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Molecular contributions to neurovascular unit dysfunctions after brain injuries: lessons for target-specific drug development

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Abstract

The revised ‘expanded’ neurovascular unit (eNVU) is a physiological and functional unit encompassing endothelial cells, pericytes, smooth muscle cells, astrocytes and neurons. Ischemic stroke and traumatic brain injury are acute brain injuries directly affecting the eNVU with secondary damage, such as blood–brain barrier (BBB) disruption, edema formation and hypoperfusion. BBB dysfunctions are observed at an early postinjury time point, and are associated with eNVU activation of proteases, such as tissue plasminogen activator and matrix metalloproteinases. BBB opening is accompanied by edema formation using astrocytic AQP4 as a key protein regulating water movement. Finally, nitric oxide dysfunction plays a dual role in association with BBB injury and dysregulation of cerebral blood flow. These mechanisms are discussed including all targets of eNVU encompassing endothelium, glial cells and neurons, as well as larger blood vessels with smooth muscle. In fact, the feeding blood vessels should also be considered to treat stroke and traumatic brain injury. This review underlines the importance of the eNVU in drug development aimed at improving clinical outcome after stroke and traumatic brain injury.

Keywords

aquaporin-4; blood–brain barrier; cerebral blood flow; edema; expanded neurovascular unit; ischemic stroke; matrix metalloproteinases; nitric oxide; tissue plasminogen activator; traumatic brain injury

Classically, the neurovascular unit (NVU) is defined by the structural cellular composition of neurons, astrocytes and endothelium. The authors recently contributed to a revised concept of the ‘classical’ NVU, which expands the structural matrix to include the capillary level, together with blood vessels, smooth muscle cells and cerebral arteries, thus naming it the vascular neural network or expanded NVU (eNVU) (Figure 1) [1]. The authors pursue the main idea that any new treatment targeting the eNVU (from the feeding arteries to capillary level) would improve the outcome after ischemic stroke and traumatic brain injury (TBI) not only at the bench, but also at the bedside.

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Ischemic stroke and TBI are the two most frequent acute brain injuries, associated with vascular dysfunction, in addition to neuronal cell death. For several years, therapeutic development focused on direct preservation of neurons, which ultimately failed as a clinical application for both TBI and stroke. With cerebral blood vessels becoming an important contributor of the NVU, the vasculature became a new therapeutic target alongside neuroprotective approaches. The goal of this review is to highlight some of the molecular pathways involved in NVU dysfunction after stroke and TBI. These molecular pathways are potential candidates for development of new therapies to protect structural and functional blood–brain barrier (BBB) properties, improve brain perfusion and, ultimately, contribute to neuronal survival.

NVU as a physiological entity for brain disorders

Definition & history

The NVU is a physiological unit with specific structural and functional components. It encompasses endothelial cells, pericytes, smooth muscle cells, astrocytes and neurons. These cells are part of a vast network, interacting together for precise regulation of brain tissue perfusion, as well as a functionally sound BBB [2].

Originally, the NVU was described as a tripartite unit consisting of cerebral blood vessels, neurons and astrocytes, collectively regulating cerebral blood flow (CBF) [3]. After 2002, the NVU definition specified the blood vessel component as endothelial cells [101], and this new NVU, composed of neurons, endothelial cells and astrocytes, became a new and attractive therapeutic target in the stroke research community. However, in reality, the NVU with the endothelium, astrocytes, neurons and, sometimes, pericytes, is an extensive network corresponding to the capillary bed. The inclusion of the vascular tree in its entirety was recently proposed as a revised concept, termed the vascular neural network or eNVU (Figure 1) [1]. This new paradigm resumes the original concept of the NVU, with the addition of arterial smooth muscle cells, arterial/venous endothelial cells and perivascular nerves from the larger blood vessels. Therefore, improved knowledge of relationships between eNVU cell types can assist with development of new promising drugs, which may ultimately benefit neurons. eNVU drug targets address the primary sites of brain injury, could provide longer-lasting neuroprotection and may have additive effects alongside direct neuronal preservation strategies, thus ultimately enhancing clinical benefits. As most of the major ischemic stroke events occur upstream of the capillary bed, the eNVU should be considered as a therapeutic target in stroke pathophysiology [1]. Similarly, during TBI pathophysiology, all the vascular tree is affected by the force exerted on the brain, not only capillary vessels. Thus, the eNVU is also relevant when considering the development of new drugs to treat TBI pathophysiology. These drugs would not only be able to protect neurons and glia, but they would also act on components of larger feeding blood vessels (smooth muscle cells and nerves) to improve cerebral circulation.

