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Fibronectin induces the perivascular deposition of cerebrospinal fluid-derived amyloid- β in aging and after stroke

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Abstract

Cerebral amyloid angiopathy occurs after stroke, but the mechanism underlying the initial amyloid- β deposition is not fully understood. This study investigates whether overexpression of fibronectin and its receptor, integrin- $\alpha 5$, induces the perivascular deposition of CSF-derived amyloid- β after stroke in young and aged animals. We found that stroke impaired the bulk flow of CSF into the brain parenchyma, and further showed that perivascular amyloid- β deposition was enhanced in aged animals with stroke, which co-localized with integrin- $\alpha 5$ in the basement membrane. Furthermore, we found that stroke dramatically increased the cortical levels of fibronectin and integrin- $\alpha 5$, with further increases in integrin- $\alpha 5$ in aged animals with stroke. Fibronectin bound amyloid- β *in vitro*, and fibronectin administration increased amyloid- β deposition *in vivo*. Finally, aging and stroke impaired performance on the Barnes maze. These results indicate that fibronectin induces the perivascular deposition of amyloid- β , and that increased integrin- $\alpha 5$ further ‘primes’ the aged brain for amyloid- β binding. This provides a novel molecular and physiological mechanism for perivascular amyloid- β deposition after stroke, particularly in aged individuals.

1: INTRODUCTION

Ischemic stroke is a leading risk factor for dementia, with over a third of stroke survivors experiencing cognitive decline within 10 years of injury (Ivan et al., 2004; Sigurdsson et al., 2017). While the mechanism of post-stroke dementia is not known, studies of brain pathology in stroke survivors indicate a markedly increased rate of vascular A β pathology consistent with CAA (Gorelick et al., 2011; Akoudad et al., 2015; Matthews et al., 2009; Schneider et al., 2007). CAA likely contributes to cognitive decline via several mechanisms, including impaired perfusion of small vessel beds (Miners et al., 2016; Maier et al., 2014), reduced vessel integrity (Carrano et al., 2012, 2011; Hartz et al., 2012) as well as impaired neurovascular coupling (Maki et al., 2014; Peca et al., 2013; Williams et al., 2017)

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Therefore, reducing the development of post-stroke CAA may help to improve cognitive function in stroke survivors.

While CAA is a pervasive finding in stroke survivors, the pathophysiological mechanisms underlying progressive vascular A β accumulation remain understudied. Interestingly, recent studies suggest that the clearance of A β ₁₋₄₀ from blood vessels is mediated via a perivascular route of CSF flow, termed the glymphatic system (Iliff et al., 2012). During glymphatic flow, the pulsation of arteries pushes CSF from the meningeal cisterns along penetrating arterioles and eventually capillary beds through the perivascular space (Iliff et al., 2013), which is bordered on either side by the basement membrane (BM) and astrocytic endfeet (Hawkes et al., 2013, 2011). This process of bulk flow is disrupted in young mice with acute carotid ligation (Iliff et al., 2013), and in middle-aged animals with multiple microinfarction (Wang et al., 2017; Venkat et al., 2017), but the chronic impact of middle cerebral artery (MCA) occlusion on CSF flow within the aged brain is not known. Additionally, the impact of ischemia on the clearance of A β ₁₋₄₀ from the cerebrovascular BM remains understudied. Interestingly, Peng *et al.* (2016) showed that in addition to clearing A β ₁₋₄₀ from the brain, the glymphatic system can also redistribute CSF A β ₁₋₄₀ back into the brain along perivascular routes, and selectively accumulates in the brains of aged APP/PS1 mice, and colocalizes with pre-existing cortical amyloid plaques. Taken together with clinical data showing that CSF A β ₁₋₄₀ levels fluctuate over the course of disease in dementia patients (Shoji and Kanai, 2001), these findings suggest that CSF A β ₁₋₄₀ may deposit within perivascular regions involved in glymphatic flow. While increased perivascular redistribution of CSF A β ₁₋₄₀ could lead to CAA, the molecular and cellular factors that induce A β ₁₋₄₀ deposition in CAA, especially in the context of ischemic stroke, remain understudied.

The perivascular flow of CSF may be dependent on the composition of the cerebrovascular BM, which consists of a glycosylated protein matrix. This protein matrix may act as a filtration path that could restrict, in part, the redistribution of solutes dissolved in the CSF. We postulate that this novel BM function may become dysregulated after ischemic stroke, which stimulates the overexpression of fibronectin and its receptor, α 5 β 1 integrin. Fibronectin is a 440 kD BM glycoprotein that links cell membrane integrins to HSPGs (Huveneers *et al.*, 2008; Mahalingam *et al.*, 2007; Wang *et al.*, 2010). Importantly, fibronectin is also expressed in CAA plaques (Wyss-Coray *et al.*, 2000) and exhibits affinity for aggregated A β (Briknarová *et al.*, 2003; Maas *et al.*, 2008). However, it is not known whether fibronectin influences the cerebral distribution of CSF A β ₁₋₄₀ *in vivo*, which could be especially relevant in the aged brain, where the complex process of BM remodeling could become dysregulated following ischemia. Therefore, we hypothesize that stroke-induced increases in BM fibronectin leads to perivascular deposition of CSF-derived A β ₁₋₄₀ by direct binding.

In the present study, we show that both aging and focal stroke inhibit the bulk flow of CSF along perivascular routes within the brain, which is associated with selective impairment of cognitive function. Furthermore, we also demonstrate that CSF A β ₁₋₄₀ is abnormally deposited within the BM after stroke in aged animals, and that this deposition strongly correlates with the peri-infarct BM labeled by lectin. We identified that fibronectin and the

integrin- α 5 receptor subunit are upregulated BM proteins in aged animals with stroke, which enhanced the deposition of CSF A β _{1–40}. Based on our findings, we propose targeting fibronectin as a strategy to treat age and stroke-related CAA.

2: MATERIALS and METHODS

2.1: Animals.

