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Activation of calpain-1 in myelin and microglia in the white matter of the aged rhesus monkey

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Abstract

Ultrastructural disruption of myelin sheaths and a loss of myelin with age are well-documented phenomena in both the human and rhesus monkey. Age-dependent activation of calpain-1 (EC 3.4.22.52) has been suggested as a plausible mechanism for increased proteolysis in the white matter of the rhesus monkey. The present study documents activation of calpain-1 throughout brain white matter in aged animals, evidenced by immunodetection of the activated enzyme as well as a calpain-derived spectrin fragment in both tissue section and Triton X-100-soluble homogenate of subcortical white matter from the frontal, temporal, and parietal lobes. Separation of myelin fractions from brain stem tissue into intact and floating myelin confirmed previous reports of an age-related increase in activated calpain-1 in the floating fraction. Measurements of calpain-1 activity using a fluorescent substrate revealed an age-related increase in calpain-1 proteolytic activity in the floating myelin fraction consistent with immunodetection of the activated enzyme in this fraction. Double-immunofluorescence demonstrated co-localization of activated calpain-1 with human leukocyte antigen-DR (HLA-DR), a marker for activated microglia, suggesting that these cells represent the major source of the increase in activated calpain-1 in the aging brain. These data solidify the role of calpain-1 in myelin protein metabolism and further implicate activated microglia in the pathology of the aging brain.

Keywords: aging, microglia, myelin, oligodendrocyte, proteolysis, spectrin.

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Normal brain aging has been associated with significant imaging, ultrastructural, and biochemical changes that occur primarily in the white matter of the brain. Most notably, a finding of an age-dependent loss of white matter volume by magnetic resonance imaging (MRI) in humans (Albert 1993) was supported by histochemical and biochemical reports of myelin loss (Kemper 1994; Sloane *et al*. 2003). Microglial activation within the white matter has been described in the aged human (Mattiace *et al*. 1990), monkey (Sheffield and Berman 1998; Sloane *et al*. 1999), and rat (Ogura *et al*. 1994). At the electron microscopic level, age-related change in the ultrastructure of myelin in the rhesus monkey is a well-described phenomenon throughout the CNS (Peters 2002). The loss or alteration of myelin with age could account for the observed cognitive decline that affects both humans (Inouye *et al*. 1993) and monkeys (Herndon *et al*. 1997), by hampering axonal conduction. Previous biochemical studies have shown increases in activated calpain-1 in the corpus callosum as well as in the floating myelin fraction of frontal lobe white matter of aged rhesus monkeys and postulate a role for the enzyme in age-dependent myelin protein degradation (Sloane *et al*. 2003).

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Abbreviations used: AA, arachadonic acid; AC-1, activated calpain-1; AFU, arbitrary fluorescence units; BSA, bovine serum albumin; CNPase, 2′-3′ cyclic nucleotide phosphodiesterase; DAB, 3,3-diaminobenzidine tetrahydrochloride; DTT, dithiothreitol; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; HLA-DR, human leukocyte antigen-DR; MRI, magnetic resonance imaging; MBP, myelin basic protein; MAG, myelin-associated glycoprotein; PBST, phosphate-buffered saline containing 0.1% Triton X-100; PLP, proteolipid protein; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The calpains are a family of neutral cysteine proteases, residing in the cytosol, which are encoded by 14 different genes. Calpain-1 (EC 3.4.22.52) and calpain-2 (EC 3.4.22.53) have been linked extensively to ischemic pathology in the brain (Yamashima 2000; Neumar *et al*. 2001) as well as being associated with the regulation of tau phosphorylation and neurofibrillary tangle formation in Alzheimer's disease (Taniguchi *et al*. 2001). The enzymes, calpain-1 and calpain-2, are highly similar in structure and differ in their sensitivity to calcium ion concentration (in the low and high micromolar range, respectively). Calpains are heterodimeric proteins consisting of a common 29 kDa regulatory subunit and a unique 80 kDa catalytic subunit, which upon activation undergoes autolytic cleavage, through a 78 kDa intermediate to form a 76 kDa active conformation exposing the active site. The enzyme undergoes continuous autoproteolytic events as a mechanism of inactivation. Besides regulation by cellular calcium levels, the endogenous inhibitor, calpastatin, functions to prevent calpain activation by acting as a suicide substrate (Blomgren *et al*. 1999).

Most of the known substrates for calpain that have been identified are cytoskeletal proteins. These include spectrin (Siman *et al*. 1984), tau (Johnson *et al*. 1989), actin (Potter *et al*. 1998), tubulin and glial fibrillary acidic protein (Ishizaki *et al*. 1983), neurofilaments (Banik *et al*. 1997; Stys and Jiang 2002), and microtubule-associated proteins, MAP1B and MAP2 (Fischer *et al*. 1991). Interestingly, additional substrates for calpain include the major myelin proteins, myelin basic protein (MBP; Banik *et al*. 1994), myelin-associated glycoprotein (MAG; Sato *et al*. 1984), and most recently, 2′3′-cyclic nucleotide phosphodiesterase (CNPase; Sloane *et al*. 2003). Fittingly, calpain activation has been implicated in the pathology of the demyelinating diseases: multiple sclerosis (MS; Shields *et al*. 1999) and a related animal model, experimental autoimmune encephalomyelitis (EAE; Shields and Banik 1999).

While the function of these myelin proteins is not completely understood, it is clear that the objective of the myelin membrane is to maintain its compact lamellar structure in order to ensure fast axonal conduction. The recent discovery of a C-terminal interaction of CNPase with tubulin (Bifulco *et al*. 2002) suggests that CNPase might function as a membrane linker between the lipid bilayer and the underlying cytoskeleton. In addition, reports that myelin proteolipid protein (PLP) interacts with integrin (Gudz *et al*. 2002) support the concept that myelin proteins function to interact with the cytoskeleton. Thus, it is not surprising to find myelin proteins among the substrates of calpain, the major cysteine protease involved in cytoskeletal protein degradation.

