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## Cerebral microvascular injury: a potentially treatable endophenotype of traumatic brain injury-induced neurodegeneration

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### SUMMARY

Traumatic brain injury (TBI) is one the most common human afflictions, contributing to long-term disability in survivors. Emerging data indicate that functional improvement or deterioration can occur years after TBI. In this regard, TBI is recognized as risk factor for late-life neurodegenerative disorders. TBI encompasses a heterogeneous disease process, in which diverse injury subtypes and multiple molecular mechanisms overlap. To develop precision medicine approaches where specific pathobiological processes are targeted by mechanistically appropriate therapies, techniques to identify and measure these subtypes are needed. Traumatic microvascular injury is a common but relatively understudied TBI endophenotype. In this review, we describe evidence of microvascular dysfunction in human and animal TBI, explore the role of vascular dysfunction in neurodegenerative disease, and discuss potential opportunities for vascular-directed therapies in ameliorating TBI-related neurodegeneration. We discuss the therapeutic potential of vascular-directed therapies in TBI and the use and limitations of preclinical models to explore these therapies.

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#### DECLARATION OF INTERESTS

Dr. Diaz-Arrastia is a member of the Professional Advisory Board of Neural Analytics, Inc., and he also holds an equity position in that company.

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## Keywords

traumatic brain injury; cerebrovasculature; Alzheimer's disease; neurodegenerative disease; blood-brain barrier; biomarker

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## INTRODUCTION

Traumatic brain injury (TBI) is a prevalent condition affecting all ages, races, and socioeconomic classes throughout the world. In the US alone, there are at least 2.8 million Emergency Department visits for TBIs annually, though many more TBIs likely go unreported (Taylor et al., 2017). The incidence of TBI is disproportionately higher in low and middle-income countries, where TBIs are the leading cause of death and disability in young adults (Maas et al., 2017). Because head injuries often affect young people in their most productive years, the cumulative loss of productivity is high compared to other injuries and illnesses (Max et al., 1991). Even mild TBI (mTBI), often termed concussion, which accounts for 80–90% of all TBIs, results in a tremendous societal and economic burden and accounts for up to 44% of the total lifetime costs of TBI (Max et al., 1991). As the population ages, TBIs from falls in older persons are becoming increasingly prevalent, and are associated with more morbidity and mortality due to coexisting medical illness, anticoagulant use, and slower recovery trajectories (Peters and Gardner, 2018). Public health initiatives focused on injury prevention through bike helmets, fall prevention, seat belt use, sport impact policy changes and other public safety measures have been very effective in reducing the incidence of and the mortality associated with severe TBI (Taylor et al., 2017). Clinical management has focused on controlling intracranial pressure and cerebral edema (Chesnut et al., 2012), oxygen deprivation (Okonkwo et al., 2017), or brain metabolic demand (Andrews et al., 2015) in cases of severe injury, though the effects of these interventions on clinical outcomes has been disappointing (Maas et al., 2017). Furthermore, there are no therapies that diminish the burden of disability resulting from mild TBI, which has historically been ignored by the health care system (Levin and Diaz-Arrastia, 2015).

TBI has traditionally been conceptualized as a primary injury event, caused by an initial mechanical impact, followed by secondary insults due to the molecular and cellular responses in reaction to the primary injury. Secondary injury can propagate a trauma-induced cascade in the surrounding brain tissue. This secondary injury, even in the most severe cases, was thought to extend only for a few weeks or at most a month, followed by a trajectory of recovery that was generally thought to be largely complete within months or at most, one year. Evidence accumulated over the past decade has led to a recognition that for many patients, the consequences of TBI continue to evolve long after the acute period of initial recovery (Wilson et al., 2017). Longitudinal studies have shown that outcomes following TBI are not fixed, as there can be improvement and/or deterioration in neurological functioning many years after injury (Corrigan and Hammond, 2013; Wilson et al., 2017). TBI can therefore be best conceptualized as a chronic health condition triggered by the injury which initiates long-lasting and still poorly understood downstream events that can impact brain functioning for decades (Corrigan and Hammond, 2013), having life-long effects on multiple health outcomes (Masel and DeWitt, 2010).

Given this complexity, there is an urgent need to better understand the specific pathophysiological mechanisms contributing to TBI-related dysfunction in the both acute and chronic phases of disease. In animal models, interventions aimed at molecular targets involved in secondary injury have been successful in limiting the extent of injury and improving neurologic recovery (Marklund et al., 2006; McIntosh et al., 1998). These results provide convincing proof of principle that effective therapeutic intervention is possible, but therapeutic efficacy has yet to be achieved in the human condition. In this review, we specifically focus on TBI-related injury of the cerebral microvasculature, discuss the relationship of vascular injury to chronic neurodegenerative sequelae of TBI, and highlight opportunities for preclinical and clinical studies to improve our understanding of this disease process and promote the development of effective therapies.

## **TRAUMATIC VASCULAR INJURY FOLLOWING TBI**

### **The neurovascular unit**

The brain is critically dependent on a steady blood supply that is acutely responsive to the constantly changing metabolic demands of the brain tissue. To accomplish this, the brain parenchyma is served by a vascular network of arteries, arterioles, capillaries, venules, and veins that runs approximately 400 miles in length (Sweeney et al., 2018). Several cell types distributed along this network act in concert to regulate cerebral blood flow, vascular permeability, and micronutrient supply. Collectively, these cells are termed the neurovascular unit (NVU) (Shlosberg et al., 2010). The NVU consists of the endothelial lining of the blood vessels, smooth muscle cells (at the artery/arteriole/venule levels), and pericytes (at the capillary level; Figure 1A), which help to regulate vascular tone, neurons, and perivascular astrocytes. The endothelial cells form a monolayer consisting of intercellular tight and adherens junctions to form the blood-brain barrier (BBB), 85% of which is provided by the capillary endothelium (Sweeney et al., 2018b). This BBB forms an exquisitely regulated barrier between the systemic vasculature and the brain parenchyma. All components of the NVU undergo continuous crosstalk under normal physiological conditions to form an integrated and keenly responsive system to changing cerebral and systemic factors. This process, termed neurovascular coupling, ensures consistent cerebral blood flow and micronutrient supply across the BBB as a function of neuronal activity.

