Published in final edited form as:

Neurobiol Aging. 2012 April ; 33(4): 830.e13–830.e24. doi:10.1016/j.neurobiolaging.2011.07.006.

Astrocytes in aged non-human primate brain gray matter synthesize excess hyaluronan

Robert Cargill^{a,†}, Steven G. Kohama^{a,‡}, Jaime Struve^a, Weiping Su^a, Fatima Banine^a, Ellen Witkowski^a, Stephen A. Back^b, and Larry S. Sherman^{a,*}

^aDivision of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science, University, Beaverton, Oregon 97006

^bDepartments of Pediatrics and Neurology, School of Medicine, Oregon Health & Science, University, Portland, Oregon

Abstract

The glycosaminoglycan hyaluronan (HA) accumulates in central nervous system lesions where it limits astrogliosis but also inhibits oligodendrocyte progenitor cell (OPC) maturation. The role of hyaluronan in normative brain aging has not been previously investigated. Here, we tested the hypothesis that HA accumulates in the aging non-human primate brain. We found that HA levels significantly increase with age in the gray matter of rhesus macaques. HA accumulation was linked to age-related increases in the transcription of HA Synthase-1 (*HAS1*) expressed by reactive astrocytes but not changes in the expression of other *HAS* genes or hyaluronidases. HA accumulation was accompanied by increased expression of CD44, a transmembrane HA receptor. Areas of gray matter with elevated HA in older animals demonstrated increased numbers of olig2⁺ OPCs, consistent with the notion that HA may influence OPC expansion or maturation. Collectively, these data indicate that *HAS1* and *CD44* are transcriptionally upregulated in astrocytes during normative aging and are linked to HA accumulation in gray matter.

Keywords

hyaluronan; non-human primate; astrocytes; aging; oligodendrocyte progenitor cells

1. Introduction

Hyaluronan (HA) is a non-sulfated, linear glycosaminoglycan comprised of repeating units of (β,1→4)-D-glucuronic acid-(β,1→3)-N-acetyl-D-glucosamine (Lee and Spicer, 2000; Toole, 2001). HA is synthesized at the inner face of cell membranes by one of three transmembrane HA synthases (HAS1-3), then extruded into the extracellular matrix during chain elongation, reaching sizes greater than 10⁶ Da. In white matter, HA is localized around myelinated fibers where astrocytes deposit HA-protein complexes in the interstitial space

© 2011 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed: 505 NW 185th Avenue, Beaverton, OR 97006., Tel: 503-690-5217; Fax: 503-690-5384; shermanl@ohsu.edu.

†These authors contributed equally to this work

Conflict of interest: None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

between myelin and astrocyte processes, while in gray matter HA is a major component of perineuronal nets that surround neuron cell bodies (Asher et al., 1991; Bignami and Asher, 1992; Bignami et al., 1992; Egli et al., 1992). Following central nervous system (CNS) injury, HA is synthesized by reactive astrocytes and accumulates in glial scars and areas of astrogliosis in the injured brain and spinal cord (Back et al., 2005; Struve et al., 2005).

HA has been implicated in the maintenance of glial cell proliferation and maturation. HA maintains some cells in a state of contact inhibition of growth (Morrison et al., 2001). Consistent with these findings, HA maintains spinal cord astrocytes in a state of quiescence and may regulate glial scar formation (Struve et al., 2005; Lin et al., 2009). In addition, HA can cause remyelination failure by inhibiting oligodendrocyte progenitor cell (OPC) maturation in demyelinated CNS lesions (Back et al., 2005; Sloane et al., 2010). These findings suggest that HA may help to regulate astrogliosis while at the same time blocking OPCs from maturing into cells that can replace damaged myelin.

The role of HA in the aging nervous system has not been extensively examined. One study utilizing electrophoretic separation of glycosaminoglycans indicated a moderate increase in HA concentration in 30 month-old rat brain tissue compared to tissues from younger animals (Jenkins and Bachelard, 1988a). HA is similarly elevated in brains from patients with Alzheimer's disease (Jenkins and Bachelard, 1988b; Suzuki et al., 1965). Whether HA similarly accumulates in the primate brain with normative aging has not been previously investigated.

Most cells recognize and signal in response to HA through the CD44 family of single-span transmembrane glycoproteins. CD44 proteins have been implicated in cell-cell and cell-matrix adhesion and cell proliferation (Ponta et al., 2003). The core protein of CD44 has a molecular weight of approximately 37 kDa. However, as a result of extensive post-translational modifications and alternative RNA splicing of up to 10 variant exons, CD44 proteins range in size from 85 kDa to over 200 kDa. In the normal CNS, astrocytes and, to a lesser degree, other glial cells express the "standard" form of CD44 (CD44s, lacking variant exon-encoded sequences) while mature neurons express little or no CD44 (Asher and Bignami, 1992; Girgah et al., 1991; Jones et al., 2000; Vogel et al., 1992). Following CNS injury, CD44 expression is elevated in reactive astrocytes and a subpopulation of microglia (Jones et al., 2000). CD44 expression is also highly and persistently upregulated by glial cells in numerous inflammatory demyelinating conditions (Back et al., 2005; Girgah et al., 1991; Alldinger et al., 2000; Haegel et al., 1993) and in astrocytes associated with cortical senile plaques from patients with Alzheimer's disease (Vogel et al., 1992; Akiyama et al., 1993).

Given that HA and CD44 are elevated as a consequence of astrogliosis following CNS injury and that astrogliosis is associated with normative brain aging (Brizzee et al., 1968; Hughes and Lantos, 1987; Sloane et al., 2000; Sturrock, 1980), we hypothesized that age-related astrogliosis leads to elevated HA synthesis. We tested this hypothesis in tissues from cohorts of young, middle-aged and old rhesus macaques. Our results indicate that HA accumulates with age more in gray matter than in white matter and that this accumulation is linked to increased transcription of *HAS1* by reactive astrocytes. We also find that CD44 expression increases with age and that elevated HA and CD44 are linked to the aberrant accumulation of OPCs.

