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Research paper

Impairment of pericyte-endothelium crosstalk leads to blood-brain barrier dysfunction following traumatic brain injury

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A B S T R A C T

The blood-brain barrier (BBB) constitutes a neurovascular unit formed by microvascular endothelial cells, pericytes, and astrocytes. Brain pericytes are important regulators of BBB integrity, permeability, and blood flow. Pericyte loss has been implicated in injury; however, how the crosstalk among pericytes, endothelial cells, and astrocytes ultimately leads to BBB dysfunction in traumatic brain injury (TBI) remains elusive. In this study, we demonstrate the importance of pericyte-endothelium interaction in maintaining the BBB function. TBI causes the platelet-derived growth factor-B (PDGF-B)/PDGF receptor-β signaling impairment that results in loss of interaction with endothelium and leads to neurovascular dysfunction. Using in vivo mild (7 psi) and moderate (15 psi) fluid percussion injury (FPI) in mice, we demonstrate the expression of various pericyte markers including PDGFR-β, NG2 and CD13 that were significantly reduced with a subsequent reduction in the expression of various integrins; adherent junction protein, N-cadherin; gap junction protein, connexin-43; and tight junction proteins such as occludin, claudin-5, ZO-1, and JAM-a. Impairment of pericyte-endothelium interaction increases the BBB permeability to water that is marked by a significant increase in aquaporin4 expression in injured animals. Similarly, pericyte-endothelium integrity impairment in FPI animals greatly increases the permeability of small-molecular-weight sodium fluorescein and high-molecular-weight-tracer Evans blue across the BBB. In addition, the injury-inflicted animals show significantly higher levels of S100β and NSE in the blood samples compared with controls. In conclusion, our data provide an insight that brain trauma causes an early impairment of pericyte-endothelium integrity and results in BBB dysregulation that initiates pathological consequences associated with TBI.

1. Introduction

Pericytes in the central nervous system (CNS) plays a critical and complex regulatory role interacting with other cell types of the neurovascular unit, especially endothelial cells and astrocytes (Sweeney et al., 2016). Within the neurovascular unit, the CNS pericytes are uniquely positioned between the neurons, astrocytes and endothelial cells (Fig. 1A). Pericytes ensheath the capillary wall, making direct contacts with endothelial cells (Armulik et al., 2005; Diaz-Flores et al., 2009). Brain pericytes interact with their neighboring cells and process signals to execute diverse functional response such as regulation of blood-brain barrier (BBB) permeability, angiogenesis, clearance of toxic metabolites, capillary hemodynamic responses, neuroinflammation, and stem cell activity that are critical for CNS functions in health and disease. Pericyte-endothelial interactions play an important role in the maintenance of BBB with critical effects on the structure and function of the basement membrane and endothelial tight junction (TJ) (Daneman and Prat, 2015). Endothelial-secreted platelet-derived growth factor B (PDGF-B) binds to the platelet-derived growth factor receptor beta (PDGFR-β) on pericytes, initiating multiple signal transduction pathways regulating proliferation, migration, and pericytes recruitment to the vascular wall (Lebrin et al., 2010). Signaling mediated by PDGFR-β promotes pericyte attachment to endothelial cells, migration, and proliferation (Lindahl et al., 1999; Tallquist et al., 2003; Tallquist and Soriano, 2003).

Pericyte loss or ablation is one of the hallmarks of BBB dysfunction and has been suggested to trigger several pathological conditions such as abnormal BBB leakage, edema, micro-aneurysm formation, ischemia and so forth (Hellstrom et al., 1999; Lindahl et al., 1997). Under physiological conditions, BBB integrity is highly dependent on the ability of pericytes, endothelial cells and astrocytes to maintain a highly restricted environment in the brain against the entry of blood-borne

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factors and circulating immune cells. In previous studies, we have demonstrated that the induction of oxidative stress activates TGF-β1 (Patel et al., 2017) and matrix metalloproteinases (MMPs) that lead to disruption of BBB, and induction of inflammatory signaling following TBI (Abdul-Muneer et al., 2017b; Abdul-Muneer et al., 2017d). Another study demonstrated that MMP-9 induces migration of the pericytes from the endothelium leading to pericyte loss and disruption of BBB (Takata et al., 2011). Recent studies in adult and aging brain demonstrated that pericyte is required for capillary perfusion, cerebral blood flow (CBF) and BBB integrity (Bell et al., 2010). Furthermore, in 2010,