Wild-type young (2 – 3 month-old) and aged (18 – 20 month-old) male C57BL/6 mice were obtained from Charles River and the National Institute of Aging, respectively. Mice were kept on a 12-hour light-dark cycle, and fed standard dry chow with *ad libitum* access to food and water. Mice were examined regularly by trained members of the lab and the veterinary staff for any signs of illness, as well as gross pathology at the time of sacrifice. Importantly, all studies were carried out in accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health: Reporting of In Vivo Experiments (ARRIVE) Guidelines. The protocols were approved by the UTHealth Animal Care and Use Committee and carried out in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Animals were randomized to experimental conditions, and investigators were blinded to treatment.

2.2: Permanent distal middle cerebral artery occlusion.

Mice were subjected to permanent DMCAO as previously described (Doyle & Buckwalter, 2014). Briefly, mice under isoflurane anesthesia were placed in a sterile surgical field, and body temperature maintained at 37°C. Following this, the right dorsolateral cranium was shaved and aseptically prepared. Then, local anesthetic was injected (Marcaine, 0.05 mL), the skin and underlying temporalis muscle were incised, and a 2 mm burr hole was drilled to expose the MCA, which was then cauterized to induce ischemia. After successful cauterization, the burr hole was closed with dental cement, the temporalis muscle repaired with Vetbond and the skin incision closed with Vicryl 5–0 nylon sutures. Sham surgeries were performed following an identical procedure, except that no cauterization was performed. Ischemia was confirmed by Laser Doppler flow measurement immediately following MCA cauterization.

2.3: Behavioral tests.

2.3.1: DigiGait.—The impact of aging and stroke on motor function was assessed using DigiGait (Mouse Specifics) at 3 and 7 DPI in a double-blinded manner. Mice were first acclimated to the system, and then the speed was gradually increased to 15 centimeters/second. Gait was recorded for 60 seconds, and the degree of left forepaw rotation was measured using automated DigiGait analysis software over 10 steps by a separate blinded investigator.

2.3.2: Barnes Maze.—Cognitive function was assessed at 28 DPI using a one day training-testing paradigm, modified from previously described work (Attar et al., 2013). Following habituation, mice were trained to find the escape hole in an illuminated, elevated Barnes maze (Mouse Specifics). This training was repeated 4 times, and then tested 4 hours

later in a 3 minute probe trial. The latency to find the escape hole, number of errors and total distance moved were quantified using EthoVision XT software in a blinded manner.

2.3.3: Open Field.—Exploratory behavior was assessed at 28 DPI by open field testing. Mice were allowed to explore a 40 cm² opaque chamber for 5 minutes. Absolute distance traveled (centimeters) was recorded and quantified using EthoVision XT software in a blinded manner.

2.4: Preparation of fluorescent tracers.

aCSF containing Na⁺ (150 mM), K⁺ (3 mM), Ca²⁺ (1.4 mM), Mg (0.8 mM), P (1.0 mM) and HCO₃ (24.1 mM) was prepared in sterile distilled water on the day of the experiment. Stock solutions of lysine-fixable cascade blue dextran (CB-d3, 3,000 MW, anionic, 4% stock, Thermo Fisher), fluorescein isothiocyanate dextran (FITC-d2000, 2,000,000 MW, anionic, 4% stock, Thermo Fisher) and AlexaFluor-647-conjugated or FITC-conjugated recombinant human A β _{1–40} (647-A β _{1–40} or FITC-A β _{1–40}, 100 μ M stock, Anaspec) were then prepared in aCSF. Tracers were then combined to a final solution of 2% CB-d3, 1% FITC-d2000 and 10 μ M 647-A β _{1–40}, or 10 μ M FITC-A β _{1–40}. In other experiments, recombinant human fibronectin (R&D Systems, cat. 4305-FN) or aCSF was added to the tracer mixture to a concentration of 500 nM and incubated for 1 hour at room temperature prior to infusion. Tracer mixtures were kept on ice for up to 4 hours prior to use, and vortexed for 5 seconds immediately before infusion. To measure fluorescent background signal, zero control animals received aCSF with no tracers.

2.5: Tracer injection.

Mice were deeply anesthetized with an intraperitoneal injection of Ketamine-Xylazine dissolved in 0.9% saline (120 mg/kg Ketamine, 10 mg/kg Xylazine), then placed in a stereotaxic frame. The skin over the cervical spine was then shaved, aseptically prepared and incised. Following this, the needle was inserted through the superficial neck musculature and into the cisterna magna as previously described (Iliff et al., 2012). Tracer was slowly infused into the cisterna magna (1 μ L/min) over 10 minutes, after which mice were transferred to a warmed cage. Then, 30 minutes after the initiation of the injection, mice were sacrificed by Avertin overdose (250 mg/kg), followed by transcardial perfusion with 0.2% heparin-PBS, then 4% PFA. Tissues were dissected and stored in 4% PFA until later use. In total, each animal received 4.3 μ g of A β _{1–40}, contained within 10 μ L of tracer volume.

2.6: Fluorescent microscopy and tracer imaging.

Following perfusion, brains were extracted and post-fixed for 24 hours in 4% PFA. Then, brains were dehydrated in 30% sucrose, frozen to –20°C and sectioned to 24 μ m (immunohistochemistry/cresyl violet staining) or 100 μ m (tracer quantification) sections. Sections were then mounted onto slides and coverslipped. For experiments involving colocalization with lectin, sections were first stained with *Lycopersicon esculentum* (Tomato) lectin (see Immunohistochemistry). Sections were imaged under fluorescence with a Leica DMi8 microscope at 10X magnification. A blinded investigator imaged the lateral cortex (0.38 mm anterior to Bregma, 2.5 mm lateral, 1.5 mm deep), which corresponds to 8.1 mm from the injection site within the cisterna magna. Then, 100 μ m Z stack images were

obtained, and maximum intensity projections performed with LAS X software. Images were then quantified in ImageJ (see Data Analysis).

2.7: Immunohistochemistry.