2. Materials and methods

2.1 Animals and tissue preparation

The tissues used in this study were obtained through the non-human primate Aging Resource at the Oregon National Primate Research Center. All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center and the Oregon Health & Science University, in accordance with the NIH *Guide for the care and use of laboratory animals*. Prior to postmortem brain harvesting, animals were sedated with ketamine (15–25 mg/kg im) followed by sodium pentobarbital (25–30 mg/kg iv), a procedure consistent with the recommendations of the American Veterinary Medical Association's Panel on Euthanasia.

For immunohistochemistry, tissues from both male rhesus macaques (*Macaca mulatta*) categorized as young (1–4 years, n=5); middle-aged (10–15 years, n=5); and old (22–30 years, n=5) as well as male and female Japanese macaques (*Macaca fuscata*) categorized as young (1–4 years; n=5); middle-aged (11–14 years, n=5); and old (24–29 years, n=5), were used in this study. Brains were flushed with 1 liter of saline at room temperature, followed by 6.5 liters of ice-cold 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Following perfusion, brains were cut into blocks and cryoprotected first in 10% then 20% glycerol, with both solutions containing 2% DMSO and 0.02M KPBS. Tissue blocks were frozen in isopentane that was cooled in a dry ice ethanol bath and then stored at –80 °C. Coronal sections of the prefrontal cortex (area 46) were cut at 25 µm on a freezing, sliding microtome and sections were stored in a cryoprotectant (glycerol, ethylene glycol mix) at –20 °C until further use. None of the brains used in this study demonstrated any gross evidence of neurodegeneration. This finding is consistent with previous studies demonstrating only very limited or no evidence of neurodegeneration in the aged macaque brain (Peters et al., 1994; Peters et al., 1997; Merrill et al., 2000; Sandell and Peters, 2003).

For biochemical and RNA analyses, brains from male rhesus macaques (young: 1–8 years, n=10; middle aged: 10–19 years, n=14; and old: 20–31 years, n=19) were flushed with 1 liter of saline through the ascending aorta, dissected, frozen in liquid nitrogen, then archived at –80 °C. Gray matter and white matter were dissected prior to Trizol lysis (see below).

2.2 Real time reverse transcription PCR

Approximately 50 mg of tissue was weighed and lysed manually in 1.0 ml of Trizol (Invitrogen) according to manufacturer's protocol. Fifty microliter aliquots from the aqueous phase were stored at –80 °C for use in HA quantification assays (below), with the remaining aqueous phase processed to precipitate RNA. Synthesis of 1 µg cDNA was performed by reverse transcription PCR primed with random hexamers. Samples were treated with DNaseI (Invitrogen) (15 min at 25 °C, 10 min at 65 °C), followed by the use of ImProm-II Reverse Transcriptase (Promega) according to manufacturer's protocol (5 min at 25 °C, 60 min at 42 °C, 15 min at 70 °C). Real-time PCR was used to quantify relative mRNA transcript levels of *CD44*, *HAS1*, *HAS2*, *HAS3*, *HYAL1*, *HYAL2* and *HYAL3* using an Applied Biosciences 7900HT. Reactions were performed in triplicate using SYBR Green I (Invitrogen) and GoTaq (Promega) in 10 or 20 µl total volume. Primers (Table 1) were designed using Primer Express 2 software (Applied Biosystems). Following confirmation of exclusivity of amplicons using BLAST, oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa), and used at a final concentration of 100–200 nM. For each reaction, 5–50 ng of cDNA was used with samples normalized to 18S rRNA. Separate experiments were run for each gene. Standard curves were constructed to confirm linearity of the assays, and sample data were analyzed using the $\Delta\Delta\text{Ct}$ method. Results for *HAS1*, *HAS2*, and *HAS3* were confirmed using TaqMan assays Hs00758053_m1, Hs01052031_m1,

and Hs00193436_m1, respectively (Applied Biosystems). The association between age and levels of each transcript were analyzed using a Pearson correlation analysis. Comparisons between groups were analyzed using a Student's *t* test.

2.3 Immunohistochemistry

For each combination of antibodies, 3 sections, several mm apart, were examined from each animal. Sections were removed from a -20°C freezer and were rinsed with several washes in 0.2M Tris buffered saline, pH 7.6. Sections were incubated in the same buffer with 0.1% TritonX-100 and 2% normal goat serum in order to enhance antibody penetration and reduce background staining, respectively.

For quantitative analysis of GFAP immunoreactivity, sections were incubated overnight at 4°C with a 1:1000 dilution mouse monoclonal anti-GFAP (clone GA5, Chemicon International, Temecula, CA) on a rotary platform. The following day, sections were rinsed several times with Tris buffer, then incubated in a 1:500 dilution of biotinylated goat anti-mouse IgG (Vector labs, Burlingame, CA) rinsed and incubated with avidin-biotin complexed with peroxidase. Following another rinse, sections were incubated with a solution of diaminobenzidine with nickel, the latter to enhance contrast. The area of GFAP immunoreactivity was measured using NIH Image. We focused our analysis on a 100x light microscopic field of the dorsal bank of area 46 along the primary sulcus, starting at the most medial point. The microscope field was captured using a CCD camera and the same size digital rectangular image of layer I–VI was measured for all sections. Three sections from each animal, each separated by approximately 1 mm, were measured and total area of GFAP coverage was averaged for the age comparison (ANOVA).

For immunofluorescence studies, primary antibodies against CD44 (1:10 Hermes 3; mouse monoclonal from ATCC), GFAP (1:2000 rabbit polyclonal; DAKO, Carpinteria, CA; 1:250, mouse monoclonal, Chemicon, Temecula, CA), Iba-1 (1:250, Wako Chemicals, Richmond, VA), NeuN (1:250; Santa Cruz Biotechnology, CA), olig2 (1:800, Millipore, Billerica, MA), HAS-1 (1:500; Santa Cruz) or biotinylated-hyaluronic acid binding protein (HABP; 1:250, U.S. Biologicals, Swampscott, MA), were used either alone or in cocktails for multiple labeling. Following an overnight incubation at 4°C , sections were rinsed in PBS at room temperature and incubated with fluorescently-labeled secondary antibodies (Alexa Fluor 488, 546; Molecular Probes, Eugene, OR) or, in the case of HABP, with streptavidin linked to Cy3 (JAX ImmunoResearch, West Grove, PA) and counterstained with DAPI to label cell nuclei. Following incubation, sections were rinsed and floated onto slides and coverslipped in aqueous mounting medium. Controls included sections incubated with no primary or secondary antibody and also with mismatch of secondary and primary antibodies. These controls all gave minimal fluorescent labeling.