Following brain injury, these normal patterns of communication among the elements of the NVU can be severely altered (Figure 1B). Disturbed NVU functioning leads to inappropriate changes in cerebral blood flow in response to the altered metabolic demands of the injured brain. Dysfunction of the BBB disrupts the extracellular environment due to protein and electrolyte leakage and can trigger other downstream processes, like microglia activation and recruitment (Shlosberg et al., 2010; Zlokovic, 2011). Emerging evidence, as we discuss below, indicates that these disruptions last far longer than previously assumed and may contribute to ongoing neuropathology long after the primary injury.

### **Evidence from human patients**

Clinical evidence indicates that microvascular dysfunction extends across the spectrum of TBI-related injury. Histologically-detected ischemic damage is seen in nearly 60% of fatal

TBI without evidence of large vessel occlusions (Graham and Adams, 1971). In moderate-severe TBI, vasospasm of larger cerebral arteries can precipitate cerebral ischemia (Martin et al., 2009), but more universally trauma-induced vascular injury occurs at the arteriole/capillary level (Bouma and Muizelaar, 1992). Rodríguez-Baeza and colleagues created vascular corrosion casts to examine the cerebral microcirculation in patients who died following severe head trauma (Rodríguez-Baeza et al., 2003). They found that arterioles and capillaries in the middle and deep cortical vascular zones showed extensive injury characterized by sunken endothelial surfaces, longitudinal folds in the vessel wall, decreased lumen diameter, and corrugations, indicating a separation between the endothelium and smooth muscle cells and a disrupted BBB. Notably, larger pial and subpial vessels were histologically normal. In nonfatal head injury, cerebral intravascular microthromboses seem to be a near universal feature; Stein et al. observed intravascular thromboses in arterioles and venules in rodent, pig, and human TBI specimens (Stein et al., 2002). Multifocal BBB disruption has been observed in American football players who have sustained subconcussive injuries (but not clinical signs of concussion) using the same dynamic contrast enhanced MRI technique utilized in studies of BBB disruption in AD (Weissberg et al., 2014). At the endothelial level, endothelin-1, a peptide that acts as a vasoconstrictor, is upregulated following TBI (Chatfield et al., 2011; Maier et al., 2007; Salonia et al., 2010) and activates vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and other inflammatory pathways (Koyama et al., 2007, 2012). These molecules, in turn, contribute to further BBB disruption.

Microvascular injury sequelae are not limited to the acute period following the primary injury. Hay et al. showed that 47% of brains examined from long-term TBI survivors (1–47 years post-injury) exhibited histological evidence of multifocal BBB breakdown, as demonstrated by fibrinogen and immunoglobulin G immunostaining, in perivascular and parenchymal gray matter (Hay et al., 2015). Tomkins et al. used contrast-enhanced MRI to evaluate subjects with relatively mild head injuries (GCS>13) assessed in the subacute (1 week)- chronic (3 months) period after injury (Tomkins et al., 2011). They found that those subjects who developed post-traumatic epilepsy were more likely to have BBB disturbance than those who had a history of TBI but did not have epilepsy (82% vs. 25%). In 4 subjects, BBB disruption was evident using this MRI technique 1.5–11 years after injury. Doherty et al. examined a case of chronic traumatic encephalopathy (CTE) using immunohistochemical techniques to examine the expression of the BBB tight junction proteins claudin-1 and zona occludens-1 (Doherty et al., 2016). Perivascular p-tau was present at sites with evidence of decreased tight junction protein expression. Fibrinogen and immunoglobulin G extravasation, indicative of BBB dysfunction, was also evident at these sites. Similarly, Tagge et al. found focal cortical lesions with perivascular accumulation of immunoglobulin G in 4 cases of mild concussive injury examined in the subacute-chronic period after injury (Tagge et al., 2018). These findings are consistent with extravasation and accumulation of serum proteins at sites of focal microvascular injury. BBB disruption in the acute period after severe brain injury, determined by a cerebrospinal fluid (CSF)/plasma protein ratio > 0.007, may correlate with worse long-term outcome (Ho et al., 2014), though the prognostic significance across the TBI spectrum needs to be further explored.

In addition to these changes at the BBB, cerebral blood flow (CBF) has been extensively studied after TBI in humans, mostly in the acute period within a few days of injury (Furuya et al., 2003; Menon, 2006) although several studies have examined CBF weeks to years after TBI (Kim et al., 2010). There is a consistent body of literature in humans indicating that deficits in CBF are common after TBI, including repetitive mTBI (Bonne et al., 2003). Studies using single photon emission computed tomography (SPECT) to measure regional CBF in patients with chronic TBI (Barkai et al., 2004; Bonne et al., 2003; Lewine et al., 2007) have consistently found regions of hypoperfusion in a subset of symptomatic TBI subjects (Raji et al., 2014). SPECT perfusion changes significantly correlated with neuropsychological or neurological deficits. Studies using Xenon-CT report similar findings (Lewine et al., 2007). In addition to nuclear medicine studies, advanced MRI techniques have also been helpful in evaluating traumatic microvascular injury. Arterial Spin Labelling (ASL) reveals alterations in global and regional resting CBF in TBI patients of all severities. Kim et al. showed that patients with chronic moderate-to-severe TBI have reduced global CBF, as well as decreased regional perfusion in the thalamus, posterior cingulate cortex, and frontal cortex (Kim et al., 2010). Regions with decreased resting CBF also had altered task-related activation during an ASL fMRI working memory paradigm in chronically injured subjects. Regional relative CBF can also be measured with perfusion-weighted imaging (Bartnik-Olson et al., 2014). The exact causes of these alterations in CBF following TBI are unclear. Decreased CBF may result from a lower metabolic demand from injured tissue, resulting in an appropriately matched reduction in blood flow. However, studies using fluorodeoxyglucose positron emission tomography (FDG-PET) have suggested that glucose metabolism is disrupted following TBI, but can be increased, decreased, or unchanged in ways that do not clearly correlate with structural abnormalities (Ito et al., 2016; Yamaki et al., 2018).

