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A Neurovascular Perspective for Long-Term Changes After Brain Trauma

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Abstract

Traumatic brain injury (TBI) affects all age groups in a population and is an injury generating scientific interest not only as an acute event, but also as a complex brain disease with several underlying neurobehavioral and neuropathological characteristics. We review early and long-term alterations after juvenile and adult TBI with a focus on changes in the neurovascular unit (NVU), including neuronal interactions with glia and blood vessels at the blood-brain barrier (BBB). Post-traumatic changes in cerebral blood-flow, BBB structures and function, as well as mechanistic pathways associated with brain aging and neurodegeneration are presented from clinical and experimental reports. Based on the literature, increased attention on BBB changes should be integrated in studies characterizing TBI outcome and may provide a meaningful therapeutic target to resolve detrimental post-traumatic dysfunction.

Keywords

Traumatic brain injury; juvenile traumatic injury; blood-brain barrier; neurovascular unit; edema; cerebral blood flow; astrocyte; aquaporin

Clinical relevance of traumatic brain injury (TBI) in adults and juveniles

Traumatic brain injury (TBI) was under-investigated for a long time, but has recently become an important injury evaluated by modern neuroscience methods, both in the clinic and in the laboratory. Unfortunately, its rising significance is a consequence of higher incidence among civilians in the pediatric to aged population [1], and among combat personnel throughout the last decade [2]. In the United States alone, the TBI annual incidence rate was 1.7 million during 2002–2006 with the populations at greatest risk being young children (<5 yrs old), followed by adolescents (15–19 yrs old), and older adults (≥75 yrs old) [3]. It is estimated that 3.2 – 5.3 million people, or 1.1% to 1.7% of the US population, currently suffer from TBI-related disability and long-term complications interfering with daily quality of life [4–8]. Although the TBI-related death rate decreased by 8.2% in the United States during the 1997–2007 decade compared to previous years [9], TBI complications remain a major public health problem. The immediate cost is debilitating at an individual and institutional standpoint, and emerging data on long-term complications holds a daunting future for patients and is likely to become a looming societal burden. The latest available data suggests that the direct and indirect costs of TBI in the USA alone in

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2000 totaled an estimated $60 billion [10], yet this may reflect only cases with emergency room visits, and may not be indicative of milder TBI which can also have long-term consequences. TBI is not only an acute event but is becoming a complex brain disease, sharing common pathways with other neurobehavioral pathophysiologies, such as stroke [11–12] and Alzheimer disease (AD) [13–15].

TBI is classically defined as brain damage resulting from an external mechanical force (e.g. rapid acceleration or deceleration), blast waves, penetration by a projectile, or direct impact, it is often accompanied by a post-injury concussion or similar form of altered consciousness [16]. TBI is clinically evaluated by the patient's neurological score on the Glascow Coma Scale (GCS) and usually a CT scan to classify it as mild, moderate, or severe injury [17]. However, severity categories are not fully standardized and may not reflect the injury's actual structural elements, especially for mild TBI. Mild TBI is common after injuries where no skull fracture occurs (e.g. blast waves), and it is possible that more advanced imaging modalities such as CT scan, MRI, and/or PET can miss underlying brain damage and pathology. Based on examples described above and as demonstrated throughout this review, increased evidence shows that consequences of TBI can be observed for several months or years after the original insults. Little is known about the long-term effects of a single injury or complications arising from co-occurrence with symptoms like post-traumatic stress disorder (PTSD), which has an increased incidence following TBI [2]. Furthermore, studies on the underlying mechanisms behind long-term functional changes are in their infancy.

The complex array of lasting physical and behavioral effects in both adults and children suffering from TBI were under-estimated until recently. Motor impairment is a common post-injury complaint; however a variety of gross motor skills generally improve while cognitive signs may linger and deteriorate over time. For example, three months after a mild TBI, 17% of children show cognitive and neurobehavioral problems [18–19] and 30% of adults perform poorly on memory tasks and report ongoing memory and concentration difficulties in daily activities [20] with many neurobehavioral problems persisting for 6–12 months [21]. For those incurring a TBI early in their lifetime or at a juvenile age, the additional years post-injury are not always a formula for continued improvement (Figure 1). It is well known that early-life TBI can be a major cause of death and disability [22–23] and lingering cognitive problems can increase mortality risk into the second and third decades if injury occurred any time prior to age 40 [24–26]. On the other hand, early-life TBI injury survivors often have later development of educational difficulties, lower employment profiles, and mental health issues [27–28]. Interestingly, the profile of mild TBI in children, aged 8–17 years at injury, had memory, psychomotor skills, speed, and language problems throughout a 12 month follow-up period that was similar to outcomes from non-TBI injuries [29]. In addition, early life TBI has been associated with deficits in problem-solving, learning and memory, aggressive personality and psychiatric distress, and increased epilepsy risk lasting months to years [21, 30–32].

Although most retrospective studies categorize data according to TBI severity, age at time of injury and number of lifetime injuries are critical components of TBI outcome. Further, very little is known about the long-term consequences of a single TBI. Across age-groups, clinical observations suggest that diminished cognitive reserve from a single TBI may lead to premature aging and neurodegeneration [33–35] which may be related to widespread volume loss in frontal and temporal areas seen on magnetic resonance imaging (MRI) one year later [36]. Repeated TBI is well established as a risk factor for dementia with multiple underlying neurodegenerative processes, as is common for sports-related events (i.e. football, boxing) [14, 37]. Evidently, survivors of TBI endure lifelong consequences that warrant close attention from the medical and scientific community. The newest research and clinical developments are oriented towards mild TBI with or without concussion, and

underlying pathophysiology which may advance as a slow and silent disease, often missed by standard hospital evaluation methods. The major issues being addressed are how to define and diagnose mild TBI, and which physiological changes contribute to behavioral dysfunctions.

