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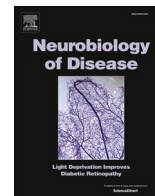
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## Mural cell dysfunction leads to altered cerebrovascular tau uptake following repetitive head trauma

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### ABSTRACT

A pathological characteristic of repetitive traumatic brain injury (TBI) is the deposition of hyperphosphorylated and aggregated tau species in the brain and increased levels of extracellular monomeric tau are believed to play a role in the pathogenesis of neurodegenerative tauopathies. The pathways by which extracellular tau is eliminated from the brain, however, remains elusive. The purpose of this study was to examine tau uptake by cerebrovascular cells and the effect of TBI on these processes. We found monomeric tau interacts with brain vascular mural cells (pericytes and smooth muscle cells) to a greater extent than other cerebrovascular cells, indicating mural cells may contribute to the elimination of extracellular tau, as previously described for other solutes such as beta-amyloid. Consistent with other neurodegenerative disorders, we observed a progressive decline in cerebrovascular mural cell markers up to 12 months post-injury in a mouse model of repetitive mild TBI (r-mTBI) and human TBI brain specimens, when compared to control. These changes appear to reflect mural cell degeneration and not cellular loss as no difference in the mural cell population was observed between r-mTBI and r-sham animals as determined through flow cytometry. Moreover, freshly isolated r-mTBI cerebrovessels showed reduced tau uptake at 6 and 12 months post-injury compared to r-sham animals, which may be the result of diminished cerebrovascular endocytosis, as caveolin-1 levels were significantly decreased in mouse r-mTBI and human TBI cerebrovessels compared to their respective controls. Further emphasizing the interaction between mural cells and tau, similar reductions in mural cell markers, tau uptake, and caveolin-1 were observed in cerebrovessels from transgenic mural cell-depleted animals. In conclusion, our studies indicate repeated injuries to the brain causes chronic mural cell degeneration, reducing the caveolar-mediated uptake of tau by these cells. Alterations in tau uptake by vascular mural cells may contribute to tau deposition in the brain following head trauma and could represent a novel therapeutic target for TBI or other neurodegenerative disorders.

### 1. Introduction

Traumatic brain injury (TBI) is the result of a sudden trauma to the brain that significantly disrupts brain function (Blyth and Bazarian, 2010). One of the long-term consequences of repetitive head trauma is the accumulation of hyperphosphorylated tau (Stern et al., 2011) and the presence of tau-reactive neurofibrillary tangles (Blaylock and Maroon, 2011) and neuropil threads (Omalu et al., 2011). Elevated levels of phosphorylated tau have also been observed in various animal

models of TBI by our group (Ojo et al., 2013; Ojo et al., 2016) and others (Goldstein et al., 2012; Huber et al., 2013; Petraglia et al., 2014; Zhang et al., 2014). Tau is a cytoplasmic protein which is believed to be restricted to the intracellular compartment of neurons, except when released from dead or degenerating cells. However, more recent work has shown that tau is constitutively released into the extracellular environment under normal conditions (Chai et al., 2012; Karch et al., 2012), indicating tau secretion may be a common biological function (Medina and Avila, 2014). Furthermore, several studies have indicated

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tau pathology is propagated through extracellular tau spreading and it is believed that increased levels of monomeric tau in the extracellular environment play a major role in the pathogenesis of neurodegenerative tauopathies (Le et al., 2012; Michel et al., 2013). In fact, tau elevations in the brain extracellular space can be used to predict adverse clinical outcomes following TBI (Magnoni et al., 2012; Ost et al., 2006).

Despite the prevalence and importance of extracellular tau in normal and disease conditions, there is little understanding of how extracellular tau is processed and eliminated from the brain. A study recently demonstrated that extracellular tau is eliminated from the brain through paravascular pathways (Iliff et al., 2014). A common paravascular pathway for removing solutes from the brain involves uptake and degradation by brain vascular mural cells (pericytes and smooth muscle cells). Prior studies have shown mural cells contribute to the elimination of beta-amyloid (A $\beta$ ) from the brain, (Alcendor, 2020; Kanekiyo et al., 2012; Kirabali et al., 2019; Ma et al., 2018), which accumulates in the brains of Alzheimer's disease (AD) patients (and to a lesser extent in TBI subjects) (Tsitsopoulos and Marklund, 2013). Studying the role of brain vascular mural cells in tau uptake may be particularly relevant in head trauma as a prominent feature of TBI is the presence of perivascular tau tangles (Stern et al., 2011; McKee et al., 2009). Moreover, recent work found that paravascular tau clearance was reduced by approximately 60% following TBI and was associated with phospho-tau pathology and neurodegeneration (Iliff et al., 2014).

Mural cell loss in the brain vasculature is a common feature of many neurodegenerative disorders including AD (Bourassa et al., 2020; Sagare et al., 2013; Sengillo et al., 2013) and Amyotrophic Lateral Sclerosis (ALS) (Winkler et al., 2013), with more modest reductions also occurring in the course of normal aging (Bell et al., 2010). With respect to TBI, pericyte loss has been observed acutely following a single controlled cortical impact (Choi et al., 2016; Zehendner et al., 2015) and, more recently, several pericyte markers were found to be significantly reduced after fluid percussion injury in mice (Bhowmick et al., 2019), though more chronic time points have yet to be examined. The degeneration of mural cells in the brain may explain the progressive solute accumulation that is prevalent in neurodegenerative disorders (e.g., A $\beta$  in AD, transactive response DNA binding protein 43 (TDP43) in ALS, and pathologic tau in repetitive TBI). Furthermore, as changes in brain caveolin-1 levels have recently been shown to correlate with tau disposition in the brain (Bonds et al., 2019; Head et al., 2010), we examined caveolin-1 expression alongside tau uptake in isolated cerebrovessels. At this stage, the role of mural cells in tau uptake is poorly understood, and the long-term effects of repetitive head trauma on mural cells in the brain has yet to be fully investigated. Since these cells can be important mediators of solute disposition and accumulation in the brain, the purpose of the present study was to evaluate the interaction between tau and brain vascular mural cells and determine the status of these cells chronically following repetitive brain injury.

## 2. Materials and methods

### 2.1. Materials

Brain vascular pericytes (cat#1200), brain vascular smooth muscle cells (SMC) (cat#1100), brain microvascular endothelial cells (HBMEC) (cat#1000), astrocytes (cat#1800), and microglia (cat#1900) (all of human origin) and associated culture reagents were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Fibronectin solution (cat#F1141), poly-L-lysine solution (cat#P4707), collagenase/dispase (cat#11097113001), and Hanks' balanced salt solution (HBSS) (cat#H8264) were purchased from MilliporeSigma (St. Louis, MO, USA). Recombinant human tau-441 (rhtau) (cat#T-1001) and fluorescein-labeled A $\beta$ (1-42) (cat#A-1119) was purchased from rPep-ptide (Watkinsville, GA, USA). Lucifer yellow dextran (10 kD) (cat#D1825) and the human tau enzyme linked immunosorbent assay (ELISA) (cat#KHB0041) were purchased from Invitrogen Corp.

(Carlsbad, CA, USA). The ELISA kits for human (cat#LS-F13051) and mouse (cat#LS-F21849) alpha smooth muscle cell actin ( $\alpha$ SMC-actin) were purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA). The ELISA kits for human (cat#EHPDGFRB) and mouse (cat#MBS919047) PDGFR $\beta$  (platelet-derived growth factor receptor beta) were purchased from ThermoFisher Scientific (Waltham, MA, USA) and MyBioSource, Inc. (San Diego, CA, USA), respectively. Antibodies for N-aminopeptidase, CD13 (cat#558744), and the brain endothelial cell marker, CD31 (cat#561410), were purchased from BD Biosciences (San Jose, CA, USA). Mammalian protein extraction reagent (M-PER) (cat#78505), Halt enzyme inhibitor cocktails (cat#78442), and the bicinchoninic acid (BCA) protein assay (cat#23225) were purchased from ThermoFisher Scientific (Waltham, MA, USA).