It is known that several molecular events present in the eNVU after TBI share similarities with ischemic stroke pathophysiology. All eNVU cells are compromised after brain injury, they respond together to the primary insult and contribute to the development of secondary injury cascades [4]. Encompassed within the eNVU, the BBB is a dynamic physical and chemical entity, which is formed of endothelial cells, astrocytes and pericytes. The BBB is a key element for eNVU function through maintenance of brain homeostasis, which is critical for neuronal survival [5].

Rising challenges of stroke & TBI

Both stroke and TBI represent major public health burdens, with 7 million people affected by stroke in the USA, and 3.17–5.3 million people living with a disability as a result of TBI

[6,7]. Each year, 795,000 people visit the intensive care unit and are diagnosed with a stroke, representing an average of one victim of stroke every 40 s [7]. Among them, 87% are ischemic strokes and 13% are brain hemorrhages. In a higher range, approximately 1.7 million people visit the emergency room for a TBI in the USA, spanning a wide range of people from the pediatric to the geriatric population. TBI represents the major cause of disability and death in developed countries, accounting for 30.5% of all injury-related deaths in the USA [6]. In ischemic stroke, the lesion is a result of an occlusion of a cerebral blood vessel (cerebral artery in most cases) by a clot or a thrombus. In TBI, the lesion can result from direct impact, penetration of a projectile, blast-waves or external mechanical force that disrupts the normal function of the brain. Both stroke and TBI are considered acute brain disorders, and are without any effective treatment options for patients. Despite the difference in the origin of the pathology, stroke and TBI share several similarities regarding their pathophysiological processes.

Comparisons of eNVU pathophysiology after stroke & TBI

Although the nature of primary injury is different, both stroke and TBI are often followed by tissue damage with a central necrotic lesion. In ischemic stroke, necrosis results from a lack of energy supply, while in TBI, necrosis results from the biomechanical injury. Interestingly, clinical and experimental data indicate that the most profound reductions in CBF are found in and around contusion injuries in association with hypometabolism [8]. Moreover, it is important to highlight that the presence of impaired cerebral autoregulation after TBI suggests a vascular pathophysiology [9]. Therefore, TBI and stroke share similar secondary injuries such as loss of homeostasis, edema, excitotoxicity, oxidative stress, BBB leakage and inflammation [10,11]. Most of the secondary events affect eNVU physiology, but occur with some postinjury delay, thus allowing a window of opportunity for treatment. Importantly, decreased CBF has a direct consequence on the availability of energy substrates for neurons as well as endothelial cells. If cells lack energy to maintain BBB integrity, BBB disruption is rapidly followed by edema formation, increased intracranial pressure, and CBF will be mechanically decreased. Without adequate intervention between the time of primary injury and the initiation of secondary cascades, these secondary events cause the expansion of the primary lesion with an ischemic area in both stroke and TBI disease sequelae.

Key molecular players in eNVU dysfunction after stroke & TBI

Ischemic stroke and TBI are followed by molecular events, most of which occur in the eNVU and are common to the two brain injuries. These molecular changes are often associated with BBB dysfunction following stroke and TBI. It is important to consider that the BBB properties are present in most of cerebral blood vessels from the feeding arteries to veins, and not only at the capillary bed [12]. It is very likely that the molecular changes of BBB properties in the endothelial layer are likely to occur in the eNVU, including the large blood vessels after stroke and TBI. These changes in the BBB may be prevented or mitigated with novel drugs currently in development. Latest advances on such drugs will be discussed in the next section.

From BBB disruption to endothelial phenotypic transformation

After acute brain injury, normal brain functions are affected by secondary injuries occurring in the eNVU (Figure 2). The BBB has been shown to have a biphasic pattern in permeability changes after ischemia–reperfusion [13,14]. Early after the injury onset, the BBB exhibits changes in permeability owing to the absence of energy, with consequential dysfunctions in endothelial ionic transport and transporters. Persistent BBB opening correlates with phenotypic changes of endothelial cells after acute brain injury. Indeed, levels of tight junction proteins, such as claudin-5, are decreased after stroke and until 5 days following reperfusion in a rat model of transient middle cerebral artery occlusion [15], and 2 days after