To better understand underlying mechanisms, it is important to consider the brain's complex network of neurons as a functional structure interacting with glial cells (e.g. astrocytes) and cerebral blood vessels as part of a single physiological entity, the neurovascular unit (NVU). For TBI, primary injury is a consequence of a direct or indirect biomechanical force on brain tissues which damage this unit and its integrated cell types. The primary injury is followed by secondary events which range from changes in cerebral blood flow to excitotoxicity and apoptosis [38–41]. Interestingly, it was proposed that vascular dysfunction is also possibly associated with different forms of neurodegenerative disease [42]. The focus of this review is to evaluate the impact of TBI below the neurobehavioral surface, focusing on cerebrovascular dysfunction in the NVU, including a timeline of structural changes in the blood-brain barrier (BBB), and highlighting differences among adult and juvenile TBI (Table 1).

Neurovascular unit: a physiological unit including the blood-brain barrier

The NVU is a physiological entity that is structurally defined by interactions occurring between endothelial cells, pericytes, smooth muscle cells, astrocytes, and neurons [43]. More recently, this physiological unit was extended to include interactions with peripheral immune cells coming from the circulation [44–45] which highlights an important role of brain barriers as regulatory and dynamic physiological interfaces between the central nervous system (CNS) and blood stream. Two of the most common barriers include the BBB and the blood-cerebrospinal fluid barriers [46–47], with other important blood-CNS barriers being the blood-retinal barrier, the blood-nerve barrier, and the blood-labyrinth barrier [44].

The BBB is formed by endothelial cells and related cellular constituents of the NVU (such as astrocytes and pericytes) that contribute to regulation of cerebral blood flow and to inherent properties of the BBB. The presence and the maintenance of these barrier properties are important for brain homeostasis and for neuronal functioning. The BBB is in charge of the exchange of ions, molecules and cells between the blood circulation and brain tissue [48]. In recent reviews, the BBB at the level of the endothelial cells is subdivided it into three elemental features [48]:

- The "*physical barrier*" of the BBB is formed by junctional complexes between endothelial cells of cerebral blood vessels that prevent paracellular diffusion, forcing most substances across the endothelial barrier in order to enter or exit the brain. The junctional complexes between endothelial cells are of two types: adherens junctions (AJ) and tight junctions (TJ). AJ are formed with proteins such as platelet–endothelial cell adhesion molecule (PECAM) and vascular endothelial-cadherin, which contribute to the close physical contact between endothelial cells and facilitate the formation of TJ. Disruption of AJ leads to BBB disruption and extravasation of blood components, followed by water contributing to vasogenic edema formation. The TJ are multi-protein complexes composed of claudins, occludins, and trans-membrane proteins, all linked together with zona occludens (ZO) proteins including ZO1, ZO2, ZO3 and cytoplasmic proteins. As such, ZO proteins have a scaffolding role to bind junctional molecules such as claudin and occludin together with the intracellular actin, a structural cytoskeletal protein. Disappearance of either claudin-3 or claudin-5 from TJ complexes induces a

weakness in the TJ between the endothelial cells and allows a physical disruption of the BBB.

- The "*transport barrier*" of the BBB is defined and composed of specific transport proteins located on the luminal and abluminal membranes of endothelial cells that allow the control of nutrients, ions and toxins crossing the endothelial layer between the blood-stream and brain [48–49]. Several nutrient molecules are transported by specific solute carriers such as glucose transporter 1 (GLUT 1), Monocaroxylate transporter 1 and 2 (MCT1, MCT2), L-system neutral amino acid transporter 1 (LAT 1) to supply the brain tissue. Large molecular weight solutes (e.g. large proteins and peptides) are able to cross the BBB to enter the intact CNS via endocytotic mechanisms called receptor-mediated transcytosis (RMT), such as with insulin, or adsorptive-mediated transcytosis (AMT), exemplified by albumin. In RMT, macromolecules bind specific receptors which cluster with their ligands into a vesicular caveolae that is internalized into the endothelial cell, and finally routed across the cytoplasm to be exocytosed at the appropriate location. By contrast in AMT, the molecule's extra positive charge renders it cationic, thus allowing an interaction with endothelial cell surface binding sites that induces endocytosis and subsequent transcytosis.

  On the other hand, transport can also be achieved by the ATP binding protein (ABC) family which consumes ATP to effectively transport a wide range of lipid-soluble compounds out of the brain endothelium. In the BBB, examples of ABC transporters for efflux transport are P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP) [49]. These efflux transporters are understood as "gatekeepers" of the brain, keeping tight control over substances allowed to enter the CNS through the endothelial cell barrier.

- The "*metabolic barrier*" of the BBB is a combination of intracellular and extracellular enzymes, such as P450 and monoamine oxidase, which function to inactivate molecules capable of penetrating cerebral endothelial cells.

Another important consideration is peripheral immune cells from the blood that have been observed to enter the brain at multiple time points [50]. During embryonic development, cells from the bone-marrow derived monocyte lineage enter and eventually become the brain's resident immunologically-competent microglia [50]. In the adult brain under normal physiological conditions, peripheral mononuclear cells are capable of penetrating the perivascular space in what appears to be routine immunological surveillance of the CNS [45]. However, exact mechanisms by which peripheral cells enter the brain are still a matter of discussion.

The NVU is a dynamic structure, with BBB properties that fluctuate from the time of brain development into adulthood and during the aging process at a structural and physiological level. Proteins involved in tight junction formation are observed early during brain development, with occludin and claudin-5 detected at 14 weeks in human fetus brain capillaries, thus limiting the free movement of proteins and macromolecules at primary neuro-developmental stages [49]. However, in most mammalians the BBB continues to mature after birth. A good example is in rodents, where P-gp expression in luminal endothelial cell membranes is increased at postnatal day P7 and reaches a plateau by P28 [49]. Interestingly, vascular and neuronal development are well coordinated, sharing the same transcription pathway requiring wnt/beta-catenin signaling that in vessels is essential for expression of cerebral endothelial cell-specific transporters such as Glut1, CAT1 and LAT1 [51]. Recent experiments showed the importance of interactions between pericytes, endothelial cells, and astrocytes in the development of BBB functional properties [52–53].

Interestingly, in rats the astrocytic spongiform processes needed for inter-cellular boundaries appear during post-natal week three [54], and may provide a critical period to study developmental outcomes of juvenile TBI injury. With aging, multiple structural changes occur, with recent attention paid to decreased transporters in brain aging and neurodegenerative disease such as P-gp, but also low-density lipoprotein-related protein 1 (LRP1) [55–56]. This observation is in agreement with the hypothesis of vascular dysfunction being a starting point of abnormal brain aging, AD and other neurodegenerations [42].