For the analysis of olig2 in areas with elevated HA expression, cell counts were compared between patchy areas with high HABP fluorescence vs. surrounding low fluorescence (see Fig. 6A for example). The numbers of olig2⁺ nuclei/mm² were counted. Five animals per age group were analyzed and 3 sections per animal were counted. Equivalent areas of pre-frontal cortex were analyzed in each case. Mean cell numbers were compared using a Student's *t* test.

2.4 Quantification of HA

HA was quantified from the aqueous phase of Trizol-extracted lysates of tissue samples from the same animals analyzed using real time RT-PCR using an ELISA-based assay (Corgenix, Inc.) according to the manufacturer's instructions. Lysates were diluted in 300 μl of the kit reaction buffer and duplicate 100 μl fractions were transferred into the ELISA

plate. At the end of the assay, absorbances were read at 450 nm on a 96-well plate reader. Concentrations were normalized using the measured tissue masses. The association between age and HA levels were analyzed using a Pearson correlation analysis. Comparisons between groups were analyzed using a Student's *t* test.

2.5 Western blotting

Portions of pre-frontal cortex were lysed by gentle sonication in ice-cold lysis buffer (20mM Tris-HCl pH 7.4, 25mM NaCl, 3mM MgCl₂, and 0.5% NP40 plus protease inhibitors). Lysates were cleared by centrifugation (14,000 rpm at 4°C) for 10 minutes, and the protein concentration of the resulting supernatant was obtained using a Quickstart Bradford assay (Biorad) before Laemmli buffer was added. The samples were boiled for 5 minutes before being loaded in equal concentrations onto a 7.5% SDS-polyacrylamide gel. The protein was transferred onto nitrocellulose membrane and probed overnight with the indicated antibodies at 4°C following a 1 hour incubation in blocking buffer (PBS + 0.1% Tween 20 + 10% milk). Primary antibodies included mouse-anti-human CD44 (Hermes3; 1:1000) and goat-anti-actin (Santa Cruz; 1:1000). Blots were washed and probed with HRP-conjugated secondary antibodies (BioRad; 1:1000) for 90 minutes, washed, and developed with chemiluminescent substrates (Pierce) for 5 minutes. CD44 signal was normalized to actin (as a loading control) and quantified using ImageJ software.

3. Results

3.1 Astrogliosis increases with normative aging in rhesus macaque brains

Previous studies demonstrated evidence of astrogliosis in the uninjured brains of patients with advanced aging (Nichols et al., 1993; Porchet et al., 2003) and in subcortical white matter and prefrontal cortex of aged rhesus macaques (Sloane et al., 2000; Haley et al., 2010). Here, we quantified the degree of astrogliosis, based on increased GFAP immunolabeling, in the brains of young (1–4 years old), middle-aged (10–15 years old), and old (22–30 years old) rhesus macaques that had no evident brain injuries or other pathological conditions, focusing on area 46 in the floor of the principal sulcus in the frontal lobe. GFAP immunoreactivity was high in the glial limitans (Fig. 1A, arrow) and adjacent white matter (Fig. 1B) of young animals. A significant, progressive increase in GFAP levels occurred in the inner cortical layers and white matter of middle-aged and old animals (Fig. 1C–G). In fact, cortical GFAP levels increased more than two-fold between the young and old cohorts (Fig. 1G). This increased GFAP immunoreactivity occurred in patches scattered throughout the tissue (e.g. Fig. 1C). These data confirm that astrogliosis increases in gray matter and to a lesser extent in white matter in the non-human primate prefrontal cortex with normal aging.

3.2 CD44 is transcriptionally upregulated with aging

CD44 becomes elevated on reactive astrocytes in individuals with neurodegenerative diseases and following a variety of insults to the CNS (Eggli et al., 1992; Struve et al., 2005; Jones et al., 2000; Vogel et al., 1992; Akiyama et al., 1993). We tested the possibility that CD44 expression is similarly upregulated with age-related astrogliosis. RNA samples from the gray and white matter of young, middle-aged and old animals were analyzed for levels of *CD44* transcripts by real time RT-PCR. *CD44* transcripts significantly increased in gray matter with aging (Fig. 2A; $R^2=0.5$; $p<0.001$). Some animals also demonstrated increased *CD44* transcription with aging in white matter but the trend was not significant (Fig. 2B; $R^2=0.01$). We validated these real time RT-PCR findings by analyzing CD44 protein levels in the prefrontal cortex gray matter of a subset of animals by Western blotting. There was some degree of variability in total CD44 expression in young ($n=5$) and middle-aged ($n=4$) animals, but overall old animals ($n=5$) expressed between 1.5- and 3-fold more CD44 than

young or middle-aged monkeys (Fig. 2C). By far the majority of CD44 expressed in these tissues was approximately 85 kDa, consistent with the “standard” form (CD44s) that lacks variant exon-encoded sequences. When blots were overexposed, both under-glycosylated forms of CD44 and at least two weak higher molecular weight isoforms (at approximately 120 and 160 kDa) could be detected, although the levels of these isoforms did not appear to vary with age (data not shown).

We assessed the distribution of elevated CD44 to determine if increased CD44 expression was coincident with astrogliosis. We performed double-labeling fluorescence immunohistochemistry in sections of gray matter from old animals. CD44 was elevated in areas where there were reactive astrocytes in gray matter (Fig. 2D–I; arrowheads) and in white matter (e.g. Fig. 4C–E) in older animals. CD44 immunoreactivity co-localized with GFAP⁺ astrocytes (see insets, Fig. 2G–I) but was also evident in the extracellular space surrounding astrocytes (Fig. 2I, arrows). CD44 was not expressed at detectable levels by other glial cells or neurons in the gray matter (data not shown).