The present study sought to identify if the age-dependent activation of calpain-1, previously described to occur in the corpus callosum (Sloane *et al*. 2003), is present throughout the aging CNS and which glial cell type is responsible for the increase in active enzyme. Using polyclonal antibodies against the active form of calpain-1 and calpain-derived spectrin fragments, a variety of brain areas were examined by immunoblot and immunohistochemistry for calpain-1 activation. In order to localize precisely the cellular source of active calpain-1, myelin was fractionated and calpain-1 activation was measured both by immunoblot and by detection of the cleavage of a fluorogenic substrate. Finally, co-localization by double immunofluorescence was employed to demonstrate activated calpain-1 within activated microglia.

Materials and methods

Monkeys

Twenty-four rhesus monkeys (*Macaca mulatta*) acquired from the colony of the Yerkes Regional Primate Research Center were behaviorally tested and their brains processed to evaluate age-related changes. Basic demographic data on all monkeys used in the study are presented in Table 1. All monkeys spent several years free-ranging in social groups maintained at the Yerkes Regional Primate Research Center before being housed individually for 1–3 years

Table 1 Rhesus monkeys used in study

ID No.	Age (years)	Sex
AM24	30.4	F
AM48†	30.2	M
AM63†	25.8	F
AM67*	24.0	M
AM68	26.2	M
AM69	30	M
AM73†	30.4	M
AM78	5.8	F
AM82	28.1	F
AM92	8.8	M
AM94*	6.1	M
AM95	7.8	F
AM98	28.1	F
AM107*	26	F
AM109*,†	29.6	M
AM120*	26.5	F
AM123	21	M
AM126	21	F
AM127	7.4	M
AM131	6.7	F
AM139	12.7	M
AM145*	8.8	M
AM146*,†	6.3	F
AM147*,†	4.4	F
AM148	7.3	M

*Indicates subject used in myelin purification experiment. †Indicates subject used in immunohistochemical or immunofluorescence experiments. Tissue from all subjects was used in immunoblot experiments

during behavioral testing at either the Yerkes Regional Primate Research Center or the Laboratory Animal Science Center at Boston University School of Medicine. Both facilities are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All animal protocols were approved by the Institutional Animal Care and Use Committees of both institutions and complied with the guidelines of the National Institutes of Health and the Institute of Laboratory Animal Resources Commission on Life Sciences Guide for the Care and Use of Laboratory Animals.

Prior to entry into the study, medical records were screened for any history of splenectomy or thymectomy, exposure to radiation, chronic illness including viral or parasitic infections, neurological disease, or chronic drug administration. In addition, all monkeys received medical examinations that included serum chemistry, hematology, urine, and fecal analysis before entering the study. During the course of the study, all monkeys were visually inspected on a daily basis by both animal care personnel and research technicians and were given regular TB tests and general medical exams every 4 months to ensure their continued good health. All monkeys had brain MRI scans and underwent behavioral testing to assess cognitive function (Herndon *et al*. 1997; Moss *et al*. 1997).

Tissue preparation

All monkeys were deeply anesthetized and transcardially perfused with 2–4 L of Krebs–Heinseleit buffer, pH 7.4 (6.41 mM Na_2HPO_4, 1.67 mM NaH_2CO_3, 137 mM NaCl, 2.68 mM KCl, 5.55 mM glucose, 0.34 mM $CaCl_2$, 2.14 mM $MgCl_2$) at 4°C. One hemisphere was blocked, *in situ*, in the coronal stereotactic plane, quickly removed, weighed, and then flash-frozen in −70°C isopentane (Rosene and Rhodes 1990). The blocks of tissue were stored in an ultralow freezer at −80°C. Coronal sections were cut on a cryostat into interrupted series of 15-μm thick sections spaced at 750-μm intervals. These sections were mounted onto poly-L-lysine subbed slides, rapidly dried, and then stored in the freezer until processed for immunocytochemistry. The monkey cryostat sections selected for this project were coronal sections including frontal, parietal, and temporal subfields. For biochemical assays, the remaining hemisphere was dissected into neuroanatomically relevant blocks, flash-frozen on dry ice, and stored in an ultralow freezer at −80°C. Triton-soluble homogenates were prepared by homogenization in 5 vol of phosphate-buffered saline with 1% Triton X-100 and protease inhibitors using a hand-held Teflon-coated pestle. After 30 min on ice, the homogenate was centrifuged at 16 000 *g* for 10 min and the supernatant was assayed for protein content using the BCA assay (Pierce, Rockford, IL, USA).

Myelin purification

Transverse sections of medulla approximately 5 mm in thickness and ranging from 0.1 to 0.3 g were dissected from brain stem blocks and samples were immediately processed after dissection and weighing. Myelin was isolated from white matter by disruption with a Dounce homogenizer and separation on discontinuous sucrose gradients as previously described (Norton and Poduslo 1973) to yield intact myelin at the interface between 0.85 M and 0.32 M sucrose and a floating myelin fraction floating in the 0.32 M sucrose portion (Lees *et al*. 1980; Persson and Corneliuson 1989). Intact and floating myelin fractions were assayed for total protein content using the BCA assay (Pierce). Samples of the intact myelin fraction representing 200 μg total protein were delipidated by adding 1 mL of 3 : 2 ether/ethanol, vortexed briefly, and remaining protein was pelleted by centrifugation at 16 000 *g* for 5 min at 4°C. Protein pellets were lyophilized in a speed vacuum centrifuge for 5 min and resuspended in 200 μL 2× reducing sample buffer (Laemmli 1970) before analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Immunoblotting

Triton-soluble homogenates and purified myelin fractions were treated identically during immunoblotting. In order to assure adequate randomization of samples between immunoblots, samples were loaded in ascending numerical order according to the AM identification number provided upon entry into the study (see Table 1). Samples representing 20 or 30 μg total protein were mixed with sample buffer containing β-mercaptoethanol, boiled for 5 min, centrifuged at 16 000 *g* for 5 min, and loaded on to 4–20% SDS–polyacrylamide gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and, after blocking in 5% non-fat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20), membranes were blotted with calpain-1 monoclonal antibody (1 : 500; Sigma, St Louis, MO, USA) or β-tubulin monoclonal antibody (1 : 1000; Sigma). Polyclonal antibodies specific for the active form of calpain-1 (1 : 2500; Meyer *et al*. 1996) and the calpain-derived spectrin fragment (1 : 2500; Roberts-Lewis *et al*. 1994) were also used. Depending on primary antibody type, antibody/antigen complexes were identified using peroxidase-linked goat anti-mouse IgG (1 : 10 000; KPL, Gaithersburg, MD, USA) or goat anti-rabbit IgG (1 : 10 000; KPL) and visualized using SuperSignal West Pico enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). Primary antibody dilutions were in 5% non-fat milk in TBST and 0.02% NaN_3. All washes and secondary antibody dilutions were in TBST.

