gyrus, SSC = somatosensory cortex, ITG = inferior temporal gyrus). In (a) and (c), indicated regions include four adjacent subcortical white matter regions in addition to the corpus callosum.

white matter (Figures 4c–f). Although not quantified by computerized counting methods, fewer iNOS immunoreactive cells were observed in gray matter areas compared to adjacent white matter areas (Figs. 4a–d). By Western blot, more iNOS is found in corpus callosum of an old monkey compared to a young monkey (data not shown).

To determine if nitric oxide and peroxynitrite are present in subcortical white matter, we performed NTyr immunohistochemistry on cryostat sections of monkey brain. To increase the sensitivity of NTyr antibodies, we delipidated the sections with a chloroform/methanol wash (a procedure analogous to the Folch method) [21]. Immunohistochemical staining showed that white matter contained extensive nitration in all areas examined in old monkeys (Fig. 5b). Young monkeys contained qualitatively lower levels of nitration consistent with the lower level iNOS expression and microglial activation observed in these monkeys (Figs. 4e and 5a) compared to older monkeys (Figs. 4f and 5b). Virtually all cellular structures in subcortical white matter contained immunostaining in old monkeys (Fig. 5b), whereas the nitration pattern in the young was much more sparse (Fig. 5a). Almost all NTyr was detected in structures that had the appearance of myelinated axons. At higher magnification, NTyr could be observed in myelin sheaths (data not shown). Such staining was abolished using 3-nitrotyrosine to preabsorb anti-NTyr antibodies (Fig. 5c). Because we did not anticipate a dramatic elevation in NTyr immunostaining in the old monkey, we repeated immunohistochemical experiments on six different brains on three separate occasions and found similar levels of staining each time.

4. Discussion

The principal findings of the present study were that 1) microglia activation increases with age, 2) microglial activation occurs mainly within the white matter in a diffuse fashion, and 3) the extent of microglia activation is related to the degree of cognitive impairment.

Prior research has suggested the relevance of inflammatory cells to the normal aging process. Although reactive astrocytosis has been extensively evaluated for its role in inflammatory processes associated with brain aging [36,51,

Table 2
Spearman correlations: activated microglia content and age[a]

	By percent area		By density		By percent area/cell	
Gray matter region	*r (n)*	*p*	*r (n)*	*p*	*r (n)*	*p*
Motor	0.435 (11)	NS	0.659 (11)	0.04*	−0.165 (10)	NS
Cingulate	0.641 (11)	0.04*	0.840 (11)	0.01	0.055 (10)	NS
Inferior temporal	0.709 (11)	0.02	0.533 (11)	NS	−0.085 (10)	NS
Somatosensory	0.307 (11)	NS	0.570 (11)	NS	−0.244 (10)	NS
	By percent area		By density		By percent area/cell	
White matter region	*r (n)*	*p*	*r (n)*	*p*	*r (n)*	*p*
Motor	0.824 (11)	0.01	0.602 (11)	NS	0.006 (10)	NS
Cingulate	0.613 (11)	0.05	0.713 (11)	0.02*	−0.317 (10)	NS
Inferior temporal	0.706 (11)	0.03*	0.828 (11)	0.01	−0.235 (10)	NS
Somatosensory	0.270 (11)	NS	0.745 (11)	0.02	−0.488 (10)	NS
Corpus callosum	0.857 (9)	0.02*	0.814 (11)	0.01	0.034 (9)	NS

[a] Both the density and percent area of staining of HLA-DR reactive cell bodies were assessed in four gray matter areas and five white matter areas. Measurements of each area of each monkey brain were assessed three independent times and averaged. Significance was reached for Spearman correlation between age and white matter activated microglia density and percent area HLA-DR stained in most white matter areas examined. Only three gray matter areas showed significant correlation between age and changes in HLA-DR immunostaining and this dropped to two gray matter areas when amyloid plaque bearing monkeys were excluded from analysis. Percent area/cell is the product of (percent area)/100*density.*r* represents rho, *n* represents monkey sample number, and *p* represents *p* value. NS represents not significant at $p \leq 0.05$; * indicates correlation that was nonsignificant when amyloid plaque bearing monkeys were excluded from statistical analysis.

57,85], only recently have microglia garnered much attention in the context of normal aging. Studies examining microglia have shown that increased activation occurs with age [45,47,59,62,67,68]. White matter is the site of most of the age-related microglia activation [59,67]. An increase in microglial activation and not total microglial number account for the increase in activated microglia with age in the rat [52,59]. In contrast to the normal aging process, a selective increase in gray matter microglial activation occurs in AD, whereas white matter contains similar activation levels compared to age-matched controls [47,65], suggesting that a different mechanism is involved in the activation of microglia in AD than in normal aging. One possible promoter for microglia activation is fibrillar amyloid peptide found within the amyloid plaque associated with AD [6,38,46].

