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Suppression of glymphatic fluid transport in a mouse model of Alzheimer's disease

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Abstract

Glymphatic transport, defined as cerebrospinal fluid (CSF) peri-arterial inflow into brain, and interstitial fluid (ISF) clearance, is reduced in the aging brain. However, it is unclear whether glymphatic transport affects the distribution of soluble A β in Alzheimer's disease (AD). In wild type mice, we show that A β 40 (fluorescently labeled A β 40 or unlabeled A β 40), was distributed from CSF to brain, via the peri-arterial space, and associated with neurons. In contrast, A β 42 was mostly restricted to the peri-arterial space due mainly to its greater propensity to oligomerize when compared to A β 40. Interestingly, pretreatment with A β 40 in the CSF, but not A β 42, reduced CSF transport into brain. In APP/PS1 mice, a model of AD, with and without extensive amyloid- β deposits, glymphatic transport was reduced, due to the accumulation of toxic A β species, such as soluble oligomers. CSF-derived A β 40 co-localizes with existing endogenous vascular and parenchymal amyloid- β plaques, and thus, may contribute to the progression of both cerebral amyloid angiopathy and parenchymal A β accumulation. Importantly, glymphatic failure preceded significant amyloid- β deposits, and thus, may be an early biomarker of AD. By extension, restoring glymphatic inflow and ISF clearance are potential therapeutic targets to slow the onset and progression of AD.

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Contribution of authors:

WP, MT, BL, YL, HM, SR and TK performed experiments.

WP, MT, BL, HM, EM, TK, SP and FD analyzed data.

MN and RD designed experiments.

RD wrote the paper.

MT, HM, HB and MN provided critical comments and edited the manuscript.

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Graphical abstract

Keywords

Convective ISF flow; astrocytes; AQP4; CSF; brain ISF clearance; lymphatic system; Glymphatic pathways; clearance; amyloid- β ; Alzheimer's disease

Introduction

While the pathogenesis of Alzheimer's disease (AD), the most common neurodegenerative disease, is still unclear, failure to efficiently eliminate potentially toxic molecules from the interstitium of the aging brain may be a contributing factor (Bateman et al., 2006; Deane et al., 2009; Nedergaard, 2013; Zlokovic, 2008a). At present there is no effective therapy to slow or prevent the biological progression of this devastating disease. Thus, there is no available approach to slow or prevent the 'AD tsunami'. However, understanding how the brain clears the interstitium of waste products could lead to the discovery of novel targets to help in delaying the onset of AD.

In peripheral organs, the very permeable capillaries and the lymphatic system facilitate rapid clearance of proteins and metabolic products from the interstitial fluid (ISF) (Michel, 1988). However, it is well established that the CNS vascular barriers restrict trafficking of polar molecules into and out of the brain, and the CNS lacks conventional lymphatic vessels (Abbott et al., 2006; Bradbury, 1985; Zlokovic, 2008b). Despite these features there is rapid clearance of metabolic waste products in the normal brain (Abbott et al., 2006; Bradbury, 1985; Deane et al., 2009; Nedergaard, 2013; Zlokovic, 2008b). The glymphatic fluid transporting pathways act as a 'pseudo-lymphatic system', and have been described as a brain-wide macroscopic system for the rapid clearance of waste from the interstitium (Iliff et al., 2012; Nedergaard, 2013). The glymphatic transport pathways consist of 1) the flow of cerebrospinal fluid (CSF) into brain via the peri-arterial space, as suggested by earlier observations (Rennels et al., 1985), astrocytic specific aquaporin 4 (AQP4)-mediated convective flow of ISF through the interstitium, and 3) clearance of molecules via the perivascular space surrounding the deep cerebral veins, and via the cervical lymph nodes (Boulton et al., 1996; Bradbury and Westrop, 1983; Brinker et al., 1997; Iliff et al., 2012; Kida et al., 1993; Plog et al., 2015; Yamada et al., 1991). AQP4, water channels, are present at high levels at the astrocytic endfeet lining the interfaces between brain tissue and fluid (Haj-Yasein et al., 2011; Mathiisen et al., 2010). Glymphatic transport and waste removal are suppressed with aging (Kress et al., 2014), which may contribute to the accumulation of

amyloid- β (A β) in brain. In the aging brain AQP4 delocalization from the endfeet to the soma of astrocytes is, in part, associated with glymphatic failure. While amyloid- β accumulation is known to cause AQP4 delocalization (Yang et al., 2011), it is unclear whether the glymphatic transport is disrupted in mouse models of AD.

Herein, we show that glymphatic transport is indeed suppressed in old APP/PS1 mice with prominent amyloid- β deposits, but most importantly, we also observe that glymphatic clearance is reduced prior to presence of substantial amyloid- β deposits in younger APP/PS1 mice when compared to age-matched control mice. Thus decline of glymphatic fluid transport may serve as an early biomarker of AD, and treatments directed at restoring normal glymphatic transport and waste removal from the brain may become a novel preventive or therapeutic approach for this dreadful disease.