Enzyme activity measurements

Measurements were made as previously described (Mallya *et al*. 1998) using the cleavage of the fluorescent dipeptide substrate Suc-Leu-Tyr-4-Methoxy-2-Naphthylamine (Suc-LY-MNA; Enzyme System Products, Livermore, CA, USA) as an indicator of calpain-1 activity. Samples of 20 μg total protein from intact and floating myelin fractions were added to a buffer solution consisting of 50 mM Tris pH 7.4, 50 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 1 mM EGTA, and 0.2 mM substrate [in dimethyl sulphoxide (DMSO)] in a total volume of 900 μL. The reaction was initiated by the addition of 100 μL of 50 mM $CaCl_2$ and the increase in fluorescence at 425 nm was continuously recorded for 10 min on a Hitachi F-4500 Fluorescence Spectrophotometer. For purposes of data presentation, the absolute results in arbitrary fluorescence units (AFU) were converted to μg of calpain-1 based on a linear standard curve (see inset Fig. 7) generated using 0, 100, and 200 ng of purified human calpain-1 (Calbiochem, San Diego, CA, USA) and the total protein levels resolved from myelin fractionation (Table 2).

Immunohistochemistry

Sections were briefly fixed in 4% paraformaldehyde for 5 min before treating for 15 min with 0.3% H_2O_2 solution in 0.01 M

Table 2 Total protein levels in myelin fractions from the medulla

Age group	Intact myelin protein (μg/mg wet weight)	Floating myelin protein (μg/mg wet weight)
Young	30.903	0.316
Old	30.563	4.222
	($p = 0.478$)	($p = 0.012$)

phosphate-buffered saline, pH 7.4, containing 0.1% Triton X-100 (PBST). Sections were then incubated for 30 min in PBST, to permeabilize white matter, before being transferred to 10% normal serum in PBST for 1 h. Sections were then incubated for 16 h at 4°C in either polyclonal antibody directed against activated calpain-1 (1 : 1000) or the 150 kDa calpain-derived spectrin fragment (1 : 10 000). Primary antibodies were diluted in 1% normal serum and PBST. After thorough washing, the sections were treated with appropriate biotinylated secondary antibodies for 1 h at room temperature, also in PBST containing 1% normal serum, followed by incubation in avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Peroxidase labeling was visualized by incubation in 0.01% 3,3-diaminobenzidine tetrahydrochloride (DAB) for brown staining and with the addition of 0.6% nickel ammonium sulfate for blue/black staining. Sections were then washed, dehydrated and mounted in DPX (Fluka, Buchs, Switzerland) before coverslipping.

Pre-absorption of immunostaining

The specificity of the polyclonal antibody against the active form of calpain-1 was established by pre-absorption studies using immunohistochemistry and immunoblot. Forty micrograms of human calpain-1 (Calbiochem) in 20 mM imidazole, pH 7.3 were activated by adding 1 mM $CaCl_2$ and 1 mM dithiothreitol (DTT) and incubating at 25°C for 10 min with agitation. To quench the reaction, 5 mM EGTA were added, as was 1% bovine serum albumin (BSA) to prevent enzyme autolysis. The concentration of activated calpain-1 was brought to 0.4 ng/μL by adding a buffer of 20 mM imidazole, pH 7.3 and 5 mM EGTA. An identical reaction was performed in the absence of calcium and DTT to serve as a negative control. Activation of the enzyme was determined by immunoblot using the activated calpain-1 antibody, which recognized only the samples treated with Ca^{2+}. Pre-absorption of the antibody was accomplished by incubating 20 μg of active calpain-1 with the polyclonal antibody overnight at 4°C with agitation. Entire preparation was then diluted in appropriate buffer and added to PVDF membrane or hemisphere cryostat section.

Immunofluorescence

Sections were briefly fixed in 4% paraformaldehyde for 5 min. Sections were then incubated for 30 min in PBS with 0.3% Triton X-100, to improve antibody penetration, before being transferred to 10% normal serum in PBST for 1 h. Sections were incubated 16 h at 4°C in primary antibody diluted in 1% normal serum and PBST. Double-labeling experiments utilized polyclonal antibodies directed against activated calpain-1 (1 : 500), monoclonal IgG against HLA-DR (ICN) 1 : 15, monoclonal IgG against glial fibrillary acidic protein (GFAP; Chemicon, Temecula, CA, USA) 1 : 500, or monoclonal IgG against CNPase (Sigma) 1 : 250. After thorough washing, the sections were treated with appropriate AlexaFluor secondary antibodies (Molecular Probes, Eugene, Or, USA) for 1 h at room temperature, also in PBST containing 1% normal serum. Digital images were collected using Improvision® software (v.3.1.2) under appropriate spectral filters on a Zeiss Axiovert 200M microscope with a 100 W mercury lamp, using a CCD camera. Images were stored and processed using Adobe® Photoshop v5.0.