We propose that the BBB is a central player in proper functioning of the NVU and that it maintains brain homeostasis through nutrient regulation and direct contribution to cerebral blood flow [44, 47]. Following TBI, primary injury at the moment of impact damages brain tissue by disrupting blood vessels; this event facilitates secondary injury cascades affecting NVU pathophysiology.

Acute neurovascular changes after adult and juvenile TBI

As mentioned previously, TBI is structurally characterized by a primary injury resulting from a direct or indirect biomechanical force on the brain matter itself (Figure 1). Based on the above definition, a practical and preventive solution for reducing primary injury would be to improve the use of protective head-gear during high-risk activities where TBI has a high likelihood of occurrence. In connection with the primary injury, TBI is associated with several secondary events that occur with some delay and often have an extended duration after the primary impact, thus allowing a large window of opportunity for therapeutic interventions. We have provided a timeline of events in Figure 1, summarizing the current data available from both clinical and experimental models of TBI. As depicted in Figure 1, the underlying mechanisms accounting for behavioral changes in the first days and weeks after brain trauma are various and may range from: changes in cerebral blood-flow (CBF) associated with hypo-metabolism, increased intracranial pressure, edema formation and brain swelling, as well as inflammation and related events at the molecular level such oxidative stress, excitotoxicity, apoptosis, and neuropathology [38–41]. These changes are observed in the pediatric and adult populations and in experimental models with diverse levels of severity (Table 1), although very little data exists for direct comparisons of pathophysiological cascades between the pediatric and adult populations. As briefly summarized in Table 1 and throughout the manuscript, younger individuals are more vulnerable to TBI and have more severe outcomes following injury than adults as underlined by mortality risk, neurobehavioral function, cerebral blood flow and edema.

Significant changes in CBF can lead to either decreased or increased ischemic or oligemic levels, depending on injury severity, and on the time and the anatomical location of the CBF measurement [57]. Clinical and experimental data indicate that the most profound reductions in CBF are found in and around contusion injuries. On the other hand, diffuse and mild injuries result in very low reductions or even increases in blood flow, at least at initial time points following TBI [41, 58–62]. Several works highlight modifications not only in CBF, but impaired cerebral autoregulation in adults [63] and in the pediatric population [64–67] with young age being a significant predictor of CBF dysregulation [67]. In children, this impaired cerebral autoregulation is further associated with overall poor outcome [64, 68–69], which is confirmed by studies in animal models such as the juvenile piglet with a fluid percussion injury (FPI) [70].

There are several possible molecular explanations within the NVU for TBI-related changes to CBF. Alterations to endothelin-1, decreased nitric oxide (NO) levels, cyclic guanosine monophosphate, cyclic adenosine monophosphate, and changes in K+ channel activation

can affect tone of the cerebral vasculature and therefore the cerebral autoregulatory capacity [71–74]. NO signaling has been well demonstrated as a regulator of vascular tonus in the periphery and central nervous system [75], and this molecule is synthesized from L-arginine by endothelial, neuronal, or inducible NO-synthases (NOS) [76]. Following TBI, the activity of endothelial NOS (eNOS) increases briefly for a few minutes, then decreases to ~50% of baseline for 7 days before it's levels normalize [77–78]. These decreases of constitutive NOS activity may contribute to altered CBF and cerebral autoregulation. Therapeutically, it may be possible to compensate for the decrease in NO after TBI by administration of NO donors like sodium nitroprusside (SNP), and thus improve CBF and cerebral autoregulation. Indeed, administration of SNP prevented the reduction of CBF, but could not reverse autoregulatory impairment during hypotension after FPI in the juvenile piglet model [79]. Additionally, while eNOS activity remains suppressed for up to 7 days, simultaneous increases occur for inducible NOS (iNOS) expression and activity in neurons, macrophages, neutrophils, astrocytes, and oligodendrocytes, reaching peak levels between 4h and 48h after injury [78, 80–82]. Unfortunately, up-regulation of iNOS results in a harmful increase of tissue NO [78], well known to contribute to neuroinflammation, apoptosis, excitotoxicity, energy depletion, and uncoupling of NOS with subsequent production of reactive oxygen species (ROS) [83–85]. These findings illustrate the complexity of the role of NO in TBI pathophysiology, and the challenges for remedial applications of NO therapies.

CBF changes after TBI may also be related to changes in basic properties of the cerebral vasculature and decreased CBF early after injury is a common signature of TBI in adults and juveniles. For example, TBI in juvenile animals impaired N-methyl-D-aspartate dependent pial dilation and reversed it to a vasoconstriction, partly mediated by tissue type plasminogen activator (tPA) through the activation of the mitogen activated protein kinase (MAPK) family including JNK and ERK [79, 86]. In a cortical contusion model, decreased CBF was characterized by lack of perfusion in the core within minutes of injury [41, 60] indicating that high reduction in CBF close to the impact site often reaches an ischemic threshold. Alternatively, other models showed a widespread reduction in CBF involving the entire brain, without reaching ischemic values in most cases, and with recovery over time [60]. In addition to dysfunction in the larger blood vessels, several studies have shown vasoconstriction, compression of microvessels by swollen astrocytes in the NVU, and obstruction of microvessels by microthromboses that may be responsible for peri-contusional ischemia. A recent study elegantly showed that immediate post-TBI decreases in peri-contusional blood flow were not caused by arteriolar vasoconstriction, but rather by injury-induced formation of microthrombi in 33% of arterioles and by rolling leukocytes and platelet activation in 70% of venules [87].

Alterations in CBF may contribute to and be exacerbated by secondary injury, as decreased blood supply is associated with reduced energy metabolism in the brain tissue of several TBI models [88–91] (Figure 1). During the first week after TBI, glucose metabolism is likewise impaired in adults and juveniles both in the clinic and laboratory models. For example, poor neurological outcome was associated with increased lactate, measured by proton spectroscopy, in infants and children 6 and 9 days after closed head injury [88, 92]. In juvenile rats, a time course of brain metabolites also revealed global increases in lactate (in both ipsi and contralateral hemispheres to the injury) at 4h until 24h after TBI [91]. Although some debate exists concerning the interplay of glucose versus lactate post-injury, increased lactate can result from increased glycolysis, a consequence of the decreased CBF described above. Altogether, these data show that TBI-induced changes in basic neurovascular properties can lead to widespread functional damage with visible consequences on tissue integrity.