Previous studies suggested that elevated CD44 expression can be observed in perivascular domains in CNS lesions (Akiyama et al., 1993). We similarly observed extensive CD44 immunoreactivity surrounding some blood vessels in prefrontal cortex of young animals (Fig. 3). In some cases, the CD44 labeling clearly co-localized with GFAP immunoreactivity intimately associated with blood vessels (e.g. Fig. 3A–C and Fig. 3G–H). In other cases, there was diffuse CD44 immunoreactivity in the neighborhood of blood vessels that did not strictly co-localize with GFAP (e.g. Fig. 3D–F). Such perivascular CD44 labeling was virtually undetectable in brain tissues from old animals.

3.3 Hyaluronan accumulates with age in prefrontal cortex gray matter

We previously found that HA accumulates in CNS lesions coincident with chronically elevated CD44 expression and astrogliosis (Back et al., 2005; Struve et al., 2005). We therefore measured the amount of HA in prefrontal cortex white matter and gray matter tissue lysates from young, middle-aged and old animals to determine if HA accumulates with increasing age in the non-human primate brain. Using an ELISA-based assay, we found that there was a significant increase in HA with age in the gray matter ($R^2=0.3$; $p<0.005$; Fig. 4A). Some animals demonstrated increased levels of HA in white matter as well, but there was no significant trend ($R^2=0.003$) (Fig. 4B). In the gray matter, HA levels ranged from approximately 20–90 ng/mg of tissue while in white matter HA was found at 60–230 ng/mg tissue.

Although HA was detected throughout the gray matter of young animals independent of CD44 immunoreactivity, HA and CD44 co-localized in older animals and both increased with advancing age (Fig. 4C–H). High HA was associated with areas of increased GFAP immunoreactivity (Fig. 4F–H, insets) including patchy areas throughout prefrontal cortex (data not shown), consistent with the notion that HA accumulates in areas of astrogliosis. Variable HA levels were detected in white matter with some areas showing very high HA in oldest-old animals (e.g. the “tract” areas in Fig. 4F–G) while other areas of white matter had very low HA levels (data not shown). This variable distribution of elevated HA in white matter from old animals may explain the variable distribution of HA observed in the ELISA assays.

3.4 HAS1 but not other HA synthases or hyaluronidases are transcriptionally upregulated with aging

To determine whether age-related HA accumulation might be due to increased transcription of HA synthases or reduced expression of hyaluronidases, we performed real time RT-PCR

assays to assess changes in HAS and hyaluronidase transcripts in RNA isolated from prefrontal cortex white matter and gray matter as above. Although there were no significant changes in *HAS2*, *HAS3*, or any of the hyaluronidases, we observed a significant age-dependent increase in *HAS1* transcripts ($R^2=0.3$; $p<0.009$) with age in prefrontal cortex gray matter (Fig. 5A, B). There was also a trend towards increased *HAS1* expression with aging in white matter ($R^2=0.3$; $p<0.1$) but no changes in other *HAS* genes or hyaluronidases (data not shown).

Like elevated HA, HAS1 protein was highest in patchy areas throughout the gray matter of aged animals and co-localized with GFAP immunoreactive astrocytes in areas with astrogliosis (Fig. 5C–E). HAS1 was not expressed by oligodendrocytes, neurons or microglia (Fig. 5F–H). Taken together, these data suggest that *HAS1* transcription increases with aging, and that HAS1 expression is elevated on reactive astrocytes where HA accumulates.

3.5 Olig2⁺ cells accumulate in areas where HA accumulates

In the adult brain, the olig2 transcription factor is typically expressed by cells in the oligodendrocyte lineage and by NG2⁺ glial progenitor cells that differentiate into oligodendrocytes following demyelinating insults (Rivers et al., 2008; Islam et al., 2009; Geha et al., 2010). Olig2⁺ cells accumulate in a rodent model of Alzheimer's disease (Buffo et al., 2005), suggesting that amyloid-induced damage results in the expansion or recruitment of OPCs. HA can inhibit the maturation of OPCs (Back et al., 2005; Sloane et al., 2010). Such progenitors are present in both white and gray matter (e.g. Dimou et al., 2008). We therefore tested whether olig2⁺ cells accumulate in areas with elevated levels of HA in tissue sections from old animals. Although olig2-immunoreactive cells were present throughout gray matter and white matter, these cells preferentially accumulated in areas of gray matter where HA was elevated (Fig. 6A–D). HA-high areas demonstrated up to 40% ($p<0.01$) more olig2⁺ cells than adjacent HA-low areas. These findings are consistent with the hypothesis that HA accumulation may influence the accumulation of OPCs in the aging brain.

4. Discussion

These studies demonstrate that chronically elevated CD44 expression and HA accumulation occur in the non-human primate CNS with normal aging. High CD44 immunoreactivity and HA accumulation occurred in areas that demonstrated elevated GFAP expression, consistent with the notion that CD44 and HA are chronically elevated as a result of age-related astrogliosis. Although we found that *HAS2* and *HAS3* are expressed at higher levels than *HAS1* at all ages, HA accumulation with aging occurred coincident with elevated *HAS1* transcription while levels of the other *HAS* genes and *HYAL* genes did not change with age, suggesting that increased *HAS1* expression contributes to age-related HA accumulation. Finally, HA accumulation correlated with elevated numbers of olig2⁺ cells, consistent with the idea that HA may influence the maturation of cells in the oligodendrocyte lineage. These findings raise numerous new questions about how astrogliosis influences the aging CNS, and what roles changes in the extracellular matrix play in age-related alterations to cell survival, proliferation and activity in the brain.