Statistical analysis

Immunoblots were imaged by scanning autoradiographs using a Hewlett-Packard ScanJet 4C scanner and quantitation was performed prior to any manipulation of raw images. Digital images of autoradiographs from different blots were cropped for the purpose of presentation only. Resulting TIFF files were imported into Scion Image v4.02 and pixel density of bands of interest was measured using the Gelplot2 macro function. Resulting raw pixel density values were imported into Microsoft Excel for statistical analysis. Raw pixel density values were averaged for both age groups and fold expression differences compared. In order to compare results from immunoblots from different areas of the brain, it was necessary to standardize raw pixel densities. This was done using a standard z-score transformation for each region whereby the average pixel density for all animals in that region was used as the mean. Resulting z-scores were then averaged across brain regions and the cumulative difference between young and old animals established. Statistical significance was determined using a two-tailed Student's test assuming unequal variance with significance determined by $p < 0.05$.

Results

Age-related increases in active calpain-1

Immunohistochemical analysis using a polyclonal antibody against active calpain-1 revealed a significant increase in staining within white matter regions of aged animals (Figs 1d and f) compared to young (Figs 1c and e). Comparable regions of gray matter from both young (Fig. 1a) and old (Fig. 1b) animals revealed no significant immunostaining and no differences in staining patterns between age groups. Two general types of white matter staining were apparent in sections from aged animals. First, staining of cells having the morphologic appearance of microglia (inset Fig. 1f) was common. While also occasionally present in younger animals, these characteristic cellular profiles were considerably more frequent in older animals. Second, larger areas of staining, without clear cell profiles, were also apparent in sections from aged animals in association with microvessels (data not shown).

The specificity of immunostaining was established by pre-absorption where the polyclonal antibody to activated calpain-1 was pre-absorbed with human calpain-1 activated by Ca^{2+}. Pre-absorption of immunoblot reactivity of both purified active enzyme and active calpain-1 present in white matter homogenates was achieved (Fig. 1g). Equal loading

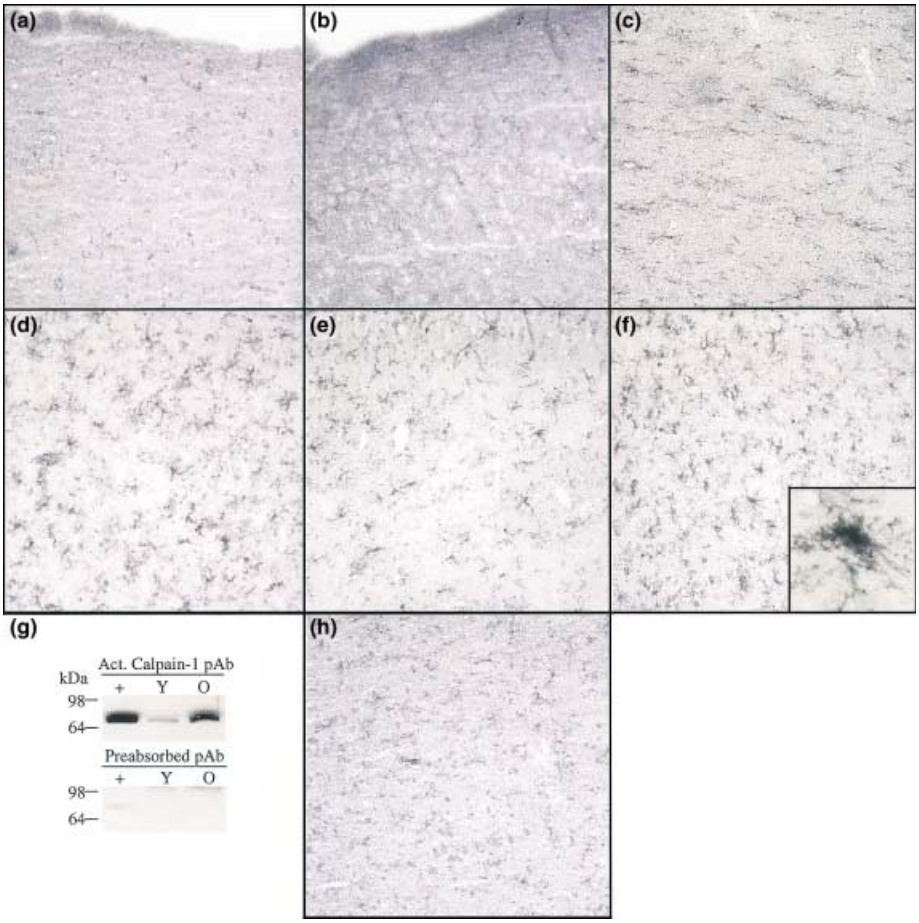

Fig. 1 Age-dependent increase in activated calpain-1 occurs predominantly in white matter regions. Immunohistochemical analysis of coronal sections of temporal lobe in young ($n = 4$) and old ($n = 4$) monkeys reveals a general lack of staining within gray matter regions in both young (a) and old (b) animals. An age-related increase in immunostaining was obvious when comparing white matter in young (c) and old (d) monkeys. Similar analysis in coronal sections of parietal lobe white matter in young (e) and old (f) monkeys highlights the predominance of immunostaining in aged animals. High magnification of active calpain-1-labeled cells (inset f) demonstrates typical morphology of microglia. Pre-absorption with 20 μg of purified calpain-1 activated in the presence of calcium (indicated by +) revealed a marked decrease in immunoreactivity by immunoblot (g) with white matter homogenates of young (Y) and old (O) monkeys. Pre-absorption also eliminated the characteristic immunostaining normally observed in the white matter of old monkeys (h). (a–f) 40× magnification; (Inset f) 400× magnification.

was confirmed by colloidal Coomassie staining of the gel post-transfer. The immunohistochemical staining for activated calpain-1 in white matter in the aging animal was similarly abolished after pre-absorption (Fig. 1h).

Using the same polyclonal antibody against the 76 kDa active form of calpain-1, immunoblot analysis of Triton X-100-soluble homogenates prepared from subcortical white matter from the frontal, temporal, and parietal lobes reveals clear association of increased calpain-1 activation with age throughout the white matter (Fig. 2). Fold expression differences in the level of activated calpain-1 were greatest in temporal lobe white matter ($p = 0.028$), though also increased but not significantly in parietal and frontal lobe white matter. To generate a global quantitative value of the increase in activated calpain-1 in aged brain, raw densitometry data from these immunoblots were standardized against the mean value for all animals from each brain area. Following this z-score conversion of activated calpain-1 immunoreactivity in all three regions of subcortical white matter examined, the cumulative average z-scores for young ($n = 10$) and old ($n = 12$) animals were calculated and are presented in Fig. 5. A significant difference ($p = 0.014$) in activated calpain-1 immunoreactivity was found between young and old animals.