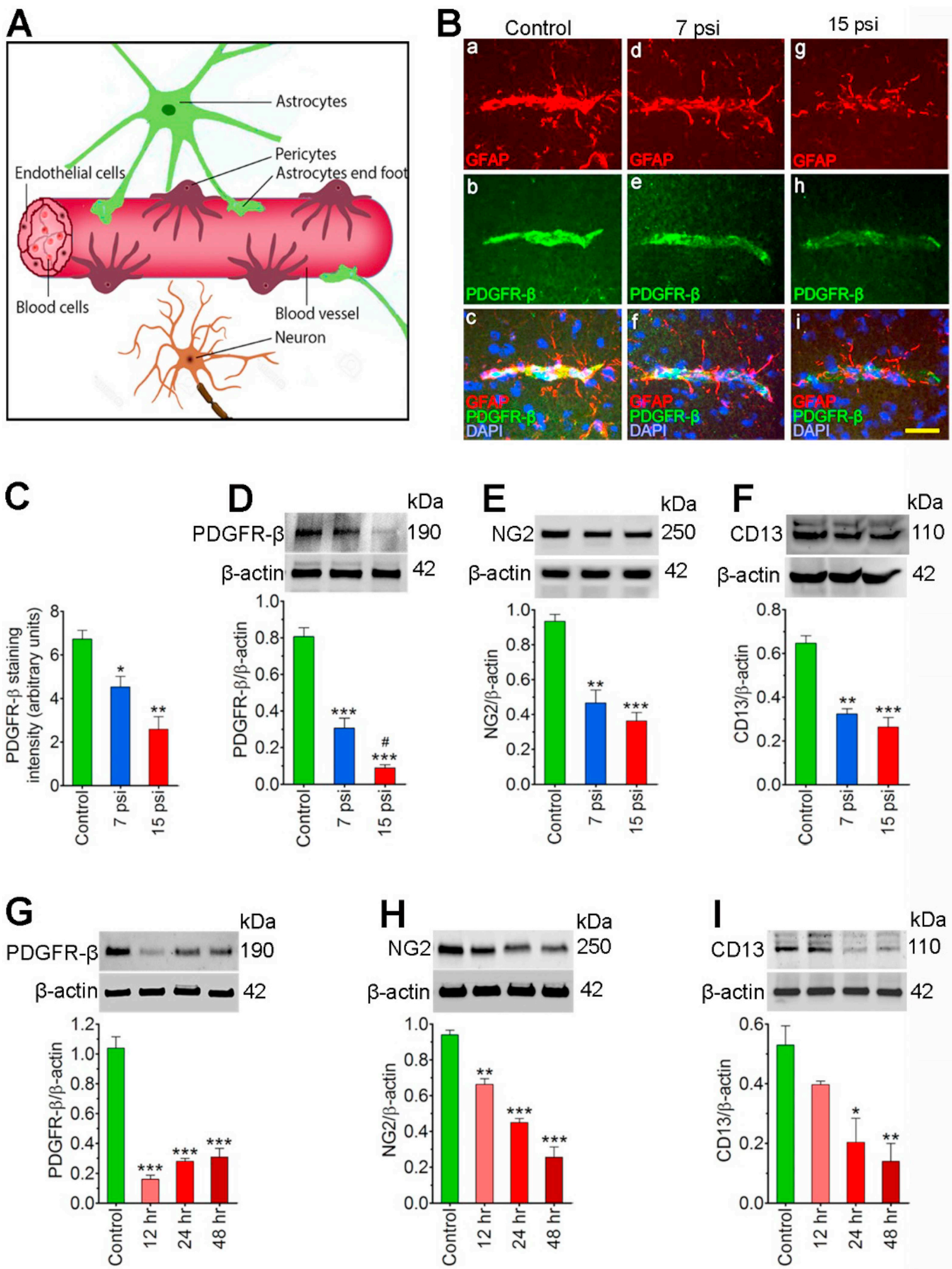

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Fig. 1. TBI results in loss of pericyte coverage. (A) Schematic representation of the blood-brain barrier (BBB)/ neurovascular unit. The BBB is formed by the brain endothelial cells connected by astrocytes, pericytes, and blood vessel neurons. The end foot of astrocytes and pericytes makes intimate contact with the cerebrovascular endothelium plays a critical role in the normal function of the BBB. (B) Immunofluorescent staining of GFAP (red) merged with PDGFR-β (green) and DAPI (blue) in mouse brain cortex tissue samples of uninjured control, 7 psi, and 15 psi injury 24 h after injury. Scale bar = 20 μm. (C) Quantification of PDGFR-β staining analyzed using ImageJ software. Values are mean ± SEM and n = 6/group. $^{*}p < .05$, $^{**}p < .01$ versus control. (D–F) Western blot analysis of PDGFR-β (D), NG2 (E) and CD13 (F) in mouse cortical tissue lysates from uninjured control, 7 psi, and 15 psi injury 24 h after injury. (G-I) Western blot analysis of PDGFR-β (G), NG2 (H) and CD13 (I) and their representative β-actin expression at different time points (0, 12, 24, 48 h) in mouse cortical tissue lysates of 7 psi injury samples. Bar diagram represents the results expressed as a ratio of pericyte markers and β-actin bands. All values are represented as mean ± SEM and n = 7/group. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus control and $^{\#}p < .05$ versus 7 psi injury in D-E. $p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus 0 h in G-H. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Armulik et al. and Daneman et al. independently showed that pericytes are required for BBB function during development using pericyte-deficient mouse models resulting from defective PDGF/PDGFR-β signaling (Armulik et al., 2010; Daneman et al., 2010). In an aging brain, pericyte loss and subsequent increase of vessel permeability promote neuroinflammation and neurodegeneration (Sengillo et al., 2013). In spite of the important role of brain pericytes in human health, the molecular mechanisms that regulate their development, survival, and distribution remain poorly understood.

In the present study, we propose pericytes loss following TBI is a consequence of down-regulation in the PDGF-B/PDGFR-β signaling pathway that results in the impairment of pericyte-endothelium interaction in BBB and leads to neurovascular dysfunction. Here, we show that PDGF-B/PDGFR-β signaling is critical for pericyte maintenance and is dispensable for BBB integrity. Pericyte loss following TBI results in significant reduction in the expression of pericyte markers such as PDGFR-β, NG2 (chondroitin sulfate proteoglycan 4) and CD13 (alanyl (membrane) aminopeptidase) and leads to permeability of BBB marked by a significant increase in Aquaporin4 (AQP4). Moreover, our data also provide strong evidence that mechanical disruption of vascular integrity and/or increased permeability with functional changes at the BBB occurring after trauma leads to subsequent reduction in the expression of extracellular matrix (ECM) proteins such as N-cadherin and Connexin-43 that connect endothelium and pericyte and TJ proteins such as Occludin, Claudin 5, ZO-1 and JAM-a. Hence, restoration of pericyte ablation using a pharmacological approach and keeping pericyte-endothelium integrity following TBI presents a new therapeutic possibility in maintaining BBB integrity and thus provides a better avenue for the treatment of TBI-related neurological disorders.

2. Materials and methods

2.1. Reagents

The primary antibodies rabbit anti-PDGFR-β (Cat. No: 3169; RRID: AB_2162497), anti-NG2 (Cat. No: 4235; RRID: AB_2087604), anti-CD13 (Cat. No: 13721), anti-N-cadherin (Cat. No: 13116; RRID: AB_2687616), anti-connexin-43 (Cat. No: 3512; RRID: AB_2294590), and anti-integrin α6 (Cat. No: 3750S; RRID: AB_2249263) were purchased from Cell Signaling Technology, Danvers, MA. Antibodies anti-integrin α3β1 (Cat. No: ab217145), anti-integrin β1 (Cat. No: ab183666), anti-occludin (Cat. No: ab31721; RRID: AB_881773), anti-claudin-5 (Cat. No: ab15106; RRID: AB_301652), anti-ZO-1 (Cat. No: ab59720; RRID: AB_946249), and anti-aquaporin (AQP4) (Cat. No: ab46182; RRID: AB_955676) were purchased from Abcam, Cambridge, MA. Antibodies mouse anti-JAM-a (Cat. No: SAB4200468; Sigma-Aldrich, St. Louis,MO), and anti-β-actin (Cat. No: MA5-15739; RRID: AB_2537666; Thermo Scientific, Rockford, IL), and anti-PDGF-B (Cat. No: MAB1739; RRID: AB_2299429; R and D Systems, Minneapolis, MN) were also used in this work. All secondary Alexa Fluor-conjugated antibodies, and DAPI were purchased from Invitrogen (Carlsbad, CA, USA).

2.1.1. Fluid percussion injury (FPI)

FPI and sham surgery procedures were performed in 9-week old C57/BI6 mice (20–25 g; Taconic Biosciences, Hudson, NY) based on our standard protocols previously described (Bhowmick et al., 2018a,b; Patel et al., 2017). Briefly, under ketamine/xylazine (mixture of 80 mg/kg ketamine and 10 mg/kg xylazine) anesthesia, a 3-mm craniotomy was performed at 3.0 mm posterior from and 3.5 mm lateral from the bregma in a stereotaxic device to create a circular window exposing the intact dura of the brain. A hollow Luer-Lok syringe hub was then secured over the craniotomy window by Cranioplastic cement (AM Systems Carlsborg, WA) to ensure effective fluid transmission and support. During the surgery process, body temperature (T_b) was continuously monitored and maintained within normal ranges (36.5–37.5 °C) by a feedback temperature controller pad (model TC-1000; CWE Ardmore, PA). 24 h later, the animal was anesthetized with 5% isoflurane until the foot-pinch reflex stopped. The animal was then attached to a digitally controlled fluid percussion injury (FPI) system-FP302 (AmScien Instruments, Richmond, VA) via the syringe hub and the fluid pressure was applied at 7–8 psi and 15–18 psi (8 animals each) with a pressure rise time of 8 ms. The animal was monitored for the duration of the apnea, loss of consciousness (hind-paw withdrawal) and latency to the occurrence of the self-righting reflex immediately after sham or FPI injury to access acute injury severity (Shultz et al., 2011). On resumption of breathing, the syringe hub was removed and the wound sutured and closed. Similarly, control animal underwent the same procedures as the FPI injury-mouse except that the fluid pulse was not given. We used a total of 24 mice (8/group) in injured and control groups for this study. Additionally, another 20 animals (5/group) were used for 0, 12, 24, and 48 h study. This time course study was conducted in 7 psi FPI animals only. 0, 12, 24 and 48 h following TBI injury, the animals were perfused transcardially with ice-cold 1× phosphate-buffered saline (PBS; pH 7.2–7.4) followed by 4% paraformaldehyde in PBS. Brains were removed, post-fixed in 4% paraformaldehyde for 4 h at 4 °C then immersed in 30% sucrose until the brains sank to the bottom of the sucrose solution. Brain tissues were dissected out, embedded in an OCT (optimal cutting temperature) compound and stored at -80 °C until analysis.

2.2. Western blotting

For western blotting, tissue samples (~50 mg) were collected below the injury from the neocortex and sonicated in homogenizing buffer (Thermo Scientific, Rockford, IL) containing a mixture of protease inhibitor (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 14,000 rcf for 10 min at 4 °C to remove unbroken cells and debris. The resulting supernatant collected was then quantified for total protein concentration using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL). Immunoblots were performed by resolving the protein (20 μg) in 4–15% gradient SDS-PAGE gel (Biorad, Hercules, CA) and the blots were transferred onto a nitrocellulose membrane, and blocked with superblock (Thermo Scientific, Rockford, IL) for 1 h. The membranes were incubated with primary antibodies against PDGFR-β, NG2, CD 13, PDGF-B, N-cadherin, connexin-43, Integrin α6, Integrin α3β1, Integrin β1, occludin, Claudin-5, ZO-1, JAM-a, AQP4 and β-actin (1:1000; Thermofisher, St. Louis, MO) for overnight at 4 °C. After washing the membrane three times at 5-min intervals, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:5000; Fisher Scientific, Rockford, IL). The membrane was rinsed 3 times with TBS-tween for 5 min at RT. Protein bands were detected using chemiluminescence western blot detection reagents (Abnova, Walnut, CA) and scanned with an imager, Syngene gel documentation system (Frederick, MD). The optical density was quantified as arbitrary densitometry intensity units using the ImageJ software package (NIH). Protein of interest was normalized and quantified using β-actin as a loading control. We used 6–8 animals' tissue samples for a single protein.