a cortical cold-injury model in rat [16]. The dimerization of claudin-5 is also compromised and may further contribute to weaknesses of endothelial tight junctions [17]. Dysfunction of the BBB may continue via degradation of the extracellular matrix. Indeed, energy failure leads to early activation of proteases, such as matrix metalloproteinases (MMPs), and tissue plasminogen activator (tPA; see the 'Early postinjury changes in MMPs & tPA at the eNVU' section for the molecular mechanisms). In addition, oxidative stress occurring in the eNVU is associated with nitric oxide (NO) dysfunction and further contributes to pathophysiology of the BBB. Loss of BBB integrity directly impacts nearby astrocytes and their physiology, with significant contribution to the edema process (see the 'Early postinjury changes in MMPs & tPA at the eNVU' section for details). Following these events, inflammatory cascades proceed and include cytokine release, endothelial adhesion molecule induction and extravasation of neutrophils, which together contribute to this second phase of increased BBB permeability and additional secondary activation of proteases such as MMPs [18,19]. All these changes are not only observed in the capillary bed, but are also present in postcapillary venules and veins. For example, extravasation of neutrophils occurs in venules and veins, which are part of the eNVU.

However, it is important to underline that much is known regarding BBB disruption, but very little is known about BBB recovery and the process of returning to a 'normal' or balanced physiological state. Most secondary events are studied during the first days after injury and little is known about what happens in the eNVU during a longer period. However, it is well described that long-term risks of developing epilepsy and cognitive impairment are increased after stroke and TBI [20–22], as well as the increased risk of cognitive decline and development of Alzheimer's disease following TBI [23]. Interestingly, BBB opening or dysfunction remains visible 4 weeks after primary injury in a rat stroke model [4]. Concerning TBI, in a juvenile rat model of controlled cortical impact (CCI), the BBB has been shown to remain open for up to 7 days. However, even when the BBB is no longer physically disrupted, phenotypic changes of endothelial cells remain. A recent study shows that 2 months after a juvenile rat model of CCI, levels of claudin-5 are elevated in the penetrating blood vessels compared with sham animals, while levels of P-glycoprotein (P-gp) are decreased [24]. P-gp is an endothelial protein implicated in β -amyloid trafficking and it is known to be decreased during normal aging and Alzheimer's disease [25]. Decreased levels of P-gp 2 months after TBI highlight long-term consequences of juvenile TBI upon neuropathology such as that found in Alzheimer's disease. Interestingly, claudin-5 expression is increased in the endothelial cells of the penetrating blood vessels, suggesting differential long-term phenotypic transformations between the capillary bed and larger blood vessels [24].

Early postinjury changes in MMPs & tPA at the eNVU

As mentioned previously, endothelial cells need energy to maintain BBB properties and ionic gradients. Therefore, prolonged energy absence contributes to early BBB alteration with several underlying mechanisms, including early activation of proteases such as MMPs and tPA. During necrotic phases, cytokines, such as TNF and IL-1, are released by astrocytes, microglia, endothelial cells and neurons, and contribute to the initiation of neuroinflammatory cascades [26,27]. These cytokines are known to induce microglial activation and expression of cell adhesion molecules, such as ICAM-1, VCAM-1, platelet type (P-selectin) and endothelial type (E-selectin). This is followed by recruitment of leukocytes (neutrophils and monocytes) toward the ischemic endothelium, and their passage across the BBB, also called diapedesis [28]. Once in the brain parenchyma, immune cells join microglia to increase inflammatory reaction and consequential release of proinflammatory factors. Leukocytes aggravate the lesion by releasing MMPs [19], which are zinc-dependent endopeptidases involved in tissue remodeling following pathologic

conditions. MMPs are well known to increase BBB permeability by degrading the extracellular matrix [29] and by cleavage of tight junction proteins such as occludin and claudin-5 [30,31]. Among these MMPs, MMP-2, -3 and -9 are known to be elevated during pathophysiology of stroke [19,32] and TBI [33], and so to be implicated in extracellular matrix degradation, BBB disruption and edema formation. However, it should be noted that degradation of the extracellular matrix or basement membrane is not solely a deleterious process. Indeed, this matrix is composed of proteins containing domains with biological activities that are sequestered when the membrane is fully assembled. Only through MMP-mediated degradation of the matrix can these proteins liberate their active domains. For example, endostatin, arrestin, canstatin and tumstatin are collagen fragments with antiangiogenic properties that have domains liberated via MMP degradation of the extracellular matrix [34]. Interestingly, a recent study showed that one such protein, perlecan domain V, may serve as a viable therapeutic approach for stroke. Perlecan domain V is derived from the basement membrane, and is indeed upregulated during stroke, thus offering neuroprotective and proangiogenic effects [35].

tPA is well known for its implication in BBB alteration. tPA is a serine protease initially described for its role in fibrinolysis promotion [36], but since tPA is expressed in the brain parenchyma, other functions have been described in both physiology and pathology [37]. Moreover, tPA is used as the only US FDA-approved treatment for thrombolysis after ischemic stroke, but excess tPA may not be very sustainable in the CNS. Accordingly, it may induce several deleterious consequences.