HA has been implicated in regulating cell proliferation, including the proliferation of astrocytes both *in vitro* and *in vivo* (Morrison et al., 2001; Struve et al., 2005). It is possible, therefore, that HA accumulation may inhibit the proliferation of astrocytes and other cell types in areas where there is astrogliosis, thus regulating cell numbers at sites of damage (Lin et al., 2009). Alternatively, the accumulation of HA may also contribute to age-related changes in myelination. HA accumulation in inflammatory demyelinating lesions

contributes to remyelination failure by preventing the maturation of OPCs to myelin-forming cells (Back et al., 2005; Sloane et al., 2010) and could, therefore, influence myelination or myelin repair in the aged brain. Our finding of increased numbers of olig2⁺ cells in areas with elevated HA is consistent with this possibility.

Another possibility is that HA accumulation in the gray matter may influence synaptic plasticity, which declines with aging (Luebke et al., 2010). Perisynaptic HA in the hippocampus can regulate synaptic plasticity through regulation of dendritic Ca²⁺ channels (Kochlamazashvili et al., 2010). Elevated HA in aging gray matter may therefore restrict synaptic plasticity, thus contributing to age-related changes in neuronal function.

The chronically elevated levels of CD44 and HAS1 in the aging brain may result from age-related inflammatory responses in the CNS. Microglial expression of MHC class II, a marker that indicates microglial activation, is increased in the brains of aged but otherwise healthy humans, non-human primates, and rodents (Perry et al., 1993; Rogers et al., 1988; Streit and Sparks, 1997; Sheffield and Berman, 1998). The inflammatory cytokine interleukin-6 is overexpressed in the brains of healthy aged mice (Ye and Johnson, 1999), possibly resulting from age-associated increases in oxidative stress (Godbout et al., 2004) and decreased IL-10 (Ye and Johnson, 2001). Similar observations have been made for other inflammatory cytokines including IL-1 β (Murray and Lynch, 1998). Inflammatory cytokines can induce CD44 expression as well as interactions between HA and CD44 (Gee et al., 2004). Furthermore, CNS inflammation, through these and other cytokines, can lead to activation of NF- κ B, which can regulate *HAS1* transcription (Kao, 2006). It is possible, therefore, that both the upregulation of CD44 and HA accumulation are linked to age-related increases in inflammatory cytokines in the CNS.

Increased CD44 and HAS1 transcription in the CNS may also be linked to age-related cell-intrinsic changes in astrocytes. Although this possibility has not been tested, skin fibroblasts demonstrate increased HA synthesis with increasing age *in vitro* (Vuillermoz et al., 2005). Both HAS2 and HAS3 as well as CD44 are upregulated with aging in aortic smooth muscle cells (Vigetti et al., 2008). These changes could be linked to epigenetic regulation of the *CD44* and *HAS* genes. *CD44*, for example, is regulated by DNA methylation (Banine et al., 2005).

The distribution of CD44 immunoreactivity in some regions of the brain was consistent with the presence of CD44 in the extracellular space. The antibody we used for these studies recognizes an epitope on the ectodomain of CD44 that is cleaved by sequential proteolytic cleavages, followed by cleavage of the transmembrane domain by presenilin-1, a secretase whose mutation has been implicated in Alzheimer's disease (Lammich et al., 2002; Murakami et al., 2003). It is possible, therefore, that the CD44 ectodomain accumulates in some region of the brain with aging and that this accumulation is associated with age-related alterations in secretase activity.

Our finding that age-related increases in HA, HAS1 and CD44 are greater in the gray matter than in the white matter are surprising given that HA and CD44 dramatically increase in damaged brain and spinal cord white matter (e.g. Back et al., 2005; Struve et al., 2005). Interestingly, in the prefrontal cortex tissue that we analyzed, reactive gliosis was also more pronounced with aging in gray matter than white matter. However, although overall levels of HA were not increased in white matter with increasing age, HA was clearly elevated in some areas of white matter in tissues from older animals. It is possible that there are regional differences in white matter damage in the aging brain that our study would not have identified, or that such damage is highly localized. Consistent with this possibility, diffusion tensor imaging studies have found significant regional variability in age-related changes in

white matter (e.g. Kennedy and Raz, 2009). These changes may be the result of age-related vascular brain injury and resulting oxidative damage in myelin (Back et al., 2011).

In contrast to areas of astrogliosis, CD44 expression declined with age in perivascular domains in both white and gray matter. Some of this CD44 immunoreactivity co-localized with GFAP suggesting that perivascular astrocytes express CD44 that decreases with age. However, we could not rule out the possibility that at least some of these CD44⁺ cells were pericytes which have been reported to express CD44 in other tissues (e.g. Crisan et al., 2009). The notion that CD44 expression declines in pericytes with aging is interesting in light of data showing that loss of pericyte function leads to neurodegenerative changes in mice (Bell et al., 2010).

Given the broad range of biological activities ascribed to CD44 and HA, their roles in nervous system aging are likely to be complex. Chronically elevated CD44 may have effects in the CNS that do not depend on HA, and HA may act on cells through multiple transmembrane HA receptors, including the receptor for hyaluronan-mediated motility (RHAMM), which, like CD44, is expressed in the CNS (Lynn et al., 2001). Nonetheless, our findings raise the intriguing possibility that CD44 and HA may be part of the underlying molecular alterations in the aging brain that result in long-term alterations in cellular and cognitive function.

Acknowledgments

This work was supported by NIH grants AG23280, T32 AG023477, AG031892 and P51RR000163.