Fixed brain sections (24 μ m) were mounted onto slides and incubated with *Lycopersicon esculentum* lectin (1:100, λ = 594, Vector Laboratories, cat. DL-1177), anti-fibronectin (Rabbit, 1:100, abcam, cat. ab2413), anti-integrin- α 5 (Rabbit, 1:100, abcam, cat. EPR7854) or anti-GFAP-Cy3 (Mouse, 1:1000, Millipore Sigma, cat. C9205,) antibodies. Then, sections were incubated with secondary antibodies (Goat anti-Rabbit (Abcam, Alexa-Fluor, 1:500, λ = 488, 594 or 647 nm). Finally, sections were cover-slipped with Vectashield containing DAPI (Vector Laboratories, H-1200).

2.8: Western blot.

The right cortex was perfused with heparinized PBS, homogenized and lysed in buffer containing 1% NP-40, 1 mM PMSF, cOmplete (Roche) and PhosSTOP (Roche). Following centrifugation, the supernatant was removed and diluted to 4 mg/mL in 4x Laemmli Sample Buffer (Bio-Rad, 161-0737) supplemented with 4% β -mercaptoethanol, then heated to 95°C for 5 minutes. Following sample preparation, 10 μ L of each lysate was run on a 4–15% Criterion TGX 26-well gels (Bio-Rad, cat. 5671085). Protein bands were subsequently transferred to a PVDF membrane and probed with anti-fibronectin (Rabbit, 1:1000, abcam, cat. ab2413), anti- α 5 Integrin (Rabbit, 1:1000, abcam, cat. EPR7854) or anti-vinculin antibody (Rabbit, 1:10,000, abcam, cat. EPR8185). Blots were then probed with HRP-conjugated goat anti-rabbit secondary antibody (1:10,000, Vector Laboratories, cat. PI-1000) and imaged using a Bio-Rad ChemiDoc Imager.

2.9: Generation of radiolabeled A β _{1–40}.

Recombinant human A β _{1–40} (Anaspec, Fremont, CA) was first dissolved to a concentration of 1 μ g/ μ L in sterile, chloride-free 250 mM sodium phosphate buffer. Then, A β _{1–40} was conjugated to Iodine-125 (¹²⁵I) with chloramine T as previously described.(Urayama et al., 2008) ¹²⁵I-A β _{1–40} was separated from free ¹²⁵I by washing the mixture through a Sephadex G-10 column. Successful conjugation was measured by TCA precipitation, and fractions retaining > 90% CPM values in the solid phase were then combined and lyophilized overnight for later use.

2.10: Fibronectin-¹²⁵I-A β _{1–40} binding assay.

To measure the binding affinity of radiolabeled ¹²⁵I-A β _{1–40} for fibronectin, an *in vitro* assay was developed that relies on separation of free ¹²⁵I-A β _{1–40} (4.2 kD) from fibronectin-¹²⁵I-A β _{1–40} conjugates (> 220 kD) based on molecular weight. First, serially diluted recombinant human fibronectin (1 $\times$ 10³ to 1 $\times$ 10⁸ ng/ml) (R&D Systems, cat. 4305-FN) was incubated with ¹²⁵I-A β _{1–40} (1 $\times$ 10⁵ CPM) at room temperature for 1 hour. Reaction mixtures were then centrifuged in a molecular weight cutoff spin column (> 50 kD, Amicon Ultra, Millipore-Sigma). The columns were washed with 400 μ L of sterile PBS and centrifuged 3 times for 5 minutes at 5,000 $\times$ g. Finally, the remaining ¹²⁵I-A β -fibronectin conjugates were resuspended in sterile PBS and the amount of fibronectin-bound ¹²⁵I-A β quantified by a

gamma counter (Packard Cobra II). See Data Analysis for the calculation of the standard curve.

2.11: Measurement of plasma A β _{1–40}.

We measured plasma levels using the Invitrogen Mouse A β _{1–40} ELISA kit. Briefly, we collected heparinized plasma from mice at 30 DPI, then performed a two-fold dilution with standard buffer. Following this, the assay was performed, read, and interpreted according to the manufacturer's instructions.

2.12: Data Analysis.

2.12.1: Atrophy quantification.—High resolution images of cresyl violet-stained coronal brain sections (24 μ m) were obtained (Supplementary Fig. 1). Then, ventricular volume and hemisphere size were quantified as previously described (Harris *et al.*, 2016; Verma *et al.*, 2014). Tissue atrophy percentage was calculated by the formula: % *tissue loss* = $100 - ([\text{total ipsilateral tissue} / \text{total contralateral tissue}] \times 100)$.

2.12.2: Tracer intensity.—Tracer influx was quantified by measuring intensity as a function of tissue depth using ImageJ (Supplementary Fig. 2). Then, average intensity values were imported into Excel, and integrated into 50 μ m bins. Absolute binned intensity values were then plotted as raw intensity (arbitrary units, A.U.). To normalize potential age-induced differences in background intensity, the relative tracer intensity was calculated by subtracting each binned value from intensity values obtained from age-matched control mice that did not receive tracer injections (Intensity [A.U.]).

2.12.3: Immunohistochemical quantification.—IHC images were acquired and quantified in a double-blinded manner. Based on lectin staining, ROI's were drawn around 3 peri-infarct vessels/image across 3 brain sections for a total of 27 vessels/brain. Threshold was held constant between brains, and the area and average pixel intensity of fibronectin was measured relative to the ROI.

2.12.4: Calculation of 50% Effective Concentration (EC₅₀).—The EC₅₀ was quantified by plotting the log [Fibronectin] against the %A β binding (calculated as CPM [X]/CPM [Max]). A sigmoidal regression line was fit with Prism, and the EC₅₀ was interpolated from the curve.

2.12.5: Statistics.—The physiology data measured at different depths was analyzed by repeated measures two-way ANOVA, with factors of aging and stroke. Repeated measures one-way ANOVA was used for repeated measures with only one factor. For immunohistochemical quantification of area or intensity, as well as for analysis of band intensity of western blots, two-way ANOVA was used, examining the effects of aging and stroke on the dependent variable. All of the above analyses were followed by Sidak's post-hoc test. Scatter plots were provided and linear mixed-effects models were used to study the association between the dextran intensity and A β ₄₀ intensity, and between the lectin intensity and the tracer intensity.