Materials and Methods

Mice

Mice [of either sex, C57BL/6J, Tg(Cspg4-Ds Red.T1)1Akik/J (NG2-DsRed reporter mice), APP^{swe}/PS1^{dE9} (APP/PS1) and their littermate controls] were purchased from Jackson Laboratory (Bar Harbor, ME, USA). NG2-DsRed reporter mice are on a C57BL/6J genetic background. In mice older than 6–8 weeks, NG2-Ds-red expression is restricted to perivascular smooth muscle cells (arteries) and pericytes (Iliff et al., 2012; Zhu et al., 2008). The APP/PS1 mice are double transgenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695^{swe}) and a mutant human presenilin 1 (PS1-dE9) both directed to CNS neurons. Mice were housed in the vivarium facilities at the University of Rochester, School of Medicine and Dentistry. All animal studies were performed according to the NIH guidelines using protocols approved by the University of Rochester Committee on Animal Resource. Mice were housed depending on the experiment, and maintained on a 12:12 light/dark schedule (6 AM : 6PM) with food and water ad libitum. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection (IP).

Materials

A β 40-HiLyte Fluor 488 and A β 42-HiLyte Fluor 488 were obtained from AnaSpec (Fremont, CA, USA), and prepared as a 0.05% solution in artificial CSF (aCSF) following the manufacturer's instructions. Samples were diluted in aCSF, aliquoted, stored at -20°C and used within two days. HFIP (1, 1, 1, 3, 3, 3-hexafluoro-2-propanol) treated lyophilized A β 40 and A β 42 were also obtained from AnaSpec, dissolved in ammonium hydroxide (1%) at room temperature for 15 minutes with intermittent mixing, sonicated for 5 minutes, diluted in aCSF to 20 μM , aliquoted and stored at -80°C . Each aliquot was used immediately and only once. Cascade blue (CB) labeled dextran 10 kDa (fixable), fluorescein labeled dextran 40 kDa (fixable) and Texas Red labeled dextran 3 kDa (fixable) were obtained from Molecular Probe (Eugene, OR, USA) and dissolved in aCSF (2% solution). Lectin was obtained from Vector Laboratory (*Lycopersicon esculentum* (tomato); Burlingame CA, USA). ^{125}I -A β 40 and ^{14}C -inulin were obtained from PerkinElmer (Waltham, MA, USA). We used ^{125}I -A β 40 without added aprotinin, a potential inhibitor of

LRP1-mediated transport. ELISA kits for A β 40 (KHB 3481) and A β 42 (KHB 3441) were obtained from Invitrogen (Camarilla, CA, USA) and A β oligomer ELISA kit (BEK-2215-1P) from Biosensis (Termecula, CA, USA).

Intracisternal injections

Mice were anesthetized as indicated above, fixed to a stereotactic frame, cisterna magna exposed and cannulated with a 30G needle (Iliff et al., 2012; Kress et al., 2014). Fluorescent tracers (A β 40 or 42 HiLyte 488, 5 μ L of a 10 μ M solution in aCSF, containing CB tagged dextran (10 kDa at 1%) were injected at 1 μ L/min using a Hamilton syringe connected to a Micro Syringe pump controller (Micro 4; World Precision Instruments, Inc., Sarasota, FL, USA). We used 10 μ M A β as this was the concentration used in earlier studies on the effect of A β on cerebrovascular reactivity (Niwa et al., 2001). In a separate series of experiments, unlabeled A β (2 μ M) was also intracisternally injected (5 μ L at 1 μ L/min). To quantify the glymphatic inflow, 125 I-A β 40, at a very low concentration (10 nM, due to the greater resolution of radioactivity analysis), and 14 C-inulin (6 kDa, 1.0 μ Ci) were intracisternally injected at 1 μ L/min for 5 minutes. We used a small volume and a slow rate of injection (5 μ L at 1 μ L/min), which does not significantly increase the intracranial pressure (Yang et al., 2013), thereby, minimizing CSF reflux into the cerebral ventricles from the cisterna magna, as indicated by the lack of injected fluorescent molecules in the ependymal layer (Iliff et al., 2012).

A β diffusion from arteries

A custom ImageJ plugin program was written to analyze A β and CB-dextran transport by convective fluid flow as a function of distance from the vessel wall. In these studies, arteries were identified by DsRed expression in the NG2 DsRed reporter mice, and veins were DsRed-negative. The digital images of photomicrographs were analyzed using ImageJ. The plugin plotted the intensity value of the fluorescence with respect to distance when a line is drawn across the blood vessel identified by the NG2-DsRed. The person analyzing the images and data was blinded to the experimental condition.

Immunohistochemistry

Immunohistochemistry was performed as reported (Rangroo Thrane et al., 2013b; Wang et al., 2012). Mice were transcardially perfused with ice-cold phosphate-buffered saline (PBS, pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) followed by 4% paraformaldehyde (PFA; Sigma-Aldrich). Free floating brain sections (100 μ m) were immunostained for astrocytes (GFAP), AQP4, neurons (NeuN), human A β (6E10) and A β oligomer (A11). The tissue was blocked with 7% donkey serum for 1 hour and the primary antibodies incubated overnight. The primary antibodies were rabbit human specific anti-A β (6E10; 1:200, BioLegend, San Diego, CA, USA), goat anti-aquaporin 4 (1:200, cs9888; Santa Cruz Technology, Inc., Dallas, Texas, USA), mouse anti-GFAP (1:500, MAB360; Millipore, Billerica, MA, USA), chicken anti-NeuN (1:500, Abcam 134014, Abcam Inc., Cambridge, MA, USA), anti-A β oligomer (A11, 5 μ g/ml; Rockland, Limerick, PA, USA). Then, Fluor-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA 1:500) were added and incubated for 2 hours at room temperature (RT). Immunofluorescence was visualized using an epifluorescence microscope (Olympus BX51) with CellSens software, and a Bio-Rad

MRC500 confocal scanning head attached to an inverted microscope (IX81, Olympus, Tokyo, Japan) controlled by Olympus Fluoview 500 software. The person analyzing the images and data was blinded to the experimental conditions.