First, tPA can induce extracellular matrix degradation by promoting plasmin formation [38]. Then, tPA is able to increase MMP-9 expression and MMP-3 activity by binding to the cell surface receptors LRP (low-density lipoprotein [LDL] receptor-related protein) in endothelial cells [39]. LRPs belong to the LDL receptor family and they are mainly implicated in receptor-mediated endocytosis and cell signaling. Interaction between tPA and LRP induces Akt phosphorylation and activation of NF- κ B. Activation of these pathways leads to activation of MMP-3 and BBB disruption. Moreover, it leads to the shedding of astrocytes LRPs ectodomain and then to the detachment of astrocytic endfeet processes [40,41]. Binding of tPA with LRP would also facilitate cleavage of PDGF-CC. Activated PDGF-CC can bind to and activate PDGF-receptor α (PDGF-R α), leading to the detachment of astrocytic endfeet [42]. These events are well understood in the context of ischemic stroke owing to exogenous tPA, but could also be an important player in cases of TBI. Indeed, it has been shown that tPA activity is increased by 30% within 1–3 h after CCI in mice and returns to basal levels by 24 h [43]. Interestingly, this tPA-mediated induction of MMP-3 activity has been seen in mice 3 h following brain trauma, reaching baseline by 24 h [44]. This suggests that endogenous tPA is involved in the induction of MMPs after TBI. Moreover, knockdown of tPA has been shown to reduce vasogenic edema in a model of mice with CCI [45]. In addition to the negative consequences on the vascular bed, tPA is also known to be part of excitotoxicity poststroke, because it was shown to bind to the GluN1 subunit of the NMDA receptor in neurons and to potentiate neuronal excitotoxicity [46]. While toxicity has been well studied for stroke, there are evidences that endogenous tPA enhances NMDA receptor-induced neurotoxicity after TBI [47]. Therefore, MMPs, tPA and LRPs are some of the key elements implicated in BBB leakage and eNVU dysfunction during the postinjury acute phase.

Role of NO in eNVU changes in BBB to CBF: oxidative stress

NO was identified in 1987 as a gas acting as a regulator of vascular tonus [48]. Since then, NO has been demonstrated to be a signaling molecule implicated in several physiological functions of the brain (maintenance of CBF, neurotransmission and neuromodulation) and in pathophysiological processes, especially when CBF is dysregulated and when there is

inflammation, such as during stroke and TBI [49,50]. NO is synthesized from L-arginine by three different NO synthases (NOS). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and they are calcium dependent. Inducible NOS (iNOS) is calcium independent and is expressed in many cell type of the eNVU (astrocytes, macrophages and microglia). NO has multiple roles and functions in the eNVU. It can be toxic when it interacts directly with various protein moieties, leading, for example, to the formation of a peroxynitrite anion, which is a strong oxidant. NO can also induce an intercellular signaling pathway leading to the formation of cGMP, thus having relaxing effect on vascular smooth muscle cells [51,52].

As with stroke, during TBI, the intracellular calcium increase leads to constitutive increase in eNOS activity during the first minutes after injury. Then, activity of eNOS decreases to approximately 50% of baseline for 7 days before its levels normalize [53,54]. This decrease of eNOS activity may contribute to altered CBF and cerebral autoregulation. Following this, we can observe an approximate 50% decrease of NO levels for up to 7 days [54,55]. These changes in the cerebral autoregulation suggest the need to have a better understanding of the molecular changes in the eNVU including the penetrating and pial blood vessels. One of the potential mechanisms is possibly the transformation from the contractile to synthetic phenotype of the smooth muscle cells as it was shown on carotids after hypoxia [56].

Unlike constitutive NOS, iNOS expression and activity in neurons, macrophages, neutrophils, astrocytes and oligodendrocytes, are upregulated after stroke and TBI for up to 7 days [53,57]. This increase has been shown to be harmful since it induces a large production of NO, leading to cell damage with neuroinflammation, excitotoxicity, apoptosis, energy depletion and uncoupling of NOS with the production of reactive oxygen species.