### 2.2. Animals

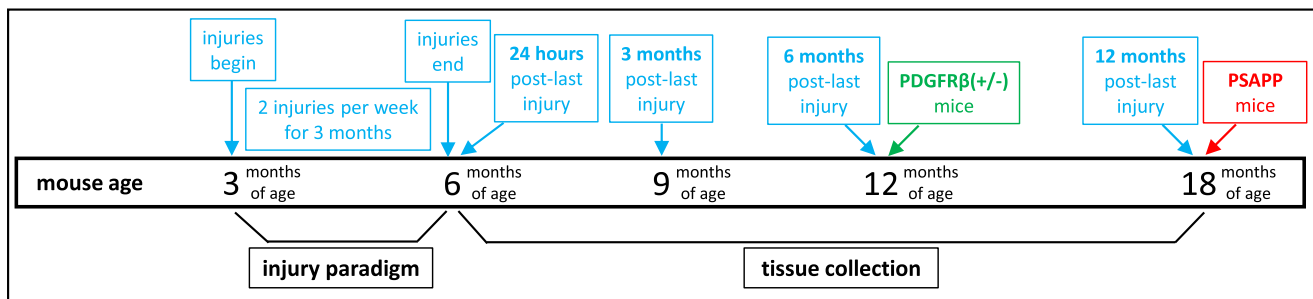
Human tau (hTau) mice (cat#005491) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The hTau mice express six isoforms of human tau on a C57BL/6 background, but do not express murine tau, as previously described (Andorfer et al., 2003). Transgenic PS1/APPsw (PSAPP) mice overexpressing the "Swedish" mutation (APP695) and mutant presenilin-1 (M146L) were used as a mouse model of Alzheimer's disease (Holcomb et al., 1998). The mural cell-depleted animals, PDGFR $\beta$ (+/-), were kindly provided by Dr. Richard Daneman (University of California, San Diego, La Jolla, CA) and were generated by disrupting PDGFR $\beta$  signaling, which leads to progressive reductions in the vascular expression of smooth muscle cells and pericytes (Bell et al., 2010; Tallquist et al., 2003). All studies used male and female mice, housed under standard laboratory conditions (23  $\pm$  1  $^{\circ}$ C, 50  $\pm$  5% humidity, and a 12-h light/dark cycle) with free access to food and water throughout the study. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Roskamp Institute.

### 2.3. Brain injury protocol

To investigate the effects of repetitive mild traumatic brain injury (r-mTBI), we used a mouse model of closed head injury as previously characterized by our group (Mouzon et al., 2012; Mouzon et al., 2014). Briefly, animals were secured in a mouse stereotaxic apparatus (Stoelting) mounted with an electromagnetic controlled impact device (Leica) and anesthetized with 1.5 l/min of oxygen and 3% isoflurane. Prior to impact, a 5 mm blunt metal impactor tip was retracted and positioned midway in relation to the sagittal suture. The injury was triggered using the myNeuroLab controller (Leica) at a strike velocity of 5 m/s, strike depth of 1.0 mm, and a dwell time of 200 milliseconds. Mice (3 months of age) received 2 injuries per week for 3 months. In this closed head injury model, there are no incisions and no craniotomy. The mouse head is shaved and the skin is retracted on either side of the brain. The impact is delivered directly to the skin on the midline of the skull. As a control, sham animals did not receive the brain injury, but were exposed to anesthesia for the same length of time as the injured mice and under the same paradigm (2 exposures per week for 3 months). Mice were euthanized at 24 h, 3 months, 6 months, and 12 months after the final brain injury or anesthesia exposure. A timeline of the injury paradigm and the timepoints for tissue collection are depicted in (Fig. 1).

### 2.4. Peptide preparation

Using a standard process to limit aggregation, as we previously described (Bachmeier et al., 2013), lyophilized A $\beta$  peptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to acquire a monomeric/dimeric sample and minimize the formation of  $\beta$ -sheet structures. Briefly, 1 mg of each lyophilized peptide was dissolved in 1 ml of ice cold HFIP. The peptides were allowed to air dry in a chemical fume hood for one hour followed by further drying in a speed-vac centrifuge for 30



**Fig. 1.** Timeline of the brain injury paradigm and tissue collection for the mouse studies. Human tau (hTau) mice at 3 months of age received 2 injuries per week for 3 months. As a control, sham animals did not receive any brain injuries, but were exposed to anesthesia for the same length of time as the injured mice and under the same paradigm (2 exposures per week for 3 months). Mice were euthanized and tissue was collected at 24 h, 3 months, 6 months, and 12 months after the final brain injury or anesthesia exposure (blue boxes). Moreover, transgenic mural cell-depleted animals, PDGFR $\beta$ (+/-) (green box), and a mouse model of Alzheimer's disease, PSAPP (red box), were euthanized and tissue collected at 12 months and 18 months of age, respectively. Of note, neither the PDGFR $\beta$ (+/-) nor PSAPP animals were administered any brain injuries. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

min. The resulting clear film was re-suspended in 100% dimethylsulfoxide to a concentration of 1 mM and stored in aliquots at  $-80^{\circ}\text{C}$ . Recombinant human tau-441 (50  $\mu\text{g}$ ) was dissolved in 1 ml of HBSS and stored in aliquots at  $-80^{\circ}\text{C}$ .

### 2.5. Tau uptake in vitro

All cells (pericytes, SMC, HBMEC, astrocytes, and microglia) were individually seeded at 50,000 cells per  $\text{cm}^2$  onto fibronectin-coated or poly-L-lysine-coated 24-well plates. Upon confluency, cells were treated with rhtau (0.5, 5, and 50  $\text{ng/ml}$ ) for 1 h at  $37^{\circ}\text{C}$ . Following the treatment period, the extracellular media was removed, and the cell monolayer washed with ice-cold HBSS. Cell lysates were collected using lysis buffer (M-PER) supplemented with phenylmethanesulfonyl fluoride (1 mM) and Halt protease and phosphatase inhibitor cocktail. The cell lysates were analyzed for total tau content by ELISA and normalized to total protein content using the BCA protein assay. It should be noted that significant tau degradation is not expected to occur within the time frame of the tau uptake studies. That said, a limitation of this approach is that any tau fragments that may be produced would not be distinguished and any disruptions to the tau epitope could prevent tau detection by the ELISA antibody altogether.

### 2.6. Isolation of brain fractions

Various brain fractions, including the cerebrovasculature, were isolated from mouse brain tissue as characterized and described by our group previously (Bachmeier et al., 2014). Briefly, the entire mouse brain (minus the cerebellum and brain stem) was freshly collected (300 mg) and ground in ice-cold HBSS with 6–8 passes of a Teflon pestle in a glass Dounce homogenizer. An equal volume of 40% dextran solution was added to the brain homogenate for a final concentration of 20% dextran and immediately centrifuged at 6000g for 15 min at  $4^{\circ}\text{C}$ . This procedure results in a pellet at the bottom of the container (cerebrovasculature) and a compact mass at the top of the solution (parenchyma) separated by a clear dextran interface (soluble fraction, i.e., non-cell associated). As we are interested in the response of both pericytes and smooth muscle cells, we used whole vascular preparations, containing vessels of a variety of sizes (microvessels, arterioles, etc.). As a result, in addition to mural cells, these preparations likely contain brain endothelia and potentially astrocytes. The freshly isolated cerebrovessels were collected and immediately used for the ex vivo studies described below.

### 2.7. Tau uptake, mural cell marker and caveolin-1 expression ex vivo

In line with the in vitro studies above, tau uptake was evaluated in cerebrovessels isolated from 1) r-mTBI (24 h, 3 months, 6 months, and 12 months post-last injury), 2) PDGFR $\beta$ (+/-) (12 months of age), and 3) PSAPP (18 months of age) mice. The PDGFR $\beta$ (+/-) and PSAPP animals were examined at 12 months and 18 months of age, respectively, as prior reporting has shown mural cell disruption at these ages or younger (Sagare et al., 2013; Bell et al., 2010). Additionally, these ages match the respective ages of the r-mTBI animals at 6 and 12 months post-last injury, which allows for comparisons of mural-cell dysfunction between the mouse models while excluding age as a confounding factor. The sample sizes for the r-sham and r-mTBI groups were the same for each timepoint: 24 h ( $n = 5$ ), 3 months ( $n = 4$ ), 6 months ( $n = 6$ ), and 12 months ( $n = 4$ ). The sample sizes for the PDGFR $\beta$ (+/-) and wild-type littermate groups were  $n = 4$  each, while the PSAPP and wild-type littermate groups were  $n = 6$  each. Freshly isolated cerebrovessels were treated with 5  $\text{ng/ml}$  rhtau for 1 h at  $37^{\circ}\text{C}$ , which is the reported concentration of tau in human ISF (Magnoni et al., 2012; Marklund et al., 2009). In addition, cerebrovessels isolated from a separate cohort of r-mTBI animals at 12 months post-injury were treated with either 2  $\mu\text{M}$  fluorescein-labeled A $\beta$ (1–42) or 10  $\mu\text{M}$  lucifer yellow dextran for 1 h at  $37^{\circ}\text{C}$ . For this study, the sample sizes for the r-sham and r-mTBI groups were the same for each probe: lucifer yellow dextran ( $n = 4$ ), fluorescein-labeled A $\beta$ 1-42 ( $n = 5$ ). Following the treatment period, the extracellular media was removed, and the cerebrovessels were washed with ice-cold HBSS. Cell lysates were collected using lysis buffer (M-PER) supplemented with phenylmethanesulfonyl fluoride (1 mM) and Halt protease and phosphatase inhibitor cocktail. The cell lysates were analyzed for total tau content,  $\alpha\text{SMC-actin}$ , PDGFR $\beta$ , and caveolin-1 by ELISA, while the fluorescein-labeled A $\beta$ (1–42) and lucifer yellow dextran were analyzed using a microplate fluorescence reader. All samples were normalized to total protein content using the BCA protein assay.