Effect of A β on glymphatic CSF inflow into brain

We used 10 μ M A β 40 or A β 42 as reported in brain superfusion studies (Niwa et al., 2001). In the glymphatic distribution of A β experiments (first series), 5 μ L of aCSF, containing A β (10 μ M) and dextran (1%; fluorescein 40 kDa and Texas Red, 3 kDa, both lysine fixable; Molecular Probe), were injected, intracisternally, at 1 μ L/min and after 30 minutes the brain fixed by PFA perfusion and 100 μ m brain sections analyzed. In a second series of experiments, the A β (5 μ L of the 10 μ M solution) was pre-infused over 40 minutes prior to the infusion of the A β and fluorescence dextran solution, as above. The degree of tracer permeation throughout the brain was evaluated ex vivo by an epifluorescence microscopy. Multichannel whole-slice montages were acquired via the CellSens software (version 1.12, Olympus CellSens Software) in the green and red emission channels, at a magnification of 4x. Exposure and gain were chosen based on the control brain slices and fixed for all experimental groups. To quantify the extent of tracer permeation throughout the brain the whole-slice montages were analyzed using ImageJ (National Institutes of Health, imagej.nih.gov/ij/). For each slice, fluorescence emission channels were split and a whole-brain ROI was defined. The pixel area of positive fluorescence (threshold pixel intensity >80 A.U) for both green and red channel) was calculated for 6 slices from each brain and the average expressed as a percentage of the brain. The person analyzing the images and data was blinded to the experimental condition.

Dot blot assay

A β 40 and A β 42 samples were prepared as described above, and diluted to 10 μ M. Each sample was separated into two: one before centrifugation (before) and the other for centrifugation (after). For centrifugation, samples were spun at 100,000 g at 4°C for 1 hour to separate fibrils. Supernatants and the un-centrifuged samples were blotted onto nitrocellulose membranes (Bio-rad; 10 μ L per dot). The membranes were allowed to dry for 1 hour at RT and then blocked in 5% nonfat dry milk, containing 50 mM Tris-HCl, 0.5 M NaCl, 0.05% and Tween-20, at pH 7.4 (TBS-T) for 1 hour at RT. The membranes were then incubated with one of the following primary antibodies: mouse anti-amyloid- β 1–16 from clone 6E10 (BioLegend, 830003, 1:1000), rabbit anti-amyloid- β oligomers (A11, Rockland, 200-401-E88, 1:1000) or rabbit anti-amyloid- β fibrils (clone M87, Millipore, MABN638, 0.1 μ g/mL) for 1 h at RT in TBS-T. Membranes were then washed three times for 10 min each in TBS-T prior to incubating with either a goat anti-mouse horseradish peroxidase (HRP; Santa Cruz, sc-2031, 1:300)- or goat anti-rabbit HRP- conjugated (Santa Cruz, sc-2030, 1:300) secondary antibody for 1 h at RT. After washing, the blot was developed using Western Lightning Plus-ECL (Perkin Elmer) for 2–5 min and the film was exposed for 30 seconds. Dots were scanned and analyzed with the Dot Blot Analyzer plugin for ImageJ (NIH). The percent of A β species in each sample was estimated (since different antibodies were used for each amyloid- β form) by using the following formula: Total A β (6E10) = monomeric A β + oligomeric A β (A11) + fibrillar A β (M87).

Brain clearance

To quantify solute inflow into brain from CSF and clearance from the brain, radio-labeled tracers were used since the data generated for A β clearance were similar using radio-labeled molecules and by using ELISA (Bell et al., 2007). ^{125}I -A β 40 (10 nM) and ^{14}C -inulin (6 kDa, 1.0 μCi) were stereotactically microinjected into the right frontal cortex, as we recently reported (Xie et al., 2013). Briefly, a stainless steel guide cannula (Plastic One, Roanoke, VA, USA) was implanted stereotactically into the left frontal cortex of anesthetized mice (2% isoflurane) with the coordinates of the cannula tip at 1.0 mm anterior and 3.5 mm lateral to the bregma, and 1.5 mm below the surface of the brain. Animals were allowed to recover after surgery and the experiments performed 18–24 hrs after the guide tube cannulation, as reported (Deane et al., 2004). In each mouse, a small volume of aCSF (0.5 μL), containing ^{125}I -A β 40 (10 nM) and ^{14}C -inulin (1.0 μCi), was simultaneously injected (33 GA cannula, Plastic One) into the brain ISF over 5 minutes. At the end of the experiments (30 min) the brain was removed and prepared for radioactivity analysis. Samples were first counted for gamma radioactivity (^{125}I -A β 40) using a Wizard Automatic Gamma Counter (Wallac 1471) and counts corrected for trichloroacetic acid (TCA) precipitability. All samples were solubilized in 0.5 ml tissue solubilizer (Solvable, Perkin Elmer) overnight followed by the addition of 5 ml of scintillation cocktail (Ultima Gold, PerkinElmer). Samples were then analyzed in a liquid scintillation counter for ^{14}C - radioactivity (LS6500 Multi-purpose Scintillation Counter (Beckman Coulter, GA, USA)). *Calculations:* The percentage of radioactivity remaining in the brain (% recovery) after microinjection was determined as % recovery in brain = $100 \times (N_b/N_i)$, where, N_b is the radioactivity remaining in the brain at the end of the experiment and N_i is the radioactivity injected into the brain ISF, i.e., counts per minute (cpm) for ^{125}I - (TCA-precipitable) and the disintegration per minute (dpm) for ^{14}C -. The TCA precipitable ^{125}I -A β 40 in brain was unchanged compared to the injectate (Deane et al., 2008; Deane et al., 2004). Total clearance was determined as 100-% recovery. ^{14}C -inulin was used as an inert polar molecule which is neither transported across the BBB nor retained by the brain, and thus, its clearance provides a measure of the ISF bulk flow only.