3: RESULTS

3.1: Aging and DMCAO impaired the perivascular flow of cerebrospinal fluid.

Previous work has shown that aging impairs glymphatic flow (Kress et al., 2014), and that multiple microinfarctions disrupt CSF flow in middle-aged mice (12 months-old) (Venkat et al., 2017; Wang et al., 2017). However, it was not known whether a single focal stroke, modeled by DMCAO, induces glymphatic impairment, or if this would further worsen underlying impairments in aged mice (> 18 months of age). Therefore, to assess the impact of aging and DMCAO on overall glymphatic flow, we injected fluorescent tracer into the cisterna magna at 30 days post-DMCAO, and quantified the distribution 30 minutes later (Fig. 1A–B, Supplementary Fig. 2). We found that CSF influx, measured as the distribution of dextran-derived fluid space markers, occurred along the superficial surfaces of the brain (Supplementary Fig. 2). Furthermore, we found that the distribution of both low (CB-d3, 3 kD) and high (FITC-d2000, 2000 kD) molecular weight dextran colocalized with lectin-stained vessels, supporting a perivascular route for CSF influx (Fig. 1A). When raw CB-d3 distribution values were examined, we found reduced CSF flow occurred in aged animals at baseline (Supplementary Figure 2D, iii, Main Effect: Age, $[F(1, 19) = 6.2, p = 0.022]$). We then measured how CSF influx changed in the injured cortex, relative to the contralateral hemisphere, and found the distribution of CB-d3 was inhibited by DMCAO in both young and aged animals (Fig. 1C, i., $[F(1, 18) = 52.3, p < 0.001]$). Taken together, these data indicate that DMCAO produced a unilateral impairment of bulk CSF influx through the lectin-stained BM, even in aged mice, which exhibit baseline reductions in CSF flow.

3.2: DMCAO increased the perivascular distribution of cerebrospinal fluid $A\beta_{1-40}$, which worsens with aging.

Previous work has shown that the glymphatic system provides a perivascular route for the deposition of CSF $A\beta_{1-40}$ in mouse models of Alzheimer's disease with pre-existing $A\beta$ pathology (Peng et al., 2016), but it was not known whether stroke would induce *de novo* $A\beta_{1-40}$ deposition, given the absence of pre-existing $A\beta$ plaques in wild-type mice. Therefore, to determine whether stroke selectively induced the 'trapping' of CSF $A\beta_{1-40}$, we first examined whether DMCAO increased the total amount of $A\beta_{1-40}$ deposition within the brains of young and aged wild-type mice by quantification of 647- $A\beta_{1-40}$ distribution. While we did not observe any changes in aged animals at baseline, we found an interesting interaction effect, which showed a paradoxical increase in 647- $A\beta_{1-40}$ distribution in aged animals with DMCAO (Fig. 1C, ii., $F[1, 18] = 21.7, p < 0.001$). Importantly, these differences between young and aged mice with DMCAO were not associated with significant differences in tissue atrophy ipsilateral to the injury (Supplementary Figure 1B, $p = 0.96$), suggesting that other mechanisms, aside from bulk flow, could play an important role in determining the fate of CSF $A\beta_{1-40}$. Taken together, these data indicate that both aging and DMCAO induced the abnormal deposition of CSF $A\beta_{1-40}$, with a major effect of DMCAO on the injured hemisphere, and a major effect of aging on the contralateral hemisphere.

Then, to determine whether stroke preferentially affects the distribution of the $A\beta_{1-40}$ isoform, we compared the distribution of 647- $A\beta_{1-40}$ to FITC- $A\beta_{1-42}$ in aged animals with

sham and DMCAO in a separate cohort (Fig. 2A). This analysis confirmed that 647-A β_{1-40} was increased in aged animals with DMCAO (Fig. 2B, i., $p = 0.015$), with no significant impact on FITC-A β_{1-42} distribution (Fig. 2B, ii., $p = 0.596$). Normalization of these values confirmed that 647-A β_{1-40} /FITC-A β_{1-42} ratio was significantly increased with DMCAO (Fig. 2B, iii., $p = 0.045$). Given that the same amount of A β_{1-40} and A β_{1-42} were infused into the brain, and that the proteins are similar in molecular weight, these data indicate a somewhat specific effect of DMCAO on the transport of CSF A β_{1-40} through the aged BM after DMCAO, which we next explored by correlation analysis.

3.3: Aging decoupled A β_{1-40} transport from CSF flow.

To examine whether abnormal A β_{1-40} deposition is related to the bulk flow of CSF, the average intensity of 647-A β_{1-40} and CB-d3 (CSF fluid space marker) were compared within each 50 μm bin for the ipsilateral hemisphere (5 bins/animal) (Supplementary Fig. 3A – B). Comparison of CB-d3 and 647-A β_{1-40} influx curves revealed similar distribution kinetics as a function of tissue depth in young sham animals (Supplementary Fig. 3A). Furthermore, linear regression revealed a positive correlation (non-zero slope) of CB-d3 and 647-A β_{1-40} distribution in young sham (Supplementary Fig. 3C, i., $[t(21) = 5.89, p < 0.001]$) and young stroke animals (Supplementary Fig. 3C, ii., $[t(25) = 3.25, p = 0.003]$). However, CB-d3 distribution did not predict A β_{1-40} distribution in aged sham $[t(17) = 0.9, p = 0.379]$ or aged stroke animals $[t(17) = 1.89, p = 0.076]$. These findings indicate that age-induced disruption of bulk CSF influx did not predict the behavior of A β_{1-40} , implying that other mechanisms, such as altered diffusion of A β_{1-40} through the BM, may have played a more important role in determining CSF A β deposition with aging, which may be further changed by DMCAO.