Postinjury edema process

Formation of brain edema is a common feature in both stroke and TBI and has a crucial impact on patient morbidity and mortality [58]. Brain edema contributes to secondary injuries by decreasing CBF [59]. Indeed, the increase in net brain water content leads to an increase in tissue volume, accelerates herniation and, thus, mechanically decreases brain perfusion.

After brain injuries, cerebral edema is traditionally explained by two mechanisms, cytotoxic and vasogenic edema. Cytotoxic edema is defined by intracellular water accumulation without BBB disruption, whereas vasogenic edema appears after BBB leakage. This leads to a diffusion of blood proteins in the parenchyma followed by water accumulation in the extracellular space.

However, recent observations made regarding the molecular mechanisms of edema formation and BBB properties, suggest that this explanation is too simple and a third type of edema occurs, ionic edema [58,60]. This revision proposes a novel order of events.

After the primary injury, the initial anoxic edema, corresponding to the cytotoxic edema, occurs within minutes after decrease of the CBF and is characterized by swelling of the astrocytes and neuronal dendrites. The absence of oxygen and energy nutrients induces a disruption of the cellular ionic gradients and leads to entry of ions into cells, accompanied by water entry, which induces cellular swelling. Aquaporin (AQP) channels may facilitate water entry via AQP4 on astrocytic endfeet [61].

The second step is the evolution toward the ionic edema, where the absence of oxygen and nutrients further alters the ionic gradients of endothelial cells, including their transcapillary flux of Na^+ [60,62]. This could contribute to the early transient leakage of the BBB, with the

consequence of further entry of water through endothelial cells resulting in brain swelling [13,63].

The final step in this cascade of events is the development of vasogenic edema, where there is disruption of cerebrovascular endothelial tight junctions, leading to increased permeability to albumin and other plasma proteins [64].

Regarding water movement during this process, the discovery of AQP channels in astrocytes suggests that these channels could be a key molecular player. AQP1, AQP4 and AQP9 have been identified in the rodent brain, where they play different roles. AQP1 in epithelial cells of the choroid plexus contributes to cerebral spinal fluid formation, and AQP9 in astrocytes and catecholaminergic neurons would play a role in brain energy metabolism. However, AQP4, the most abundant AQP in the brain, is present in astrocyte endfeet around all the brain vessels and contributes primarily to water homeostasis in the eNVU. AQP4 is clearly involved in the edema process [65]. Moreover, its expression has been shown to be changed in many pathologic conditions like subarachnoid hemorrhage, spinal cord injury (SCI), TBI and stroke [58].

AQP4 is present in astrocyte endfeet in the vicinity of the vascular bed [66] and its level of expression changes after TBI and stroke with differences in the time course and pattern. There is abundant literature studying the changes of this water channel in parallel to edema development [65]. To summarize, increased AQP4 levels are observed between 1 and 48 h after cerebral ischemia [67], whereas contrasting results are seen after a TBI, with some studies reporting an increase in AQP4 [68] and with others reporting a decrease [69]. Altered expression levels in the time after injury appear to be model dependent. With regard to this observation, some studies showed discrepancies in the role of AQP4 after injury such as SCI [70,71] outlining the complexity of the role of AQP4 in the edema process. Indeed, AQP4 deletion in AQP4-knockout mice (AQP4^{-/-}) is protective with a decrease of edema formation and lower lesion size in a model of SCI [71]. However, using a similar model and same transgenic mice, a second set of data showed a functional improvement for the wild-type mice compared with AQP4^{-/-} after contusion SCI, suggesting that AQP4 plays a protective role by facilitating the clearance of excess water [70].

This beneficial role of the AQP4 in water clearance was proposed for several years and suggested by several immunohistochemistry studies showing increased AQP4 expression in parallel to water clearance in both stroke and TBI models [13,72–74]. This hypothesis was supported by several studies showing, using a drug, that edema is decreased during times when levels of AQP4 increase [13,74,75]. For example, post-TBI edema was decreased using sulforaphane in correlation with an increase in AQP4 expression [75]. However, the question of the role of AQP4 in water clearance after brain injury has not been elucidated until now as there is no specific drug to block these channels.

In the laboratory, the authors developed the approach to use small interferent RNA targeting AQP4 expression (siAQP4) to silence the level of AQP4 expression [61]. Using this technique, the authors showed that the early decrease of AQP4 improves the functional outcome after TBI in rodents [76]. The authors' results strongly support the hypothesis that the presence of AQP4 early after brain injury contributes to edema formation, while presence of AQP4 at later stages may assist in edema resolution. These reports show the multifaceted role of AQP4 in the NVU for appropriate maintenance of water homeostasis under normal and abnormal conditions. Thus, roles of AQP4 in brain pathologies and in the NVU need to be more intensively studied.