In the present study, our data confirm that microglial activation increases with age as revealed by an increase in activated microglial cell density using HLA-DR and iNOS immunohistochemistry. We have further shown that microglial activation occurs principally within white matter in a diffuse manner. Statistical analysis showed a positive cor-

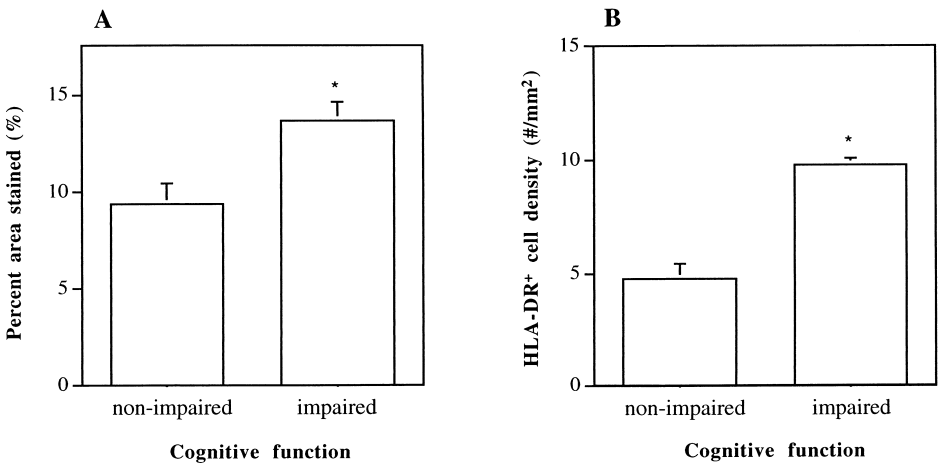

Fig. 3. Correlation of age and cognitive function with activated microglia content. HLA-DR cell density (#/mm^2) and percent area staining for HLA-DR (%) was assessed as described for five or six monkeys, respectively. Measurements were averaged and impaired monkeys (cognitive score $\geq$ 2 SD) were compared to old cognitively nonimpaired monkeys (cognitive score $<$ 2 SD). There was a significant difference between microglial levels of old cognitively nonimpaired and impaired monkeys in the cingulate gyrus subcortical white matter (a) and the corpus callosum (b) by ANOVA (indicated by *) ($p < 0.05$). Activated microglia density values are displayed in (b) while percent stain values are displayed in (a). Values represent the mean $\pm$ SE.