2.3. Immunofluorescence and microscopy

Immunofluorescence staining was performed in 10 μm thickness cryostat-sectioned coronal brain tissue sections as previously described (Abdul Muneer et al., 2012, 2013, 2017a,c; Patel et al., 2017). Briefly, the brain sections were washed with 1× PBS and fixed in 4% paraformaldehyde for 20 min at 25 °C. The sections were then immersed in 1× PBS (pH 7.4) for 5 min, and then blocked in 3% normal goat serum containing 0.1% Triton X-100 (5% heat-inactivated BSA, PBS at pH 7.4) for 1 h at RT. The tissue sections were then incubated for 24 h at 4 °C with the primary antibodies against PDGFR-β, PDGF-B, N-cadherin, connexin-43, occludin, Claudin-5, AQP4, GFAP, and vWF. All antibodies were used at a concentration of 3.0 μg/mL. Cells or tissue sections were then washed in 1× PBS at room temperature for 15 min and incubated with secondary antibodies (Alexa Fluor 488 or 594 conjugated with anti-mouse or anti-rabbit immunoglobulin G (IgG); 1:500 dilution or 4 μg/mL) for 1 h and mounted with 5–10 μL immunomount containing DAPI (Invitrogen) on a slide.

As previously described (Abdul-Muneer et al., 2017c; Bhowmick et al., 2018a,b; Patel et al., 2017), for semi-quantitative analysis of the protein of interest was captured using the Eclipse TE200 fluorescent microscope (Nikon, Melville, NY) and NIS Elements software (Nikon, Melville, NY) in three channels, DAPI (cell nuclei), Alexafluor-488 (green colour emission), and Alexafluor-594 (red colour emission). For imaging, the area of image capture was randomized and was performed by a researcher that was blinded to the experimental conditions. The analysis was performed on at least 4 tissue samples for immunofluorescent staining and captured 6 images from a single sample (slide). Just below the injury or the peri-lesion area, approximately, 1.5 mm^2 area was covered for the analysis. For consistency during image capture, same parameters of camera and software including brightness of the excitation light, the detector sensitivity (gain), or the camera exposure time across the samples or tissue sections for in vitro and in vivo studies was used. The intensity of immunostaining was analyzed by ImageJ (NIH) software (Bankhead, 2014). To correct for uneven illumination in fluorescence images, uniform dark background for all images were kept.

2.4. BBB permeability assay

The effect of 7 and 15 psi FPI on BBB permeability was examined by sodium fluorescein (Na-Fl) and Evan's Blue (EB) tracer dye mixtures (5 μM each) using our animal model of infusion into the common carotid artery (Abdul Muneer et al., 2012, 2013; Alikunju et al., 2011). 24 h after FPI, we infused Na-Fl/EB directly into the right common carotid artery and two hours after the infusion, the animals were perfused transcardially with ice-cold 1× phosphate-buffered saline (PBS; pH 7.2–7.4), followed by 4% paraformaldehyde in PBS. The brains were then removed, dissected, weighed, and homogenized in 600 μl 7.5%(*w/v*) trichloroacetic acid (TCA). Resulting suspensions were divided into two 300 μl aliquots. One aliquot was neutralized with 50 μl of 5 N NaOH and fluorescence was measured on a multifunctional Promega microplate reader (excitation 485 nm, emission 535 nm) to determine Na-Fl concentration. The second aliquot was centrifuged for 10 min at 10,000 rpm and 4 °C, and the EB concentration in the supernatant was measured by absorbance spectroscopy at 620 nm. A standard curve was generated using serial dilutions of EB/Na-Fl solution in 7.5%TCA. We used separate 15 mice (5/group) for this experiment.

2.5. Analysis of cerebral edema

Brain water content was evaluated using the wet/dry-weight method, a reliable measure of posttraumatic brain edema (Armulik et al., 2010). Briefly, 24 h post FPI, brains were rapidly removed from the skull, and the bilateral brains were separated. Both were placed separately into preweighed aluminum foil and samples were then placed in an oven for 72 h at 90 °C and reweighed for dry-weight content. We calculated the percentage of water content using the following equation: ([wet weight—dry weight]/wet weight) × 100. We used separate 17 mice for this experiment, and the number of mice used in each group for brain edema study was as follows: for control (n = 6), for 7 psi FPI (n = 6), and for 15 psi FPI (n = 5).

2.6. Enzyme-linked immunosorbent assay (ELISA)

To determine the cerebral vascular BBB leakage as well as neuronal damage by FPI, we analyzed the neuronal and astrocyte-specific marker proteins in blood serum protein samples from control mice and injury mice subjected to 7 psi and 15 psi FPI. Using commercial ELISA kit, the level of S100β (Abnova, Littleton CO, USA) and neuron-specific enolase (NSE) (Alpha Diagnostic, San Antonio, Texas, USA; Cat. No 0050) were analyzed in tissue lysates as per manufacturer's instructions.

2.7. Statistical analysis

Data processing and statistical analyses were performed using Microsoft Office Excel 2010 and Graph PadPrism6. Data were analyzed with one-way or two-way ANOVA with repeated measures. Significant main effects or interactions were subjected to Bonferroni's correction. The statistical significance level was accepted at the 95% confidence level. Data are expressed as mean ± SEM, and $P < 0.05$ were considered for statistical significance.

3. Results

3.1. TBI down regulates pericyte markers

Our first aim was to show the expression level and role of pericytes in the integrity of BBB, and how brain injury compromises this integrity. Using immunofluorescence imaging analysis for PDGFR-β, a specific marker for pericytes (Winkler et al., 2010), we show that PDGFR-β co-localizes with pericytes on brain capillaries, as illustrated in a brain cortex tissue section of injured and uninjured mice (Fig. 1B). We have not seen the expression of PDGFR-β in other cell types such as neurons, astrocytes, or endothelial cells, as reported (Winkler et al., 2010). In addition, the expression level of PDGFR-β significantly reduced in 7 psi and 15 psi compared to uninjured control samples (Fold change = 1.48, $p < 0.05$ and 2.59, $p < 0.01$; Fig. 1B and C). Further, in immunofluorescence staining with anti-GFAP (astrocyte-specific marker), we noticed that the structural integrity of BBB was lost since tight connections of astrocytes to endothelial cells were lost in 7 psi TBI samples, and the loose connections of astrocytes to endothelial cells exacerbated in 15 psi compared to uninjured samples (Fig. 1B). Next, using western blotting, we analyzed the expression level of PDGFR-β and other well-established pericyte markers, NG2 and CD13 in mouse brain cortex tissue lysate. Western blotting data show that the expression of PDGFR-β, NG2, and CD13 were significantly reduced in 7 psi and 15 psi FPI samples compared to uninjured controls (Fold change for PDGFR-β, NG2, and CD13 at 7 psi and 15 psi are 2.61 and 9.00, 1.98 and 2.58, 2.03 and 2.50, respectively; Fig. 1D–F).

Next, we examined the temporal resolution of expression of these three proteins at 0 (immediately after injury), 12, 24, and 48 h following FPI conducted at 7 psi pressure. In mouse brain tissue lysates, western blotting data show that the level of PDGFR-β decreases the most at 12 h, and then a slow increase was noticed at 24 h and 48 h after

FPI ($p < 0.001$). However, all these values were highly significant when compared with 0 h samples (Fig. 1G), whereas the expression of NG2 and CD13 continuously and significantly reduced at all these three time periods (Fig. 1H and I).