Future perspective: potential therapeutic targets

This section will focus on different drugs that affect the eNVU on the endothelium, as well as on the smooth muscle and astrocyte layers, which compose the eNVU. For example, tPA is the unique drug used in ischemic stroke to dissolve blood clots in the vasculature. However, its side effects on adjacent vasculature, as well as brain cells (astrocytes and neurons), reveal the importance of considering the eNVU as a functional unit for drug development. As it is illustrated in this part, drugs such as tPA are also very likely to have effects on the larger blood vessels. It is important to consider that any changes in endothelial cells would have effects on the smooth muscle layer in larger blood vessels, as well as on astrocytes in the capillary bed. Similarly, changes in astrocytes would have consequences on physiological properties of the smooth muscle layer of the large blood vessels and endothelial cells at the capillary level.

tPA: from side effects to benefits

tPA, owing to its role in fibrinolysis and its capacity to degrade blood clots, is the only available thrombolytic treatment for acute ischemic stroke [36]. If patients are treated during the therapeutic window (4.5 h), injured vessels have a chance to be reperfused and CBF can be restored in the ischemic area [77]. The ischemic zone can be rescued and clinical outcome can be improved.

However, tPA is a complex multifaceted protein that also displays prohemorrhagic and neurotoxic effects within the eNVU [37]. Several strategies tend to avoid those deleterious effects of tPA. It was shown that tPA-S481A, a catalytically inactive tPA variant, can reduce neurotoxicity induced by tPA in TBI. tPA-S481A competes with endogenous tPA and prevents tPA-mediated NMDA receptor overactivation [47]. An immunotherapy strategy has also been developed to block the interaction between tPA and the GluN1 subunit of the NMDA receptor. This antibody (anti-ATD GluN1) binds to the aminoterminal domain of GluN1, prevents potentiation of excitotoxicity induced by tPA and lowers PDGF-CC activation [78]. Another strategy has been designed in the context of ischemic stroke to produce tPA mutants capable of inducing fibrinolysis, but unable to induce neurotoxicity [79]. Moreover, owing to its modifications, such tPA mutants should be able to lower PDGF-CC activation and limit BBB opening.

Despite neurotoxic and prohemorrhagic effects of tPA, new studies also underline the complex and multiple roles of tPA. Indeed, tPA has been shown to promote phenotypic switching of vascular smooth muscle cells and type I collagen deposition following deep vein thrombosis [80]. These phenomena participate in venous wall remodeling, showing the importance to consider eNVU and not only the capillary bed. These data also strongly suggest that application of tPA could be beneficial long after stroke and even TBI. The new observations underline the possibility of using this FDA-approved drug in the context of 'neurovascular regeneration' later after a stroke or a TBI.

Modulation of NO levels & NOS activity

As previously described, NO and NO signaling can have dual effects during pathophysiology of ischemic stroke and TBI. Briefly, decreases of NO levels may be deleterious for the CBF regulation whereas increases of NO via iNOS upregulation would be more harmful because of reactive oxygen species formation.

Therapeutically, it may be possible to compensate for decreases in the NO signaling pathway after stroke and TBI by administration of NO donors, such as sodium nitroprusside (SNP) and, thus, improve CBF and cerebral autoregulation [81,82]. Indeed, administration of SNP can prevent the reduction of CBF after stroke or TBI. However, SNP could not

reverse impaired cerebral autoregulation during hypotension after fluid percussion injury in the newborn pig [81]. This therapeutic strategy could only be applicable during the early stage of TBI pathology, as previously described for stroke [82]. As discussed in this review, other molecular changes may occur in the vascular bed, including smooth muscle differentiation. Interestingly, a recent study showed early changes in the expression contractile proteins in smooth muscle cells after TBI [83].

The use of inhaled NO donors seems to be promising since it has been recently shown that it can lead to arteriolar dilatation and improvement of cerebral perfusion in a model of permanent middle cerebral artery occlusion in sheep [84]. Improvement of CBF was also seen in a mouse model of CCI with a decrease in intracranial pressure. Long-term treatment with NO inhalation (24 h) was beneficial showing decreased lesion volume and improved neurological outcome after 7 days. Interestingly, no adverse effect was observed concerning cerebral autoregulation, blood pressure, or oxidative damage [85].