As observed in stroke, TBI is associated with an inflammatory response in the NVU involving leukocyte accumulation in the brain tissue. More specifically in TBI, leukocyte infiltration is observed at 12 hours until several days after the injury. However, leukocyte recruitment is preceded by the activation of pro-inflammatory cytokines secreted by the glial cells, such as tumor necrosis α (TNFα), interleukin 1β (IL1β) and interleukin 6 (IL6). These cytokines activate the expression of specific leukocyte adhesion factors on the endothelial cells, such as intercellular adhesion molecules (ICAMs) and E-selectin [93]. In addition, these pro-inflammatory cytokines may trigger chemokine synthesis, such as interleukin 8 (IL8) and macrophage inflammatory proteins (MIPs), which serve as chemo-attractive molecules contributing to the migration of leukocytes from the blood stream into the central nervous system [93]. Thus, the intracerebral accumulation of leukocytes is considered part of the secondary injury cascade following the disruption of the BBB and vasogenic edema.

Inflammatory responses after TBI can contribute to oxidative stress, as leukocytes themselves can produce free radicals such as superoxide dismutase (SOD) and nitric oxide [93]. In turn, free radicals induce lipid peroxidation and damage several cell types in the NVU, including the endothelial cells of the cerebral microvasculature. While leukocyte inhibition is beneficial toward improved neurological outcome for stroke [93], a similar treatment is not beneficial for TBI and must be evaluated in the context of differences between the nature of the primary injury. While there are many similarities between stroke and TBI at the molecular level, TBI often involves the local destruction of the blood vessels and presence of bleeding which contrasts with cerebral blood flow and reperfusion patterns during stroke.

A notable consequence of TBI-induced changes in CBF and infiltration of peripheral immune cells is the formation of brain edema, which is a common feature of acquired brain injury and has a crucial impact on morbidity and mortality [94]. A local perturbation of the brain environment usually induces regional edema [95], which leads to expansion of brain volume. These events have a vital influence on morbidity and mortality as they increase intracranial pressure (ICP), accelerate herniation, and contribute to secondary injuries such as ischemia [96]. Despite its complexity, brain edema has been defined as an increase in net brain water content, which leads to an increase in tissue volume [97]. TBI in infants and children is more frequently associated with severe and widespread brain swelling than in adults [98–99]. Two mechanisms may account for these age-related differences: changes of CBF post injury in the young and developmental and mechanical properties of the brain and skull [100]. Experimental studies have suggested that post-TBI edema in the immature brain also may be related to enhanced diffusion of excitotoxic neurotransmitters, an intensified inflammatory response, and increased BBB permeability [100].

In the past decade, the discovery of brain aquaporin (AQP) channels in astrocytes suggests a new hypothesis that water channels contribute substantially to edema formation and may potentially account for the exaggerated injury response seen in pediatric patients. This suggestion is supported by experimental studies which demonstrate that AQP4 expression increases with development [101] and that brain water content is higher in the young rat compared to the adult [102]. AQP1, AQP4, and AQP9 have been identified in the rodent brain [103] and disordered expression of these AQPs has been found in several conditions such as stroke, trauma, brain tumors, and subarachnoid hemorrhage (SAH) [104]. AQPs in the rodent brain are thought to play an important role in extracellular water homeostasis and sustaining normal neuronal activity [105] and are likely involved in water movement during the formation and resolution of cerebral edema. AQP9 has been observed in astrocytes and in catecholaminergic neurons [103] and its astrocytic expression is increased after stroke [106] but little is known about the role of AQP9 in brain disorders [107].

One of the most intensely studied brain AQPs is AQP4, which is found on astrocytic end-feet [94]. Notably, astrocyte end-feet are a critical part of the NVU and cover ~98% of the endothelial vascular wall in the BBB [108]. AQP4 has altered expression after trauma [109–111], ischemia [106, 112–113] and human SAH [104]. After transient cerebral ischemia in mice, we showed AQP4 expression peaked at 1h and 48h, temporally coinciding with maximal hemispheric swelling [106]. This temporal evolution of AQP4 differs from that seen in trauma where there is an initial decrease at 48 hours, followed by an increase [109–110, 114]. In contrast, AQP9 after ischemia shows a significant induction at 24h that increases gradually, with no correlation to the degree of swelling [106], suggesting that AQP4 but not AQP9 plays a more direct and significant role in edema formation. In adult trauma models, the role of AQP4 remains unclear. However, the absence of AQP4 in AQP4-knockout (KO) mice is protective in decreasing edema formation and lesion size in a model of spinal cord injury (SCI) [115]. This data contrasts with a recent paper showing a better functional recovery for the wild type mice compared to the AQP4-KO animals after contusion SCI, suggesting that AQP4 plays a protective role by facilitating the clearance of excess water [116]. Indirectly in support with this study, post TBI edema was decreased using sulforaphane, an abundant isothiocyanate present in cruciferous vegetables such as broccoli, which correlated with an increase in AQP4 expression [117]. In contrast, progesterone administration reduced edema at 24h and 72h after TBI but was only associated with a decrease in AQP4 expression at 72h [118]. Recently, brain AQP4 expression was decreased *in vivo* after injection of small interference RNA (siRNA) against AQP4 [119]. The next step will be to inject siRNA against AQP4 to prevent edema formation after TBI.

These reports show the multifaceted role of AQP4 in the NVU to appropriately maintain water homeostasis under normal and abnormal conditions. An important clinical question relates to the role of each AQP in edema formation and resolution profiles of different injuries and/or diseases. One reason for these discrepancies between AQP4 expression and TBI-induced edema may be due to differences in the experimental models. Overall, it appears that the presence of AQP4 is deleterious in the formation of cytotoxic edema, whereas the presence of AQP4 is important in resolution of vasogenic edema occurring after initial opening of the BBB post-injury [94]. These findings strengthen the importance of studying AQP4 expression independently in each model and determining its precise role in edema formation, to gain a better understanding of AQP4 modulation of edema depending on injury type and developmental age.