3.2. TBI impairs PDGF-B/PDGFR-β signaling pathway

PDGF-B/PDGFR-β signaling is essential for pericyte recruitment during angiogenesis. Brain pericytes, particularly, regulate key aspects of neurovascular function. Recent studies using pericyte-deficient mouse models ensuing from defective platelet-derived growth factor (PDGF) signaling established that brain pericytes are essential for blood-brain barrier (BBB) function during development (Armulik et al., 2010; Daneman et al., 2010). Pericytes, which express PDGFR-β, are dependent on endothelium-derived PDGF-B for proliferation and migration. In this study, we analyzed the expression of PDGF-B/PDGFR-β signaling and its impairment following TBI. Immunofluorescence data show that the expression level of PDGF-B significantly reduced in 7 psi and 15 psi FPI samples compared to uninjured controls (Fold change = 2.02, $p < 0.001$ and 2.97, $p < 0.001$; Fig. 2A and B). A similar trend was observed in the expression of PDGF-B protein in western blotting in mouse brain cortex tissue lysates. Here, the expression of PDGF-B protein significantly reduced in 7 and 15 psi samples compared to uninjured (Fold change = 2.64, $p < 0.01$ and 4.75, $p < 0.001$; Fig. 2C). In Fig. 1B and D, we have shown the expression of PDGFR-β by immunofluorescence staining and western blotting.

Next, we also examined the temporal resolution of expression of PDFG-B protein at 0, 12, 24, and 48 h following FPI conducted at 7 psi pressure. In mouse brain tissue lysates, western blotting data show a similar trend of result observed in PDGFR-β. Here, the level of PDGF-B decreases the most at 12 h, and then a slow increase was noticed at 24 h and 48 h after FPI ($p < 0.001$). However, all these values were highly significant when compared with 0 h samples (Fig. 1G) as we observed in PDGFR-β.

3.3. Decrease in pericytes down-regulates the expression of basement membrane proteins

Pericytes are located directly on the capillary wall and share a common basement membrane with endothelial cells (Winkler et al., 2011). Both pericytes and endothelial cells are attached to ECM proteins of the basement membrane by different integrins (Diaz-Flores et al., 2009; Stratman et al., 2009), adherent junctional protein, N-cadherin and gap junction protein, connexin-43 (Winkler et al., 2011) (Schematic representation is shown in Fig. 3A). To test the hypothesis that TBI compromises the expression level of integrins and affects the integrity of the BBB, we analyzed the expression level of three different integrins such as integrin α6, integrin α3β1, and integrin β1 by western blotting. The expression level of all these three high molecular weight integrins was significantly reduced in 7 psi and 15 psi 24 h after the injury when compared with uninjured control samples (Fold change for integrin α6, integrin α3β1 and integrin β1 at 7 psi and 15 psi are 4.24 and 9.18, 2.71 and 3.83, 1.63 and 3.38, respectively; Fig. 3A–C); however, the level of statistical significance was varied in these integrins.

Pericytes share a basement membrane with endothelial cells and form direct synaptic-like peg-socket focal contacts with endothelium through N-cadherin and connexins, allowing exchanges of ions, metabolites, second messengers, and ribonucleic acids between the two cell types (Armulik et al., 2011). N-cadherin is an adherent junctional protein that is expressed in brain endothelial cells during brain angiogenesis (Gerhardt et al., 2000). Connexin-43 is a member of transmembrane proteins that form gap junctions. It is one of the dominant brain connexins, which is important for the diffusion of cytosolic factors such as ions and second messenger signaling molecules (Harris, 2007). Since N-cadherin and connexin-43 have significant roles in maintaining the BBB integrity (Zhao et al., 2015), next, we analyzed the level of expression of these two proteins 24 h after FPI. Our double immunostaining results show that the expression of N-cadherin significantly reduced in 7 psi and 15 psi injured samples compared to uninjured controls (Fold change = 1.62, $p < 0.05$ and 2.43, $p < 0.01$; Fig. 4A and B). The expression of N-cadherin clearly co-localized with vWF, a specific endothelial cell marker, in 10 μm brain tissue sections.

A similar trend of results was observed in the expression of connexin-43, and it was significantly decreased in injured samples ($p < 0.01$) when compared to uninjured controls (Fold change = 2.15, $p < 0.01$ and 3.87, $p < 0.001$; Fig. 4C and D). The expression of connexin-43 also co-localized with vWF in brain microvessels. To validate the immunostaining results, tissues collected below the injury area were subjected to western blotting. A significant down-regulation of N-cadherin and connexin-43 proteins were observed at 24 h post-injury compared to uninjured controls (Fold change for N-cadherin and

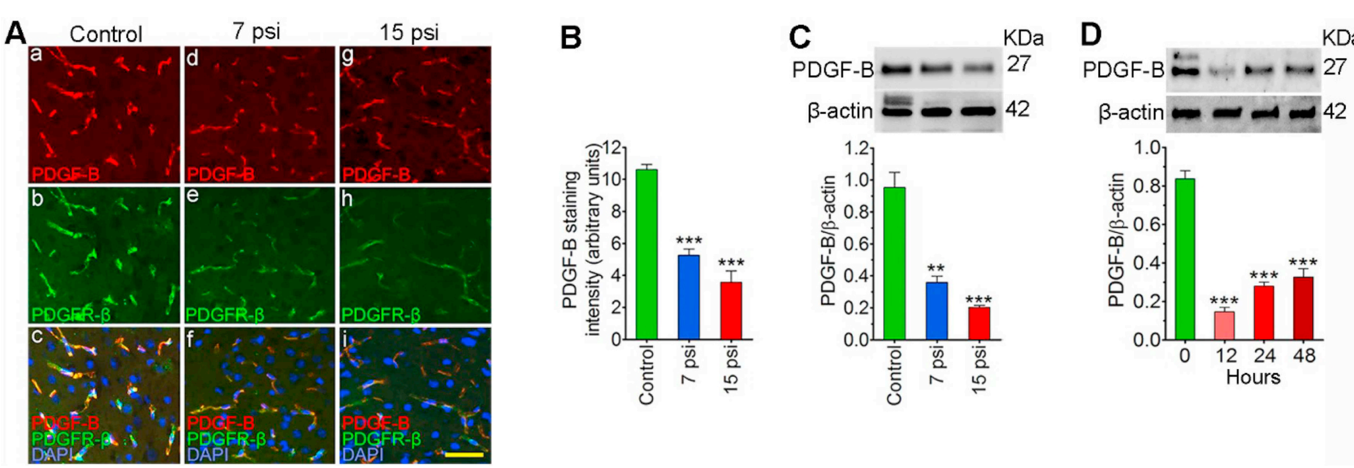

Fig. 2. Brain injury impairs PDGFR-β/PDGF-B signaling pathway. (A) Immunofluorescent staining of PDGF-B (red) merged with PDGFR-β (green) and DAPI (blue) in mouse brain cortex tissue samples of uninjured control, 7 psi, and 15 psi injury 24 h after injury. Scale bar = 40 μm. (B) Quantification of PDGF-B staining analyzed using ImageJ software. Values are mean ± SEM and n = 6/group. ***$p < .001$ versus control. (C) Western blot analysis of PDGF-B in mouse cortical tissue lysates from uninjured control, 7 psi, and 15 psi injury 24 h after injury. (D) Western blot analysis of PDGF-B and its representative β-actin expression at different time points (0, 12, 24, 48 h) in mouse cortical tissue lysates of 7 psi injury samples. Bar diagram represents the results expressed as a ratio of PDGF-B and β-actin bands. All values are represented as mean ± SEM and n = 7/group in A and B, and n = 5/group in C. **$p < .01$, ***$p < .001$ versus control in A and B, and versus 0 h in C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