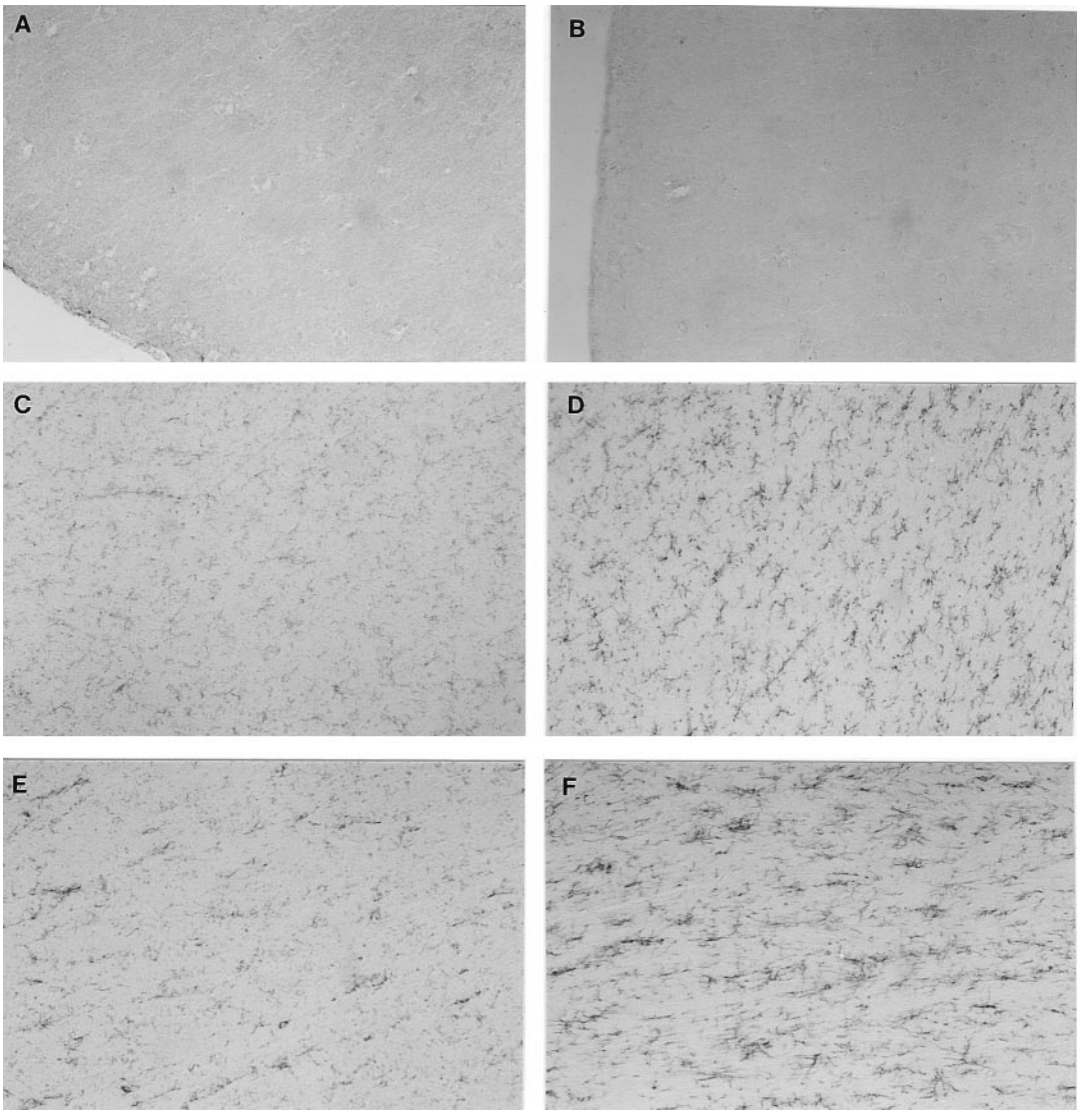

Fig. 4. iNOS expression as a function of age in white matter. Formaldehyde-fixed coronal cryostat sections from a 7-year-old monkey (a, c, e) and a 30-year-old monkey (b, d, f) were stained with iNOS mAb. Representative staining of frontal lobe gray matter (a, b), frontal lobe subcortical white matter (c, d), and corpus callosum (e, f) is shown (100×).

relation between increased age and increased density and percent area occupied by HLA-DR$^+$ cells in most areas of white matter, but not in gray matter. The increase in calculated density and percent stain of HLA-DR$^+$ microglia indicates that activation of microglia in white matter is not constitutive [45], but is minimally present at young ages and significantly increases with age. It is possible that the low level of microglial activation seen in white matter in young individuals may be partly a reflection of the fact that most microglia normally reside in white matter in an inactivated state.

We also observed a less dramatic increase in microglial activation in gray matter. Although activated microglia levels were elevated in the gray matter of one old monkey, activated microglia numbers appear to be significantly influenced by amyloid plaque content (Table 2). It is possible that activated microglia numbers increase in a topographic association with amyloid plaque content (preliminary data) [69] and we are currently examining this possibility.

Because microglial activation was chiefly localized to subcortical white matter, it is likely that the source of activation is the white matter itself. In other studies by our group, we have reported several relationships between myelin breakdown and cognitive dysfunction. By electron microscopy, breakdown of cortical myelin sheaths with axonal sparing is easily detectable in old monkeys [64]. Although a preliminary finding, cognitive dysfunction appears to correlate well with myelin sheath degeneration both in gray and white matter [63]. In addition, myelin breakdown products are significantly elevated in old cognitively impaired monkeys compared with old nonimpaired monkeys (J. Sloane et al., unpublished data). Coupled with the present study, these