The studies above describe alterations in resting CBF following TBI. In addition, cerebrovascular reserve, or the ability of the cerebral vasculature to react to vasodilatory or vasoconstrictive stimuli, termed cerebrovascular reactivity (CVR), can be altered after TBI. Breath holding (resulting in induced hypercapnia), hyperventilation, CO<sub>2</sub> inhalation, or acetazolamide administration can be used in conjunction with non-invasive imaging techniques to assess CVR (Kassner and Roberts, 2004). Transcranial Doppler (TCD) ultrasound, near infra-red spectroscopy (NIRS) and magnetic resonance imaging (MRI) have been the most popular methods to examine post-TBI CVR in recent studies. TCD offers the advantage of very high temporal resolution but suffers from poor spatial resolution. A large prospective study of 299 acute TBI patients assessed the incidence of cerebral vasospasm via TCD (Oertel et al., 2005). Nearly half of the patients met at least one TCD criterion for vasospasm. After the acute stage, studies of professional boxers exposed to repetitive mild TBI showed that CVR was reduced in the subacute period when measured with both TCD and NIRS particularly in boxers who had experienced the highest mTBI exposures (Bailey et al., 2013). In the boxers, lower CVR measurements correlate with worse neurocognitive dysfunction and were inversely correlated with head injury exposure. A recent meta-analysis identified three studies examining sport-related concussion and CVR via TCD in 42 athletes (primarily boxers and hockey players) and 33 healthy controls (Gardner et al., 2015). All three studies found decreased CVR in the acute period after a mTBI.

NIRS has been used to study CVR after TBI as well as other neurologic conditions (Lee et al., 2009; Rodriguez Merzagora et al., 2014; Zweifel et al. 2010). Changes in CBF and associated changes in tissue concentrations of oxy- and deoxy-hemoglobin can be measured by NIRS. Like TCD, NIRS offers a very high temporal resolution, and while spatial resolution is superior to TCD, it is not as high as MRI. NIRS allows for reliable CVR measurements over time during dynamic challenges that are independent of hemoglobin concentration, skull thickness and extracranial circulation (Kainerstorfer et al., 2015). NIRS has been used to assess the CVR index in 37 acute TBI patients in the intensive care unit (Diedler et al., 2011). NIRS has also been carried out in the chronic stage of TBI. In chronic moderate and severe TBI patients (mean 18 years after TBI), CVR remained significantly reduced compared to 15 age-sex matched controls (Rodriguez Merzagora et al., 2014), indicating that CVR disruption is a long-lasting deficit in at least a subset of patients. Notably, CVR abnormalities corresponded to cognitive performance.

CVR can also be measured with MRI coupled with a hypercapnia challenge (Amyot et al., 2017; Mutch et al., 2016a, 2016b). CVR is more sensitive than CBF for discriminating between injured and uninjured participants. Even in the absence of frank structural or CBF abnormality, impaired response to induced hypercapnia (end-tidal CO<sub>2</sub> = 50 mmHg) can be observed years after even relatively minor injuries (Amyot et al., 2017; Haber et al., 2018), indicating that cerebrovascular reserve is chronically impaired in some patients following TBI. It should be noted that, while these responses are abnormal in TBI subjects compared to healthy, uninjured control subjects, they do not address whether the brain tissue served by these vessels experiences metabolic distress in the form of impaired oxygen and glucose delivery.

### **Evidence from preclinical animal models**

Animal models of head injury have furthered our understanding of the vascular sequelae of TBI. Animal models allow studies of TBI-related vascular pathology using tools that are not practical or ethical in human patients. In animal models, BBB extravasation is typically studied histologically. Common methods include immunostaining for endogenous markers such as immunoglobulin G (IgG) and fibrin, or fluorescent detection of exogenous Evans blue or sodium fluorescein dyes (Qin et al., 2017; Yao et al., 2015; Zhiyuan et al., 2016). Additional tools include calculation of brain water content for evidence of edema (wet/dry weight ratio), vascular density through immunohistochemistry, or biochemical changes in tight junction protein or mRNA expression in whole, half, or micro-dissected brain lysates (Alluri et al., 2016; Blixt et al., 2015). Emerging functional assessments focus on CBF and intracranial pressure, and can be measured by ultrasonogram (Clevenger et al., 2015) and telemetry (Kawoos et al., 2016) respectively. As this field advances, a focus on simple, translational biomarkers to assess cerebrovascular impairment that can be applied clinically as well as in animal models are highly desirable.

Common vascular phenotypes following rodent and porcine fluid percussion injury (FPI), controlled cortical impact (CCI), blast, and other closed head injury (CHI) models include increased albumin permeability (Bhowmick et al., 2019; Johnson et al., 2018a; Qin et al., 2017; Yao et al., 2015; Zhiyuan et al., 2016), increased leukocyte adhesion to the vascular

endothelium (Li et al., 2017; Schwarzmaier et al., 2013), decreased expression of tight junction proteins (Alluri et al., 2016; Bhowmick et al., 2019; Blixt et al., 2015), widening of intracellular junctions (Sangiorgi et al., 2013), acute pericyte loss (Washington et al., 2018), and disrupted cross-talk between pericytes and endothelial cells (Bhowmick et al., 2019). CBF decreases after rotational injury and FPI (Clevenger et al., 2015; Wang et al., 2016), whereas intracranial pressure has been reported to increase following blast injury (Kawoos et al., 2016) and CCI (Moon-Massat et al., 2017). Both phenomena are hypothesized to result from endothelial disruption and dysfunction that subsequently affects the homeostatic regulation of vascular tone (Villalba et al., 2017).

Injury-induced decreases in CBF and microvascular circulation can result in microthrombi formation (Schwarzmaier et al., 2016) and tissue hypoxia (Bragin et al., 2017). Compared to sham animals, animals exposed to CCI had a greater incidence of stationary leukocyte-platelet aggregates in parenchymal capillaries (Schwarzmaier et al., 2016). Microthrombi aggregation can lead to secondary injuries including hematoma formation and edema. Beyond local changes, vasogenic edema can also cause gross structural changes akin to those seen in humans after TBI, such as hemispheric expansion and midline shift, after CCI (Liu et al., 2017) or CHI (Blixt et al., 2015). Models of milder head impacts, designed to simulate concussive injuries, have shown similar disturbances of the microvasculature, including endothelial irregularities, dysmorphic capillaries, and disruption of pericytes and perivascular astrocytes at the ultrastructural level (Tagge et al., 2018). Thus, vascular pathology following TBI in animal models can be substantial, depending on injury type and severity, much like their human analogues, though exactly how the post-injury cerebrovascular phenotype varies based on the mechanism of injury induction is not yet known.