References

- Akiyama H, Tooyama I, Kawamata T, Ikeda K, McGeer PL. Morphological diversities of CD44 positive astrocytes in the cerebral cortex of normal subjects and patients with Alzheimer's disease. *Brain Res.* 1993; 632(1–2):249–59. [PubMed: 7511977]
- Alldinger S, Fonfara S, Kremmer E, Baumgartner W. Up-regulation of the hyaluronate receptor CD44 in canine distemper demyelinated plaques. *Acta Neuropathol (Berl).* 2000; 99(2):138–46. [PubMed: 10672320]
- Asher R, Bignami A. Hyaluronate binding and CD44 expression in human glioblastoma cells and astrocytes. *Exp Cell Res.* 1992; 203(1):80–90. [PubMed: 1426053]
- Asher R, Perides G, Vanderhaeghen JJ, Bignami A. Extracellular matrix of central nervous system white matter: demonstration of an hyaluronate-protein complex. *J Neurosci Res.* 1991; 28(3):410–21. [PubMed: 1713274]
- Back SA, Tuohy TM, Chen H, Wallingford N, Craig A, Struve J, Luo NL, Banine F, Liu Y, Chang A, Trapp BD, Bebo BF Jr, Rao MS, Sherman LS. Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. *Nat Med.* 2005; 11(9):966–72. [PubMed: 16086023]
- Back SA, Kroenke CD, Sherman LS, Lawrence G, Gong X, Taber E, Sonnen J, Larson E, Montine TJ. White matter lesions defined by diffusion tensor imaging in older adults. *Ann Neurol.* 2011 In press.
- Banine F, Bartlett C, Gunawardena R, Muchardt C, Yaniv M, Knudsen ES, Weissman BE, Sherman LS. SWI/SNF chromatin-remodeling factors induce changes in DNA methylation to promote transcriptional activation. *Cancer Res.* 2005; 65(9):3542–7. [PubMed: 15867346]
- Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, Zlokovic BV. Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron.* 2010; 68(3):409–27. [PubMed: 21040844]
- Bignami A, Asher R. Some observations on the localization of hyaluronic acid in adult, newborn and embryonal rat brain. *Int J Dev Neurosci.* 1992; 10(1):45–57. [PubMed: 1376955]

- Bignami A, Asher R, Perides G. The extracellular matrix of rat spinal cord: a comparative study on the localization of hyaluronic acid, glial hyaluronate-binding protein, and chondroitin sulfate proteoglycan. *Exp Neurol*. 1992; 117(1):90–3. [PubMed: 1377637]
- Brizzee KR, Sherwood N, Timiras PS. A comparison of cell populations at various depth levels in cerebral cortex of young adult and aged Long-Evans rats. *J Gerontol*. 1968; 23(3):289–97. [PubMed: 5663608]
- Buffo A, Vosko MR, Ertürk D, Hamann GF, Jucker M, Rowitch D, Götz M. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. 2005; 102:18183–8.
- Crisan M, Chen CW, Corselli M, Andriolo G, Lazzari L, Péault B. Perivascular multipotent progenitor cells in human organs. *Ann NY Acad Sci*. 2009; 1176:118–23. [PubMed: 19796239]
- Dimou L, Simon C, Kirchhoff F, Takebayashi H, Götz M. Progeny of Olig2-expressing progenitors in the gray and white matter of the adult mouse cerebral cortex. *J Neurosci*. 2008; 28(41):10434–42. [PubMed: 18842903]
- Eggl PS, Lucocq J, Ott P, Graber W, van der Zypen E. Ultrastructural localization of hyaluronan in myelin sheaths of the rat central and rat and human peripheral nervous systems using hyaluronan-binding protein-gold and link protein-gold. *Neuroscience*. 1992; 48(3):737–44. [PubMed: 1376458]
- Gee K, Kryworuchko M, Kumar A. Recent advances in the regulation of CD44 expression and its role in inflammation and autoimmune diseases. *Arch Immunol Ther Exp (Warsz)*. 2004; 52(1):13–26. [PubMed: 15053229]
- Geha S, Pallud J, Junier MP, Devaux B, Leonard N, Chassoux F, Chneiweiss H, Dumas-Duport C, Varlet P. NG2+/Olig2+ cells are the major cycle-related cell population of the adult human normal brain. *Brain Pathol*. 2010; 20:399–411. [PubMed: 19486010]
- Girgah N, Letarte M, Becker LE, Cruz TF, Theriault E, Moscarello MA. Localization of the CD44 glycoprotein to fibrous astrocytes in normal white matter and to reactive astrocytes in active lesions in multiple sclerosis. *J Neuropathol Exp Neurol*. 1991; 50(6):779–92. [PubMed: 1748883]
- Godbout JP, Berg BM, Kelley KW, Johnson RW. alpha-Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6 secretion in primary murine microglia and in brain. *J Neuroimmunol*. 2004; 149(1–2):101–9. [PubMed: 15020070]
- Haley GE, Kohama SG, Urbanski HF, Raber J. Age-related decreases in SYN levels associated with increases in MAP-2, apoE, and GFAP levels in the rhesus macaque prefrontal cortex and hippocampus. *Age*. 2010; 32:283–296. [PubMed: 20640549]
- Haegel H, Tolg C, Hofmann M, Ceredig R. Activated mouse astrocytes and T cells express similar CD44 variants. Role of CD44 in astrocyte/T cell binding. *J Cell Biol*. 1993; 122(5):1067–77. [PubMed: 8354694]
- Hughes CC, Lantos PL. A morphometric study of blood vessel, neuron and glial cell distribution in young and old rat brain. *J Neurol Sci*. 1987; 79(1–2):101–10. [PubMed: 3612167]
- Islam MS, Tatsumi K, Okuda H, Shiosaka S, Wanaka A. Olig2-expressing progenitor cells preferentially differentiate into oligodendrocytes in cuprizone-induced demyelinated lesions. *Neurochem Int*. 2009; 54:192–8. [PubMed: 19070638]
- Jenkins HG, Bachelard HS. Developmental and age-related changes in rat brain glycosaminoglycans. *J Neurochem*. 1988; 51(5):1634–40. [PubMed: 3139839]
- Jenkins HG, Bachelard HS. Glycosaminoglycans in cortical autopsy samples from Alzheimer brain. *J Neurochem*. 1988; 51(5):1641–5. [PubMed: 3139840]
- Jones LL, Liu Z, Shen J, Werner A, Kreutzberg GW, Raivich G. Regulation of the cell adhesion molecule CD44 after nerve transection and direct trauma to the mouse brain. *J Comp Neurol*. 2000; 426(3):468–92. [PubMed: 10992250]
- Kao JJ. The NF-kappaB inhibitor pyrrolidine dithiocarbamate blocks IL-1beta induced hyaluronan synthase 1 (HAS1) mRNA transcription, pointing at NF-kappaB dependence of the gene HAS1. *Exp Geront*. 2006; 41:641–47.
- Kennedy KM, Raz N. Pattern of normal age-related regional differences in white matter microstructure is modified by vascular risk. *Brain Res*. 2009; 1297:41–56. [PubMed: 19712671]