Lectin administration

Lectin (*Lycopersicon esculentum* (tomato); Vector Laboratory Burlingame CA, USA) was administered during the cardio-perfusion stage of the experiment. The mice were cardio-perfused with cold PBS containing the lectin (0.02 mg/ml) at 2 ml/min for 10 min. This was then followed by perfusion with PFA (4% in PBS), as described above.

Methoxy-X04 administration

Methoxy-X04 (Tocris Bioscience, Bristol, UK) was administered intraperitoneally (IP; 10 mg/kg) and after 60 minutes A β 40 was intracisternally injected. The brain was analyzed after an addition 30 minutes.

Brain extraction

Frozen cerebral cortex (150 mg) was homogenized in ice-cold TBS buffer (1 ml; 20 mM Tris-HCl, 150 mM NaCl, pH 7.4), containing complete protease inhibitor (Roche Applied

Sciences), and centrifuged at 16,000 g for 30 minutes at 4°C. The supernatant was used to determine levels of soluble A β . This pellet was immediately re-suspended in 5 M guanidine-HCl, sonicated, mixed for 3 hrs, centrifuged at 10,000 g for 30 minutes at 4°C and the supernatant collected and used to determine levels of insoluble A β . For soluble A β oligomers, frozen cerebral cortex was homogenized in TBS containing 1% Triton-X and complete protease inhibitor and centrifuged at 16,000 g for 30 minutes at 4°C. The supernatant was used to determine levels of soluble A β oligomers.

A β ELISA

Levels (pmol/g brain tissue) of human A β 40, A β 42 and soluble A β oligomers were determined using ELISA kits [A β 40 (KHB 3481) and A β 42 (KHB 3441); Invitrogen (Camarilla, CA, USA)] and following the manufacturers' instructions. Levels of A β were determined from the standard curves. Since soluble A β oligomers vary in size and number of oligomers the unit is arbitrary and is indicated as equivalent to A β . Brains from wild type mice were used as controls and the signal subtracted from that obtained from the APP/PS1 mice.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by post hoc Tukey test, or Student's t test. The differences were considered to be significant at $p < 0.05$. All values were expressed as mean $\pm$ SEM.

Results

Earlier reports have shown that intracisternal injection of tagged molecules, such as inulin, dextran and ovalbumin, enter the brain via the peri-arterial space and are cleared by convective driven glymphatic transport through the interstitium (Iliff et al., 2012; Kress et al., 2014). However, it is unclear whether glymphatic transport characteristics of A β 40 and A β 42 are similar. This is important to establish because, in contrast to A β 40, A β 42 is a CSF biomarker of AD and a toxic molecule (Blennow, 2004; Fagan et al., 2006; Fagan et al., 2014; Zetterberg et al., 2007). We simultaneously injected, intracisternally, A β 40 HiLyte Fluor 488 (A β 40-488) or A β 42 HiLyte Fluor 488 (A β 42-488) and an inert reference molecule, labeled dextran 10 kDa (CB-dextran), and mapped their distribution in brain sections (Figure 1A). NG2-dsRed reporter mice were used to clearly identify arteries, as reported (Iliff et al., 2012). Interestingly, the distribution of CSF-derived A β 42-488 within brain was mostly confined to the arteries when compared to A β 40-488, while CB-dextran distribution was similar in both experiments (Figure 1B–J). The diffusion of A β 40 from the brain surface and glymphatic transport along the penetrating arteries into brain was more efficient than A β 42 while CD-dextran transport was similar in both A β 40 and A β 42 experiments (Figure 1B–C). Accordingly, the CB-dextran distribution areas were grouped together and compared to that of A β 40 and A β 42 (Figure 1E–F). While A β 40-488, A β 42-488 and CB-dextran were all present along the penetrating arteries (Figure 1G–H), glymphatic transport of A β 42 within the parenchyma was restricted (Figure 1I–J). When compared to A β 40, the reduced A β 42 glymphatic inflow may reflect its greater propensity to oligomerize into larger fibrils (Supplementary Figure 1), which would reduce A β 42

inflow, since molecular weight is known to limit the brain-wide glymphatic transport (Iliff et al., 2012). In addition, A β /receptor interactions may also interfere with A β glymphatic transport compared to the inert reference dextran molecule. To confirm that the Alexa 488 tagging of A β was not altering the glymphatic transport of A β , we injected untagged A β 40 or A β 42 (2 μ M), intracisternally, and mapped its distribution pattern by immunolabeling with a human specific anti-A β antibody (6E10), which does not react with mouse A β (Supplementary Figure 2). In this setting, A β 40 and A β 42 were also present along the penetrating arteries, within the parenchyma, and associate with brain cells (A β 40>A β 42), such as neurons (Figure 2 A–P). Again, A β 42 distribution was almost totally restricted to the penetrating artery when compared to A β 40 (Figure 2 E/H and M/P), similar to that shown in Figure 1 H–J). The association of CSF-derived A β 40 with cells within the parenchyma may reflect their expression of A β receptors (Deane et al., 2004; Herz, 2009; Holtzman et al., 2012; Kanekiyo et al., 2012).