The post-traumatic changes described in the NVU are mostly observed during the first week after injury. Additional observations during this time-point include BBB dysfunction involving a physical “opening” of the barrier shortly after TBI (see below for details). However, the evolution of these changes in NVU over a long period of time is still unknown. As mentioned in the introduction, additional evidence is also needed to show whether one TBI can induce long-term changes to the NVU that ultimately affect behavioral outcome.

BBB changes over time: TBI and neurodegeneration

As described, TBI pathophysiology directly affects normal brain processes such as blood flow, inflammation, swelling, and incurs structural damage. It is important to address whether these changes affect behavioral outcome for months and years after the initial impact. One central player in the midst of these processes is the brain vasculature, yet these factors are not collectively studied, especially relating to juvenile injury, developmental age, and effects on the BBB. A recent review asks “what happens to cellular inward and outward transfer mechanisms following trauma and in neurodegenerative diseases?” [47]. To engage

comprehensive answers, outlines for emerging solutions are given by the international blood-brain barrier society as they review how BBB involvement can play a role in AD, Parkinson disease (PD), multiple sclerosis (MS), stroke, epilepsy, tumors, as well as HIV-infection and psychiatric disorders [44, 108]. Currently, comparable research endeavors are scarce in TBI, especially so following a single injury early in life. Lasting BBB alterations may very likely form the structural and physiological basis for the clinical and experimental reports indicating long-term functional damage from TBI.

TBI's effect on brain barriers is likely to differ according to injury model, age at injury, and severity of impact, and has mostly been studied during early time points after injury. These BBB changes can range from complete rupture and leakage to simply an altered biochemical and/or functional profile. Thus, although the BBB is often referred to as "open" or "closed", these terms may have misleading connotation, as all brain barriers are dynamic structures, and there are specific proteins/structures involved at these dynamic stages.

TBI induces altered properties of the endothelial cells at the structural level. Research reports highlight disruption of tight junctions, transporters/channels, basal lamina and pericytes, and astrocytic foot processes within the NVU in experimental models. At early post-injury time-points, BBB opening can be visualized with stains like Evans blue or anti-IgG staining. It is thought that TBI induction of BBB "opening" occurs within the first day after injury and contributes to vasogenic edema formation [120]. For a long time, the opening of the BBB was considered a transient event that normalized within one week. However, a recent study has shown that the BBB remains open as late as 30 days after the insult in a stroke model [121]. We show coronal sections from juvenile rats receiving a controlled cortical impact (CCI) at post-natal day 17 and evaluated at several timepoints (Figure 2). IgG extravasation levels are high near the injury site and surrounding tissue at 1 and 3 days, and are substantially lower at 7 days, although they remain high close to the site of the impact (Figure 2). At 60 and 180 days after juveniIe impact, IgG is not detected near the injury site and staining is only observed in the regions without a barrier, such as the median eminence.

In addition to these measures of BBB permeability, some studies report continued alteration in tight junction proteins, such as claudin 5. At multiple time-points post-injury, BBB dynamics often coincide very well with the expression levels of tight junction markers. In the short term after injury, pial and intracerebral vessels have decreased claudin 5, as evidenced at 2 days after cortical cold injury model in rats [122], within 120 hours after focal MCAO ischemia in adult rats [123], and shortly after exposure of human brain endothelial cells to alcohol [124]. When the overt BBB opening resolves at later time-points, claudin 5 (among other BBB proteins) is up-regulated 1–2 weeks post-injury after sustained epidural compression of rat somatosensory cortex [125]. In another model, claudin 5 remained elevated as late as 4 and 8 weeks in mice infected with the T. canis larvae, which readily crosses the BBB [126]. Thus, it appears that claudin 5 decreases when the BBB is open, and increased claudin 5 expression occurs only when BBB opening resolves, possibly indicative of BBB repair after TBI.

For transporters, a recent study has looked at monocarboxylate transporters (MCTs). These proteins are expressed on membranes of endothelial cells, neurons, microglia, and astrocytes [127]. MCT levels in brain and vasculature decline with cerebral maturity in humans and rodents, but MCT in brain lysates increased in the first week post-CCI in young adult rats at P35 and P75 [128]. In a comparable study, only young rats had improvements in behavior and preserved cortical tissue after a 1-week post-injury ketogenic diet which increased MCT levels [129]. In general, studies on BBB proteins are scarce for TBI, and more studies would be suitable to address the role of other proteins that coordinate tight junction formation such

as occludin, fibronectin, type IV collagen, actin, ZO-1, ZO-2, JAM, and cingulin, to name a few. TBI may affect other transporters that are critical in the maintenance of the BBB functions. In addition, the role of pericytes long after TBI remains to be explored, as function of these cells are important during development and the juvenile injury time-point [52–53].

TBI, especially early life injury, may disrupt the NVU in such a way to accelerate brain aging and promote aberrant protein accumulation to ensue and persist over time. Several groups have elegantly shown the importance of a triad of endothelial cell proteins in this regard, as mediators of protein trafficking across the BBB: P-gp, LRP1, and the receptor for advanced glycation end products (RAGE) [130–132]. While P-gp is well known as an efficient gatekeeper on the luminal side and often pharmaceutically by-passed to allow efficient drug delivery, its expression is influenced via several distinct molecular pathways and its putative role in disease and post-trauma is just emerging [133]. With normal aging, P-gp and LRP1 are decreased on endothelial cells in aged human and AD brains as well as in aging rodents [55–56]. However, senescence accelerated mice (SAMP8) display increased P-gp and reduced RAGE profiles, possibly as an effort to compensate functional abnormalities and improve clearance and/or prevent brain entry of toxic substances [134–135]. In addition, P-gp knock-out models have increased Aβ deposition after injection of Aβ in the brain [136], suggesting P-gp as a key player in the clearance of Aβ and other toxic substances from the brain parenchyma.