Age-related increases in calpain-derived spectrin fragmentation

Activation of calpain-1 has been shown to lead to spectrin proteolysis, resulting in a unique 150 kDa calpain-derived fragment (Johnson *et al.* 1991). Immunohistochemical

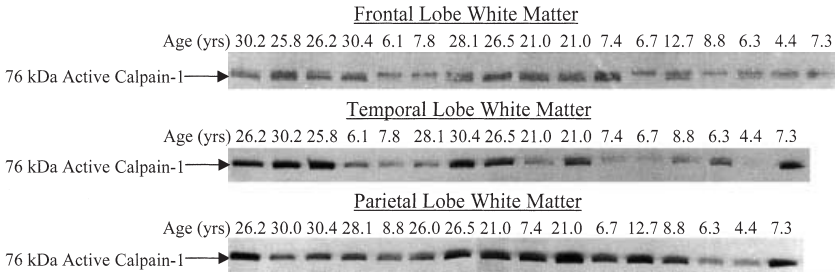

Fig. 2 Immunoblots showing age-dependent activation of calpain-1 in white matter. Homogenates of subcortical white matter from frontal, temporal, and parietal lobes from old ($n = 12$) and young ($n = 10$) monkeys were immunoblotted for activated calpain-1. The lower band representing the 76 kDa active enzyme is highlighted by the arrow.

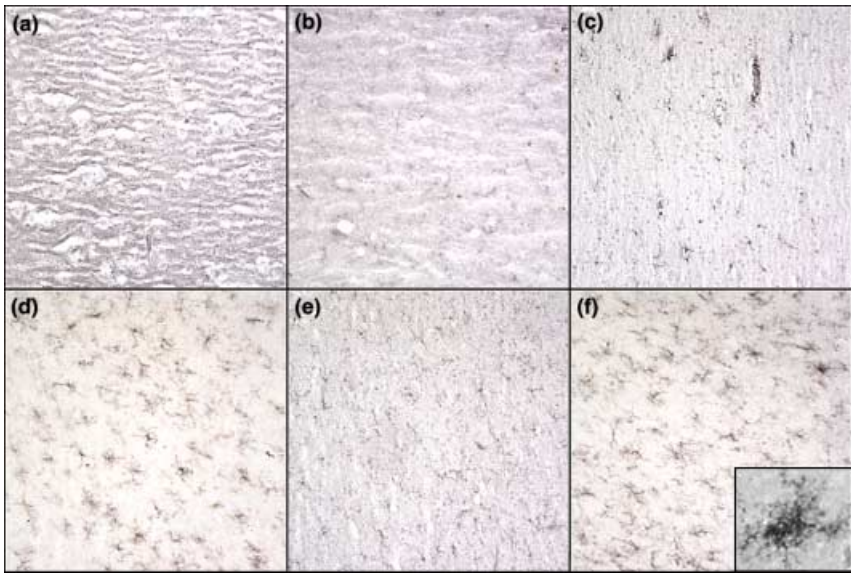

Fig. 3 Age-dependent increase in calpain-cleaved spectrin staining within white matter regions. Immunohistochemical analysis of coronal sections of temporal lobe in young ($n = 4$) and old ($n = 4$) monkeys reveals no detectable staining in gray matter of either young (a) or old (b) animals. An increase in immunostaining is confined largely to white matter in young (c) and old (d) animals with an obvious age-related increase. Similar analysis in coronal sections of parietal lobe in young (e) and old (f) monkeys confirms the result seen in the temporal lobe. 40× magnification. High magnification of typical immunostaining (inset f) illustrates the morphology of cells stained for calpain-derived spectrin fragments; 400× magnification.

analysis using a polyclonal antibody against the 150 kDa calpain-derived cleavage product of spectrin revealed an increase in staining within white matter regions of aged animals (Figs 3d and f) compared to young animals (Figs 3c and e). Comparable regions of gray matter from both young (Fig. 3a) and old (Fig. 3b) animals revealed no significant immunostaining and no differences in staining patterns between age groups. Cellular profiles with the morphologic appearance of microglia were characteristic of the immunostaining observed in aged animals (Fig. 3g and inset). Sections from young animals were not free of this characteristic staining; however, it was considerably less frequent. These cellular profiles appear similar to those observed in immunostaining with the activated calpain-1 antibody. As with the activated calpain-1 antibody, staining was also present within the microvasculature.

Using the same polyclonal antibody against calpain-cleaved spectrin, immunoblot analysis of Triton-soluble homogenates prepared from subcortical white matter from the frontal, temporal, and parietal lobes demonstrates an age-dependent association (Fig. 4). Fold expression differences in the level of calpain-derived spectrin fragmentation were greatest in parietal lobe white matter ($p = 0.029$), though also increased but not significantly in temporal and frontal lobe white matter. To generate a global quantitative value of the increase in calpain-derived spectrin fragmentation in aged brain, raw densitometry data from these immunoblots were standardized against the mean value for all animals from each brain area. Following this z-score conversion of calpain-derived spectrin fragment immunoreactivity in all three regions of subcortical white matter examined, the cumulative average z-scores for young ($n = 10$) and old ($n = 12$) animals were calculated and are presented in Fig. 5. This analysis revealed a trend ($p = 0.07$) towards more calpain-derived spectrin fragmentation in older animals; however, this result lacked statistical significance.