Since bulk flow did not significantly predict CSF A β_{1-40} deposition in disease states, we then broadly assessed whether components of the cerebrovascular BM are associated with CSF A β_{1-40} accumulation *in vivo*. To accomplish this, the average intensity of 647-A β_{1-40} and CB-d3 were each compared to the expression of glycosylated BM proteins, measured by the intensity of 594-lectin staining (Supplementary Fig. 4A). Interestingly, linear regression of 594-lectin vs. 647-A β_{1-40} intensity revealed a highly significant positive correlation in all experimental groups (Supplementary Fig. 4C, i. – iv.; young sham, $[F(1, 23) = 10.1, p < 0.001]$; young stroke, $[F(1, 27) = 67.91, p < .0001]$; aged sham, $[F(1, 19) = 26.4, p < 0.001]$; aged stroke, $[F(1, 19) = 17.61, p < 0.001]$). In contrast, linear regression of 594-lectin vs. CB-d3 intensity (used as a fluid space marker and to control for potential non-specific trapping within the extracellular space) revealed a positive relationship in young sham animals (sham, Supplementary Fig. 4B, i., $t(23) = 3.7, p = 0.001$), but not in young stroke animals (Supplementary Fig. 4B, ii., $[t(27) = 0.4, p = 0.685]$). Additionally, in aged animals, 594-lectin intensity did not correlate with CB-d3 intensity in sham (Supplementary Fig. 4B, iii., $[t(19) = 0.9, p = 0.384]$) or stroke mice (Supplementary Fig. 4B, iv., $[t(19) = 0.5, p = 0.619]$). Overall, these data showed that BM proteins were more strongly associated with 647-A β_{1-40} than CB-d3 controls, indicating an association of lectin-stained BM proteins on A β_{1-40} levels. This difference was further exacerbated by aging and DMCAO.

3.4: BM fibronectin and integrin- α 5 expression increase following stroke.

We next examined how the expression of fibronectin changes in the context of the perivascular space, bordered by astrocytic endfeet and the lectin-stained BM matrix. Immunohistochemistry revealed increases in fibronectin expression at 7 DPI (Fig. 3A). To confirm these findings, total protein levels of fibronectin and integrin- α 5 were then assessed by western blot of the peri-infarct cortex at 7 DPI (Fig. 3B, Supplementary Figure 7). Stroke increased fibronectin expression (Fig. 3C, i., [F(1, 19) = 63.8, $p < 0.001$]), with no additional effects of aging [F(1, 19) = 0.06, $p = 0.81$] or interaction [F(1, 19) = 0.1, $p = 0.733$]. In contrast, integrin- α 5 levels were increased by both DMCAO (Fig. 3C, ii., [F(1, 19) = 34.6, $p < 0.001$]) and aging [F(1, 19) = 5, $p = 0.038$], with no significant interaction effect [F(1, 19) = 1.5, $p = 0.232$]. These data suggest that perivascular fibronectin deposition occurred within 7 DPI, which was also accompanied by further increases in integrin- α 5 expression in aged animals with DMCAO. We also observed acute reductions in LRP1 following DMCAO in aged animals (Supplementary Figure 5), which may further impair the washout clearance of A β ₁₋₄₀ from the cerebrovascular BM across the blood-brain barrier in the aged brain. We also observed an increase in plasma A β ₁₋₄₀ levels with aging (F[1, 22] = 15.5, $p < 0.001$), however, this was not altered by ischemia at 30 DPI (Supplementary Figure 6). These changes could reflect reduced systemic clearance in aged animals, or increased shedding of amyloid precursor protein in peripheral tissues.

3.5: Cerebrospinal fluid-derived amyloid- β ₁₋₄₀ colocalized with fibronectin, the integrin- α 5 subunit and lectin-stained basement membrane components, which increased with DMCAO.

Based on our data showing that aging and DMCAO cause increased association of CSF A β ₁₋₄₀ with the lectin-stained cerebrovascular BM, we next assessed the contribution of fibronectin and the integrin family of receptors to this phenotype. In addition to fibronectin, we selected integrin- α 5 because previous work has shown that it is strongly expressed in the cerebrovascular BM (Huang et al., 2015; Milner et al., 2008), regulates the binding of multiple β subunits to fibronectin (Benito-Jardón et al., 2017) and is associated with increased A β accumulation in disease states (Jullienne et al., 2014).

Image quantification at 30 DPI revealed that DMCAO increased the expression of FITC-lectin-stained BM components (Fig. 4B, ii., [F(1, 19) = 5.756, $p = 0.0269$]). Aging also exhibited a trend for increased FITC-lectin staining, but failed to reach statistical significance [F(1, 19) = 3.252, $p = 0.0872$]. Additionally, we found that perivascular fibronectin staining increased with DMCAO in both young and aged animals (Fig. 4B, i., F[1, 12] = 19.5, $p < 0.001$) and colocalized with the lectin-stained BM (Fig. 4A). Furthermore, intracisternally injected FITC-A β ₁₋₄₀ colocalized with fibronectin within the BM across all conditions, and was most apparent in aged animals with DMCAO (Fig. 4A). This suggested that fibronectin was a marker of sites within the BM that were predisposed to A β ₁₋₄₀ deposition, which further increased with aging and DMCAO.

Despite observing colocalization with A β ₁₋₄₀, we did not observe a consistent increase in fibronectin expression in aged animals. Therefore, we next explored the impact of aging and DMCAO on its major receptor, integrin- α 5, to determine whether this could further

modulate A β ₁₋₄₀ distribution in the aged brain. We found that perivascular integrin- α 5 staining in the aged brain further increased with DMCAO, and colocalized with the lectin-stained BM (Fig. 5A). Furthermore, intracisternally injected FITC-A β ₁₋₄₀ colocalized with integrin- α 5 within the BM across all conditions, and was, again, most apparent in aged animals with DMCAO (Fig. 5A). Total expression of vascular integrin- α 5 was highest in aged animals with DMCAO (Fig. 5B), with main effects of DMCAO ($F[1, 12] = 344.6$, $p < 0.001$) and aging ($F[1, 12] = 15.1$, $p = 0.002$) on increased expression, with a significant interaction effect ($F[1, 12] = 27.9$, $p < 0.001$). Increased expression of integrin- α 5 could alter the affinity of the BM by complexing with its major ligand, fibronectin. Alternatively, it could possess additional binding sites for A β ₁₋₄₀.