Animal studies have reported increased expression of matrix metalloproteinase-9 (MMP-9) post-CCI (Alluri et al., 2016; Shi et al., 2015; Tao et al., 2017; Washington et al., 2018). Further, compared to wild-type controls, MMP-9 knockout mice displayed attenuated vascular permeability measured by extravasation of exogenous fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) following CCI (Muradashvili et al., 2016), which correlated with improved behavioral performance on cognitive tasks. Suppression of MMP-9 also mitigated long term gross tissue damage following CCI (Shi et al., 2015). Inhibition of claudin-5, a tight junction protein important for small molecule transport across the BBB, using a short interfering RNA approach increased water transport across the BBB leading to cerebral edema, suggesting that claudin-5 may be an attractive target for acute management of TBI-related BBB dysfunction (Campbell et al., 2012). Inhibition of endothelin-1 receptors decreased expression of VEGF and MMPs and decreased brain edema in a FPI mouse model (Michinaga et al., 2018), similar to the human condition. Aquaporins are also an integral part of vascular homeostasis and are postulated to have a role in water influx and efflux. Genetic deletion of aquaporin-4 (AQP-4) prevented cytotoxic edema and reduced intracranial pressure after CCI, but did not affect extravasation outcomes or CSF tracer uptake (Smith et al., 2017; Yao et al., 2015). Notably, AQP-4 expression is altered in human AD and animal AD models and may play a role in A $\beta$  clearance and degradation (Yang et al., 2016). It is important to note, however, that the mechanisms by

which CSF and interstitial fluid flow and perivascular drainage pathways govern the clearance of brain waste products, before and after TBI, is not fully understood.

## TBI-RELATED NEURODEGENERATION

Neurodegenerative disease following TBI was first described in professional boxers in the 1920's who sustained repeated head trauma (Martland, 1928). Termed dementia pugilistica, it was appreciated that a history of head impacts is associated with the development of AD and other dementias (Barnes et al., 2018; Gardner et al., 2014a), PD (Gardner et al., 2018), and/or amyotrophic lateral sclerosis (Sweeney et al., 2018) later in life. Though far from universal (Weiner et al., 2017), the possibility of these long-term consequences are understandably of great concern to patients and their families. Epidemiological studies aimed at better understanding this relationship have been mixed. It has long been recognized that moderate and severe TBI in early and mid-life is associated with an increased risk of late-life dementia, with relative risks (RRs) in the order of 2.5–5.0 (Fleminger et al., 2003; Guo et al., 2000; Plassman et al., 2000). Several prospective observational studies failed to establish a relationship between mTBI, which is a far more common injury, and later-life dementia (DamsO'Connor et al., 2013; Mehta et al., 1999; Weiner et al., 2017). More recently, careful epidemiologic studies indicate that mTBI is also associated with increased risk of dementia, with RRs in the order of 1.3–2.0 (Gardner et al., 2014; Nordström et al., 2014; Wang et al., 2012). RRs for those who sustain multiple mild TBIs approach those associated with a single severe TBI (Nordström et al., 2014). Additional, detailed prospective studies will be critical to better understand the patient and injury characteristics that associate with later neurodegeneration.

It is often asserted that TBI-associated dementia is similar to Alzheimer's disease (AD) (Fleminger et al., 2003), which is the most common type of dementia in the general population. However, prior studies on the etiology of dementia associated with TBI have used chart reviews or clinical interviews for dementia ascertainment, which are recognized to have a low specificity (Kukull et al., 1990). No prior study of TBI-associated dementia has used pathologic confirmation of the dementia subtype, which is recognized as the gold standard. Furthermore, no prior studies have used modern neurodiagnostic tools such as neuroimaging or biomarker assays in CSF, serum, or plasma, which are recognized to provide refinements over the clinical diagnosis alone (Jack et al., 2011; Walhovd et al., 2010). Despite the substantial social burden imposed by the long-term neurodegeneration after TBI, very little is known about the pathophysiology involved, there are no biomarkers that are prognostic for identifying individuals at risk for progressive neurodegeneration, and as a consequence, there are no effective therapies to prevent or slow this process. It is unclear whether brain trauma in early or mid-life leads to an acceleration and higher risk of AD-type pathology, or whether TBI-associated dementia is a distinct pathologic entity.

Much recent attention has focused on the neurodegenerative sequelae of those exposed to mild repetitive subconcussive injuries, such as those sustained as a result of multiple sport-related impacts similar to those diagnosed with dementia pugilistica in Martland's original case series (Changa et al., 2018). Now called CTE, the diagnosis can only be made upon neuropathological examination after death. While there has been much lay media attention



on the clinical features of CTE, which can include memory impairments, personality changes, and neuropsychiatric disturbances, the clinical criteria have not been rigorously defined. The importance of accurately portraying the diagnostic challenges of CTE has recently been highlighted (Stewart et al., 2019). The neuropathology of CTE is similarly complex, consisting of deposits of tau and amyloid- $\beta$  ( $A\beta$ ), neuronal and axonal loss, and gliosis. The most consistent neuropathological characteristic of CTE neuropathology is accumulation of phosphorylated tau protein (p-tau) in perivascular regions and at sulcal depths (McKee et al., 2016). Although  $A\beta$  pathology can be observed following TBI, tau pathology is prominent both in CTE and in long-term survivors after a single moderate-severe TBI.  $A\beta$  plaques can be seen after acute TBI (Gentleman et al., 1997; Graham et al., 1995), but are more rarely seen in long-term survivors and, when present, are more likely to be in a mature fibrillary form (Johnson et al., 2012). Emerging preclinical evidence suggests that this variability in pathology may be influenced by the age at injury and underlying genetic factors, and most intriguingly, may influence post-TBI behaviors (Cheng et al., 2018, 2019).

## VASCULAR DYSFUNCTION AND NEURODEGENERATION

Given that vascular dysfunction is prevalent and persistent following mild-severe TBI and neurodegeneration is a late consequence following some TBIs, it stands to reason that the two may be mechanistically linked. To date, much of what we know about vascular dysfunction and neurodegeneration has been described in the context of AD, where there is mounting evidence that vascular dysfunction plays a role in pathogenesis (Snyder et al., 2015; Sweeney et al., 2019a). Patients with AD and related dementias frequently exhibit alterations in the brain microcirculation, including decreased capillary density, fibrohyalinosis of microvessel walls, loss of tight and adherens junctions, and increased BBB permeability (Farkas et al., 2006; Zlokovic, 2011). The neuropathological hallmarks of AD include  $A\beta$ -containing plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein. The two-hit vascular hypothesis of AD proposes that blood vessel and BBB disruption are the initiating events leading to  $A\beta$  deposition and accumulation in the brain (Sweeney et al., 2018). Recent evidence suggests that alterations in BBB permeability can be detected at the earliest stages of cognitive decline. Changes in the CSF pericyte soluble platelet-derived growth factor (PDGF) receptor- $\beta$  marker could be detected before changes in the AD biomarkers  $A\beta$  and tau were evident in subjects with very mild cognitive dysfunction (Nation et al., 2019). Observational data suggest that treatment of vascular risk factors like hypertension and hyperlipidemia are associated with a decreased risk of AD, but rigorous, prospective studies are lacking (Valenti et al., 2014). There are several different (and not mutually exclusive) ways in which vascular dysfunction contributes to neurodegenerative pathology, including hypoperfusion/ischemia, BBB breakdown, and endothelial dysfunction (Zlokovic, 2011).