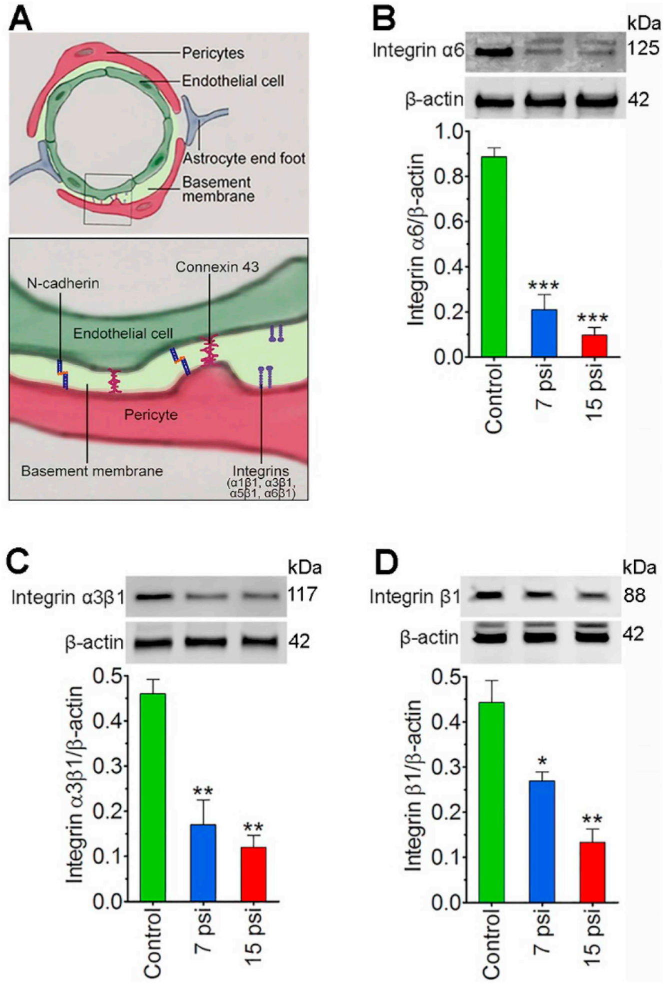

Fig. 3. Decrease in pericyte-endothelium integrity down-regulates the expression of integrin proteins. (A) Schematic representation of the interaction between endothelial cells, pericytes, astrocytes and basement membrane proteins are shown. Down picture shows how both pericytes and endothelial cells are attached to extracellular matrix (ECM) proteins of the basement membrane by different integrins, adherent junctional protein N-cadherin and gap junction protein connexin-43. (B–D) Western blot analysis of Integrin α6 (B), Integrin α3β1 (C) and Integrin β1 (D) in mouse cortical tissue lysates of uninjured control, 7 psi, and 15 psi injury 24 h after injury. Bar diagram represents the results expressed as a ratio of Integrins and β-actin bands. All values are represented as mean ± SEM and n = 6/group. *$p < .05$, **$p < .01$, ***$p < .001$ versus control.

connexin-43 at 7 psi and 15 psi are 2.87 and 4.5, and 2.21 and 5.73, respectively; Fig. 4E and F). Taken together, these results clearly indicate the essential role of pericytes in maintaining the integrity of BBB and that TBI compromises pericyte's ability to fulfill this role.

3.4. TBI downregulates the expression of tight junction proteins

Brain endothelial cells are connected with each other by different types of TJ proteins, forming the BBB (Zlokovic, 2008). Brain pericytes are necessary for the formation of the BBB by regulating important functional aspects governing BBB integrity, including the formation of tight junctions and transendothelial vesicle trafficking (Daneman et al., 2010). Here, we investigated our hypothesis that the loss of pericytes-endothelium integrity compromises the expression or the formation of TJ proteins following brain injury. In double immunostaining, we analyzed the expression of TJ protein occludin in brain tissue sections and co-localized with vWF. The expression of occludin was significantly reduced in 7 psi, and 15 psi injured samples compared to uninjured controls (Fold change = 1.67, $p < 0.05$ and 5.58, $p < 0.001$; Fig. 5A and B). In the cortex region below the site of injury, the expression of occludin was confirmed in endothelial cells by co-localizing the occludin with an endothelial cell-specific marker, vWF (Fig. 5A). Next, we analyzed the expression of claudin-5 in the cross section of intact brain microvessel and co-localized with vWF. A significant reduction in the expression of claudin-5 was noticed in 7 psi, and 15 psi injured samples compared to uninjured controls (Fold change = 1.49, $p < 0.05$ and 1.68, $p < 0.05$; Fig. 5C and D). Further, the FPI-induced decrease in occludin and claudin-5 proteins were validated by western blotting in protein samples extracted from mouse cerebral cortex tissue. A significant decrease ($p < 0.01$) in occludin and claudin-5 were found in injured samples compared to uninjured controls (Fold change for occludin and claudin-5 at 7 psi and 15 psi are 1.70 and 5.00, and 2.48 and 4.95, respectively; Fig. 5E and F).

Next, we analyzed the expression of zonula occudens 1 (ZO-1) and junctional adhesion molecule-a (JAM-a) in the protein lysates extracted from mouse cerebral cortex tissue of 7 psi and 15 psi injured and uninjured samples. Zonula occludens (ZO 1–3) are membrane-associated guanylate kinase proteins that serve as anchoring components for TJ proteins (Gonzalez-Mariscal et al., 2000). The JAMs mediate the early attachment of adjacent cells via homophilic interactions of a single membrane-spanning chain with a large extracellular domain (Del Maschio et al., 1999). JAMs (JAM-a, −b, −c) are involved in the formation and maintenance of the TJ, and they regulate the transendothelial migration of leukocytes in animal models (Del Maschio et al., 1999). Western blotting data shows that the expression of ZO-1 and JAM-a were significantly reduced in brain cortex tissue lysates of 7 psi and 15 psi injured samples 24 h after injury when compared with uninjured controls (Fold change for ZO-1 and JAM-a at 7 psi and 15 psi are 3.08 and 5.71, and 2.23 and 2.48, respectively; Fig. 5G and H).

3.5. BBB disruption causes vasogenic edema

Leakiness of cerebral vasculature due to BBB disruption is often associated with the disruption of water-channel proteins (Obermeier et al., 2013). AQP4 has been presumed to play an important functional role in the transport of water in and out of the brain parenchyma by enhancing transmembrane water flux in the neurovascular unit (Bonomini and Rezzani, 2010; Simard et al., 2003). Thus, we examined the changes in AQP-4 (a water channel protein) and the development of edema around the vasculature by immunofluorescent staining. Indeed, enhanced expression of AQP4 was observed around the perivascular region in the cortical brain tissue sections of 7 psi and 15 psi injured animals (Fig. 6A). We co-localized AQP-4 with an endothelial cell-specific marker, vWF to show that the brain edema formation is around the perivascular unit. Our data showed that AQP4 water channel activation is most likely towards the astrocyte end-feet surrounding the perivascular region (Fig. 6A). This result suggests that cerebrovascular edema formation by AQP4 activation may promote vascular fluid cavitation and neuroinflammation in FPI-induced TBI. Next, the western blot data validated the significantly enhanced expression of AQP4 in brain microvessels of 7 psi and 15 psi injured animals compared to uninjured controls (Fold change = 2.23, $p < 0.01$, and 3.68, $p < 0.001$; Fig. 6B).

The presence of AQP4 at the BBB suggests that it is important for the brain water balance and may play a critical role in brain edema (Nag et al., 2009). Increased water content is a clear evidence of BBB impairment. Thus, next we evaluated the brain edema by analyzing wet:dry weight of injured brain tissue 24 h after 7 psi and 15 psi injury and compared them to the uninjured controls. A significant increase in the water content shows the brain edema, and BBB leakiness in 7 psi and 15 psi injured brain samples (Fold change = 1.27, $p < 0.001$, and 1.40, $p < 0.001$; Fig. 6C).