- Kochlamazashvili G, Henneberger C, Bukalo O, Dvoretzskova E, Senkov O, Lievens PM, Westenbroek R, Engel AK, Catterall WA, Rusakov DA, Schachner M, Dityatev A. The extracellular matrix molecule hyaluronic acid regulates hippocampal synaptic plasticity by modulating postsynaptic L-type Ca(2+) channels. *Neuron*. 2010; 67(1):116–28. [PubMed: 20624596]
- Lammich S, Okochi M, Takeda M, Kaether C, Capell A, Zimmer AK, Edbauer D, Walter J, Steiner H, Haass C. Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Abeta-like peptide. *J Biol Chem*. 2002; 277(47):44754–9. [PubMed: 12223485]
- Lee JY, Spicer AP. Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Curr Opin Cell Biol*. 2000; 12(5):581–6. [PubMed: 10978893]
- Lin CM, Lin JW, Chen YC, Shen HH, Wei L, Yeh YS, Chiang YH, Shih R, Chiu PL, Hung KS, Yang LY, Chiu WT. Hyaluronic acid inhibits the glial scar formation after brain damage with tissue loss in rats. *Surgical Neurology*. 2009; 72:S2:50–S2:54. [PubMed: 18423545]
- Luebke J, Barbas H, Peters A. Effects of normal aging on prefrontal area 46 in the rhesus monkey. *Brain Res Rev*. 2010; 62(2):212–32. [PubMed: 20005254]
- Lynn BD, Li X, Cattini PA, Turley EA, Nagy JI. Identification of sequence, protein isoforms, and distribution of the hyaluronan-binding protein RHAMM in adult and developing rat brain. *J Comp Neurol*. 2001; 439(3):315–30. [PubMed: 11596057]
- Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev*. 2001; 15(8):968–80. [PubMed: 11316791]
- Murakami D, Okamoto I, Nagano O, Kawano Y, Tomita T, Iwatsubo T, De Strooper B, Yumoto E, Saya H. Presenilin-dependent gamma-secretase activity mediates the intramembranous cleavage of CD44. *Oncogene*. 2003; 22(10):1511–6. [PubMed: 12629514]
- Murray CA, Lynch MA. Dietary supplementation with vitamin E reverses the age-related deficit in long term potentiation in dentate gyrus. *J Biol Chem*. 1998; 273(20):12161–8. [PubMed: 9575163]
- Nichols NR, Day JR, Laping NJ, Johnson SA, Finch CE. GFAP mRNA increases with age in rat and human brain. *Neurobiol Aging*. 1993; 14(5):421–9. [PubMed: 8247224]
- Perry VH, Matyszak MK, Fearn S. Altered antigen expression of microglia in the aged rodent CNS. *Glia*. 1993; 7(1):60–7. [PubMed: 8423063]
- Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol*. 2003; 4(1):33–45. [PubMed: 12511867]
- Porchet R, Probst A, Bouras C, Draberova E, Draber P, Riederer BM. Analysis of glial acidic fibrillary protein in the human entorhinal cortex during aging and in Alzheimer's disease. *Proteomics*. 2003; 3(8):1476–85. [PubMed: 12923773]
- Rivers LE, Young KM, Rizzi M, Jamen F, Psachoulia K, Wade A, Kessaris N, Richardson WD. PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. *Nat Neurosci*. 2008; 11:1392–401. [PubMed: 18849983]
- Rogers J, Lubner-Narod J, Styren SD, Civin WH. Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol Aging*. 1988; 9(4):339–49. [PubMed: 3263583]
- Sheffield LG, Berman NE. Microglial expression of MHC class II increases in normal aging of nonhuman primates. *Neurobiol Aging*. 1998; 19(1):47–55. [PubMed: 9562503]
- Sloane JA, Batt C, Ma Y, Harris ZM, Trapp B, Vartanian T. Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. *Proc Natl Acad Sci USA*. 2010; 107(25):11555–60. [PubMed: 20534434]
- Sloane JA, Hollander W, Rosene DL, Moss MB, Kemper T, Abraham CR. Astrocytic hypertrophy and altered GFAP degradation with age in subcortical white matter of the rhesus monkey. *Brain Res*. 2000; 862(1–2):1–10. [PubMed: 10799662]
- Streit WJ, Sparks DL. Activation of microglia in the brains of humans with heart disease and hypercholesterolemic rabbits. *J Mol Med*. 1997; 75(2):130–8. [PubMed: 9083930]
- Struve J, Maher PC, Li YQ, Kinney S, Fehlings MG, Kuntz Ct, Sherman LS. Disruption of the hyaluronan-based extracellular matrix in spinal cord promotes astrocyte proliferation. *Glia*. 2005; 52(1):16–24. [PubMed: 15892130]

- Sturrock RR. A comparative quantitative and morphological study of ageing in the mouse neostriatum, indusium griseum and anterior commissure. *Neuropathol Appl Neurobiol.* 1980; 6(1):51–68. [PubMed: 7374912]
- Suzuki K, Katzman R, Korey SR. Chemical Studies on Alzheimer's Disease. *J Neuropathol Exp Neurol.* 1965; 24:211–24. [PubMed: 14280498]
- Toole BP. Hyaluronan in morphogenesis. *Semin Cell Dev Biol.* 2001; 12(2):79–87. [PubMed: 11292373]
- Vigetti D, Viola M, Karousou E, Rizzi M, Moretto P, Genasetti A, Clerici M, Hascall VC, De Luca G, Passi A. Hyaluronan-CD44-ERK1/2 regulate human aortic smooth muscle cell motility during aging. *J Biol Chem.* 2008; 283(7):4448–58. [PubMed: 18077444]
- Vogel H, Butcher EC, Picker LJ. H-CAM expression in the human nervous system: evidence for a role in diverse glial interactions. *J Neurocytol.* 1992; 21(5):363–73. [PubMed: 1607880]
- Vuillermoz B, Wegrowski Y, Contet-Audonneau JL, Danoux L, Pauly G, Maquart FX. Influence of aging on glycosaminoglycans and small leucine-rich proteoglycans production by skin fibroblasts. *Mol Cell Biochem.* 2005; 277(1–2):63–72. [PubMed: 16132716]
- Ye SM, Johnson RW. Increased interleukin-6 expression by microglia from brain of aged mice. *J Neuroimmunol.* 1999; 93(1–2):139–48. [PubMed: 10378877]
- Ye SM, Johnson RW. An age-related decline in interleukin-10 may contribute to the increased expression of interleukin-6 in brain of aged mice. *Neuroimmunomodulation.* 2001; 9(4):183–92. [PubMed: 11847480]

Fig. 1.