### Hypoperfusion/Ischemia

Neurovascular coupling, where CBF is regulated to precisely match neuronal activity and cellular metabolism in the surrounding brain tissue, is particularly relevant. For neurovascular coupling to occur properly, vascular smooth muscle cells and pericytes, at the

arteriole and capillary levels respectively, modulate vessel diameter to regulate blood flow to neurons (Hill et al., 2015; Iadecola, 2004; Kisler et al., 2017; Yemisci et al., 2009). Mild decreases in CBF affect neuronal protein synthesis and synaptic plasticity. More marked decreases in CBF decrease ATP generation, disturbs neuronal electrolyte balances, and impairs action potential propagation, resulting in severe neuronal dysfunction. When CBF is decreased by >80%, neuronal cell death ensues (Zlokovic, 2011). Reductions in CBF have been observed in individuals at high risk of AD even before the onset of cognitive decline (Ruitenber et al., 2005). In animal models, hypoperfusion can induce AD-like neuropathological changes, including accumulation of A $\beta$  and hyperphosphorylated tau, and cause CBF reductions. The mechanisms and sequelae by which cerebral blood flow reductions contribute to neuropathology are beginning to be understood, including hypoxia inducible factor-1 $\alpha$  (HIF1 $\alpha$ )-dependent increases in A $\beta$  production as well as alterations in transporters required for A $\beta$  clearance (Zhang et al., 2007). Interestingly, prior to the onset of cognitive impairment and significant cortical A $\beta$  deposition, microvascular pathologies in the form of microaneurysms, have been detected in 4–5 months old APP/PS1 mice, a preclinical model of AD (Kelly et al., 2017). In a pharmacologically induced-model of PD, acute, sub-acute, and chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure led to decreased vessel intensity, length and number within the nigrostriatal pathway (Sarkar et al., 2014). These vascular phenotypes in particular may be intimately related to detectable changes in CBF, suggesting a general vulnerability of the cerebrovasculature in preclinical models of neurodegeneration. Even in the absence of frank CBF changes, there is also evidence of impaired CVR in AD (den Abeelen et al., 2014; Yezhuvath et al., 2012), reflecting an impairment in cerebrovascular reserve. Thus, even when absolute CBF is not impaired at baseline, the vascular response may not be appropriate when physiologically stressed (Len et al., 2011; Lynch et al., 2016).

As cannabinoids similarly regulate vascular tone, mechanistic studies have explored the role of the cannabinoid receptors CB1 and CB2 in this process, including elucidating the role of a potential atypical endothelial cannabinoid receptor (Bondarenko, 2014). Cannabinoid receptor activation causes vascular relaxation through direct inhibition of smooth muscle contractility as well as stimulating the release of endothelium-derived vasodilators. Under stress conditions, however, cannabinoid receptor activation can reduce CBF. Amenta and colleagues found that mice with genetic deletion of CB2 had increased BBB extravasation, as demonstrated by sodium fluorescein uptake (Amenta et al., 2014). While little human data are available, expression of CB1 and CB2 is altered in AD brains (Aso and Ferrer, 2016; Solas et al., 2013).

### **Blood-brain barrier dysfunction**

Hypoperfusion also causes the release of reactive oxygen species that can damage the vascular endothelium and disrupt the BBB (Carvalho et al., 2009; Fernández-Checa et al., 2010). Like CBF changes, BBB dysfunction has been shown in early stages of AD as well as other neurodegenerative disorders. Montagne et al. used MRI-based methods to examine BBB leakage and found increased BBB permeability in the hippocampus and dentate gyrus during normal aging (Montagne et al., 2015). BBB disruption was associated with pericyte injury, as determined by biomarker measurements, indicating dysfunction at the level of the

capillaries. Further studies have shown that BBB breakdown, as determined using dynamic contrast enhanced MRI, is evident in patients with clinically determined mild cognitive impairment (Nation et al., 2019) and early AD (van de Haar et al., 2016). BBB breakdown has also been observed in PD, most notably in vascular PD, where vascular lesions in the striatum as well as lacunar infarcts are characteristic (Benamer and Grosset, 2009; Bertrand et al., 2008). In amyotrophic lateral sclerosis, characterized by upper motor neuron degeneration in the spinal cord, there is a similar degradation between the spinal cord and its blood supply, suggesting that BBB breakdown is common in the pathophysiology of these various neurodegenerative disorders (Engelhardt and Appel, 1990).

The mechanisms that underlie BBB dysfunction in neurodegenerative diseases are not fully understood. Expression levels of many tight junction and related proteins decrease in several neurodegenerative conditions (Yamazaki and Kanekiyo, 2017). These changes may be related to dysfunction and loss of pericytes (Bell et al., 2012), which express many of the proteins required for tight junction formation, in the aging and injured brain (Bhowmick et al., 2019). MMP activity, responsible for degrading many tight and adherens junction proteins, is increased in many of these disorders and may play important roles in BBB breakdown (Rosenberg, 2009). Decreased synthesis/increased breakdown of these proteins makes the BBB “leaky”, allowing previously excluded proteins and other circulating factors to enter the brain parenchyma, leading to an accumulation of toxic serum proteins and/or reactive cascades in the brain, potentiating further neurovascular damage, brain edema, and neuronal toxicity. A recent study using FPI to induce TBI in mice (Bhowmick et al., 2019) demonstrated that injury to pericytes causing decreased PDGF receptor- $\beta$  dependent signaling was associated with enhanced expression of AQP-4 in the perivascular region, down-regulation of basement membrane and tight junction proteins, and increased BBB permeability, as well as changes in the serum biomarkers S100B and neuron specific enolase. These studies suggest that pharmacologic approaches to maintaining pericyte-endothelium integrity following TBI represent an attractive therapeutic strategy.