### 2.8. Mural cell marker and caveolin-1 expression in human brain specimens

Human brain specimens were acquired from Dr. Thomas Beach, Director of the Brain and Body Donation Program at the Sun Health Research Institute (Sun City, AZ). Frozen human cortex samples from the inferior frontal gyrus (500 mg) were obtained from the autopsied brains of 1) non-demented control subjects (no history of TBI or AD diagnosis), 2) TBI, 3) AD, and 4) TBI and AD. For the TBI specimens, donors reported 1 or 2 brain injuries in which loss of consciousness occurred and each incident lasted less than 30 min. Moreover, as we are primarily

interested in the chronic phase post-injury, samples with a longer post-last injury period were prioritized (mean, ~40 years post-last injury). A summary of the human brain specimens is displayed in Table 1. In the same manner as the mouse ex vivo studies above, the cerebrovasculature was isolated from each human brain specimen and collected using lysis buffer (M-PER) supplemented with phenylmethanesulfonyl fluoride (1 mM) and Halt protease and phosphatase inhibitor cocktail. The cell lysates were analyzed for  $\alpha$ SMC-actin, PDGFR $\beta$ , and caveolin-1 by ELISA and normalized to total protein content using the BCA protein assay.

2.9. Flow cytometry

Fresh cerebrovascular tissue was isolated from the brains of r-sham and r-mTBI mice at 6 months post-last injury and processed as previously described (Crouch and Doetsch, 2018). Briefly, the cerebrovascular pellet (obtained as described above) was resuspended in a collagenase/dispase solution (1 mg/ml) and incubated for 1 h at 37 °C with gentle agitation. Following enzymatic digestion, the tissue was pelleted by centrifugation at 6000 rpm for 3 min and resuspended in a DNase solution (1 mg/ml, Worthington Biochemical) and subjected to further mechanical digestion via trituration with a pipette to achieve a single cell suspension. The tissue was centrifuged at 6000 rpm for 3 min, the supernatant discarded, and the resulting pellet was resuspended and stained with antibodies against the mural cell-specific N-aminopeptidase (CD13) using anti-CD13-FITC at 1:200 (BD Biosciences), and the brain endothelial cell marker (CD31) using anti-CD31-PE-CY7 at 1:500 (BD Biosciences). For live/dead cell discrimination, the viability dye propidium iodide (Sigma Aldrich) was added to the antibody cocktail. Cells were stained on ice for 30 min in the dark and resuspended in 1% BSA in HBSS. Data were acquired and analyzed using the Attune® NxT Acoustic Focusing Flow Cytometer and Attune® NxT software version 2.7 (Thermo Fisher Scientific, Waltham, MA, USA).

2.10. Statistical analyses

Quantitative data were plotted as mean  $\pm$  standard error of the mean. Statistical analysis was performed using InStat 3.0 or GraphPad Prism 8.0 (GraphPad Software, Inc). The Brown-Forsythe and Bartlett's tests were performed to ensure homogeneity of variance and the Shapiro-Wilk test was completed to assess normality. Statistical significance was evaluated using a two-way ANOVA with Bonferroni's multiple comparisons testing as indicated in the figure legends. For data that did not reflect a normal/Gaussian distribution, the Kruskal-Wallis test was utilized followed by Dunn's multiple comparisons test. Correlation analyses were evaluated using Pearson's correlation coefficient. For all analyses, a *p* value  $\leq$ 0.05 was considered statistically significant.

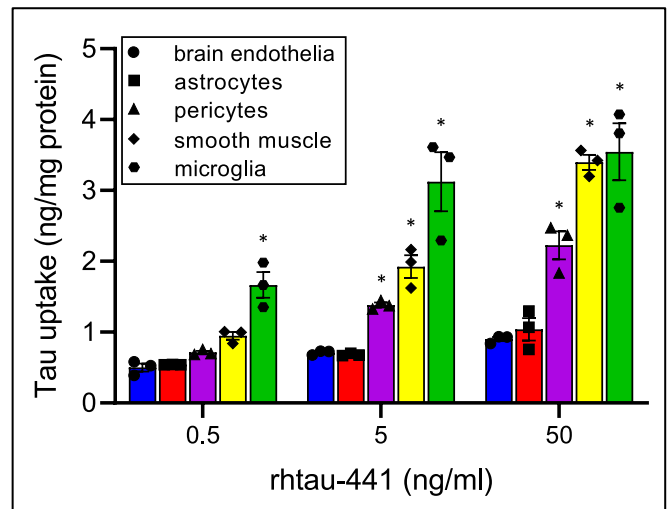
3. Results

3.1. Tau uptake in vitro

The microglia cultures showed the strongest interaction with tau at all 3 concentration (0.5, 5 and 50 ng/ml), while both the pericytes and smooth muscle cells demonstrated a dose-dependent capacity for tau uptake (Fig. 2). At the highest tau treatment concentration (50 ng/ml), the smooth muscle cells had essentially the same levels of intracellular

**Table 1**  
Characteristics of human brain specimens.

Group	Sample size	Age $\pm$ SEM (years)	Sex (M/F)	Years post-last injury $\pm$ SEM
Control	15	79.4 $\pm$ 1.9	8/7	
TBI	12	85.2 $\pm$ 1.9	6/6	38.1 $\pm$ 9.2
AD	15	82.5 $\pm$ 1.7	7/8	
AD-TBI	14	79.6 $\pm$ 2.3	7/7	41.5 $\pm$ 7.9



**Fig. 2.** Tau uptake in human non-neuronal brain cells. Cells were exposed to various concentrations (0.5, 5, and 50 ng/ml) of full length recombinant human tau (rhtau-441) for 1 h at 37 °C. Lysates were analyzed for tau content by ELISA and normalized to total protein using the BCA assay. Values represent mean  $\pm$  SEM (*n* = 3) and are expressed as ng of tau per mg of total protein. \**P* < 0.05 compared to brain endothelia as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

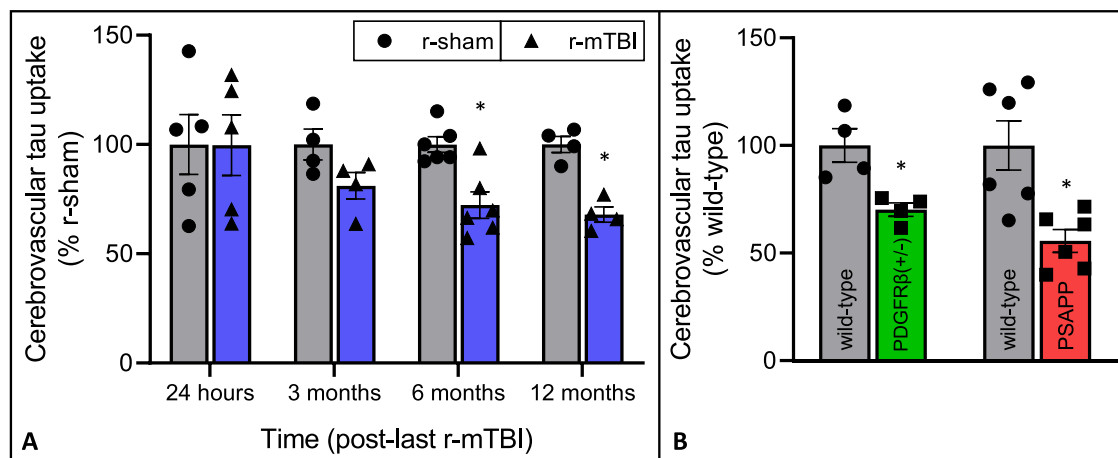
tau as the microglia. In contrast, the astrocytes and brain endothelial cells showed a lower degree of tau uptake (at any concentration) as the tau levels in these cells were 3-times lower than that observed in the other cell types (pericytes, smooth muscle cells, and microglia) at 5 and 50 ng/ml and near the background level of detection. The rank order for the in vitro uptake of tau was microglia > smooth muscle cells > pericytes  $\gg$  brain endothelia = astrocytes.