Calpain activation occurs largely in floating myelin fractions

To establish the source of the increased activated calpain observed in white matter homogenates, separation of intact from floating myelin (Lees *et al.* 1980) was performed using the myelin purification technique of Norton and Poduslo (1973). Purification of these myelin fractions from

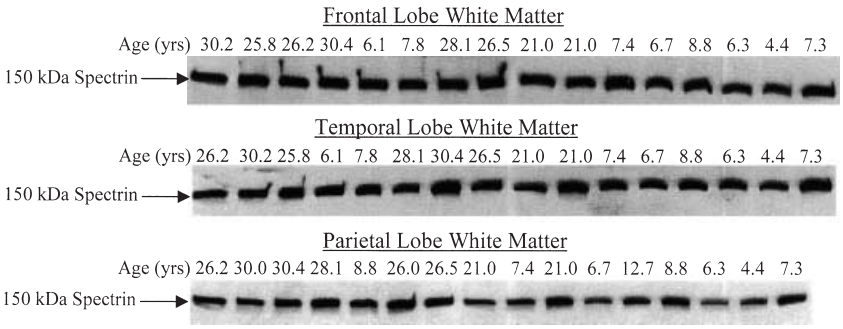

Fig. 4 Immunoblots showing age-dependent increase in calpain-derived spectrin fragmentation in white matter. Homogenates of subcortical white matter from frontal, temporal, and parietal lobes from old ($n = 12$) and young ($n = 10$) monkeys were immunoblotted for the 150 kDa calpain-derived spectrin fragment.

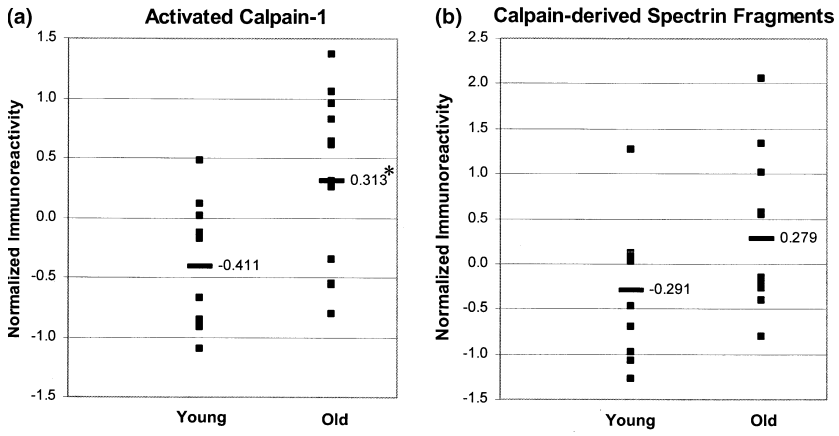

Fig. 5 Quantitation of active calpain-1 and calpain-derived spectrin fragments in white matter regions. Densitometric measurement of immunoreactivity for both active calpain-1 (a) and calpain-derived spectrin fragments (b) from each cortical area (frontal, temporal, parietal) was normalized based on the average immunoreactivity for that brain area for each animal and the resulting z-scores for each animal from each brain area was averaged. This cumulative average z-score of each animal from all cortical areas was plotted and the average cumulative z-score for each age group is indicated by the bar and value. Asterisk indicates statistically significance at the $p < 0.05$ level using a Student's *t*-test.

transverse sections of medulla from four young and four old animals (see Table 1), resulted in consistently greater amounts of floating myelin in a fraction that had significantly higher protein content in aged animals as described previously in frontal lobe white matter (Sloane *et al.* 2003). Samples of medulla were chosen to test the hypothesis that calpain-1 activation is occurring throughout white matter areas and provide an ample source of tissue for myelin purification. Immunoblot analysis of the intact myelin fraction revealed that calpain-1 is contained within the myelin membrane; however, the level of active calpain-1 in this fraction is minimal and does not increase with age (Fig. 6a). In contrast, in the floating myelin fraction, most of the calpain-1 was also detected using the antibody against the active form of the enzyme (Fig. 6b). As shown by immunoblot, the concentration of active calpain-1 in the floating fraction was comparable in young and old animals. However, the absolute amount of the active enzyme in the fraction was 13-fold higher in old animals because of the higher protein content of the fraction generated from aged animals (Table 2).

Consistent with these findings were changes in the activity of calpain-1 in the intact and floating myelin fractions determined by the degradation of a fluorescent substrate (Fig. 7). Based on a standard curve of substrate cleavage generated using purified human calpain-1 (inset Fig. 7), AFUs were scaled to the total amount of active calpain-1 present in each myelin fraction (in ng equivalents). Measurements represent the average of four animals in each age group assayed in two independent experiments. Calpain-1 activity measured within floating myelin is markedly higher in aged animals than in young, demonstrating statistical significance ($p = 0.008$). The overall level of enzyme activity appears much higher in the intact myelin fraction because of the 7.5-fold higher level of protein present in intact versus floating myelin (Table 2). However, there was no statistical difference between young and old animals in the level of calpain-1 activity in the intact myelin fraction.

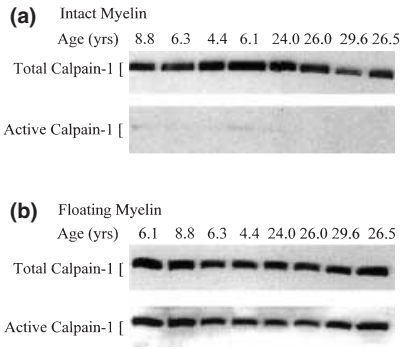

Fig. 6 Active calpain-1 localizes to floating myelin. Myelin was isolated from samples of medulla from young ($n = 4$) and old ($n = 4$) animals using sucrose density centrifugation. This procedure results in an intact myelin fraction (at the sucrose interface) as well as a floating myelin fraction (above interface). Activated calpain-1 was detectable at similar concentrations in the floating myelin fraction of young and old animals with minimal detection of the active enzyme in the intact fraction. Absolute levels of active enzyme differed greatly between young and old animals due to a 13-fold increase in total protein levels in the aged floating fraction.