Astrogliosis as a function of age in the nonhuman primate prefrontal cortex (area 46) gray matter (A, C, E) and white matter (B, D, F). GFAP immunoreactivity (DAB-nickel), in young Japanese macaques was high in the glial limitans (A, arrow) and white matter (B). Increased GFAP immunoreactivity was observed in the mid-cortical layers by middle-age (C, arrow), and progressed with age (old, E). Levels remained elevated in the white matter of middle-aged and old animals (D and F, respectively). The area of GFAP coverage (pixels) in the prefrontal cortex increased significantly (ANOVA, $p < 0.05$) and progressively with age (old > middle-aged > young, $p < 0.01$ for all comparisons).

Fig. 2.

CD44 is transcriptionally upregulated in gray matter with aging. (A) Real-time RT-PCR analysis of CD44 transcript levels in gray matter RNA. (B) Real-time RT-PCR analysis of CD44 transcripts in RNA from white matter. (C) Left panel: Western blots of prefrontal cortex lysates showing levels of CD44s (the standard form of CD44) from animals at different ages. Actin is used as a loading control. Note that lanes ran somewhat unevenly due to a “smile effect”. Right panel: Examples of the full blots from a young and an old animal, overexposed to reveal low, unchanging levels of apparent CD44 splice variants (CD44v) and a band corresponding to the size of underglycosylated CD44 (CD44u). (D–I) Immunohistochemical analysis of CD44 expression in a 30-year-old animal (D, G) in prefrontal cortex gray matter, showing co-localization with areas of elevated GFAP (E, H) immunoreactivity (merged images, F, I). D–F are 10x images; G–I are 20x images. Insets in G–I are 40x images of glial processes. Arrowheads show areas of CD44 and GFAP co-localization. Arrows show areas of diffuse CD44 expression not associated with cells.

Fig. 3.

Colocalization of GFAP and CD44 in the vasculature of the macaque prefrontal cortex. In areas containing a low density of immunoreactivity, blood vessels containing both GFAP (A, D, G) and CD44 (B, E, H) could be observed, with some overlap (C, F, I). These isolated structures were seen at a very low frequency. Arrows indicate blood vessels near the middle of gliotic areas. Scale bars: A–F, 10 μ m; G–I, 2 μ m.

Fig. 4.

HA accumulates with aging in prefrontal cortex gray matter. (A) ELISA assay for total HA concentrations in prefrontal cortex gray matter. (B) ELISA assay for HA concentrations in white matter. (C–E) Immunohistochemical staining (10x) for CD44 (green) of area 46 in young (C), middle-aged (D), and old (E) animals. Insets show high power (40x) images taken from the cortex. (F–H) Immunohistochemical staining of area 46 for GFAP (green) and HA (red) in young (F), middle-aged (G) and old (H). Overlapping labeling is shown in yellow. The insets are high power (40x) images of cortex. Exposure times for all images were equivalent.

Fig. 5. *HAS1* is transcriptionally upregulated with age in macaque prefrontal cortex gray matter. (A) Real time RT-PCR analysis of *HAS1*, *HAS2*, and *HAS3* expression in RNA from prefrontal cortex gray matter. (B) Real time RT-PCR analysis of *HYAL1*, *HYAL2*, and *HYAL3* expression in RNA from prefrontal cortex gray matter. (C–H) Immunohistochemical analysis of *HAS1* expression in sections of prefrontal cortex gray matter from an old (age 30) animal. *HAS1* (C; red) is predominantly expressed by GFAP-immunolabeled astrocytes (D; green) as shown by double labeling (E). (F) Cells in the oligodendrocyte lineage (Olig2⁺, red) did not co-localize with *HAS1* (green). (G) Neurons (red) also did not express detectable levels of *HAS1* (green). (H) Microglia (green) also did not appear to express *HAS1* (red). Scale bar = 100 μ m.

Fig. 6. Olig2+ cells accumulate in areas with elevated HA. (A–C) Immunohistochemical labeling for HA (A; red), olig2 (B; green) and both HA and olig2 with DAPI (blue) to label cell nuclei in prefrontal cortex of a 30-year-old animal. The percentages of olig2+ nuclei in HA-high (encircled) and HA-low (areas outside of encircled areas) were calculated and compared (D). *p<0.01.

Table 1

Primers Used for Real Time RT PCR Assays

Gene	Forward Primer	Reverse Primer
<i>CD44</i>	GAAAGCTCTGAGCGTCGGAT	CAGATGGAGTTGGGCTGAATC
<i>HAS1</i>	CCTGCATCAGCGTCCTCTA	GGTTGTACCAGGCCTCAAGAAA
<i>HAS2</i>	AGCACTGGGACGAAGTGTGG	CAGATGAGGCTGGGTCAAGC
<i>HAS3</i>	CGGCGATTCTGGTGGACTA	TCCAGCACAGTGTCTCAGAGTCG
<i>HYAL1</i>	GCTGCCCTATGTCCAGATCTTC	GCTCACCCAGAGCACCACTC
<i>HYAL2</i>	GCACTCCCAGTCTACGTCTTCAC	CTGCACTCTCGCCAATGGTA
<i>HYAL3</i>	GCCTCACACACCGGAGATCT	CACCAATGGTCTGCACAAGG