3.2. Cerebrovascular tau uptake ex vivo

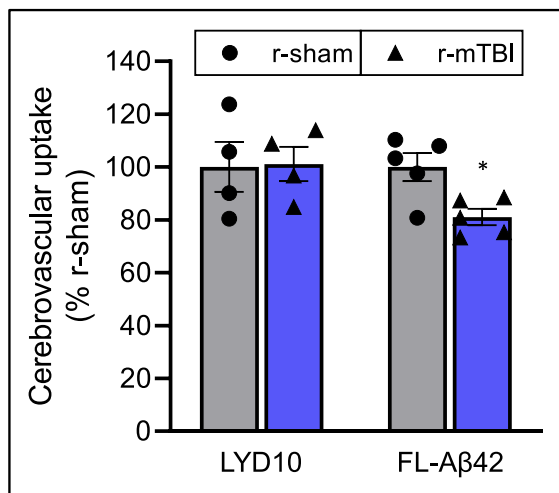
Freshly isolated cerebrovessels from r-mTBI mice showed a progressive decline in tau uptake post-last injury, resulting in a statistically significant decrease at 6 (25%) and 12 months (30%) post-last injury compared to each respective r-sham cohort (Fig. 3A). Similarly, tau uptake was also significantly diminished (30% decrease) in cerebrovessels from mural cell-depleted PDGFR $\beta$ (+/-) animals compared to age-matched wild-type littermates (Fig. 3B). Lastly, cerebrovessels from PSAPP animals demonstrated substantially less tau uptake (40% decrease) than age-matched wild-type littermates (Fig. 3C). Additionally, fluorescein-labeled A $\beta$ (1-42) uptake was decreased (20%) in cerebrovessels from r-mTBI animals at 12 months post-last injury compared to r-sham mice (Fig. 4). Lastly, no difference in the cerebrovascular uptake of lucifer yellow dextran was observed between r-mTBI and r-sham animals at 12 months post-last injury (Fig. 4).

3.3. Mural cell marker expression in r-mTBI and transgenic animals

In the brain-injured animals, expression of the mural cell markers,  $\alpha$ SMC-actin and PDGFR $\beta$ , progressively decreased post-last injury in isolated r-mTBI cerebrovessels compared to r-sham animals. For  $\alpha$ SMC-actin (Fig. 5A), a significant decrease was observed at both 6 and 12 months after the final injury (approximately 25% decrease for both compared to respective r-sham animals). For PDGFR $\beta$ , expressions levels were significantly lower (approximately 30% decrease) at 12 months post-last injury compared to r-sham animals (Fig. 6A). Similarly, in the PDGFR $\beta$ (+/-) animals (12 months of age),  $\alpha$ SMC-actin in isolated cerebrovasculature was diminished (approximately 30%) compared to age-matched wild-type littermates, but this effect did not reach statistical significance (Fig. 5B). As anticipated, a substantial decrease in PDGFR $\beta$



**Fig. 3.** Tau uptake in freshly isolated cerebrovasculature from (A) r-mTBI mice (24 h, 3 months, 6 months, and 12 months post-last injury), and (B) PDGFR $\beta$ (+/-) mice, PSAPP mice, and respective wild-type littermates. Cerebrovessels were exposed to 5 ng/ml recombinant human tau (rhtau-441) for 1 h at 37 °C. Lysates were analyzed for tau content by ELISA and normalized to total protein using the BCA assay. Values represent mean  $\pm$  SEM and are expressed as a percentage of each respective r-sham or wild-type littermate. The sample sizes for the r-sham and r-mTBI groups were the same for each timepoint: 24 h ( $n = 5$ ), 3 months ( $n = 4$ ), 6 months ( $n = 6$ ), and 12 months ( $n = 4$ ), PDGFR $\beta$ (+/-) and wild-type littermates ( $n = 4$  each) and PSAPP and wild-type littermates ( $n = 6$  each). \* $P < 0.05$  compared to each respective r-sham or wild-type littermate as determined by two-way ANOVA and Bonferroni's multiple comparisons test.



**Fig. 4.** Solute uptake in freshly isolated cerebrovasculature from r-mTBI mice at 12 months post-last injury. Cerebrovessels were exposed to lucifer yellow dextran (10  $\mu$ M) or fluorescein-labeled A $\beta$ 1-42 (2  $\mu$ M) for 1 h at 37 °C. Lysates were analyzed for fluorescence and normalized to total protein using the BCA assay. Values represent mean  $\pm$  SEM and are expressed as a percentage of each respective r-sham group. The sample sizes for the r-sham and r-mTBI groups were the same for each probe: lucifer yellow dextran ( $n = 4$ ), fluorescein-labeled A $\beta$ 1-42 ( $n = 5$ ). \* $P < 0.05$  compared to each respective r-sham as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

levels (approximately 40% reduction) was observed in the PDGFR $\beta$  (+/-) animals compared to age-matched wild-type littermates (Fig. 6B). Lastly, in the PSAPP mouse AD model (18 months of age), both  $\alpha$ SMC-actin and PDGFR $\beta$  were significantly reduced (approximately 30% decrease) in isolated cerebrovessels compared to age-matched wild-type littermates (Figs. 5C and 6C). In combining the tau uptake and mural cell expression datasets, we observed a strong correlation between cerebrovascular tau uptake and mural cell marker expression ( $\alpha$ SMC-actin,  $P = 0.0195$ ; PDGFR $\beta$ ,  $P = 0.0335$ ) following repetitive trauma to the brain (Fig. 7).

#### 3.4. Mural cell marker expression in human brain specimens

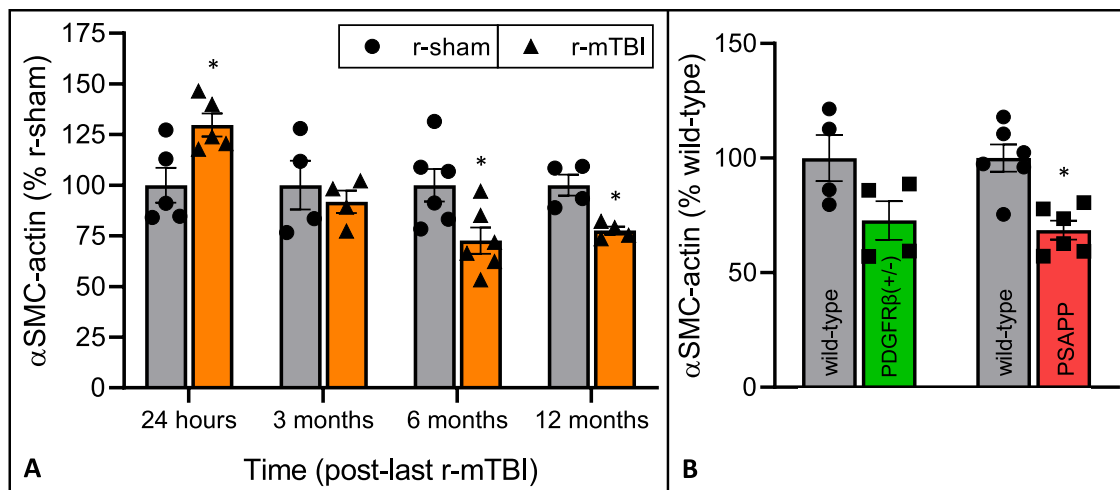
In isolated cerebrovasculature from human brain cortex,  $\alpha$ SMC-actin levels in AD specimens were approximately half that observed in control specimens (Fig. 8A), and in the TBI specimens a reduction in  $\alpha$ SMC-actin was also observed (approximately 25% compared to control brains), but this comparison was not statistically significant. While not quite as diminished as the AD group,  $\alpha$ SMC-actin expression in the cerebrovasculature from the TBI-AD group was significantly lower than that found in control brains (Fig. 8A). For PDGFR $\beta$  expression in isolated cerebrovessels, the levels in AD specimens were less than half that observed in control brains (Fig. 8B). In the TBI specimens, a reduction in PDGFR $\beta$  expression was apparent (approximately 15% compared to control), but was not statistically significant. Lastly, in the AD-TBI group, PDGFR $\beta$  levels were significantly reduced by 35% compared to control specimens (Fig. 8B).