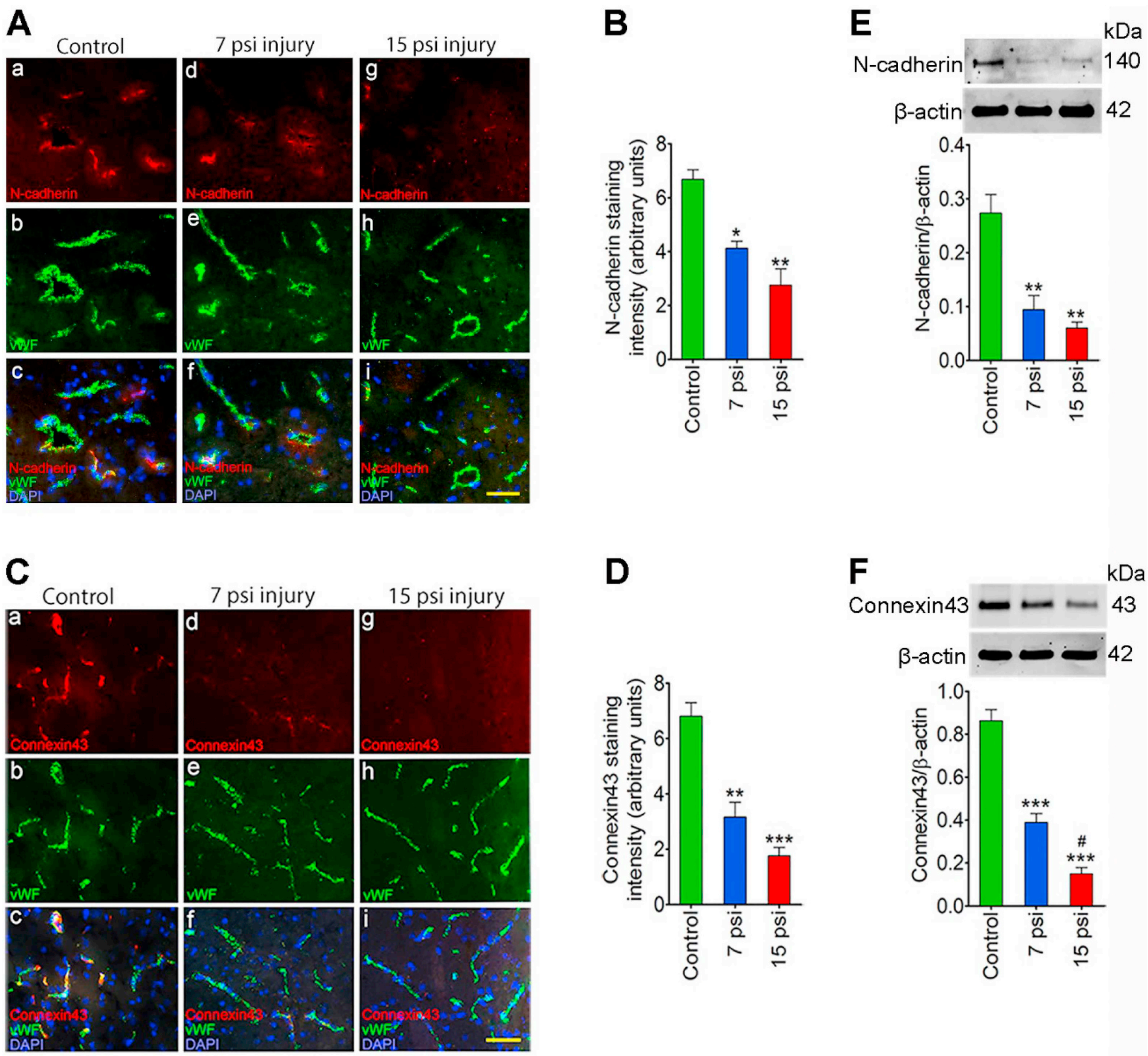

Fig. 4. Impairment of pericyte-endothelium integrity down-regulates the expression of adherent junctional protein, N-cadherin and gap junction protein, connexin 43. (A and C) Immunofluorescent staining of N-cadherin (red) (A) and Connexin43 (red) (C) merged with vWF (green) and DAPI (blue) in mouse brain cortex tissue sections of uninjured control, 7 psi, and 15 psi injury 24 h after injury. Scale bar = 80 μm. (B and D) Quantification of N-cadherin (B) and Connexin43 (D) staining analyzed using ImageJ software. Values are mean ± SEM and n = 6/group. *p < .05, **p < .01, **p < .001 versus control. E-F, Western blot analysis of N-cadherin (E) and Connexin43 (F) in mouse cortical tissue lysates from uninjured control, 7 psi, and 15 psi injury 24 h after injury. Bar diagram represents the results expressed as a ratio of N-cadherin and Connexin-43 to β-actin bands. All values are represented as mean ± SEM and n = 7/group. *p < .05, **p < .01, ***p < .001 versus control; #p < .05 versus 7 psi injury. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Loss of TJ integrity enhances permeability and leakiness of BBB

Disruption of cerebral vascular barrier integrity as a result of TJ proteins, and adherens and gap junction proteins damage was assessed by leaking in and leaking out of biomarkers across the BBB. Disruption of BBB integrity was assayed by analyzing the permeability of Na-Fl/EB tracers across the BBB. Leaking out of brain matters into the blood circulation after 7 psi and 15 psi FPI was analyzed by detecting S100β and neuronal-specific enolase (NSE) in blood samples that are commonly used in TBI event (Berger et al., 2005). We observed that mild (7 psi) and moderate (15 psi) injury greatly increased the permeability of small molecular weight NaFl (MW = 376) and large molecular weight tracer EB (MW = 961) across the BBB compared with respective controls (Fold change for NaFl and EB at 7 psi and 15 psi are 1.70 and 2.06, and 1.98 and 2.60, respectively; Fig. 7A and B). Similarly, 7 psi and 15 psi injured animals also showed significantly higher levels of S100β and NSE in the blood samples compared with controls (Fig. 7C and D). 1.82 times of S100β was found in 7 psi ($p < 0.05$) whereas the level of S100β in 15 psi was around 2.25 times ($p < 0.01$) compared to uninjured controls (Fig. 7C). However, the level of NSE was approximately 2.85 times ($p < 0.05$) and 3.78 times ($p < .01$) in 7 psi and 15 psi respectively, compared to controls (Fig. 7D). The elevation of S100β and NSE in the plasma of TBI animals clearly indicated that degenerated/injured glial/neuronal cell body contents were leaked out of the brain into the circulation.

4. Discussion

Pericytes within the neurovascular unit are capable of regulating many neurovascular functions, including development and maintenance of structural elements of the BBB, integrity of BBB, vascular stability and angiogenesis, and regulation of blood flow at the capillary