Aberrant protein accumulation in the brains of TBI patients, especially regarding the Aβ peptide, may be mediated by efficiency of protein trafficking at the BBB. Recent findings propose that sporadic Aβ accumulation within the brain depends largely on the effectiveness of its clearance through the BBB [130, 137]. Following TBI, several reports indicate acute increases of Aβ and its parent protein beta-amyloid precursor protein (APP) in the brain, however a recent review suggests that Aβ dynamics are more complex and sensitive to altered brain activity patterns following injury [138]. Transient Aβ increases have been reported after TBI in several APP transgenic models for either several hours and days [139–141], weeks [139, 142], or months [142–144]. Notably, a single controlled cortical impact (CCI) in non-transgenic C57Bl/6 mice showed Aβ increased and normalized within one week [145]. As previously mentioned, TBI postmortem and experimental studies, at both early and delayed timepoints after injury, have consistently shown evidence of increased neuropathology for Aβ, as well as elevated tau and alpha-synuclein [13–15, 35, 37].

The important consequences of cerebrovascular dysfunction and the role of the BBB in aging and AD are recently gaining momentum. SAMP8 and aged controls both show increased BBB disruption at 12 months of age, but only SAMP8 animals exhibit additional vascular Aβ [146], indicating the close relationship of BBB disruption and pathology development. Recent findings propose that sporadic Aβ accumulation within the brain depends largely on the effectiveness of its clearance through the BBB [132, 137]. In an opinion paper on the amyloid cascade hypothesis of AD [147], ongoing research suggests that vascular damage may either initiate amyloid damage or be caused by it. While the endogenous role of Aβ in rodents (or any other species) is still unknown, recent reports highlight that Aβ may serve as a signaling molecule. Notably for the barriers perspective, since the BBB prevents blood-to-brain cholesterol transport, neurons may release Aβ as a signal to astrocytes about neuronal need for cholesterol. This was demonstrated by the decreased expression of ABCA1 cholesterol transporter in transgenic mice and in cultured astrocytes with elevated Aβ levels [148]. But it would also be worthwhile to evaluate other pathways in TBI that may be related to brain aging and disease. For example, oxidative stress occurs in human brain aging and AD [149–152]. In the first week post-injury, TBI in 12- or 24-month aged rats caused more oxidative damage to lipids (4-hydroxynonenol,

acrolein) and reduced antioxidant enzyme activity compared to TBI in 3-month young rats [153]. Other changes may occur in the inflammatory profile, as with age there is a shift in human hippocampus and cortex from an anti- to pro-inflammatory mRNA expression profile [154]. In addition to Aβ protein, other neurodegenerative proteins may accumulate that are also found in TBI cases and models, such as tau and alpha synuclein [155–156]. A recent review also highlights AD and related *in vitro* and *in vivo* models exhibiting widespread dysfunction of the neurovascular network [157]. Collectively, these observations suggest that BBB integrity represents a complex and dynamic pattern that merits attention at many stages of aging, disease, and also in trauma.

Summary and significance

While advancing knowledge about neurodegenerative diseases, the overwhelming neurocentric focus has not resulted in effective therapeutics capable of modifying disease outcomes, such as in AD. In regard to the literature this last decade, non-neuronal cells, such as astrocytes and endothelial cells, are emerging as important players that account for disruption to the NVU, which can predispose the CNS to neurodegenerative processes as it was previously addressed in rodent AD models. For TBI, the clinical reports lead us to hypothesize that an early life injury would elicit enduring behavioral deficits that are associated with permanent changes to molecular pathways that persist into adulthood. To date, few experimental TBI studies have focused on the timeline and/or pattern of deficits in association with specific molecular alterations for pediatric models. Very little is known about whether juvenile injury mimics adult injury patterns and the nature of the connections to acceleration of neurodegenerative disease. However, literature from neurodegenerative studies serve as a guide in formulating the hypothesis that long-term deficits in BBB function after TBI accrue over time to ultimately cause damage at a behavioral and molecular level (Figure 3). In addition, early life trauma at a critical developmental stage may result in a new baseline expression of molecular players at the BBB and within the NVU. This new baseline may be impaired compared to normal, cannot be functionally and structurally maintained over time, and is ultimately pathological and permissive to the acceleration processes of brain aging (Figure 3).

In addition to the well established BBB changes immediately post-injury, long term evaluation of the BBB and NVU properties are necessary to understand their roles during brain development, normal aging, and disease and/or injury. Although the BBB is the most abundant brain barrier regarding its large surface area, other brain barriers should receive close attention since patterns of barrier disruption may depend on the nature and location of TBI barrier breakdown. For example, the meningeal barrier between the skull and the brain surface is highly complex and the least studied of the brain barriers; yet it may play the most critical role for TBI injuries, as this barrier is the nearest to the external surface and likely the first area of damage in most TBI injuries. Altogether from a BBB perspective, it appears that the brain is especially vulnerable to TBI during two time points: early in life when the BBB is still undergoing development, and later in life when the aging process causes BBB structure and function to decline (as proposed in Figure 3). This is supported by the clinical data showing that the most vulnerable populations in the event of a TBI are the young and aged [3].

We propose that a possible root cause of observed clinical patterns of long-term behavioral dysfunction from TBI could be the result of an abnormal BBB, where compromised vascular integrity persists over time and hinders full recovery. The data outlined above provide a strong indication that TBI may promote BBB dysfunction which progresses over time to affect protein trafficking/clearance, and ultimately accelerate brain aging and/or disease pathophysiology (Figure 3). Further, target-specific studies should address whether these

changes are interconnected, or whether they are parallel events occurring independently of one another in the same trauma-affected brain. There is a need to build on the existing platform and take advantage of the generous window of opportunity offered by both acute and long-term modifications available for study following brain trauma. On the other hand, just as ongoing BBB alterations can promote dysfunction, treatments that preserve vascular networks may contribute to preserved tissue and neuronal viability, leading to physiological improvement and restored cognitive function.