3.5: Fibronectin treatment increased the perivascular A β ₁₋₄₀.

Previous work has suggested that plasma fibronectin may bind serum amyloid species via the fibronectin type I domain (Maas et al., 2008), but there are a lack of studies examining its potential affinity to the soluble A β ₁₋₄₀ fragment, especially in the context of the perivascular microenvironment. Therefore, to first assess the binding characteristics of fibronectin to A β ₁₋₄₀, recombinant fibronectin was incubated with ¹²⁵I-A β ₁₋₄₀ (Fig. 6A, i.). We found that fibronectin bound A β ₁₋₄₀ *in vitro* with an EC₅₀ of 216 nM (Fig. 6A, ii.). Then, to determine whether fibronectin impacted CSF flow or A β ₁₋₄₀ deposition, fibronectin or vehicle control was incubated with CB-d3 and 647-A β ₁₋₄₀, then injected intracisternally (Fig. 6B). Fibronectin treatment increased the 647-A β ₁₋₄₀/CB-d3 ratio (Fig. 6C, [$F(1, 8) = 11.2$, $p = 0.01$]), which decreased with tissue depth [$F(4, 32) = 18.1$, $p < 0.001$]. Furthermore, an interaction between fibronectin treatment and tissue depth was also observed [$F(4, 32) = 3.1$, $p = 0.028$]. Treatment did not affect total CB-d3 ([$F(1, 8) = 0.05$, $p = 0.835$]) or 647-A β ₁₋₄₀ influx ([$F(1, 8) = 0.6$, $p = 0.468$]). These data demonstrate that fibronectin-A β ₁₋₄₀ binding occurred, and that this interaction was sufficient to induce the selective accumulation of A β ₁₋₄₀ within the superficial cortex, without impacting bulk CSF influx.

3.6: Aging and DMCAO induced impairments in motor function, cognition, and exploratory behavior.

Previous work has shown that young mice develop cognitive impairment after DMCAO (Doyle et al., 2015). To evaluate whether aging and DMCAO induced similar behavioral changes in our animals, we tested mice on DigiGait, Barnes maze and open field (Supplementary Figure 1C–D). We found that stroke induced left forepaw rotation at 3 DPI (Supplementary Figure 1C, [$F(1, 31) = 21.4$, $p < 0.0001$]), which resolved by 7 DPI ([$F(1, 21) = 1.6$, $p = 0.214$]). Aging also induced mild forepaw rotation at 3 DPI [$F(1, 31) = 4.7$, $p = 0.037$] and 7 DPI [$F(1, 21) = 5.6$, $p = 0.028$]. No significant interaction of aging and DMCAO were observed at 3 DPI [$F(1, 31) = 0.9$, $p = 0.35$] or 7 DPI [$F(1, 21) = 1$, $p = 0.333$]. These data indicate that both aging and DMCAO induce motor impairment, with DMCAO-induced motor deficits recovering by 7 DPI.

Aging increased the latency to find the escape hole on the Barnes maze (Supplementary Figure 1D, [$F(1, 26) = 8.3$, $p = 0.008$]). No major effect of DMCAO on latency was observed [$F(1, 26) = 1.2$, $p = 0.287$], likely due to a trending interaction between aging and DMCAO

[$F(1, 26) = 3.3$, $p = 0.082$), as DMCAO worsened Barnes maze performance in young (Sidak test, $p = 0.049$), but not aged animals ($p = 0.8784$). Finally, aging reduced total movement on open field testing ($F(1, 26) = 12.6$, $p = 0.002$), with no effect of DMCAO [$F(1, 26) = 0.003$, $p = 0.96$] or interaction observed [$F(1, 26) = 0.06$, $p = 0.817$]. Taken together, these data indicate that cognitive deficits existed in conditions of reduced CSF flow, even when motor deficits were no longer detectable by gait measurement.

4: DISCUSSION

Stroke dramatically accelerates the rate of cognitive decline in patients, suggesting that it induces a chronic neurodegenerative cascade that continues for years after the initial insult (Gorelick et al., 2011; Sigurdsson et al., 2017). While this cascade is associated with increased A β deposition in peri-infarct vessel beds, the mechanism of CAA induction is not known. Previous work has shown that stroke is associated with increased A β expression in the thalamus (Mäkinen et al., 2008; van Groen et al., 2005) and peri-infarct regions (Garcia-Alloza et al., 2011) in animal models. However, the chronic molecular mechanisms and physiological processes favoring A β deposition after stroke are understudied. In addition, there is a lack of studies that examine perivascular A β pathology (CAA) in animal models of stroke. As such, our findings illuminate multiple facets of a potential new mechanism of CAA development, including both the physiological route of the initial perivascular A β deposition after stroke, as well as the specific extracellular matrix components that pathologically enhance this A β deposition after stroke. A better understanding of how aging and stroke impact these processes is critical to developing new and effective therapies to combat CAA in at-risk stroke patients.

The impact of ischemia on glymphatic physiology is an understudied area of study that continues to grow in importance to our aging population. One previous study found that acute carotid ligation in young mice reduced CSF flow acutely due to reduced pulsatility of the distal vessel beds (Iliff et al., 2013). A subsequent study of young rats with multiple microinfarction found focal disruptions in AQP4 expression and CSF flow at 14 DPI (Venkat et al., 2017). Finally, another recent study found that multiple microinfarction induced the focal trapping of CSF solutes, which was worse in middle-aged mice (Wang et al., 2017). However, impacts on the transport of A β_{1-40} were not directly explored in this study, which we found to distribute differently than dextran particles.