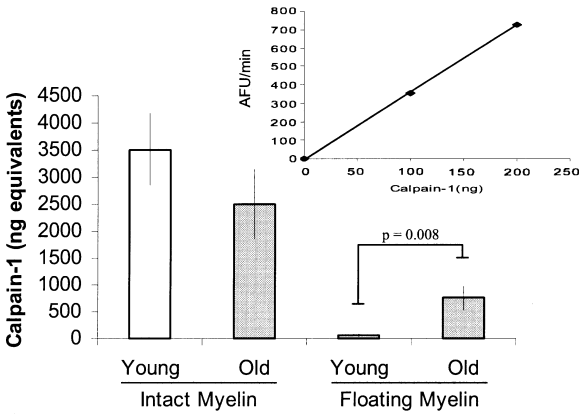

Fig. 7 Calcium-dependent proteolytic activity of calpain-1 is increased in the floating myelin fraction of old monkeys. Calpain-1 activity was measured in both intact and floating myelin fractions by monitoring cleavage of a fluorescent substrate (Suc-LY-MNA) in the presence of 5 mM Ca^{2+}, 1 mM EDTA, and 1 mM EGTA. Active calpain-1 in ng equivalents per total myelin isolation was derived from a standard curve created with known amounts of purified calpain-1 (inset). The amount of total protein isolated in the intact myelin fraction is markedly higher than that of the floating myelin fraction (Table 2). Thus, the amount of active calpain-1 present in intact myelin is observed to be higher than that of the floating myelin fraction. Asterisk indicates statistically significance at the $p < 0.05$ level using a Student's *t*-test.

Active calpain-1 localizes to microglia, not oligodendrocytes or astrocytes

In order to clarify further the cellular origin of the increase in active calpain-1 seen in aged animals, co-localization by immunofluorescence was attempted using whole hemisphere cryostat sections of rhesus monkey brain. Activated calpain-1 staining was predominantly associated with activated microglia, identified by the expression of HLA-DR. Two representative cells double-labeled for activated calpain-1 (Figs 8a and d) and HLA-DR (Figs 8b and e), found in subcortical white matter are shown in Figs 8(c and f). In contrast, cells labeled with CNPase (large arrow, Figs 8h and i), an oligodendrocyte marker, did not demonstrate significant co-localization with those labeled for activated calpain-1 (small arrow, Figs 8g and i). Likewise, cells labeled with the astrocytic marker GFAP (Figs 8j and k) in subcortical white matter, did not show co-localization with activated calpain-1 immunostaining.

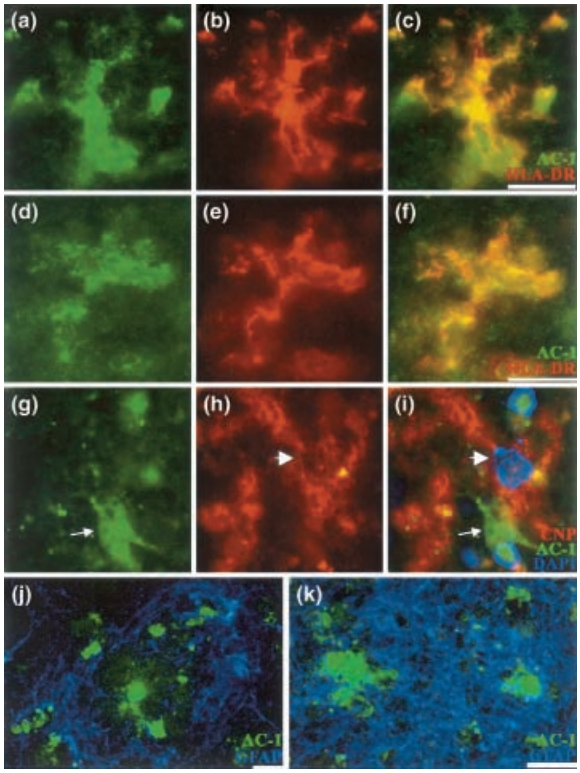

Fig. 8 Active calpain-1 localizes to microglia and not oligodendrocytes or astrocytes by immunofluorescence. White matter regions of coronal sections of monkey brain were examined for co-localization of activated calpain-1 with different glial cell types using known markers. Cells immunoreactive for activated calpain-1 (a and d, green) were identified as HLA-DR-positive microglia (b and e, red). Co-localization of the staining is shown in (c) and (f). Immunostaining for activated calpain-1 (small arrow; g and i, green) was not apparent in CNPase-positive oligodendrocytes (large arrow; h and i, red) or in GFAP-positive white matter astrocytes (j and k, blue). (a–i, k) 160× magnification; (j) 100× magnification; scale bar = 5 μm.

Discussion

Disruption of myelin in the aging CNS is a postulated mechanism of age-related cognitive decline, supported by

findings of both ultrastructural and biochemical changes in myelin in the aging rhesus monkey (Peters *et al*. 1996; Sloane *et al*. 2003). The importance of calpain in the degradation of myelin proteins has been documented in MS (Shields *et al*. 1999) and its animal model, EAE (Shields and Banik 1999). Previous studies in our laboratory have documented an age-related increase in the active form of calpain-1 in the corpus callosum of aging monkeys in association with enhanced degradation of several myelin proteins (Sloane *et al*. 2003). The present studies indicate the activation of calpain-1 is a phenomenon that occurs throughout the white matter of the aging CNS. Immunohistochemical staining for activated calpain-1 revealed a predominance of white matter staining that was specifically pre-absorbed with active enzyme. Immunoblotting with the same antibodies confirmed the immunostaining result with an age-dependent increase in active calpain-1 in homogenates of subcortical white matter from three different brain regions. These findings are generally supportive of the concept that, at least in the aging rhesus monkey, the most significant age-related changes occur in brain white matter and not in neurons. While calpain plays a critical role in tau phosphorylation in Alzheimer's disease via the degradation of the cdk5 inhibitor, p35 (Kusakawa *et al*. 2000; Lee *et al*. 2000; Nath *et al*. 2000; Taniguchi *et al*. 2001), the present findings suggest an additional important function of calpain-1 relevant to the normal aging brain.