#### 3.5. Flow cytometry and immunophenotypic analysis of cerebrovascular mural cells and endothelia

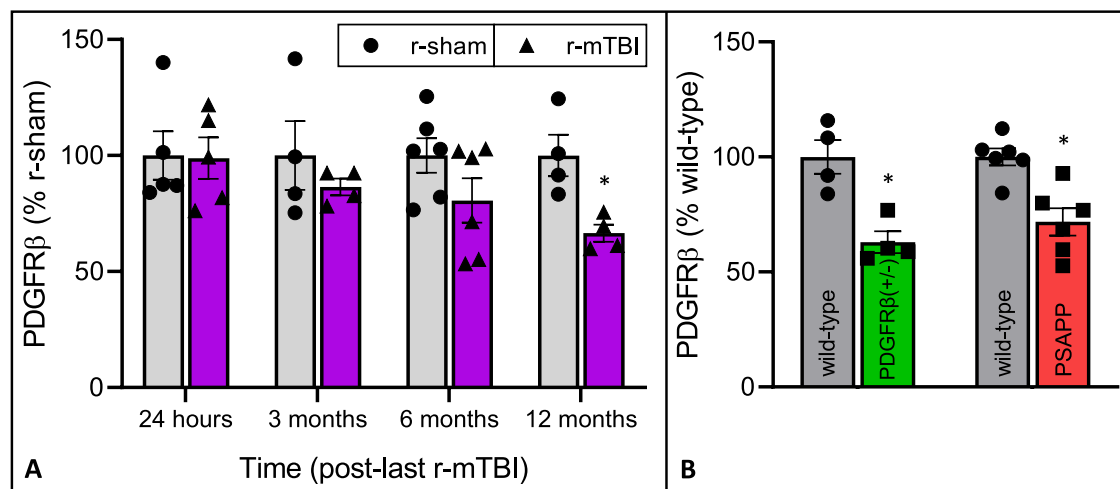
The number of CD31 + ve endothelial cells and CD13 + ve mural cells in freshly isolated cerebrovasculature from r-sham and r-mTBI animals (6 months post-injury) were quantified and expressed as a percentage of the total number of gated events (Fig. 9). No significant difference was identified in the percentage of CD31 + ve endothelial cells (Fig. 9F) or CD13 + ve mural cells (Fig. 9G) when comparing r-sham and r-mTBI cerebrovessels.

#### 3.6. Caveolin-1 expression in animal and human cerebrovasculature

Caveolin-1 expression was significantly decreased in isolated r-mTBI cerebrovessels compared to r-sham animals (35%) at 12 months post-last injury (Fig. 10). Similar reductions in cerebrovascular caveolin-1 expression were observed in both the PSAPP and PDGFR $\beta$ (+/-) animals compared to their respective wild-type littermates (Fig. 10). With respect to the human brain specimens, cerebrovascular caveolin-1 levels were significantly reduced in the TBI brain specimens (40%), while nearly 2-times less caveolin-1 was detected in the AD cerebrovessels in relation to the control brain specimens (Fig. 11). The combination of TBI and AD had the lowest levels of cerebrovascular caveolin-1 demonstrating a 4-fold decrease when compared to the control brains (Fig. 11).



**Fig. 5.** Mural cell marker expression ( $\alpha$ SMC-actin) in freshly isolated cerebrovasculature from (A) r-mTBI mice (24 h, 3 months, 6 months, and 12 months post-last injury), and (B) PDGFR $\beta$ (+/-) mice, PSAPP mice, and respective wild-type littermates. Lysates were analyzed for  $\alpha$ SMC-actin by ELISA and normalized to total protein using the BCA assay. Values represent mean  $\pm$  SEM and are expressed as a percentage of each respective r-sham or wild-type littermate. The sample sizes for the r-sham and r-mTBI groups were the same for each timepoint: 24 h (n = 5), 3 months (n = 4), 6 months (n = 6), and 12 months (n = 4). PDGFR $\beta$ (+/-) and wild-type littermates (n = 4 each) and PSAPP and wild-type littermates (n = 6 each). \*P < 0.05 compared to each respective r-sham or wild-type littermate as determined by two-way ANOVA and Bonferroni's multiple comparisons test.



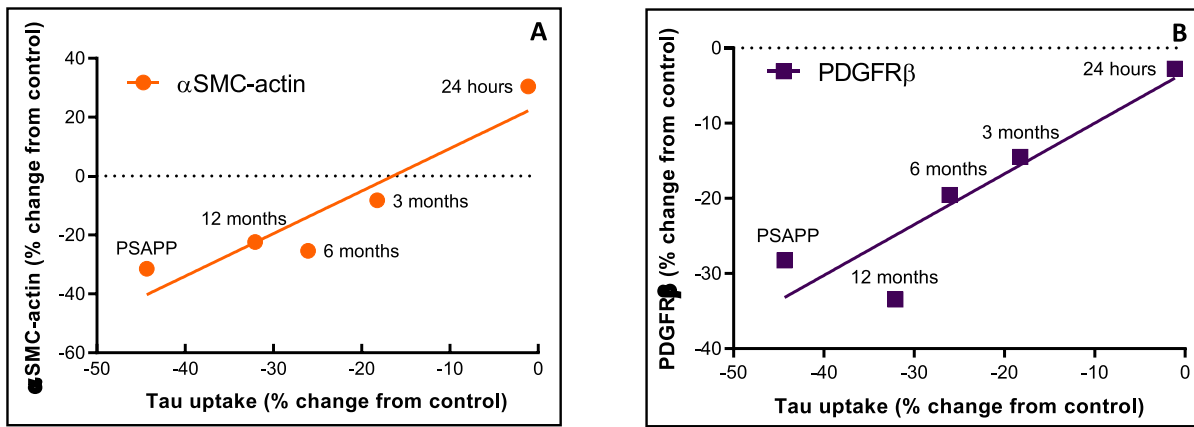
**Fig. 6.** Mural cell marker expression (PDGFR $\beta$ ) in freshly isolated cerebrovasculature from (A) r-mTBI mice (24 h, 3 months, 6 months, and 12 months post-last injury), and (B) PDGFR $\beta$ (+/-) mice, PSAPP mice, and respective wild-type littermates. Lysates were analyzed for PDGFR $\beta$  by ELISA and normalized to total protein using the BCA assay. Values represent mean  $\pm$  SEM and are expressed as a percentage of each respective r-sham or wild-type littermate. The sample sizes for the r-sham and r-mTBI groups were the same for each timepoint: 24 h (n = 5), 3 months (n = 4), 6 months (n = 6), and 12 months (n = 4). PDGFR $\beta$ (+/-) and wild-type littermates (n = 4 each) and PSAPP and wild-type littermates (n = 6 each). \*P < 0.05 compared to each respective r-sham or wild-type littermate as determined by two-way ANOVA and Bonferroni's multiple comparisons test.

**4. Discussion**

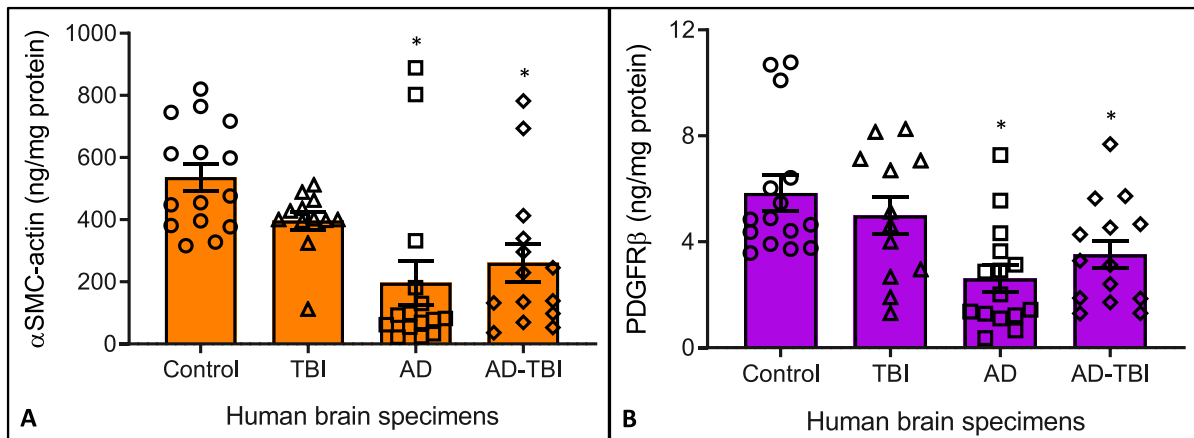
Brain vascular mural cells are an essential component of the neurovascular unit, which couples neuronal activity to vascular function (ElAli et al., 2014) in regulating brain homeostasis (Hill et al., 2014). Specifically, mural cells perform a number of diverse functions including: cerebral blood flow regulation, blood-brain barrier (BBB) maintenance, endothelial cell regulation and angiogenesis, and phagocytosis of extracellular solutes (Dalkara et al., 2011; Kolinko et al., 2018). As a result of their broad function and importance to cerebrovascular health, brain mural cells have been implicated in a variety of neurological pathologies (Hill et al., 2014; Dalkara et al., 2011). In the current studies, isolated cerebrovessels from human AD brain specimens and an AD mouse model (PSAPP) showed significant reductions in mural

cell markers (PDGFR $\beta$  and  $\alpha$ SMC-actin) compared to age-matched human control brains and wild-type animals, respectively, consistent with prior reports of vascular mural cell disruptions in AD brains (Bourassa et al., 2020; Sagare et al., 2013; Sengillo et al., 2013).