In addition to these studies, our work adds to the scientific knowledge in this area in three important ways. First, ours is the first to examine the chronic impact of cortical infarction on glymphatic flow in aged animals (> 18 months), which may have more relevance to elderly survivors of ischemic stroke than previous studies. Second, our study directly examines the impact of aging and ischemia on the transport of A β_{1-40} through the BM, which has not been adequately explored as a regulator of glymphatic flow in disease states. While one previous study examined the glymphatic transport of A β_{1-40} in animal models of Alzheimer's disease without ischemia (Peng et al., 2016), ours differs due to the use of ischemic injury, as well as the association with changes in BM composition. We have found that aging and ischemia interact to increase the distribution of CSF A β_{1-40} through the BM. Furthermore, we found that this occurred in wild-type animals, without preexisting amyloid

pathology, suggesting that other ECM proteins play a role in the initiation of vascular amyloid pathology. These chronic ischemia-induced changes could predispose the aged brain to the development of CAA plaques, although additional studies in animal models of CAA would be required to verify this interpretation. Finally, we newly found that fibronectin plays a role in regulating the CSF transport of A β ₁₋₄₀, which is modulated by the increased integrin- α 5 expression in aged animals. We verified this using colocalization studies in young and aged animals with stroke, as well as infusion of fibronectin-A β ₁₋₄₀ conjugates into naive mice. While previous studies have shown that fibronectin can bind aggregated amyloid species *in vitro* (Briknarová et al., 2003; Maas et al., 2008), ours is the first to show that this affinity regulates the glymphatic transport of soluble A β ₁₋₄₀ *in vivo*, and that ischemia-induced increases in A β ₁₋₄₀ distribution can be attributed to this phenomenon.

While it is generally accepted that CAA preferentially affects arterioles over venules, the pathophysiological mechanism of arterial A β deposition is not well understood. Interestingly, our findings suggest that stroke is associated with a chronic impairment of glymphatic flow and the distribution of CSF A β ₁₋₄₀ in peri-infarct vessel beds. This process is associated with both motor and cognitive impairment. Importantly, impairment of CSF flux extends to 30 DPI and is associated with the ‘trapping’ of CSF-derived A β in the perivascular space. While aging and stroke reduce CSF influx, they paradoxically enhance the distribution of A β within the superficial cortical layers. Furthermore, we found that aging and stroke disrupt the bulk transport of A β , decoupling it from CSF flow. Our data further show that A β levels strongly correlate with the expression of lectin-stained BM proteins. This suggests that when perivascular solute flux is reduced with aging and stroke, even low levels of CSF-derived A β transport permit accumulation of A β within the brain over time. Taken together, these findings demonstrate that CSF A β can be abnormally deposited in the brain along perivascular routes, suggesting a new pathophysiological mechanism that may contribute to the development of CAA in stroke survivors.

Previous work has shown that CAA occurs in the context of ECM remodeling, but it is not known whether perivascular A β disposition is a cause or a consequence of this remodeling process. The current study highlights BM fibrosis as a potential early contributor to CAA pathology after stroke, which modulates the transport of CSF A β by direct binding. We hypothesized that BM remodeling after stroke includes chronic changes in both fibronectin and a subunit of its receptor, integrin- α 5, which together ‘prime’ the BM matrix for the binding of CSF-derived A β . Indeed, the present study indicates that stroke induces the chronic deposition of large, extracellular fibronectin aggregates as well as associated lectin-stained BM components, such as heparan sulfate proteoglycans. Furthermore, we found that fibronectin directly binds A β , and treatment with fibronectin increases the capacity of the BM for CSF A β sequestration, especially within superficial cortical layers. Importantly, this research also identifies integrin- α 5 as an important regulator of A β deposition, which is further enhanced with aging. Our study also shows that integrin- α 5 is expressed within the lectin-stained BM after stroke, and sensitizes the BM to the binding of fibronectin-A β aggregates.

Overall, these findings clearly demonstrate that stroke induces upregulation of fibronectin and integrin- α 5 within the BM of peri-infarct vessels, and that their interaction determines

the amount and location of perivascular CSF A β deposition after stroke. This novel molecular mechanism may provide an important point of intervention in the pathophysiology of CAA, which could be targeted with specific inhibitors of fibronectin or integrin- α 5. Additionally, the role of stroke-related endogenous trophic factors, such as TGF- β , in stimulating BM remodeling by endothelial cells, pericytes or astrocytes remains an understudied area, and may provide significant potential for alternative therapeutic targets for reducing CSF A β deposition.

In addition to better understanding the role of lectin-stained glycosylated proteins such as heparan sulfate proteoglycans in modulating CSF A β deposition, further work must be done to determine the role of other factors that may be responsible for modulating A β transport with disease. For example, the impact of stroke on the tortuosity of brain geometry is not known, but could also play a role in trapping CSF A β in addition to specific protein-protein interactions (Brunden *et al.*, 1993; Morris *et al.*, 2016; van Horssen *et al.*, 2001). Another potential contributor to stroke-induced trapping of CSF A β includes changes in the diffusibility of A β itself. Importantly, the free diffusion of molecules in the brain is governed by Brownian movement, which occurs as a function of the hydrodynamic radius of solutes (Syková and Nicholson, 2008). Therefore, the misfolding and recombination of distributed A β into larger oligomers likely reduces the diffusibility of A β through the brain parenchyma (Barrow & Zagorski, 1991; Funahashi *et al.*, 1994; Syková & Nicholson, 2008). This could be of particular importance in perivascular regions affected by stroke, as increased hypoxia and acidic pH within the stroke penumbra may stimulate the further aggregation of A β (Barrow & Zagorski, 1991; Funahashi *et al.*, 1994). Taken together with our results showing increased affinity of the BM for A β after stroke, a reduced threshold for A β aggregation could provide a potential feed-forward mechanism for the induction of CAA in stroke survivors.