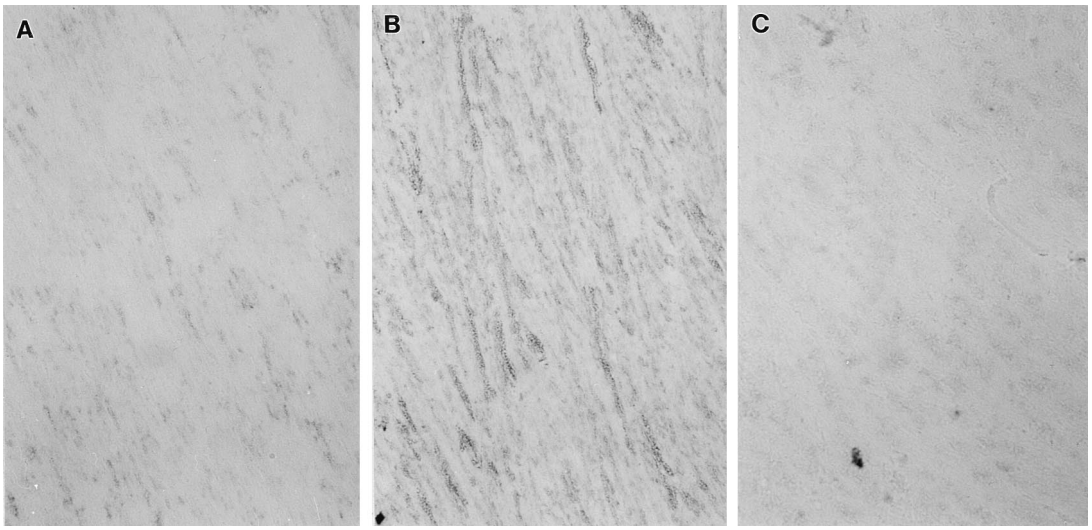

Fig. 5. Tyrosine nitration as a function of age in corpus callosum. Formaldehyde-fixed cryostat sections of a 7-year-old monkey (a) and a 30- year-old monkey (b) were delipidated using the Folch method. Sections were then rehydrated and stained with anti-NTyr antibodies (100×). NTyr immunoreactivity seen in (b) could be preabsorbed by 10 mM NTyr (c)(100×).

findings suggest that microglial activation and myelin loss may be central to age-associated cognitive loss in the rhesus monkey.

Although microglial activation may be a result of white matter degeneration, it is possible that this activation actually contributes to or exacerbates age-related myelin pathology. Hence the role of activated microglia in age-dependent white matter damage and cognitive loss is currently unclear.

On the one hand, myelin breakdown may lead to activation of microglia, which function to clear the white matter parenchyma of myelin breakdown products. By electron microscopy, our group has previously shown that microglia (and astrocytes) contain electron dense inclusions that have the appearance of lipid-rich myelin remnants [62]. Others have shown that microglia possess the capacity to phagocytose myelin in vitro and that phagocytosis is mediated by opsonization [17,53,73,81]. Activation of microglia occurs rapidly after myelin is phagocytosed [81]. Thus, microglia may partly function to remove degenerating myelin from the brain parenchyma.

Alternatively, activated microglia can also initiate or contribute to white matter injury by release of toxic molecules. In the process of clearing myelin and aiding with repair, microglia may cause injury to surrounding oligodendrocytes and myelin sheaths. Several microglial-secreted molecules, such as TNF-α [29,42], complement [82], and nitric oxide [48,49] are known oligodendrocyte toxins. Each of these molecules is secreted from activated microglia and seems to be preferentially toxic to oligodendrocytes or myelin.

The present study is the first to examine cognitive deficits in the context of changes in microglial activation in the normal aging of the brain. Significant correlations between cognitive function and activated microglia density were attained with two white matter but no gray matter regions, suggesting a possibly important relationship between white matter microglial activation and age-associated cognitive deficits. However, a more comprehensive study will be undertaken in the future to test the strength of correlations between microglial activation and the age-related cognitive decline seen in the rhesus monkey.

In this study, we showed increases in iNOS and NTyr content in subcortical white matter with age. In agreement with the changes in activation state of microglia with age, the density of $iNOS^+$ cells qualitatively increased with age. The content and distribution of NTyr was similar with the pattern of microglial activation and iNOS expression. The presence of NTyr indicates that both increased generation of nitric oxide and peroxynitrite in subcortical white matter occurred with age. Thus, several lines of evidence strongly suggest that brain aging is linked to increased microglia activation, iNOS expression, nitric oxide generation, and localized nitric oxide-mediated protein nitration.

Nitric oxide and peroxynitrite may be responsible for much of the oxidative stress observed with age [12,13]. Peroxynitrite seems capable of generating a number of oxidation products, such as lipid peroxides, protein carbonyls, and oxidized DNA [18,24,30,32,34,66,77] that are also observed in the aged brain [4,8,11,58,71]. In the context of normal brain aging, we have preliminary evidence suggesting increased NTyr content with age in purified myelin (data not shown). In addition, we have found several candidate proteins that are especially susceptible to nitration. Other experiments are currently being conducted to assess further the role of nitric oxide in age-related white matter damage.

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