Another way to improve CBF and cerebral autoregulation would be through activation of eNOS and/or downregulation of iNOS activity. Upregulation of eNOS activity and expression can be achieved using HMG-CoA reductase inhibitors (statins). Indeed, in a model of middle cerebral artery occlusion in spontaneous hypertensive rats, intravenous administration of pravastatin 10 min after surgery induces upregulation of eNOS expression and activity, and reduces brain edema. Pravastatin is one of the two hydrophilic statins suitable for intravenous injection, with the other being rosuvastatin. The upregulation of eNOS would lead to improvement in endothelial function, vasodilation, and so to the maintenance of blood supply around the primary lesion. With time, this would rescue damage in the ischemic penumbra, limit the extension of the lesion core and improve neurological outcome [86]. In addition, statins are interesting components because they are able to inhibit excitotoxicity [87], to prevent glutamate-induced BBB disruption in an *in vitro* model [88], to decrease tPA-induced MMP-9 synthesis in rat astrocytes [89], and to decrease neuroinflammation by limiting NO production via the decrease of iNOS expression [90].

A very recent study showed that eNOS activation could be obtained in human vascular endothelial cells by the use of persimmon (an orange berry) extracts. These bioactive extracts would increase NO availability and decrease secretion of endothelin-1, suggesting a better blood supply [91].

Drugs against AQP4

AQP4 should be considered as a potential therapeutic target to reduce edema in patients with cerebrovascular disease or trauma [72]. However, following treatment of stroke, heterogeneous alterations in AQP4 expression demonstrate the complexity of modifying edema reduction. For example, hypertonic saline treatment induces increased or decreased AQP4 expression after stroke [92]. It appears that the expression of AQP4 is dependent on the stroke model used, further complicating pharmacological manipulation for modulating this protein.

Currently there are no specific inhibitors to block the AQP4 channel, and such a compound is essential for evaluating the role and treatment of edema. Recent studies have proposed a range of compounds that may block AQP4, including butenamide which blocks the AQP4 channel and water permeability in oocytes [93]. However, butenamide is also an inhibitor of Na–Cl–K cotransporter expressed in endothelial cells, thus complicating *in vivo* validation [62]. Butenamide prevents edema formation in stroke models [62,94], which correlates with decreased AQP4 expression [94], with the caveat that these efficacious results may be due to partial inhibition of the endothelial cell Na–Cl–K cotransporter.

Other investigators have proposed acetazolamide, a sulfonamide carbonic anhydrase inhibitor, as an inhibitor of water permeability associated with AQP1 and AQP4 [95]. However, it was reported that acetazolamide has no effect on water permeability [96]. Two other inhibitors belonging to sulfonamide carbonic anhydrase inhibitor class, methazolamide and valproic acid, have also been tested, but without clear benefits [95]. Finally, erythropoietin has been shown to indirectly decrease the permeability of AQP4 via activation of metabotropic glutamate receptors [97]. Development of specific drugs targeting AQPs is required to further demonstrate the role of these channels in water modulation in cerebrovascular disease.

The approach that consists in targeting AQP4 RNA expression with RNA interference (siAQP4) in a restricted area (lesion site) is another way to study roles of AQP4 during pathology. The study in our laboratory shows that the 30% decrease in AQP4 expression induced by siAQP4 at the lesion site after a rat model of rat juvenile TBI leads to decreased edema formation and BBB disruption, increased microglial activation, and reduced astrogliosis and neuronal death [76]. Beyond studying AQP4 implication during TBI, siAQP4 serves as a potential treatment after TBI since it reduces edema and improves functional recovery even 60 days after injury.

Conclusion

In this review, we outline the fact that in order to protect the CNS after acute brain injuries such as stroke and TBI, more than the classical NVU, the eNVU should be considered in preclinical studies and targeted for drug development. In support of our recent proposed idea [1], tPA was shown at long-term point to be involved in vascular remodeling by changing the phenotype of smooth muscle cells, and some statins are also acting on feeding arteries [80], improving the CBF [86]. As indicated earlier, it is important to remember that most of the cerebral endothelial cells exhibit BBB characteristics, from the capillary bed to the penetrating blood vessels [12]. Therefore, BBB disruption and edema formation are very likely to occur in the larger feeding blood vessels, as well as in the capillary bed. Endothelial dysfunction in larger blood vessels would have toxic effects on smooth muscle layer. In support of the changes observed in endothelial cells of the larger blood vessels, we observed phenotypic transformation after TBI in penetrating blood vessels with increases of claudin-5 expression in the endothelium [24]. Moreover, the whole cerebrovascular tree is engulfed by astrocytes, which also sustain major changes in terms of protein expression after stroke or TBI [98]. The changes in astrocytes properties may also have consequences on the adjacent cells, such as endothelial cells in capillary bed, and also on smooth muscle cells in the larger blood vessels. All these interactions are so far understudied in stroke and TBI pathophysiology.