The molecular and cellular factors that regulate the perivascular transport of A β ₁₋₄₀ from the CSF to the brain parenchyma is emerging as an exciting new area of study, which could eventually have a major impact on our current understanding of the pathophysiology of CAA. Further work is required to determine the nature of the cross-talk between the ECM and A β at both early and late stages of CAA plaque formation. While our current work primarily focused on the early molecular and physiological mechanisms of A β distribution in aging and stroke, we also found that glymphatic impairment was associated with impaired performance on the Barnes maze. Further work in Alzheimer's disease models is required to determine whether progressive A β deposition leads to accelerated cognitive decline in aged animals with stroke. Similar characterization of ECM changes and associated CSF A β deposition in these transgenic animal models may help to shed light on the directionality of pathology development, as well as the generalizability of our findings to other causes of CAA and cognitive impairment. It is our hope that a better understanding of the basic pathogenesis of CAA will lead to better treatments for both prevention and reversal of this disease process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS:

Aβ	Amyloid- β
Aβ₁₋₄₀	Amyloid- β ₁₋₄₀
A.U.	Arbitrary Units
aCSF	Artificial Cerebrospinal Fluid
BM	Basement Membrane
CB	Cascade Blue
CAA	Cerebral Amyloid Angiopathy
CPM	Counts Per Minute
DPI	Days Post-Injury
DMCAO	Distal Middle Cerebral Artery Occlusion
EC₅₀	Effective Concentration 50
FITC	Fluorescein Isothiocyanate
GLM	General Linear Model
GFAP	Glial Fibrillary Acid Protein
HSPG	Heparan Sulfate Proteoglycan
¹²⁵I	¹²⁵ Iodine
MCA	Middle Cerebral Artery
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
TGF-β	Transforming Growth Factor- β
TCA	Trichloroacetic Acid

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Highlights:

- Fibronectin and integrin- $\alpha 5$ increase in the vascular basement membrane after stroke.
- This process worsens with aging, inducing CSF amyloid- β deposition.
- Perivascular remodeling may be a viable target in treating cerebral amyloid angiopathy.

Figure 1. Aging and DMCAO disrupted the transport of cerebrospinal fluid and enhanced A β_{1-40} deposition in the superficial cortex.

(A.) Representative images of tracer uptake (cascade blue dextran [CB-d3, 3 kD]; 647-A β_{1-40} [647-A β_{1-40} , 4.5 kD]; FITC-dextran [FITC-d2000, 2000 kD]) and colocalization with lectin-stained BM proteins. (B.) (i.) Representative 10X magnification images from young sham and DMCAO animals showing axis of tracer quantification, (ii.) Comparison of intensity tracings from young sham and DMCAO animals. (C.) Quantification of (i.) CB-d3 tracer uptake and (ii.) 647-A β_{1-40} from ipsilateral (injured/sham) cortex, 1 – 250 μ m depth.

Data are normalized to contralateral hemisphere. Data are analyzed by two-way ANOVA with Sidak's post-hoc test. Mean $\pm$ SE (n=5–8 per group). *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar = 50 μ m.

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Figure 2. DMCAO selectively increased the uptake of A β_{1-40} in the injured cortex of aged animals.

(A.) Representative images comparing the distribution of 647-A β_{1-40} to FITC-A β_{1-42} in aged animals treated with sham or DMCAO at 30 DPI. (B.) Quantification of (i.) 647-A β_{1-40} uptake, (ii.) FITC-A β_{1-42} uptake and (iii.) 647-A β_{1-40} /FITC-A β_{1-42} ratio. All data are acquired from 1 – 250 μ m depth of superficial cortex, and normalized to the contralateral hemisphere. Mean $\pm$ SE (n=5 per group). Data are analyzed by Student's t-test, *p < 0.05.

Figure 3. DMCAO increased the expression of fibronectin and integrin- α 5 at 7 days post-injury. (A.) Representative images of fibronectin expression in young and aged animals, taken at 7 DPI (40X magnification). (B.) Representative blots and quantification of fibronectin, integrin- α 5 and vinculin (loading control) expression of these proteins at 7 DPI. Data are analyzed by two-way ANOVA with Sidak's post-hoc test, Mean $\pm$ SE (n=5–7 per group). *p < 0.05, ***p < 0.001. Scale bar = 50 μ m.

Figure 4. Fibronectin expression colocalized with sites of cerebrospinal fluid A β_{1-40} deposition. (A.) Colocalization of intracisternally injected A β_{1-40} with fibronectin occurs within the lectin stained BM at low levels in young sham mice, which worsens with aging and DMCAO. Low-power images were acquired at 10X magnification, and high power images were acquired at 40X magnification. (B.) Quantification of (i.) fibronectin and (ii.) lectin staining at 30 DPI. Scale bar = 200 μ m. Data are analyzed by two-way ANOVA with Sidak's post-hoc test. Mean $\pm$ SE (n=3–5 per group). *p < 0.05, **p < 0.01.

Figure 5. Integrin- $\alpha 5$ expression colocalized with sites of cerebrospinal fluid $A\beta_{1-40}$ deposition. (A.) Colocalization of intracisternally injected $A\beta_{1-40}$ with integrin- $\alpha 5$ occurs within the lectin stained BM at low levels in young sham mice, which worsens with aging and DMCAO. Low-power images were acquired at 10X magnification, and high power images were acquired at 40X magnification. **(B.)** Quantification of integrin- $\alpha 5$ staining at 30 DPI. Data are analyzed by two-way ANOVA with Sidak's post-hoc test. Mean $\pm$ SE (n=3–5 per group). Data are analyzed by two-way ANOVA with Sidak's post-hoc test. *p < 0.05, **p < 0.01. Scale bar = 200 μ m.

Figure 6. Fibronectin conjugation altered the perivascular transport of Aβ₁₋₄₀.

(A.) (i.) Experimental design of *in vitro* fibronectin binding assay. (ii.) Binding kinetics of fibronectin and ¹²⁵I-Aβ₁₋₄₀. (B.) Representative images showing increased distribution of fibronectin-Aβ₁₋₄₀ conjugates compared to unconjugated Aβ₁₋₄₀ following intracisternal injection into naïve young mice. (C.) Comparison of (i.) CB-d3 uptake, (ii.) 647-Aβ₁₋₄₀ uptake and (iii.) 647-Aβ₁₋₄₀/CB-d3 ratio. Data are analyzed by Student's t-test. Mean ± SE (n=5–6 per group). *p < 0.05.