As vascular mural cell perturbations are evident in AD and other neurodegenerative disorders (Winkler et al., 2013), we sought to interrogate the state of the mural cell population following trauma to the brain, particularly at more chronic timepoints post-injury, as such studies are currently lacking. In the acute phase post-injury, prior reporting showed numerous mural cell markers (including PDGFR $\beta$ ) were diminished in mouse cortical tissue up to 48 h following fluid percussion injury (Bhowmick et al., 2019). In this same post-injury window (48 h), at the ultrastructural level, it was found that a subset of pericytes associated with the microvasculature underwent a form of



**Fig. 7.** Correlation between mural cell expression and tau uptake in cerebrovessels isolated from r-mTBI (24 h, 3 months, 6 months, and 12 months post-last injury) and PSAPP mice. Cerebrovascular expression of (A)  $\alpha$ SMC-actin, and (B) PDGFR $\beta$  was plotted versus tau uptake ( $\alpha$ SMC-actin,  $r = 0.93$ ; PDGFR $\beta$ ,  $r = 0.91$ ). Values represent the mean % change from each respective control (r-sham or wild-type littermates).  $\alpha$ SMC-actin ( $P = 0.0195$ ) and PDGFR $\beta$  ( $P = 0.0335$ ) as determined by a two-tailed Pearson correlation.



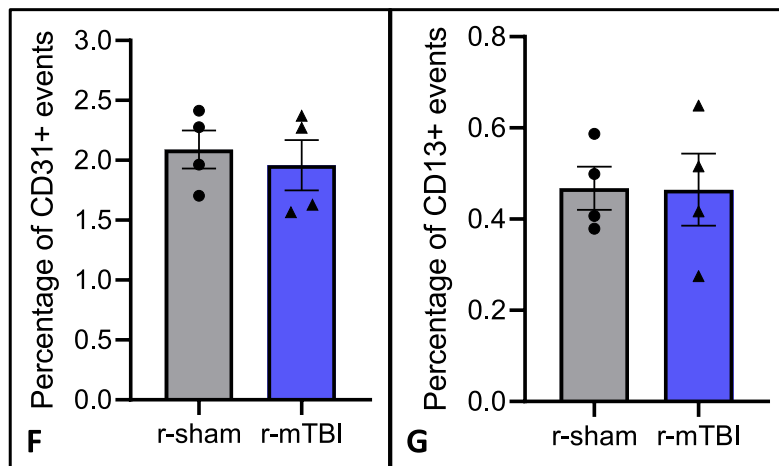
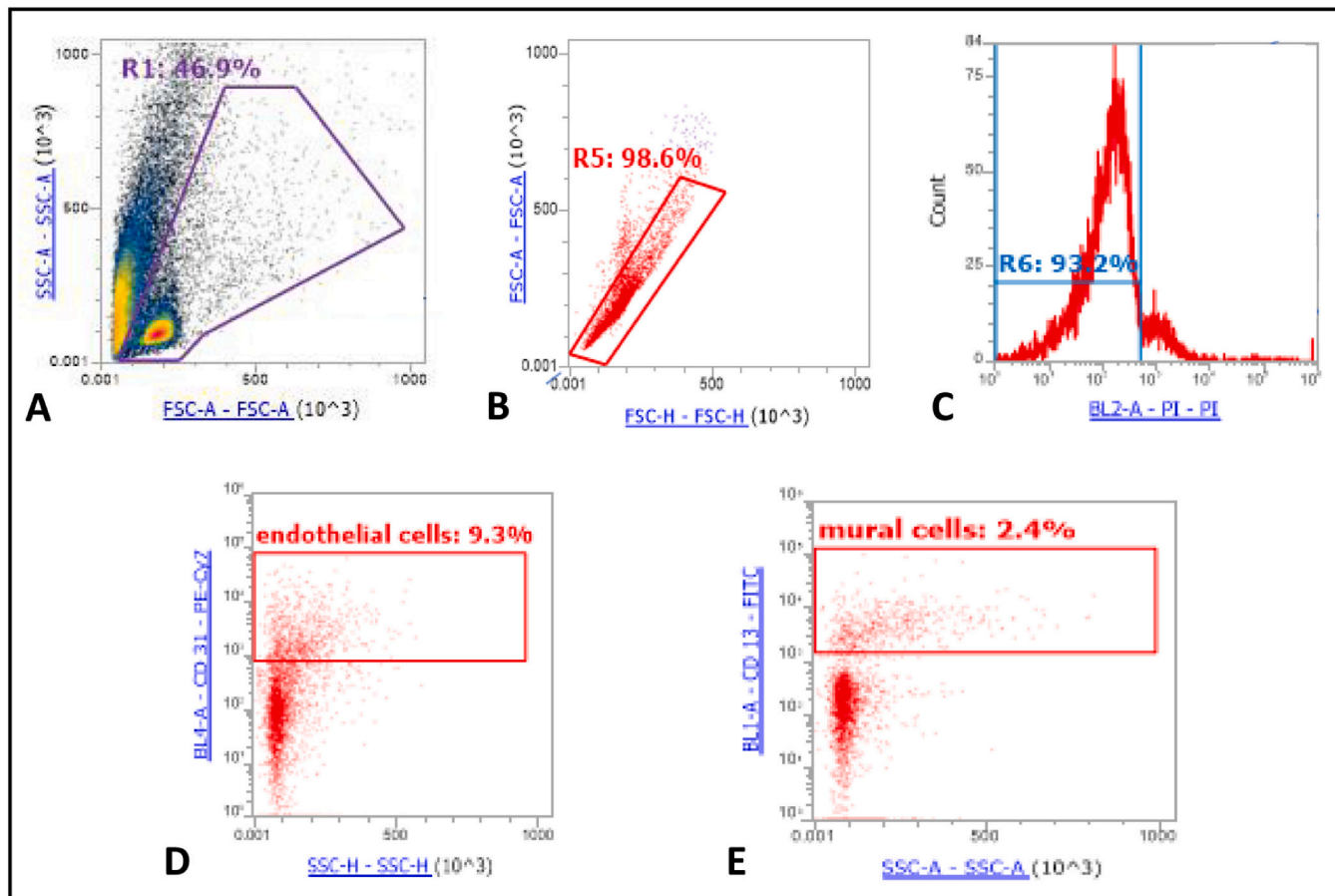
**Fig. 8.** Mural cell marker expression in cerebrovasculature isolated from human brain cortex derived from, 1) non-demented control subjects (no history of TBI or AD diagnosis), 2) TBI, 3) AD, and 4) TBI and AD. Lysates were analyzed for (A)  $\alpha$ SMC-actin, and (B) PDGFR $\beta$  by ELISA and normalized to total protein using the BCA assay. Values represent mean  $\pm$  SEM and are expressed as ng per mg of total protein. Control ( $n = 15$ ), TBI ( $n = 12$ ), AD ( $n = 15$ ), TBI-AD ( $n = 14$ ). \* $P < 0.05$  compared to control as determined by the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

cellular degeneration, while another group of pericytes migrated from the endothelium in response to traumatic injury (Dore-Duffy et al., 2000). Thal and colleagues observed a rapid decline in PDGFR $\beta$  within the first 12 h after injury, however by day 5 post-TBI, not only did these levels resolve but several mural cell markers were significantly elevated compared to control conditions (Zehendner et al., 2015). In our mouse model of r-mTBI, we observed an initial increase in  $\alpha$ SMC-actin compared to r-sham animals (24 h post-injury), akin to that reported after TBI in prior studies (Dore-Duffy et al., 2011). However, as the post-injury time increased in our studies, both mural cell markers (PDGFR $\beta$  and  $\alpha$ SMC-actin) progressively devolved to levels comparable to that observed in the AD animal model. These findings were consistent with our previous observations using the same r-mTBI paradigm in an aged wild-type cohort (12 months of age) in which both PDGFR $\beta$  and  $\alpha$ SMC-actin were significantly decreased at 7 months post-injury, as determined through immunoblotting (Lynch et al., 2016). Furthermore, we interrogated these same markers in cerebrovessels isolated from human brain specimens and observed a decrease in both mural cell markers in the TBI brains compared to control specimens, though these analyses did not reach statistical significance. It is worth noting that only 1 of the 12 TBI specimens had at least one apolipoprotein E4 (apoE4) allele, which is a genetic risk factor for AD (Kim et al., 2009), while 11 of the 14 AD-

TBI specimens were apoE4 positive, and is a likely reason the AD-TBI individuals converted to AD versus the TBI-only subjects. Additionally, a glaring difference between the human TBI specimens and the r-mTBI animal model is the number of brain injury exposures (1–2 TBI in the human specimens vs. 24 TBI in the mouse cohort). The r-mTBI paradigm intends to model the frequency of injuries experienced over the course of an entire career, such as contact sports athletes or military personnel. The more pronounced mural cell marker changes in the r-mTBI animal cohort versus the human TBI specimens could be the result of a higher injury frequency, though further investigation is certainly warranted.