As a whole unit, endothelial cells, smooth muscle cells, astrocytes, neurons are implicated in stroke and TBI pathophysiology. Therefore, future studies and drug development should be on this intricate network of cells in order to improve cerebral circulation, avoid excitotoxicity, edema, inflammation and finally to protect neurons and improve recovery not only early after injury but also in the long term.

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Executive Summary

Ischemic stroke & traumatic brain injury affect the expanded neurovascular unit

- Similar cascades of secondary injury events occur in the expanded neurovascular unit (eNVU) during acute brain injuries, such as blood–brain barrier (BBB) disruption, edema, excitotoxicity, oxidative stress and neuroinflammation.

BBB sustains changes during & long after brain injuries

- The BBB is disrupted early after the onset of stroke and traumatic brain injury (TBI) owing to the lack of energy and the activation of proteases, such as tissue plasminogen activator (tPA) and matrix metalloproteinases, further affecting extracellular matrix and tight junction proteins such as claudin-5 and occludin.
- The BBB shows long-term changes with phenotypic alteration of endothelial cells after injury. For example, levels of P-glycoprotein are decreased and levels of claudin-5 are increased 60 days after TBI in rats. Long-term changes in the BBB may increase risk of developing neuropathology found in Alzheimer's disease.

Edema process implicates the water channel AQP4

- Although discrepancies exist in this field, there are growing evidences showing that AQP4 has an important role in edema formation.

Nitric oxide is implicated in eNVU & cerebral blood flow changes after brain injury

- Nitric oxide (NO) plays multiple roles in the eNVU including cerebral blood flow regulation and enhancement of inflammatory processes. During stroke and TBI, modification in endothelial and inducible NO synthases may contribute to dysregulation of cerebral blood flow and neuroinflammation.

Multiple roles of tPA in the eNVU

- tPA is the only US FDA-approved treatment for thrombolysis during ischemic stroke, but shows deleterious effects with high risk of hemorrhagic transformation and neurotoxicity. Promising strategies have developed to avoid proneurotoxic and prohemorrhagic effects of tPA. Moreover, tPA's influence on vascular remodeling could be used in the context of 'neurovascular regeneration' poststroke or TBI.

NO modulation & cerebral blood flow regulation

- Several strategies are interesting and promising:
 - The use of inhaled NO donors leading to better cerebral perfusion and decreased lesion volume after stroke and TBI;
 - Statin treatment leading to increase of endothelial NO synthases activation and maintenance of blood supply around the primary lesion. In addition, statins are able to inhibit excitotoxicity, prevent glutamate-induced BBB disruption, decrease tPA-induced MMP-9 synthesis and decrease neuroinflammation by limiting NO production via the decrease of inducible NO synthases expression.

Drugs against AQP4

- Specific pharmacological agents against AQP4 are needed for a better understanding of its roles in the eNVU and for potential treatments of edema. Targeting AQP4 expression using siRNA targeting AQP4 could be a treatment to limit edema during stroke and TBI.

Figure 1. The expanded neurovascular unit concept

The expanded neurovascular unit consists of the 'classical' neurovascular unit with capillary endothelial cells, neurons, astrocytes and pericytes, but it also includes the vascular tree with arterial smooth muscle cells, arterial and venous endothelial cells, and perivascular nerves from larger vessels.

Figure 2. Similarities and differences in primary and secondary injuries during pathophysiology of ischemic stroke and traumatic brain injury

Both brain injuries start with different primary events leading to a very similar cascade of secondary events encompassing BBB disruption, excitotoxicity, oxidative stress, inflammation, neuroinflammation and edema formation, with an increase of ICP followed by a decrease of the CBF and the formation of an ischemic area. For the first few days, these events contribute to the persistent opening of the BBB. In the longer term, these secondary events lead to functional deficits and could lead to the development of cognitive impairment or other pathologies, such as Alzheimer's disease or epilepsy.

BBB: Blood-brain barrier; CBF: Cerebral blood flow; ICP: Intracranial pressure.