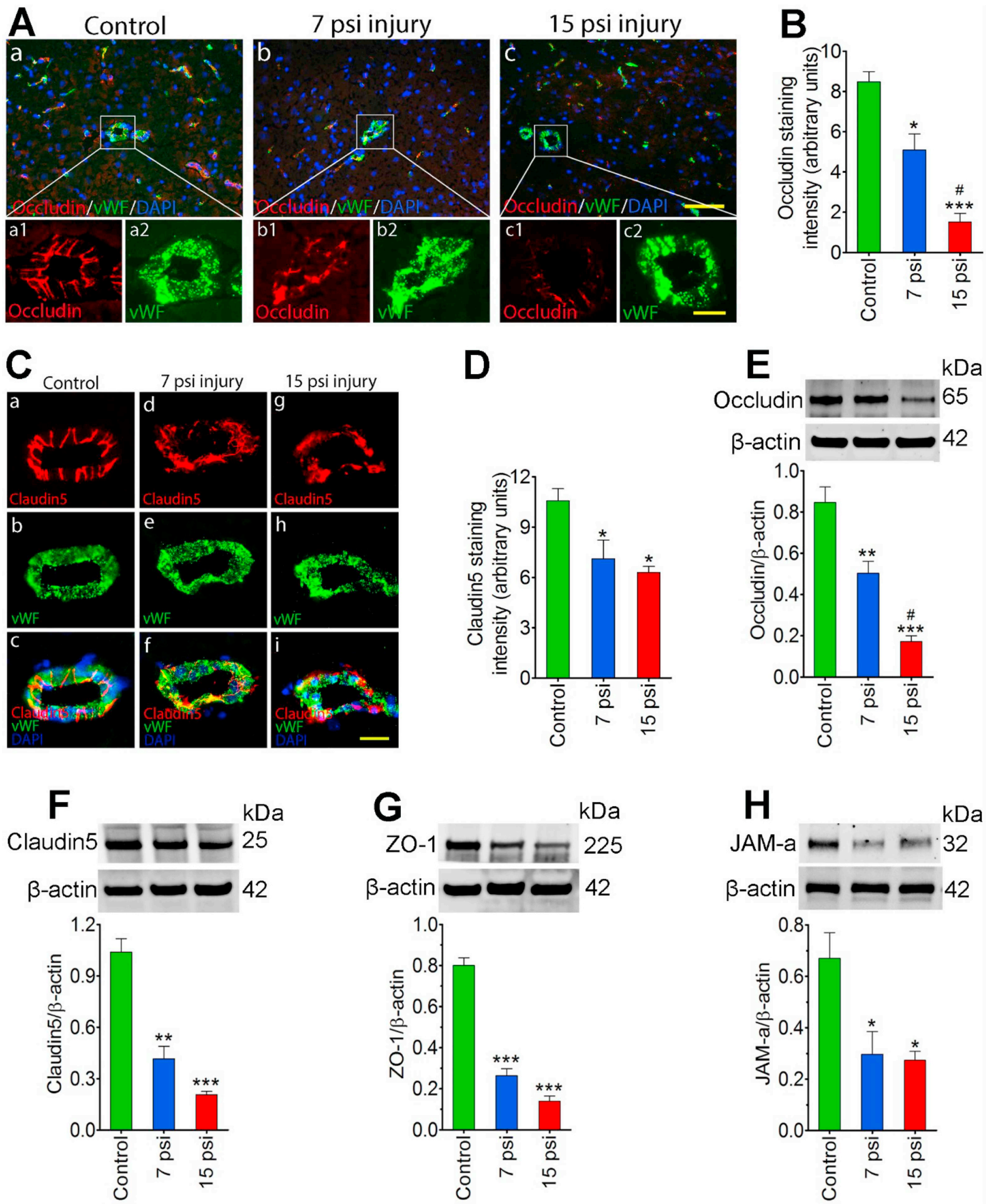

Fig. 5. TBI downregulates the expression of tight junction proteins. (A) Immunofluorescent staining of Occludin (red) merged with vWF (green) and DAPI (blue) in mouse brain cortex tissue sections of uninjured control, 7 psi, and 15 psi injury 24 h after injury. Scale bar = 80 μm in a, b and c, and 10 μm in a1, a2, b1, b2, c1, and c2. (C) Immunofluorescent staining of Claudin-5 (red) merged with vWF (green) and DAPI (blue) in the cross-section of intact brain microvessels of uninjured control, 7 psi, and 15 psi injury 24 h after injury. Scale bar = 10 μm in all panels. (B and D) Quantification of Occludin (B) and Claudin-5 (D) staining analyzed using ImageJ software. Values are mean ± SEM and n = 5/group. *p < .05, **p < .01, **p < .001 versus control; #p < .05 versus 7 psi. (E and H) Western blot analysis of Occludin (E), Claudin-5 (F), ZO-1 (G) and JAM-a (H) in mouse cortical tissue lysates from uninjured control, 7 psi, and 15 psi injury 24 h after injury. Bar diagram represents the results expressed as a ratio of TJ proteins to β-actin bands. All values are represented as mean ± SEM and n = 7/group. *p < .05, **p < .01, ***p < .001 versus control; #p < .05 versus 7 psi. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

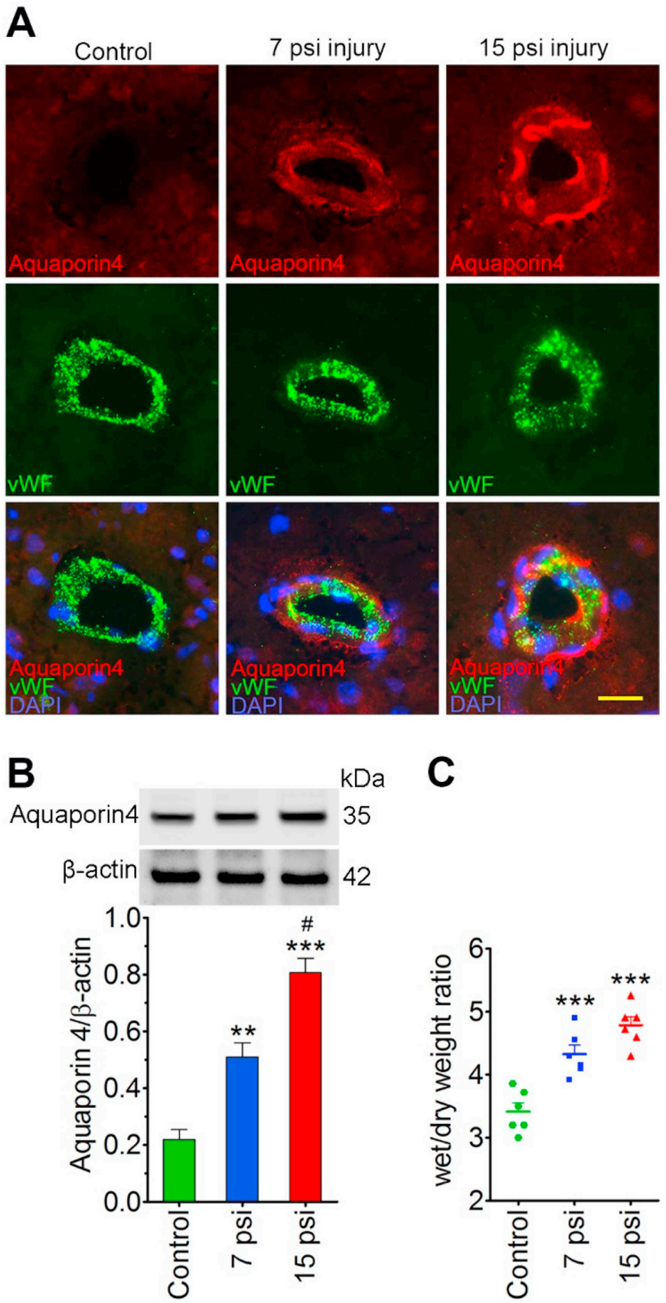

Fig. 6. BBB disruption causes vasogenic edema following TBI. (A) Immunofluorescent staining of Aquaporin4 (red) merged with vWF (green) and DAPI (blue) in mouse brain cortex tissue sections of uninjured control, 7 psi, and 15 psi injury 24 h after injury. Scale bar = 10 μm. (B) Western blot analysis of Aquaporin4 in mouse cortical tissue lysates from uninjured control, 7 psi, and 15 psi injury 24 h after injury. Bar diagram represents the results expressed as a ratio of Aquaporin4 to β-actin bands. All values are represented as mean ± SEM and n = 7/group. $^{**}p < .01$, $^{***}p < .001$ versus control; $^{\#}p < .05$ versus 7 psi injury. (C) Water content was measured by wet/dry ratios to assess BBB leakage in uninjured control, 7 psi, and 15 psi injury mice 24 h after injury. $^{***}p < .001$ versus control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

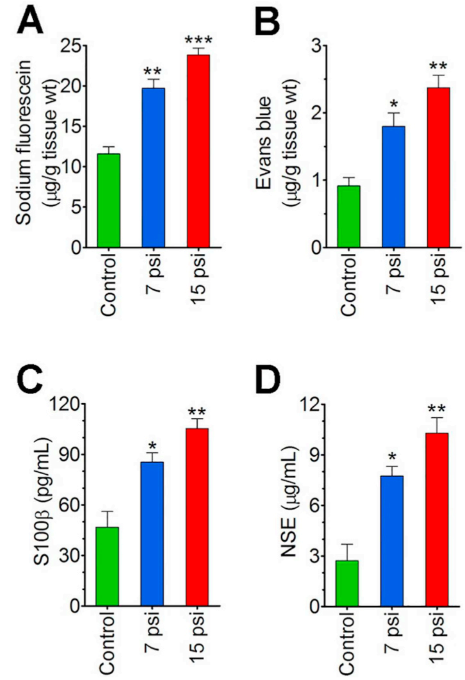

Fig. 7. Loss of integrity enhances permeability and leakiness of BBB. (A-B) Graphical representation of in vivo permeability to show the leakage of Evans Blue (EB, 5 μM) (A) and Sodium fluorescein (Na-Fl, 5 μM) (B) in uninjured, 7 psi, and 15 psi injury mice. The carotid artery of mice was exposed and infused EB-NaFl mixture and collected the brain after one hour of infusion and processed for BBB permeability assay as given in materials and methods. (C-D) ELISA shows the levels of S100β (C) and NSE (D) in the blood serum of uninjured, 7 psi, and 15 psi mice. The blood serum was collected 24 h after FPI. Values are the mean ± SEM and n = 6/group. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

level (Winkler et al., 2011). Pericytes are required for the formation of an integral part of BBB, the tight junctions, and transendothelial vesicle trafficking (Armulik et al., 2010; Daneman et al., 2010). Pericytes exert their physiological functions by communication with endothelium and other cells of the neurovascular unit by several signal transduction cascades (Fisher, 2009). In the present study, using the FPI model in mice, we demonstrate the loss of pericytes and its consequence in the impairment of crosstalk among the other cells, dysfunction of BBB causing its integrity, brain edema, and leakage of cerebral vasculature following TBI. To the best of our knowledge, the present findings are the first to describe the role of pericytes in the mechanisms of impairment of structure and function of BBB following TBI.