While our findings revealed changes in key mural cell markers following head trauma, particularly in the chronic phase post-injury, what remains unclear is whether these alterations are indicative of cellular degeneration or cellular loss. In human AD brains, PDGFR $\beta$  levels were significantly reduced in the cortex and hippocampus (Sengillo et al., 2013), and both the mural cell number and vessel coverage was found to be reduced in mouse and human AD brains compared to control, using the mural cell-specific marker CD13 (Bourassa et al., 2020; Sagare et al., 2013; Sengillo et al., 2013). With respect to TBI, controlled cortical impact induced mural cell loss by 3 days after TBI in the perilesional cortex and ipsilateral hippocampal areas, as PDGFR $\beta$  was found to be colocalized with several indicators of cell death (Choi

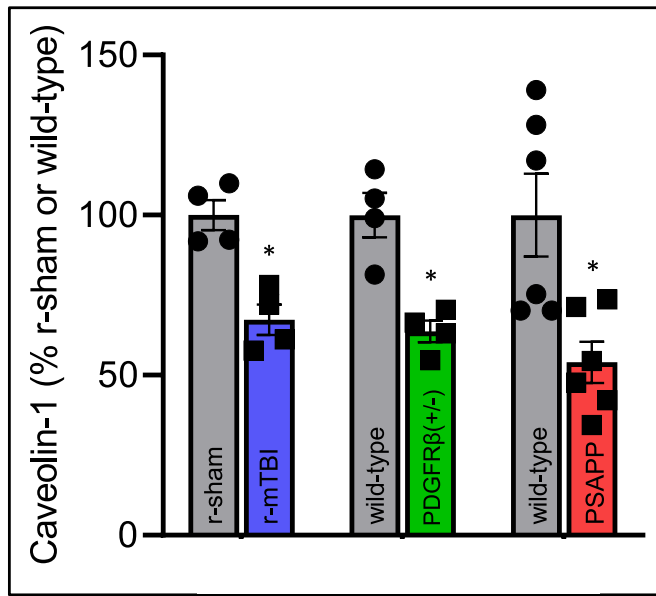




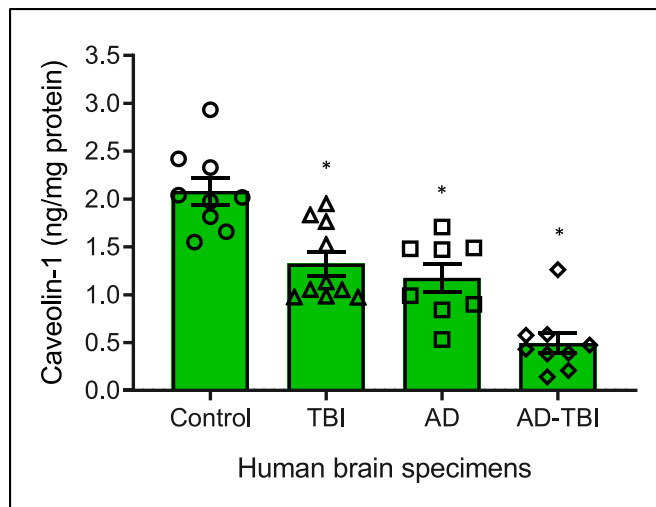
**Fig. 9.** Flow cytometry analysis of brain endothelia and mural cells in freshly isolated cerebrovasculature from r-sham and r-mTBI mice at 6 months post-last injury. The scatter plot diagrams represent the gating strategy used during the identification and analysis of CD31 + ve endothelial cells and CD13 + ve mural cells. Cellular debris was excluded through gating based on (A) particle size (forward scatter area FSC-A) and particle complexity (side scatter area SSC-A). Gating based on (B) FSC-H (H- height) and FSC-A (A- area scaling) selected for single cell populations, while live cells were identified through (C) negative propidium iodide staining. The resulting sample populations were gated based on (D) positive expression of PE-Cy7 indicating CD31 + ve endothelial cells, and (E) positive expression of fluorescein isothiocyanate (FITC) indicating CD13 + ve mural cells. The percentages refer to the proportion of endothelia or mural cells in relation to the previous parent gate. Figs. F and G represent the percentage of CD31 + ve endothelial cells and CD13 + ve mural cells, respectively, in relation to the total number of gated events in r-sham and r-mTBI cerebrovessels. Values represent mean  $\pm$  SEM (n = 4). \*P < 0.05 compared to r-sham as determined by a one-way ANOVA followed by post-hoc test correction for false discovery rate using the Bonferroni correction.

et al., 2016), while a separate report showed CD13 levels were significantly diminished 48 h after fluid percussion injury (Bhowmick et al., 2019). In our studies, we performed flow cytometry to assess the state of the mural cell population after r-mTBI and found no change in the of

number of CD13+ cells between r-sham and r-mTBI cerebrovessels at 6 months post-injury. Furthermore, we recently reported no difference in mural cell vessel density between r-sham and r-mTBI animals (up to 9 months post-injury) based on CD13 vessel coverage using confocal



**Fig. 10.** Caveolin-1 expression in freshly isolated cerebrovasculature from 1) r-mTBI mice (12 months post-last injury), 2) PDGFRβ(+/-) mice and wild-type littermates, and 3) PSAPP mice and wild-type littermates. Lysates were analyzed for caveolin-1 by ELISA and normalized to total protein using the BCA assay. Values represent mean ± SEM and are expressed as a percentage of each respective r-sham or wild-type littermate. The sample sizes for the r-sham and r-mTBI groups were the same for the 12 month timepoint (n = 4 each). PDGFRβ(+/-) and wild-type littermates (n = 4 each) and PSAPP and wild-type littermates (n = 6 each). \*P < 0.05 compared to each respective r-sham or wild-type littermate as determined by two-way ANOVA and Bonferroni's multiple comparisons test.



**Fig. 11.** Caveolin-1 expression in cerebrovasculature isolated from human brain cortex derived from, 1) non-demented control subjects (no history of TBI or AD diagnosis), 2) TBI, 3) AD, and 4) TBI and AD. Lysates were analyzed for caveolin-1 by ELISA and normalized to total protein using the BCA assay. Values represent mean ± SEM and are expressed as ng per mg of total protein. Control (n = 9), TBI (n = 10), AD (n = 8), TBI-AD (n = 9). \*P < 0.05 compared to control as determined by the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

microscopy (Lynch et al., 2020). Thus, while mural cell loss may occur acutely following brain trauma (Choi et al., 2016; Bhowmick et al., 2019), there does not appear to be an overt reduction in the number of mural cells in the chronic phase post-injury, but rather a progressive

decline in key mural cell proteins (i.e., PDGFRβ and αSMC-actin), which are expressed in both pericytes and smooth muscle cells (Alarcon-Martinez et al., 2018; Hellstrom et al., 1999; Skalli et al., 1989). It has been demonstrated that the PDGF pathway is tightly regulated and maintenance of the PDGFRβ receptor is necessary for mural cell function and survival (Bell et al., 2010; Winkler et al., 2014). Similarly, alterations in smooth muscle-actin expression have been associated with changes in mural cell phenotype including cell contraction, migration, and survival (Ahmed and Warren, 2018). Smooth muscle-actin is an important component of mural cell contractility (Ahmed and Warren, 2018; Hamilton et al., 2010) and it has been suggested that the vasomotion wave initiated by contractile smooth muscle cells of cerebral arteries contributes to the clearance of fluid and solutes from the brain (Aldea et al., 2019). In fact, it was shown that reductions in arterial pulsatility decrease both the paravascular (Iliff et al., 2013) and perivascular (Carare et al., 2008) movement of solutes in the brain. As such, the chronic disruptions in PDGFRβ and SMA we observed following repetitive head trauma may impact vasomotion during neurovascular coupling and the perivascular elimination of tau from the brain.