In addition, the present study also employed the use of a polyclonal antibody against the calpain-derived spectrin fragment to demonstrate an age-dependent increase in spectrin proteolysis occurring largely in white matter regions by immunohistochemistry, thus confirming the results found with direct analysis of activated calpain-1 by using an endogenous substrate. The pattern of immunostaining was highly similar using either antibody, demonstrating that, where activated calpain-1 was found, evidence of its proteolytic activity can also be detected in the form of spectrin fragments. These findings are consistent with the finding of increased calpain-1 activity in microglial cells and may suggest an increase in the turnover of the cytoskeleton in these cells. However, it is not possible to rule out that these spectrin proteolytic fragments may have been derived from myelin and/or the oligodendrocyte as spectrin has been shown to interact with MAG in paranodal loops of myelin (Trapp *et al*. 1989).

An age-dependent increase in calpain-1 activation within the white matter can be presumed to have profound effects on myelin proteins, which serve as substrates for the enzyme, as well as cytoskeletal proteins such as spectrin. A physiologic turnover of the myelin membrane may be a necessary feature of aging, in which calpain-1-mediated proteolysis of myelin and cytoskeletal proteins is required to facilitate the creation of new, more stable membrane segments. In this case, it could be expected that this calpain-1-mediated myelin protein turnover would be carried out by the oligodendrocyte. Exposure of rat sciatic nerves to ionomycin, a calcium ionophore, resulted in vesicular disruption of the myelin sheath, implicating the role of myelin-derived calcium-dependent proteolytic activity in myelin membrane alteration (Schlaepfer 1977). Interestingly, complement activation leading to sublytic membrane attack complex (MAC) formation results in arachidonic acid (AA) mobilization and leukotriene B4 (LTB4) formation in an oligodendrocyte cell line (Shirazi *et al*. 1987, 1989). The release of AA metabolites requires extracellular Ca^{2+} influx shown to occur through C5b-8 or C5b-9 transmembrane channels (Seeger *et al*. 1986), thus providing a potential mechanism for calpain activation in oligodendrocytes. In the present study, however, the active form of calpain-1 was not detectable in intact myelin fractions by immunoblot, despite the presence of detectable amounts of proenzyme. Calpain-1 activity was present in intact myelin fractions but only after the addition of adequate levels of calcium and measured only a third of the absolute activity present in floating myelin. In addition, failure to co-localize activated calpain-1 in oligodendrocytes by immunostaining further suggests the age-dependent activation of calpain-1 originates from another cell type within white matter.

From a pathologic perspective, the age-dependent activation of calpain-1 may represent an exacerbation of an enzymatic system requiring precise control to prevent unwanted proteolysis, whereby the degradation of myelin proteins is deleterious. While the role of activated microglia in the aging brain has not been clearly established, they are known to increase in number with age as well as increase their expression of iNOS (Sloane *et al*. 1999), leading to higher levels of reactive oxygen species and the release of various cytokines. These inflammatory mediators have been documented to induce both ultrastructural and biochemical changes in the myelin membrane (Smith *et al*. 1999). Macrophages have also been documented to release inflammatory AA metabolites in response to late complement components (Hansch *et al*. 1984). This sublytic complement activation, via the classical pathway, can be triggered by central myelin membrane fragments in the absence of IgG (Cyong *et al*. 1982; Vanguri *et al*. 1982). Thus, complement component signaling and resulting fluctuations in intracellular calcium levels could provide a mechanism for calpain-1 activation within microglia.

Floating myelin has been previously described (Lees *et al*. 1980) and the protein content of this fraction increases dramatically with age in frontal lobe myelin (Sloane *et al*. 2003) and in brain stem myelin, as demonstrated in the present study. Using metabolic labeling studies, degraded myelin proteins originally found in the intact fraction can be demonstrated within the floating fraction (Persson 1991; Persson and Karlsson 1991; Persson *et al*. 1992). Localization of active calpain-1 to floating myelin, by both

immunoblot and enzymatic activity assay, coupled with co-localization to activated microglia, suggest that this active calpain-1 is serving to degrade myelin proteins and may be derived from microglia. These cells have been shown to phagocytose myelin debris and proteolyze myelin proteins, such as MBP (Copelman *et al*. 2001). In the Long Evans shaker rat, a long-lived myelin mutant, myelin degeneration is accompanied by microglia activation with evidence of myelin phagocytosis occurring within these cells (Zhang *et al*. 2001). Furthermore, Lewis rats with experimental optic neuritis demonstrated increased calpain expression in activated microglia (Banik and Shields 1999). In the present study, with detectable levels of active calpain-1, age-dependent activated microglia may be degrading phagocytosed myelin fragments. These myelin fragments when altered due to myelin protein degradation may localize to the floating myelin fraction, thus accounting for the increase in detection of the active enzyme in the fraction. Cultured microglia respond to interferon-α exposure both by increasing the phagocytosis of myelin as well as the proteolytic secretion of enzymes which can be inhibited by calpain inhibitors (Smith *et al*. 1998). In addition, active lymphoid cells have been shown to secrete calpain-2 capable of MBP degradation, into culture media, despite the lack of a signal peptide sequence (Deshpande *et al*. 1995). Activated microglia in the aging brain may also be capable of calpain-1 secretion; however, the present study suggests that the enzyme is localized intracellularly. Reactive astrocytes, known to be present in the white matter of aging monkeys (Sloane *et al*. 2000), have also been shown to increase expression of calpain in immune-mediated models of myelin damage (Banik and Shields 1999). However, double-labeling experiments revealed that astrocytic expression of activated calpain-1 contributed minimally, if at all, to the observed age-dependent increase of the enzyme within brain white matter.

In summary, an age-related increase in calpain-1 activity throughout myelinated regions of the brain, particularly within the floating myelin fraction, further supports a role for this enzyme in myelin protein degradation. Detection of the active enzyme within activated microglia is consistent with previous studies describing an age-dependent increase in these cells and highlights their importance in mediating age-related changes in myelin. Further studies are needed to elucidate the precise mechanism of microglial activation with age and to investigate if pharmacologic blockade of calpain may be beneficial.

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