There are several molecular markers that have been developed for pericytes (Armulik et al., 2005; Diaz-Flores et al., 2009; Krueger and Bechmann, 2010). However, not all markers are useful from a practical perspective, PDGFR-β, NG2, CD13, αSMA (alpha-smooth muscle actin), and desmin are currently validated and often-used pericyte markers (Huang et al., 2010; Kunz et al., 1994; Lindahl et al., 1997; Winkler et al., 2010). Among these, PDGFR-β appears to be the most specific and reliable, and abundantly expressed in pericytes (Armulik et al., 2011). In this study, we used PDGFR-β, NG2, and CD13 to show the pericyte loss following TBI. Our results show that the expression of all these three markers was significantly reduced in 7 psi and 15 psi injured samples. However, when we analyzed the temporal resolution of these markers at 0, 12, 24 and 48 h, the expression of PDGFR-β was different from that of the other two makers. The expression of PDGFR-β decreased maximum at 12 h and then slowly increased at 24 h and 48 h after FPI, but not as the level of the uninjured samples. Interestingly, we observed a similar trend of the result in the temporal resolution of PDGF-B protein. Even though we do not know the exact reason for this change, one possibility is that the initial disturbed PDGF-B/PDGFR-β signaling slowly rebuilds as time passes. Moreover, the reason for the similar trend of results in PDGFR-β and PDGF-B could be non-availability of the PDGFR-β receptor for the ligand PDGF-B. A decrease in

PDGFR-β expression has been previously reported in models of brain trauma (Zehendner et al., 2015). This decrease in pericyte-specific marker has been suggested to reflect a less differentiated phenotype of these cells (Thanabalasundaram et al., 2011). To recruit pericytes around vessels, brain endothelial cells produce PDGF-B binding to PDGFR-β on pericytes (Bergers and Song, 2005). This receptor possesses significant functions in attracting pericytes to brain endothelium, thereby promising BBB maturation. Additional research into more thoroughly characterizing the heterogeneity of pericyte function along the vascular network is essential for the ultimate understanding of these clearly important cells in the NVU.

The critical part of this study is the analysis of the impairment of PDGF-B/PDGFR-β signaling following FPI. PDGF-B/PDGFR-β signaling is essential for CNS pericytes recruitment (Bjarnegard et al., 2004; Enge et al., 2002). Endothelial secreted PDGF-B binds to the PDGFR-β on pericytes, initiating multiple signal transduction pathways regulating proliferation, migration, and recruitment of pericytes to the vascular wall (Armulik et al., 2005; Lebrin et al., 2010). Interestingly, in immunofluorescent staining, our results show that the injury-mediated diminution of PDGFR-β expression on pericytes affected the expression of PDGF-B on endothelial cells causing impairment of PDGF-B/PDGFR-β signaling. Further, we validated the expression of these two proteins by western blotting. In 2003, Tallquist et al. reported that the number of pericytes presented within the embryonic neural tube correlates with the abundance of PDGFR-β and the number of PDGFR-β signal transduction pathways available for activation(Tallquist et al., 2003). Moreover, the sustained PDGF-B/PDGFR-β signaling in the adult CNS is required for pericyte cell survival (Bell et al., 2010; Geraldes et al., 2009). However, the clear evidence of impairment of PDGF-B/PDGFR-β signaling by the loss of pericytes have established by Lindahl, and Hellstrom using transgenic mice (Hellstrom et al., 1999; Lindahl et al., 1997). Moreover, two 2010 reports demonstrated that brain pericytes are required for BBB function during development using pericyte-deficient mouse models resulting from defective PDGF-B/PDGFR-β signaling (Armulik et al., 2010; Daneman et al., 2010). These works lead us to investigate the dysfunction of BBB causing its integrity, brain edema, and leakage of cerebral vasculature due to loss of pericyte-endothelium interaction following TBI.

Down-regulation of PDGF-B/PDGFR-β signaling is one of the initial cascades associated with brain injury that results in impairment of membrane basement proteins (Arimura et al., 2012). In our study, results indicate that following mild and moderate FPI, perturbation of membrane basement proteins such as integrins are sufficient to affect the integrity of the BBB. Both pericytes and endothelial cells are attached to ECM proteins of the basement membrane by different integrins (Diaz-Flores et al., 2009; Stratman et al., 2009). Endothelial cells and pericytes both express a subset of different mammalian integrins (Albelda et al., 1989; Smith et al., 1996). Here, we analyzed the expression level of three different integrins such as integrin α6, integrin α3β1, and integrin β1; and we provide evidences of integrins mediated anchoring of brain endothelial cells or pericytes to the ECM and their role in the regulation of BBB TJ integrity and permeability. Down-regulation in the expression of integrin proteins reflects a consequence of pericyte loss that correlates to BBB dysfunction. In Osada et al., 2011., show that abrogating β1-integrin-mediated adhesion of brain endothelial cells to collagen IV, a basement membrane component, induced the loss of junctional localization of claudin-5 and causes increased permeability of BBB (Osada et al., 2011). The observations made by Osada et al. direct our attention to the critical role of pericytes/ECM interactions in maintaining BBB properties in the neurovascular unit. Further studies are required to understand the intracellular signals elicited by the integrin-mediated interaction of pericytes and/or endothelial cell to the ECM that ultimately leads to BBB TJ stabilization.

In the neurovascular system, we showed that how the loss of pericyte-endothelium crosstalk affects the expression of N-cadherin, the adherens junction protein (Gerhardt et al., 2000; Li et al., 2011) and the connexin-43 (CX43) hemichannels that form astrocyte-endothelial gap junctions (Chew et al., 2010) and regulates the structural and functional stability of BBB. We found down-regulation of N-cadherin and connexin-43 expressions following 7 psi and 15 psi FPI samples. Since pericytes and endothelial cells are directly connected by N-cadherin and connexin-43 in the neurovascular unit, the loss of pericytes evidently affects the stability and integrity of BBB. Similarly, previous studies have demonstrated that reduction in pericyte coverage in brain injury due to a reduction in PDGFR-β were accompanied by a diminution of TJ protein expression, an indicative of BBB compromise (Luissint et al., 2012). Loss of brain pericyte-endothelium integrity has been shown to increase transendothelial fluid flow (Armulik et al., 2010) and paracellular transport as a result of reduced TJ protein expression (Bell et al., 2010), both causing BBB disruption. Thus, the maintenance of the adherens, gap, and tight junctions between different cell types within the NVU is essential for CNS vascular homeostasis.

It is evident that mild and moderate FPI causes cerebral vascular injury, thus, severe injury is expected to exacerbate cerebral vascular injury. Here, we demonstrate the pathophysiological evidence that disruption of the BBB and perivascular components by mild and moderate FPI contributes to vasogenic edema. The association of cerebral vascular injury with a concomitant reduction of the BBB TJ proteins and the perivascular units with subsequent enhanced AQP4, a water channel protein, justified the notion that vascular barrier break down precedes edema formation. AQP4 has been presumed to play an important functional role in the transport of water in and out of the brain parenchyma by enhancing transmembrane water flux in astrocytes (Simard et al., 2003). Previously, we have reported that the loosening of BBB is mediated by oxidative stress-induced MMPs and fluid channel AQP4 activation in the perivascular units in blast model of mild TBI (Abdul-Muneer et al., 2013). Our findings suggest that TBI promotes edema formation and BBB leakiness by down-regulating integrins, TJ proteins, and adherens and gap junction proteins.

We further interconnected pericyte-endothelium integrity loss and BBB leakage by validating the detection of biochemical and pathological biomarkers with evidence of enhanced BBB permeability (Na-Fl/EB tracers), and neuro-astroglial degeneration as evident by the elevated leakage of NSE/S100β into the bloodstream. Leakage of brain matters from the cerebrospinal fluid into the blood circulation is possible only if the BBB function is impaired and brain cells (pericytes, neurons, astrocyte) in the proximity of the perivascular area are either injured or dead. In summary, our results indicate that both mild and moderate TBI can diminish BBB supportive functions of pericytes and endothelial cells and promote their proinflammatory phenotype, vasogenic edema, and cerebral vascular BBB leakage. Such crucial changes could lead to neurodegeneration in TBI. Importantly, a better understanding of pericyte alterations may provide opportunities for new treatments using this cell type as a target.

Conflict of interest

The authors state that they have no conflict of interest.

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