Topical application of A β (μ M range) is known to reduce cerebral blood flow (CBF) (Niwa et al., 2001), and this could alter glymphatic peri-arterial inflow of CSF. However, in our studies neither A β 40 nor A β 42 (10 μ M; 5 μ L) appeared to affect glymphatic transport of dextran into brain (Figure 3 A–F). Nevertheless, we tested the effect of A β pre-infusion, as reported (Niwa et al., 2001), on glymphatic transport (Figure 3G). For these experiments, A β was pre-infused for 40 minutes, and A β 40, but not A β 42, reduced the glymphatic transport of the dextran (3 kDa and 40 kDa) into brain (Figure 3 H–L). Specifically, A β 40 reduced CSF inflow into the cortex but not into the hippocampus due, perhaps, to the low but detectable levels present in this region (Supplementary Figure 3). Collectively, our data show that soluble A β in CSF enters brain via the penetrating arteries and, thus, may contribute to the accumulation and toxicity of amyloid- β , in the long-term. We evaluated glymphatic transport in the APP/PS1 mice with and without prominent amyloid- β deposits in comparison to control littermates.

We quantified the glymphatic inflow of A β and inulin from CSF into brain using a radiolabeled approach, which allows the use of tracer levels of A β (Deane et al., 2012; Iliff et al., 2012; Xie et al., 2013; Zlokovic, 2008a). Artificial CSF, containing 125 I-A β 40 (monomeric, 10 nM) (Deane et al., 2004) and 14 C-inulin (1.0 μ Ci; the reference molecule), was injected intracisternally and after 30 minutes the brain collected and analyzed for radioactivity. First, the data showed that the inflow of A β and inulin was reduced with aging, as reported for other molecules (Kress et al., 2014). Second, inflow of A β 40 and inulin was reduced in the APP/PS1 mice when compared to their controls but more so in the mice with prominent amyloid- β deposits i.e., old mice (2-fold) compared to young mice (1.5-fold; Figure 4A–C). We also quantified A β clearance from the frontal cortex using the same radiolabeled approach (Deane et al., 2012; Iliff et al., 2012; Kress et al., 2014; Singh et al., 2013; Xie et al., 2013). CSF (0.5 μ L) containing 125 I-A β 40 (monomeric, 10 nM) and 14 C-inulin (1.0 μ Ci) was microinjected into the frontal cortex and after 30 minutes the brains were collected for analysis of the remaining radioactivity (Figure 4D). While the clearance of both A β and inulin was reduced in the APP/PS1 mice when compared to their controls, the effect was greater for inulin (2-fold) compared to A β (1.2-fold; Figure 4E–F), suggesting that reduced ISF convective flow may promote greater A β /receptor interactions, due to slower transit time, and possibly influencing its clearance. A β clearance from brain is

due to receptor mediated transport at the blood-brain barrier (BBB) and bulk flow of ISF, which are reduced with aging (Banks et al., 2003; Deane et al., 2004; Jaeger et al., 2009; Kress et al., 2014; Zlokovic, 2008a). Since CSF-derived A β may interact with the amyloid- β deposits, A β 40-488 was injected intracisternally and its association with endogenous amyloid- β mapped in the older APP/PS1 mice (Figure 5). A clear interaction between the CSF-derived A β 40 and the endogenous A β plaques within the parenchyma and vasculature was observed (Figure 5A–H), suggesting that A β 40 adhering to existing amyloid- β plaques may promote further amyloid- β accumulation. Collectively, by extension, the data show that glymphatic activities are disrupted in AD, and before the presence of significant amyloid- β deposits as seen in the younger APP/PS1 mice compared to their controls (Figure 4).

It was shown that amyloid- β accumulation is linked to the loss of AQP4 polarization at the astrocytic endfeet in the TgArcSwe mouse model of AD (Yang et al., 2011). Our recent studies also found that the loss of AQP4 polarization at the endfeet processes of reactive astrocytes suppresses glymphatic transport (Iliff et al., 2014; Kress et al., 2014). Here, we show that astrocytes were in a reactive state in the vicinity of amyloid- β deposits in APP/PS1 mice (Supplementary Figure 4A–F). This condition is associated with loss of AQP4 polarity and suppression of glymphatic transport (Iliff et al., 2014; Kress et al., 2014). Interestingly, soluble A β oligomers, a more toxic form of A β than amyloid- β plaques (Selkoe, 2001), were also observed in the vicinity of AQP4/GFAP-positive astrocytes along vessels but not in the parenchyma (Supplementary Figure 5A–H). Since A β species may disrupt glymphatic transport their levels were also determined. Compared to the younger mice, the levels of soluble and insoluble A β 40 and A β 42 were increased by about 2-fold in the old mice (Figure 6A–D), while the levels of soluble A β oligomers were considerably increased (6-fold) in the older mice (Figure 6E), as reported (Bruggink et al., 2013). Thus soluble A β oligomers may also contribute to the progressive failure in glymphatic transport in the aging brain. Figure 7 shows a schematic diagram of our working model of glymphatic fluid of A β in AD brains.