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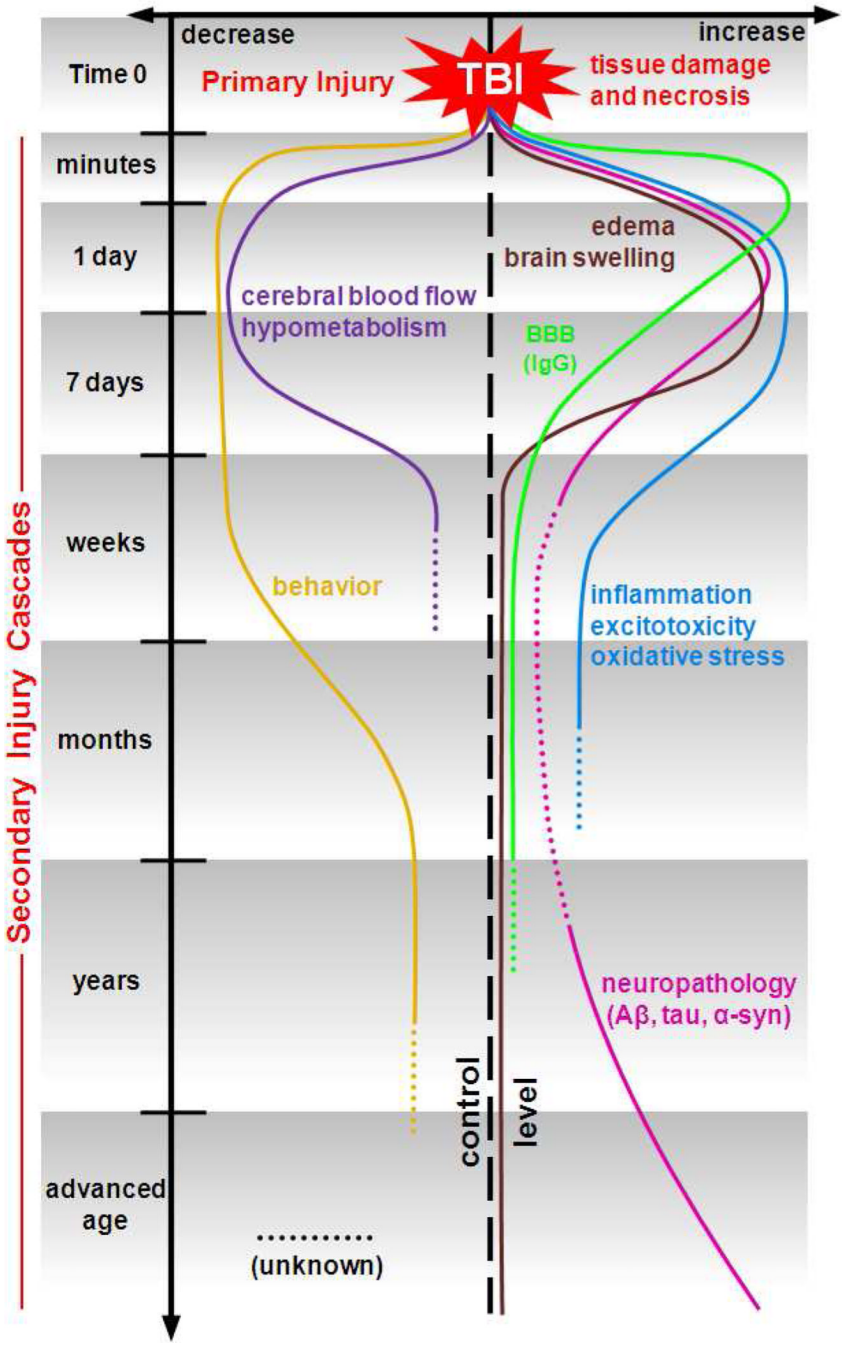

Figure 1. Summary timeline of pathophysiological cascades following TBI

Existing clinical and experimental literature agree on a fairly consistent description of the course of events within days and weeks following TBI (solid lines) compared to expected control levels over time (dashed line in center). The primary injury is depicted as the moment of impact at Time 0, which can result in tissue damage and necrosis to the NVU, often with the local destruction of the blood vessels and presence of bleeding. However, further clinical studies with experimental verifications are necessary to address the nature and time-line of several secondary injury cascades and related long-term modifications post-TBI which are currently unknown (dotted lines).

The schematic conveys a generalized post-injury timeline where behavior (gold line) is used to denote a wide range of motor and neurobehavioral dysfunctions. Thus, behavioral impairment occurs within minutes of most TBI injuries and partial recovery may occur in the weeks and months following injury, depending on the specific behavior, with data indicating continued neurobehavioral impairment years later, and little data available for post-TBI assessment during advanced age. Some possible underlying mechanisms accounting for the acute phase of behavioral impairment in the first week post-TBI may be: decreased cerebral blood flow and hypometabolism (purple line), increased edema and brain swelling (brown line), increased BBB permeability measured with staining of IgG extravasation (green line), increased inflammation, excitotoxicity, and oxidative stress (blue line), and increased neuropathological accumulation of proteins associated with neurological disease (pink line).

Notably, some post-TBI changes are transient and return close to control levels, such as edema and brain swelling (brown line) and BBB permeability (green line). On the other hand, long-lasting behavioral dysfunction might be explained by one or all of the remaining pathophysiological cascades that are not short-lived, but stabilize at a new substandard level. Thus, there is a need and an opportunity to explore these cascades for the development of new drugs targeting long-term dysfunctions after TBI.