Proper functioning of the BBB is required not only to protect the brain from peripheral toxins but also for appropriate removal of proteins and other substances from the brain parenchyma (Sweeney et al., 2019b). In animal models and humans with AD, A $\beta$  clearance through the BBB is disrupted, promoting the aggregation of amyloid deposits within the brain parenchyma and, in particular, within the vessel walls of the cerebrovasculature, a phenomenon known as cerebral amyloid angiopathy (CAA) (Mawuenyega et al., 2010; Pflanzner et al., 2010; Shibata et al., 2000). Apolipoprotein E (apoE) is the principal lipid carrier in the brain and modifies A $\beta$  accumulation in AD (Kim et al., 2011) in part through its actions on the cerebrovasculature (Liu et al., 2009; Sagare et al., 2012; Zhao et al., 2018). There are 3 isoforms of apoE (apoE2, apoE3 and apoE4), with apoE4 being associated with impaired A $\beta$  metabolism and increased AD risk (Roses et al., 1996). Recently, Robert et al. used a tissue engineering approach to show that apoE4 is less efficient at promoting A $\beta$  transport across a synthetic blood vessel when compared to other apoE isoforms (Robert et al., 2017), supporting a mechanism by which apoE4 increases susceptibility to neurodegenerative disease.

## Endothelial dysfunction

Endothelial cells may contribute in other ways to neurodegenerative pathology in addition to BBB breakdown. When cerebral microvessels were isolated from the brains of autopsy specimens with AD, but not age-matched control brains, they were found to release higher levels of the inflammatory mediators interleukin (IL)-1 $\beta$ , IL-6, and monocyte chemoattractant protein-1 (Grammas and Ovase, 2001) as well as VEGF, MMP-9 and angiopoietin-2 (Thirumangalakudi et al., 2006). Endothelial cells derived from AD microvessels synthesize thrombin, a protein with known neurotoxic effects (Yin et al., 2010). High levels of thrombin were detected in the blood vessel walls from these AD subjects, but not controls, and could also be detected in the CSF. In 2 transgenic mouse AD models, the cerebrovasculature was found to express thrombin, tumor necrosis factor- $\alpha$ , IL-6, MMP-9, and IL-1 $\beta$ . When this so-called “vascular activation” phenotype was targeted pharmacologically using sunitinib, a multikinase inhibitor targeting VEGF and PDGF receptors, expression of these molecules was blunted and cognitive behaviors improved in these animals (Grammas et al., 2014). *In vitro*, sunitinib blocked endothelial cell release of MMP-9, TNF- $\alpha$  and A $\beta$ , suggesting its effects are mediated at the level of the endothelium.

## LOOKING TO THE FUTURE: VASCULAR DIRECTED THERAPEUTICS FOR TBI

While direct links between traumatic injury of the cerebral vasculature and neurodegenerative disease in humans have not yet been established, the evidence presented above suggests the hypothesis that TBI-related neurodegeneration may be, at least in part, a consequence of chronic microvasculopathy, akin to vascular dementia and the vascular contributions to dementia (Iadecola, 2004; Sweeney et al., 2019a). Together, these clinical and preclinical observations suggest that therapies targeting the cerebral microvasculature are promising areas of investigation in TBI-related neurodegenerative disorders.

Animal studies have been widely used to provide target validation and proof-of-concept efficacy data for TBI therapeutics and there are a number of promising options that influence the microvasculature (Figure 2). Pre-treatment with aspirin, a non-steroidal anti-inflammatory drug, inhibited thrombosis of parenchymal vessels in a subdural hemorrhage injury model, preventing secondary injury due to hypoperfusion and ischemia (Wang et al., 2018). Activated protein C (APC), a serine protease that has anti-inflammatory, anti-thrombotic, and neurodegenerative effects, has been shown to promote angiogenesis and improve outcomes in a CCI model of TBI (Petraglia et al., 2010). Recently, 3K3A-APC, an agonist of the APC receptor protease activated receptor-, was shown to decrease hemorrhage rates when administered following human ischemic stroke treated with intravascular thrombolytic agents or mechanical thrombectomy (Lyden et al., 2019). These data suggest that this agent may prove efficacious and safe for the treatment of TBI-related injury.

A number of agents have been found to impact BBB permeability after TBI. Co-treatment with lithium and valproic acid, clinically-approved therapies for the treatment of mood disorders and epileptic seizures, have shown promise in attenuating BBB damage measured by IgG extravasation and also improving long-term functional recovery after CCI (Yu et al.,

2013). Other methods of ameliorating TBI-induced pathophysiology include inhibiting endogenous MMP-9, VEGF and endocannabinoid degradation pathways. Acute MMP-9 inhibition, with the use of melatonin, was found to protect against CCI-induced BBB permeability and edema (Alluri et al., 2016). VEGF inhibitor treatment lowered vascular permeability to albumin and upregulated tight junction proteins following FPI (Gao et al., 2015). Additionally, inhibiting endocannabinoid degradation, namely for 2-arachidonoyl glycerol and N-arachidonylethanolamine, protected BBB integrity and rescued behavioral phenotypes after FPI (Katz et al., 2015).

Most, but not all, studies have shown an association between poor outcome after TBI and *APOE4* carrier status in humans (Lawrence et al., 2015). While the exact mechanisms underlying this difference is not known, different apoE isoforms distinctly affect BBB recovery, where human targeted replacement apoE3 mice displayed faster recovery after TBI compared to their apoE4 counterparts (Washington et al., 2018). Relative to wild-type controls, apoE-deficient mice also displayed greater axonal damage and impaired cognitive recovery after mild, repetitive closed head injury (Namjoshi et al., 2013). In mice that express high levels of human amyloid precursor protein, a model of AD pathology, exposed to CCI, expression of human apoE4 was associated with A $\beta$  deposits, visualized by Thioflavin S staining, in the dentate gyrus, an effect not observed in mice expressing human apoE3 or deficient in apoE (Hartman et al., 2002). A $\beta$  deposition occurred at a much younger age than in the same mouse model that was not exposed to TBI. As apoE4 impairs A $\beta$  clearance through the cerebrovasculature (Liu et al., 2013), these data suggest that apoE may influence neurodegenerative pathology through a vascular mechanism, a phenomenon exacerbated by TBI. The apoE mimetic peptide, COG1410, reduced BBB disruption and cerebral glucose uptake at acute time-points after CCI (Qin et al., 2017).