Discussion

A β is released during neuronal activity and is known to accumulate in the aging brain due to inefficient clearance from the ISF in animals and in human subjects (Banks et al., 2003; Cirrito et al., 2005b; Deane et al., 2009; Deane et al., 2004; Kress et al., 2014; Mawuenyega et al., 2010; Shibata et al., 2000; Zlokovic, 2008a). While accumulation of amyloid- β as extracellular deposit is one of the hallmarks of AD, the role of amyloid- β in the pathogenesis of AD is still unclear since amyloid- β is also present in the brain of non-AD subjects (Herrup, 2015; Musiek and Holtzman, 2015). Nevertheless, it is undisputed that continuous removal of A β from the aging brain is important to prevent the accumulation of A β neurotoxins.

It is believed that A β in CSF is derived mainly from brain (Fagan and Holtzman, 2010). Thus, CSF levels of A β 42 are used as a biomarker of AD, which is reduced as AD progresses presumably due to its accumulation within brain (Blennow, 2004; Fagan et al., 2014; Zetterberg et al., 2007). However, the fate of CSF A β is unclear. The ultimate drainage of CSF into blood facilitates the clearance of soluble molecules, including A β (Abbott et al.,

2006; Bradbury, 1985; Iliff et al., 2012; Johanson et al., 2008; Pollay, 2010). However, our data demonstrate that CSF may serve as a medium for the delivery of A β to brain via the glymphatic inflow pathway, the peri-arterial space, in the same direction as blood flow, as reported for other solutes, such as dextran and ovalbumin (Iliff et al., 2012), lipophilic molecules (Rangroo Thrane et al., 2013a), tau (Iliff et al., 2014) and apoE (Deane et al., 2015). Earlier reports suggested that A β transport along the peri-vascular space occurs in the opposite direction to blood flow (Hawkes et al., 2011; Rolyan et al., 2011; Thal et al., 2007; Weller et al., 1992). While it is possible that A β trafficking in the perivascular space may be bidirectional, more investigation will be needed to fully clarify this. We speculate, that circulation of A β via the glymphatic fluid transporting system may contribute to the formation of amyloid- β plaques in the long-term, particularly to cerebral amyloid angiopathy (CAA) and ultimately to lower CSF A β 42 levels observed in progressing AD. Intriguingly, the greater inflow of A β 40 compared to A β 42 along the arteries could also explain the A β 40 and A β 42 composition (A β 40>A β 42) in cerebral amyloid CAA (Kumar-Singh, 2008; Van Dorpe et al., 2000). The concentration of A β 40 in CSF is greater than that of A β 42 in both normal and AD subjects with an A β 42/A β 40 ratio of 0.3 and 0.17, respectively (Oe et al., 2006; Schoonenboom et al., 2005). Since glymphatic fluid transport from CSF into brain depends on bulk flow the amount of A β entering brain would reflect the concentration of soluble A β species in CSF.

Interestingly, the differences in glymphatic distribution of A β in brain (A β 40>A β 42) corroborate earlier reports on their respective ISF clearance pattern, which demonstrated that A β 40 elimination is greater than that of A β 42 (Banks et al., 2003; Deane et al., 2004). The restricted distribution of A β within brain when compared to dextran (cascade blue) may reflect A β /A β receptor interactions, which promote cellular interaction, retention within brain or clearance (Deane et al., 2004; Kanekiyo and Bu, 2014; Kanekiyo et al., 2013). Multiple cell types (e.g., vascular smooth muscle cells, endothelial cells, astrocytes, microglia and neurons) express several types of A β receptors, such as low-density lipoprotein receptor-related protein-1 (LRP1), receptor for advanced glycation end products (RAGE) and α 7 nicotinic acetylcholine receptor (Clifford et al., 2008; Deane et al., 2005; Kanekiyo and Bu, 2014; Kanekiyo et al., 2013). As A β flows along the perivascular space, capillaries and veins interaction with LRP1, for example, may lead to cellular uptake and transcytosis into blood (Cirrito et al., 2005a; Deane et al., 2005; Deane et al., 2004; Kanekiyo et al., 2012). A β 40 clearance is rapid since 60% of the injected dose is eliminated within 30 minutes (Deane et al., 2004; Xie et al., 2013). This may explain the low levels of A β 40 present in brain after intracisternal injection. Other cells within the brain parenchyma also express receptors, which could interact with A β and promote uptake and retention (Kanekiyo and Bu, 2014; Kanekiyo et al., 2013). The unexpected reduced levels of A β 42 entering brain from the CSF is likely due to higher levels of fibrils since its more prone to oligomerization when compared to A β 40 (Selkoe, 2001). Thus, the lower levels of monomeric A β 42 compared to A β 40 may explain its reduced brain-wide distribution. Earlier studies have documented that molecular weight inversely controls glymphatic transport (Iliff et al., 2012). Interestingly, pre-treatment with A β 40, but not A β 42, caused suppression of glymphatic CSF inflow, which may contribute to the long-term effect of A β on the suppression of glymphatic transport with aging (Kress et al., 2014) and in AD. A β 40

in CSF or blood reduces CBF by inducing vasoconstriction (Niwa et al., 2001; Paris et al., 2000; Suo et al., 1998) due to interaction with receptors, such as RAGE (Deane et al., 2003), generation of free radicals (Niwa et al., 2001) and proinflammatory molecules (Paris et al., 2000). Therefore, the increase in A β 40 levels prior to substantial amyloid- β deposits may contribute to the reduction of glymphatic fluid transport. Thus, the distribution of CSF-derived A β into brain may depend on their species and on the duration of A β 40 exposure.