(TBI: traumatic brain injury; NVU: neurovascular unit; BBB: blood-brain barrier; IgG: immunoglobulin G; Aβ: beta-amyloid; α-syn: alpha synuclein)

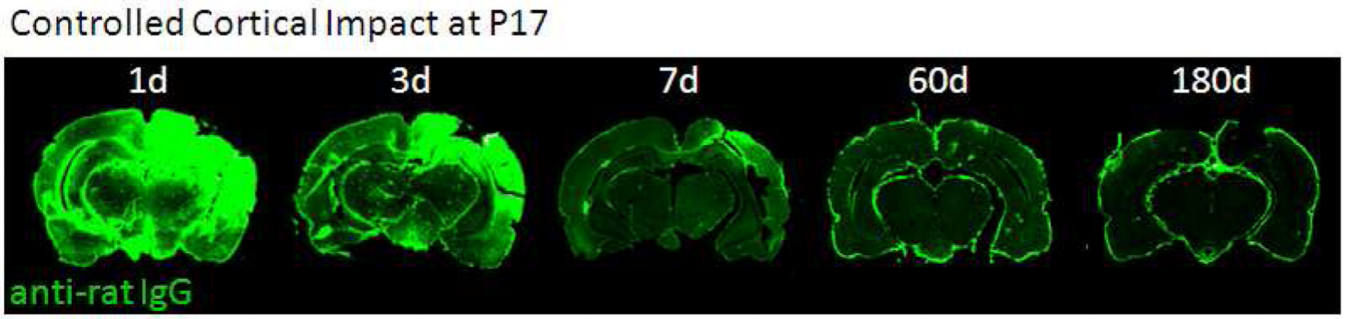

Figure 2. Timecourse of IgG extravasation following juvenile TBI

Coronal sections of rats given a controlled cortical impact (CCI) at a juvenile age, post-natal day 17 (P17), are evaluated after 1, 3, 7, 60, and 180 days post-injury. The CCI was given to the right somatosensory cortex overlying the hippocampus (top right of each coronal section). IgG extravasation levels are elevated (bright green staining) near the injury site and surrounding tissue at 1 and 3 days, much lower at 7 days, with the exception of high levels retained close to the impact site. At 60 and 180 days after juvenile impact, IgG is not detected near the injury site and staining is observed in regions without a barrier, such as the median eminence.

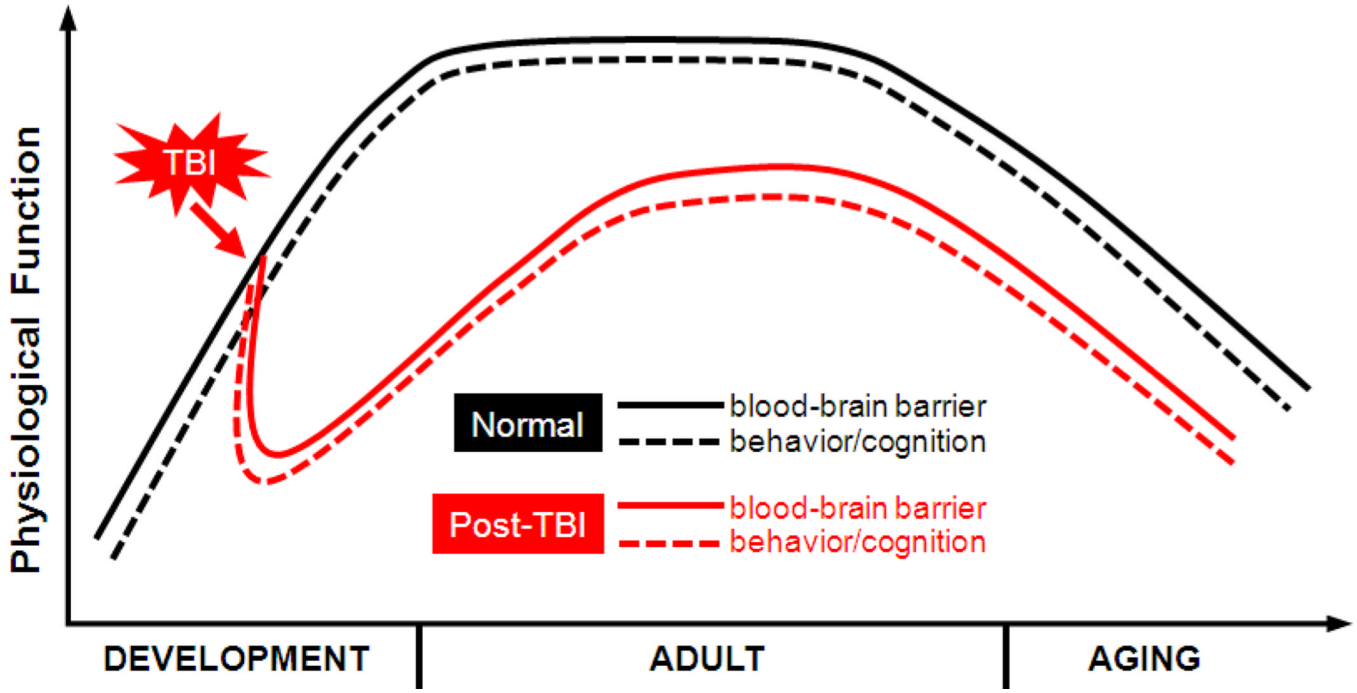

Figure 3. Proposed model of physiological function during normal conditions and after TBI

Several reports indicate that cognitive patterns may follow cerebrovascular physiological function, and this model gives a view of general temporal relationships expected in normal conditions (black solid and dashed lines) and following traumatic brain injury (TBI, red solid and dashed lines).

The reports from the literature suggest that functions of the blood-brain barrier (BBB) and behavioral/cognitive processes are interconnected, and steadily improving during early developmental stages (black solid and dashed lines). They reach a maximal point that remains a steady plateau during adult years, yet eventually succumbs to normal processes of aging that cause a decline in late adulthood, and which continues to drop with advancing age.

A TBI injury early in life at a critical developmental period causes a permanent disruption in these normal patterns. Immediately after injury, there is a temporary sharp drop in structural and physiological functions of the BBB concomitant with behavioral/cognitive processes (red solid and dashed lines). With time, recovery occurs, but in many cases it can only approach but never quite reach normal levels. Possibly, a new baseline of stable BBB and cognitive function are reached during adulthood, but this period is short-lived due to accumulating processes of aging. Thus, TBI injury has detrimental effects in the short-term which are partially restored but never fully recover over an individual's lifetime, and which may contribute to enhanced vulnerability to neurodegenerative processes, ongoing cerebrovascular dysfunction, and behavioral as well as cognitive deteriorations.

Table 1

Comparison of TBI outcome between young and adult populations

CATEGORY	TBI in Young	TBI in Adult
Mortality Risk	+ +	+
Neurobehavioral Function (motor, cognitive)	− −	−
Cerebral Blood Flow	− −	−
Edema (brain swelling)	+ +	+

+ small increase,

+ + large increase

− small decrease,

− − large decrease