Studies using exogenous administration of endothelial progenitor and mesenchymal stem cells important for blood vessel formation and maintenance have shown remarkable promise with respect to increasing microvascular density following FPI (Guo et al., 2017; Huang et al., 2013), decreasing BBB extravasation following FPI and CCI (Huang et al., 2013; Menge et al., 2014), and decreasing water content following FPI (Huang et al., 2013). Nikolian et al. found that increases in endothelial permeability following moderate-severe TBI in a swine model could be attenuated using endothelial-protective resuscitation strategies that promote barrier integrity (Nikolian et al., 2018). Similarly, one small retrospective study showed an association between marijuana use, which activates the cannabinoid system and can impact vascular tone, and improved outcome after TBI (Nguyen et al., 2014). While preliminary, these data raise the possibility that these vascular-directed therapies may serve as a therapeutic target that could prevent or limit secondary injury following TBI. It should be noted, however, that most of these trials have focused on treatment in the acute setting; whether these interventions will be durable over a longer time course or effective if instituted in the chronic phase after injury remains to be studied.

While a number of recent studies have shown preliminary translational promise, we must remember that equally compelling TBI therapies, similarly based on sound preclinical observations, have not translated to effective intervention for the human condition (Stein, 2015). The consensus of several expert panels convened to improve TBI-related clinical

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trials have emphasized the urgent need for TBI imaging and molecular biomarkers that facilitate (1) the identification of subgroups of TBI patients with specific brain pathologies who can be selected for targeted therapeutic trials, (2) confirmation that the therapy is engaging the proposed molecular target, (3) measurement of pharmacodynamics and therapeutic efficacy (Diaz-Arrastia et al., 2014; Saatman et al., 2008). In this regard, biomarkers of BBB breakdown and other vascular pathology are also being developed. The most common biofluid biomarker of BBB breakdown is the CSF:serum albumin ratio which is altered in mild cognitive impairment, Alzheimer's disease, and severe TBI (Saw et al., 2014), but not clearly altered in milder forms of TBI (Papa et al., 2015). Soluble PDGF receptor- $\beta$  is shed from pericytes and can be used to distinguish pericyte injury from injury to brain endothelial or vascular smooth muscle cells (Sagare et al., 2015). Development of markers for these other vascular cell types will be important, as will determining if biofluid levels of soluble PDGF receptor- $\beta$  correlate with TBI-related neurodegeneration, as was recently demonstrated for CSF levels in those with mild cognitive impairment (Nation et al., 2019). We also need to develop and leverage imaging biomarkers that can assess vascular damage and BBB permeability, using available techniques like NIRS, ASL, and CVR. Additional studies are required to assess longitudinal changes in these imaging markers so that they can be used to identify patients with vascular dysfunction and monitor their response to vascular-directed therapeutics. The validation and implementation of such biomarkers will be absolutely critical to make studies of long-term outcomes practically feasible.

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Another reason behind the lack of translational success may lie in the difficulty of modelling traumatic vascular injury in a rodent or porcine model. Rodents in particular possess a more robust and resilient cerebrovasculature than humans, a hypothesized consequence of differing circulating lipoproteins that affect vascular health. Specifically, as wild-type mice do not express cholesteryl ester transfer protein, they have far higher levels of vasoprotective high density lipoproteins compared to humans, and are therefore resistant to atherosclerosis (Marotti et al., 1993). Further, the method of inducing head injury is an equally important consideration. Though no single animal model has been able to capture the entire spectrum of vascular outcomes of human TBI, rotational platforms are showing promise (Clevenger et al., 2015; Johnson et al., 2018b). From a biomechanical perspective, few models are able to capture the head kinematics that contribute to TBI-induced tissue deformation. We speculate that impact-acceleration and rotation are key biomechanical features that would increase the likelihood of a pre-clinical model being sufficient to reproduce the complex vascular pathophysiology following TBI.

## CONCLUSIONS

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Preclinical models of both TBI and neurodegenerative disease should be leveraged to explore molecular mechanisms and genetic contributions to traumatic vascular dysfunction that cannot be easily explored in studies of human patients, with a focus on mechanisms that can be therapeutically targeted. Continued work to develop imaging and biochemical biomarkers that are specific to TBI-related vascular injury and can be measured reliably in both preclinical models and in human patients will be critical so that we can use these directed therapies in appropriately injured patients, monitor vascular-specific therapeutic

responses, and track recovery. Imaging and physiological monitoring tools are available to measure CBF, vascular reactivity, brain oxygen delivery, and BBB integrity in humans (Citerio et al., 2015). Recently, similar tools have been described that can be used in mouse models using advanced two-photon microscopy and intrinsic optical signal imaging to assess hemodynamic responses both regionally and individual capillaries/arterioles, regional brain tissue oxygenation, and the ability of oxygen supply the metabolic demands of the brain tissue (Kisler et al., 2018). Using these and similar tools in preclinical models will be critical to translating basic science observations into efficacious human interventions. Using what we know and are continuing to learn from neurodegenerative diseases, we will hopefully be able to lessen the vascular consequences of TBI.