The interaction of CSF-derived A β with existing amyloid- β deposits would also prevent efficient A β clearance by promoting further oligomerization and the formation of toxic A β species. The oligomerization of A β is a progressive process in which the monomeric A β (A β 42>>A β 40) oligomerize into various soluble oligomers (low but varying molecular weights), which eventually progress to fibrils and plaques (Selkoe, 2001). The soluble oligomers are believed to be the more toxic A β species since they correlate better with neurotoxicity in vivo, and are more toxic than fibrils to neurons in culture (Kayed et al., 2010; Kaye et al., 2003; Lue et al., 1999; McLean et al., 1999; Walsh et al., 2002). A β 42 present in brain ISF may, accordingly, result in formation of more neurotoxic A β oligomers (Selkoe, 2001). While many A β species may cause glymphatic dysfunction (i.e., CSF inflow and ISF clearance) in the aging brain, our data suggest that the soluble A β oligomers, which also co-localized with AQP4/GFAP-positive astrocytes, may contribute to this process by, possibly, causing delocalization of AQP4 from the endfeet to the soma of astrocytes. In addition, amyloid- β deposits markedly suppressed ISF clearance of inulin. Since the size of the interstitial space may influence ISF convective flow (Xie et al., 2013), extracellular amyloid- β aggregation may impede this solute transport due to reduced extracellular volume and/or increased tortuosity.

Recently, it was shown that glymphatic activity is profoundly suppressed in the aging brain of wild type mice mainly due to delocalization of AQP4 from the endfeet processes of astrocytes (Kress et al., 2014). AQP4, the predominant water channel in brain, is selective expressed in astrocytic endfeet (10-fold greater than that in the rest of the astrocyte (Yang et al., 2011). Earlier studies have shown that this polarized distribution of AQP4 is altered in ArcSwe (Arctic (E693G) and Swedish (K670N, M671L) mutations) mouse model of AD (Yang et al., 2011). In these mice there was less AQP4 at the endfeet that were associated with amyloid- β plaques. Interestingly, *Aqp4* gene deletion reduced A β 42-induced astrocyte activation, in vitro (Yang et al., 2012). Recently, it was shown that deletion of *Aqp4* in the APP/PS1 mice exacerbates A β accumulation in brain and contributes to memory deficits (Xu et al., 2015). Our data are congruent with these studies, but also indicated that soluble A β oligomers, a more toxic form of A β , may contribute to the suppression of glymphatic activities in aging APP/PS1 mice.

The implications of these findings are that reduced glymphatic inflow into the aging brain could restrict distribution of CSF to brain, reduce ISF convective flow and thus, clearance of A β and other neurotoxic waste products. The reduced flow of CSF along the penetrating arteries may lead to the development of CAA due to the slower transit time that promotes greater cellular binding and uptake. In addition, reduced ISF clearance would exacerbate the oligomerization and accumulation of brain A β , and consequently its toxicity due the generation of soluble oligomers. Reduced CSF inflow into brain and impeded downstream

ISF clearance appear to be critical stages, whereby the CSF-derived A β aggravates vascular and parenchymal A β accumulation and the generation of toxic species. Thus, therapeutic targets that maintain glymphatic function and the continuous removal of A β monomeric molecules from the aging CNS hold promise for preventing or delaying the accumulation of potentially neurotoxic molecules. In addition, since glymphatic activity is reduced before significant amyloid- β deposits this could be an important early indicator of the disease.

Conclusions

We show for the first time that 1) glymphatic transport, i.e., CSF inflow into brain and ISF clearance, is suppressed in a mouse model of AD (APP/PS1) 2) glymphatic transport is suppressed prior to significant accumulation of amyloid- β , 3) both A β 40 and A β 42 (a CSF biomarker of AD), circulate by glymphatic pathways but oligomerization may limit their distribution, especially for A β 42, due to increasing molecular size, 4) CSF-derived A β is taken up by cells within the parenchyma 5) CSF-derived A β 40 delivered to the parenchyma via the glymphatic transport co-localized with amyloid- β plaque, suggesting that this source of A β 40 may also contribute to plaque formation, 6) soluble A β oligomers, a more toxic forms of A β , may also contribute to glymphatic dysfunction, and 7) long-term exposure to A β 40 in the subarachnoid CSF may suppress glymphatic transport prior to the presence of substantial amyloid- β deposits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Glymphatic transport is suppressed prior to significant accumulation of amyloid- β .
2. CSF A β 40 and A β 42 circulate through the brain parenchyma.
3. CSF-derived A β is taken up by cells within the parenchyma, e.g., neurons,
4. CSF-derived A β may contribute to amyloid- β plaque formation.
5. Long-term exposure to A β 40 in CSF suppresses glymphatic transport.

Figure 1. Fluorescently-tagged A β 40 and A β 42 enter brain along the peri-arterial space and the pial membrane after intracisternal delivery

A) Schematic diagram showing the injection site. Representative images of A β 40 and A β 42 HiLyte Alexa 488 (**B**); cascade blue labeled dextran (CD-Dextran, a reference molecule; 10 kDa; **C**) and NG2-DsRed labeling of smooth muscle cells (**D**). **E**) Quantification of the areas of A β 40, A β 42 and CB-Dextran distribution. **F**) Standardization of A β distribution area as a percentage of CB-Dextran area. Peri-arterial inflow of CB-dextran and A β (**G–J**). Panel **H** shows the white boxed areas in **G**. White arrow head in panel **H** indicate the vessel magnified in panel **I**. **J**) Distribution of A β 40 or A β 42 (green) and CB-Dextran (blue) from an artery (red) shown in panel **I**. Vessel lumen in gray. Values are mean $\pm$ SEM, N=5. Scale bars: **B**, 2 mm; **H**, 500 μ m; **I**, 100 μ m. A β 40-488 or A β 42-488 and CD-dextran were intracisternally injected in NG2-DsRed reporter mice (2–3 months old) and the animals fixed with PFA 15 minutes later followed by preparation of vibratome sectioning and analysis of tracer distribution in brain sections.