As described above, vascular mural cells contribute to the phagocytosis and clearance of a variety of extracellular molecules and are important mediators of solute disposition and accumulation in the brain (Winkler et al., 2014). Several studies have demonstrated the role of mural cells in the elimination of Aβ from the brain (Wildsmith et al., 2013; Alcendor, 2020; Kanekiyo et al., 2012; Kirabali et al., 2019; Ma et al., 2018), and we found Aβ uptake was diminished in r-mTBI cerebrovessels compared to the r-sham group. Comparatively, there has been little investigation into the interaction of tau with non-neuronal cells, particularly cells associated with the cerebrovasculature. We examined the association of tau with a panel of non-neuronal cells of the brain in vitro and found that brain vascular mural cells (pericytes and smooth muscle cells) interact with tau at a level near that observed with microglia cells, which are prominent phagocytic mediators of the brain and have been shown to degrade tau species (Majerova et al., 2014). Thus, vascular brain mural cells may be involved in the uptake of tau, not unlike their role in the elimination of Aβ and other molecules from the brain. Moreover, disruption of these cells post-injury may lead to the presence of perivascular tau tangles, which is a pathological hallmark of human repetitive brain trauma (Stern et al., 2011; McKee et al., 2009). Similar to the Aβ study above, we investigated tau uptake in isolated cerebrovessels from r-mTBI mice and observed a progressive decline post-injury compared to r-sham cerebrovessels. The diminished cerebrovascular tau uptake at 12 months post-injury was similar to that observed in age-matched AD animals, both of which demonstrated a significantly reduced expression of cerebrovascular mural cell markers, as indicated above. As depicted in Fig. 7, we show a strong correlation between the expression of cerebrovascular mural cell markers and tau uptake following repetitive trauma to the brain. Moreover, the reduced cerebrovascular tau uptake in the current studies coincides with our prior work (using the same hTau mouse model and r-mTBI paradigm) where a significant increase in phosphorylated, oligomeric, and total tau levels was observed in the brain cortex post-injury compared to r-sham animals (Ojo et al., 2016). The general lack of perivascular tau in mouse TBI models compared to human TBI may be due to differences in brain anatomy (gyrencephalic vs lissencephalic) or the ratio of tau isoforms (3R vs 4R), but further investigation is needed to identify the nature of these species-related differences. Further evidence of a potential link between mural cell degeneration and tau disposition in the brain has been reported previously where an AD mouse model (which does not display tau pathology on its own) was crossed with transgenic mural cell-deficient mice and a significant elevation in tau pathology became evident in the cortex and hippocampus of the AD x mural cell-deficient crossed animals (Sagare et al., 2013). While our studies clearly show a progressive decline in markers for pericytes and smooth muscle cells following brain injury, our approaches do not identify the individual contributions of each cell type to tau uptake, outside of the in vitro tau

uptake studies, nor do they differentiate the response of each cell type to head trauma. Despite these limitations, collectively the above studies suggest a role for vascular mural cells in the uptake of extracellular tau, and that disruption of these cells could potentiate tau pathology in the brain.

To understand the mechanics of tau internalization by vascular mural cells and the influence of brain trauma on these processes, we interrogated caveolin, an endocytic component found in mural cells that has been associated with the PDGF pathway (Sundberg et al., 2009). Coinciding with the reductions in cerebrovascular mural cell markers and tau uptake following r-mTBI, we observed a significant decrease in caveolin expression in the brain vasculature of the r-mTBI animals at 12 months post-injury, on par with that observed in mouse AD cerebrovessels. Moreover, similar reductions in caveolin-1 were observed in the human TBI and human AD cerebrovasculature. Interestingly, it has been shown that a loss of caveolin-1 accelerates aging and contributes to neurodegeneration (Head et al., 2010). In particular, mice with reduced caveolin-1 brain expression exhibit a number of TBI and AD pathological features including: increased beta-amyloid (Head et al., 2010), phosphorylated tau (Bonds et al., 2019; Head et al., 2010), and astrogliosis (Head et al., 2010), alongside decreased cerebrovascular volume (Head et al., 2010) and cognitive impairment (Bonds et al., 2019) compared to wild-type animals. Correspondingly, it was observed that caveolin-1 overexpression can reduce the extent of injury and enhance functional recovery after TBI (Kellerhals et al., 2013). It is important to note that our cerebrovascular preparations likely contain brain endothelia and potentially astrocytes, which also express caveolin-1, and could contribute to the alterations in caveolin-1 we observed after r-mTBI and AD. However, any potential changes occurring in brain endothelia after TBI may not necessarily translate to tau uptake, as our *in vitro* findings suggest the interaction between tau and brain endothelia/astrocytes is minimal compared to vascular mural cells. Altogether, these findings suggest irregular PDGF signaling could impact the endocytic uptake of solutes via caveolin-1 and may explain the diminished tau uptake by the cerebrovasculature we observed in r-mTBI and AD mice.

Lastly, to more directly identify the link between mural cell disruption and tau internalization in brain vascular mural cells, we interrogated transgenic PDGFR $\beta$  animals, which display reduced vascular mural cell coverage with age, as a result of genetic modifications to the PDGF receptor (Bell et al., 2010; Tallquist et al., 2003). Cerebrovessels isolated from 12 month-old PDGFR $\beta$ (+/-) mice (same age as the 6 months post-last r-mTBI mice) showed significant reductions in PDGFR $\beta$  and caveolin-1, in addition to diminished tau uptake compared to wild-type littermates, in line with our observations in the chronic phase following r-mTBI and the AD animals. Based on these findings, changes in the PDGF receptor causes the mural cell population in the brain to devolve post-injury, potentially reducing the elimination of extracellular tau by these cells, which may contribute to the elevation of tau species in the brain that occurs following head trauma. In addition to the elimination of extracellular solutes, mural cell degeneration may be an important factor in TBI etiology overall. The most compelling evidence for this concept may be the extensive overlap in pathophysiology between the r-mTBI mice and the transgenic mural cell-depleted PDGFR $\beta$  animals. Both the r-mTBI and mural-cell depleted animals exhibit cerebrovascular abnormalities (Bell et al., 2010; DeWitt and Prough, 2003), neuroinflammation (Bell et al., 2010; Mouzon et al., 2014), white matter regression (Donovan et al., 2014; Montagne et al., 2018), and cognitive impairment (Bell et al., 2010; Mouzon et al., 2014). Importantly, it has been shown that mural cell degeneration precedes the neuroinflammatory response, neuronal decline, and cognitive dysfunction in transgenic PDGFR $\beta$  animals (Bell et al., 2010). Similarly, in human AD microvessel extracts, it was found that reduced brain mural cell levels correlated with cognitive impairment and AD diagnosis (Bourassa et al., 2020). In our prior work using this r-mTBI mouse model, we observed a decline in cognitive performance at 1 month post-injury which progressively deteriorated at 3 and 6 months post-injury

(Lynch et al., 2016; Lynch et al., 2020), in line with the mural cell degeneration we observed at these time points following r-mTBI in the current studies, though further work is needed to understand the potential correlation between brain mural cell changes and cognitive function. Collectively, these studies point toward mural cell disruption as a contributing factor in TBI pathogenesis and cognitive impairment.

## 5. Conclusion

A prominent pathological feature of repetitive head trauma in humans is the accumulation of hyperphosphorylated tau and the presence of neurofibrillary and perivascular tau tangles. Our findings indicate brain vascular mural cells interact with tau and may serve as a pathway for eliminating tau from extracellular brain fluids. Furthermore, human TBI brain specimens and a mouse model of r-mTBI demonstrate a degeneration of mural cell markers in the chronic phase post-injury, coinciding with reduced cerebrovascular caveolin-1 expression and tau uptake, features consistent with those identified in transgenic mural cell-depleted PDGFR $\beta$ (+/-) animals and an AD mouse model. Hence, as the mural cell population devolves in the aftermath of brain trauma or disease, cerebrovascular tau elimination is diminished, which may contribute to the brain deposition of tau species in TBI and other neurodegenerative diseases. Vascular mural cells have a prominent role in regulating brain function and their disruption following head trauma may be a critical driver of TBI pathophysiology and neurodegeneration.

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## Declaration of Competing Interest

None.

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