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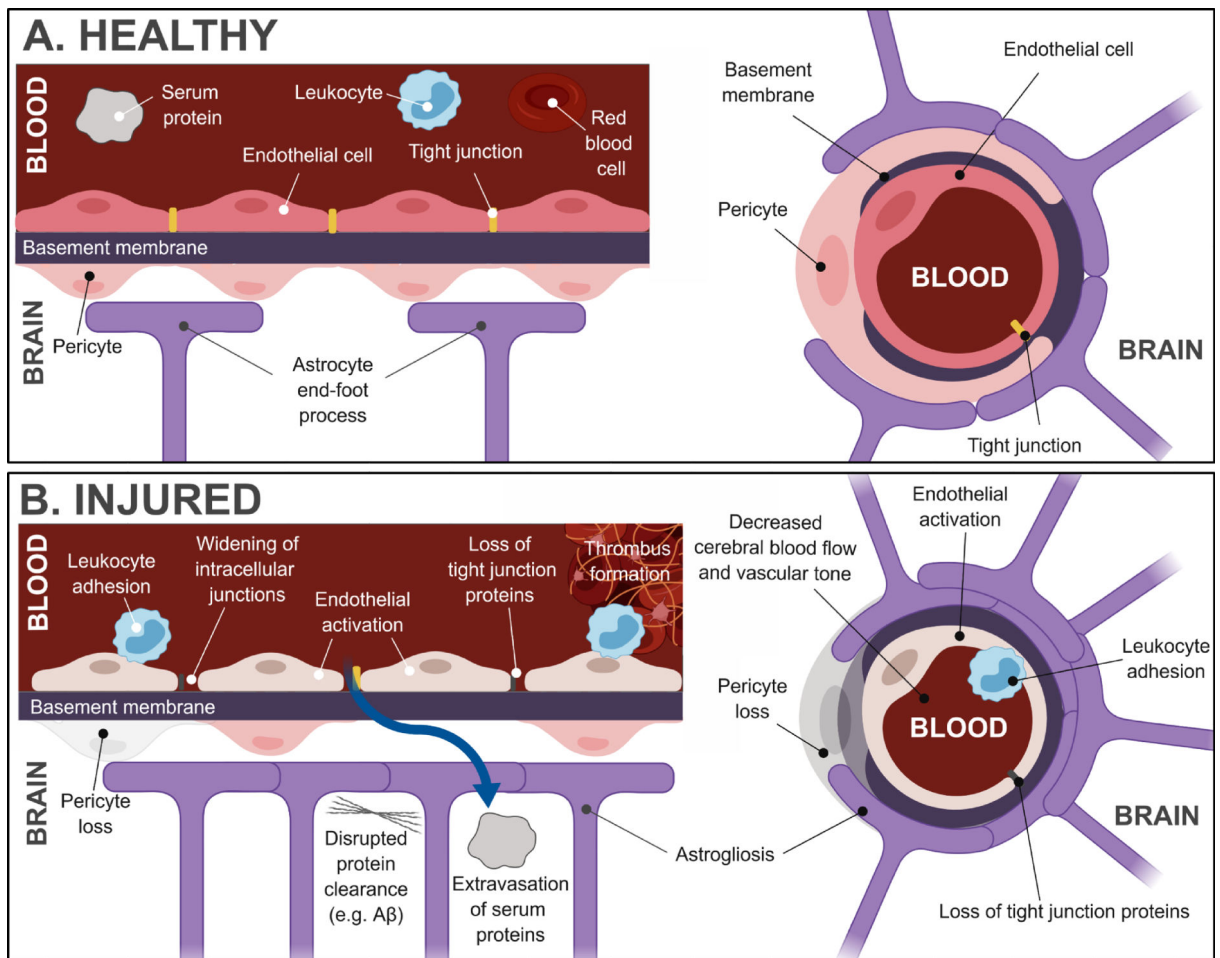
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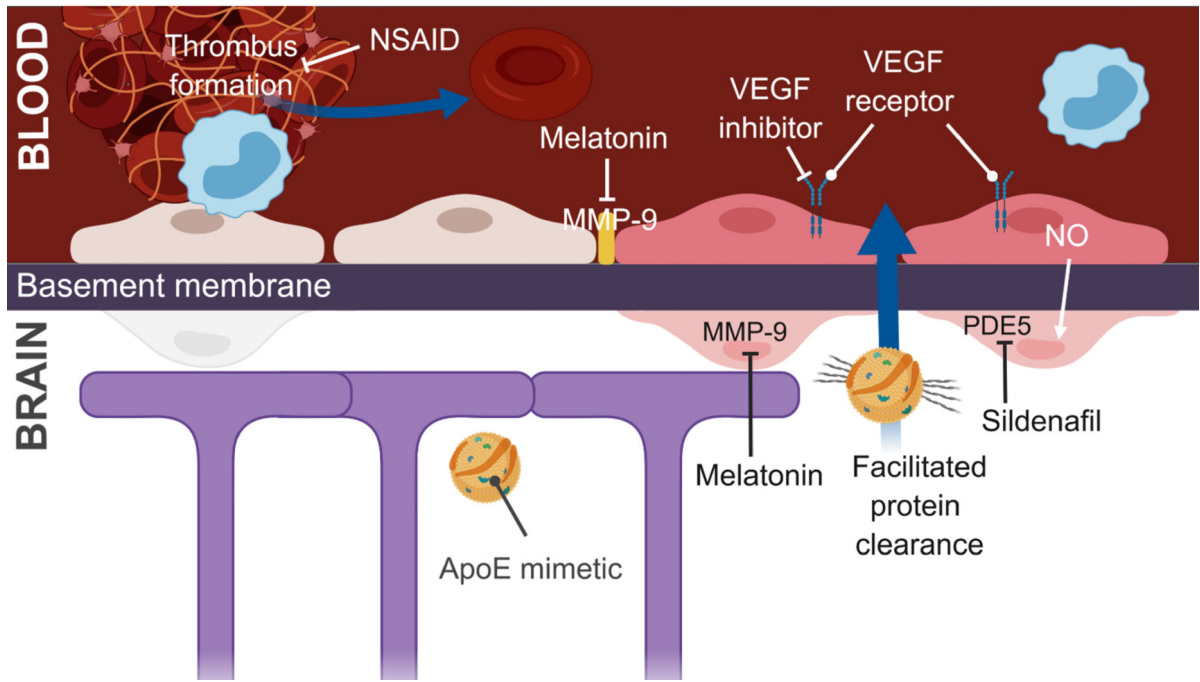
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**Figure 1: Healthy and injured neurovascular unit.**

A. At the capillary level, the neurovascular unit is composed of endothelial cells, perivascular astrocytes, and pericytes. Smooth muscle cells are present at the artery/arteriole/venule levels (not shown). The endothelial cells form a monolayer consisting of intercellular tight junctions to form the blood-brain barrier. B. Following injury, a number of vascular changes have been reported in preclinical studies, including widening of intracellular junctions, loss of tight junction proteins – both of which contribute to extravasation of serum proteins into the parenchyma – astrogliosis, pericyte loss, endothelial activation, leukocyte adhesion, thrombus formation and disrupted protein clearance.

# VASCULAR DIRECTED THERAPEUTICS



**Figure 2: Potential vascular-directed therapeutics for TMVI.**

Several therapies have shown promise in improving cerebrovascular function after TBI (see text), a number of which have known targets in the capillary, shown here. Acute inhibition of MMP-9 diminished the loss of tight junction proteins and pericytes after CCI (Alluri et al., 2016). VEGF inhibition prevented FPI-induced endothelial activation (Gao et al., 2015). Administration of an apoE mimetic facilitated protein clearance and reduced BBB disruption after CCI (Qin et al., 2017). NSAID pre-treatment has been shown to inhibit thrombosis of parenchymal vessels in a subdural hemorrhage injury model (Wang et al., 2018). Phosphodiesterase-5 inhibition using sildenafil improves cerebrovascular reactivity in humans with chronic TBI-related injuries (Kenney et al., 2018).