Figure 2. Unlabeled human A β binds to cells after intracisternal delivery

A–D). Immunolabeling of CSF injected A β 40 in brain (**A**), GFAP⁺-astrocytes (**B**), NeuN⁺-neurons (**C**) and merged images (**D**). **E–H)** Boxed area in panel A showing A β 40 association with cells, mainly neurons (white arrows). **I–L)** Immunolabeling of A β 42 (**I**), GFAP⁺-astrocytes (**J**), NeuN⁺-neurons (**K**) and merged images (**L**). **M–P)** Boxed area in panel I showing CSF A β 42 mainly restricted to vessels. Representative images from 3 wild type mice (2–3 months old). Scale bars: A and I, 0.8 mm; E and M, 25 μ m. A β 40 or A β 42 was intracisternally injected and after 30 minutes, to allow for cellular uptake, the brains were fixed and analyzed by immunolabeling.

Figure 3. Effect of Aβ on glymphatic inflow of CSF into brain

A) Schematic diagram of the experimental design. **B)** Representative images of vehicle (Veh) treated mice and mice treated with Aβ40 (**C**) and Aβ42 (**D**). Quantification of the distribution areas of Texas Red Dextran 3 kDa (**E**) and FITC-Dextran 40 kDa (**F**) from images as in panels B–D. CSF containing Aβ40 or Aβ42 (10 μM; 5 μL), FITC-Dextran 40 kDa (1%) and Texas Red Dextran 3 kDa (1%) were intracisternally injected and after 30 min the PFA fixed brain sections were analyzed. Values are mean ± SEM, N=5–6 mice per group. **G)** Schematic diagram of the experimental design. Representative images of vehicle treated mice (**H**) and mice treated with Aβ40 (**I**) and Aβ42 (**J**). Quantification of the distribution areas of Texas Red Dextran 3 kDa (**K**) and FITC-Dextran 40 kDa (**L**). First, 5 μL

of CSF containing A β 40 or A β 42 (10 μ M) was pre-infused intracisternally over 40 min, followed by CSF containing A β 40 or A β 42 (10 μ M), FITC-Dextran40 kDa and Texas Red Dextran 3 kDa (1%), and the PFA fixed brain sections were analyzed. Values are mean $\pm$ SEM, N=6–9 mice (2–3 months old) per group.

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Figure 4. Reduced CSF inflow and ISF clearance in APP/PS1 mice

A) Schematic diagram showing the injection site of the radiolabeled molecules. **B–C)** Inflow of $^{125}\text{I-A}\beta$ and $^{14}\text{C-inulin}$ from CSF into brain in young (3–4 months old; **B**) and old (**C**; 12–13 months old) APP/PS1 mice (APP) compared to their controls (C). $^{125}\text{I-A}\beta$ (10 nM) and $^{14}\text{C-inulin}$ were injected intracisternally (1 $\mu\text{L}/\text{min}$ for 5 min) and after 30 minutes the brain analyzed for radioactivity. $N=4$. **D)** Schematic diagram showing the cortical injection site. **E–F)** Clearance of $^{125}\text{I-A}\beta$ and $^{14}\text{C-inulin}$ in young (**E**; 6–8 months old) and old (**F**; 12–18 months old) APP/PS1 mice compared to their controls. $^{125}\text{I-A}\beta$ (10 nM) and $^{14}\text{C-inulin}$ were microinjected intracortically (0.5 μL ; 0.1 $\mu\text{L}/\text{min}$ for 5 min) and after 30 minutes the brain analyzed for radioactivity. $N=3-6$. Values are mean $\pm$ SEM.

Figure 5. Intracisternal injected fluorescently labeled A β 40 is associated with amyloid- β plaques in APP/PS1 mice

A–H) Endogenous A β detected with methoxy-X04 (**A, E**), cerebrovasculature delineated with intravascular injected lectin (**B,F**), CSF A β 40-488 is present in brain (colored red) (**C,G**) and merged images (**D,H**) showing co-localization of the intracisternal injected A β 40 with endogenous amyloid- β (white areas) in brain parenchyma and vessels. Representative images from 3 mice (7–9 months old). White areas show the co-localization of methoxy-X04 labeled endogenous amyloid- β plaques and injected A β 40-488. Scale bars: A and E= 50 μ m. Methoxy-X04 was administered intraperitoneally (IP; 10 mg/kg) and after 60 minutes A β 40 was intracisternally injected, and the brain analyzed after an addition 30 minutes.

Figure 6. Levels of Aβ40, Aβ42 and oligomers are increased with age in the APP/PS1 mice
Levels of soluble Aβ40 (A) and Aβ42 (B), insoluble Aβ40 (C) and Aβ42 (D) and soluble Aβ oligomers (E) in cortical brain tissues of young (3–4 months old) and old (12–13 months old) mice. N=4–6 mice per group. Values are mean ± SEM.

Figure 7. Working model for the glymphatic distribution of Aβ

Aβ from the CSF enters brain (Aβ40>Aβ42) via the peri-arterial space and associated with parenchymal cells and amyloid β deposits. Aβ42 is mainly confined to the peri-arterial space due to, possibly, to the formation of fibrils, which is larger in size.