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Blood–brain barrier, bulk flow, and interstitial clearance in epilepsy

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Abstract

Understanding the pathophysiology of epilepsy implies elucidating the neurovascular modifications occurring before or at time of seizures. Cerebrovascular dysfunction provokes or sustains seizures and loss of selective blood–brain barrier (BBB) permeability is a modulator of seizure threshold. However, cerebrovascular pathology in epilepsy extends beyond BBB “leakage” to encompass vascular and parenchymal events. Whenever abnormal accumulation of protein is observed surrounding brain blood vessels, BBB disruption (BBBD) was invoked. Recent clinical and laboratory findings challenged an exclusive role of BBBD in perivascular accumulation of serum-derived products. The circulation of interstitial fluid (ISF) and its bulk flow have emerged as candidate mechanisms which play a role in clearance of CNS waste. Although controversy exists, changes of ISF flow may contribute to CNS disorders through a mechanism encompassing incomplete parenchymal clearance and accompanying accumulation of toxic byproducts.

We summarize the evidence in favor and against ISF bulk flow and propose a scenario where abnormal ISF in the epileptic brain allows accumulation of brain protein, sustaining pathophysiology and altering the pharmacology of antiepileptic drugs. We also describe the methods routinely used to dissect out the contribution of BBB-dependent, vascular or paracellular mechanisms to altered neuronal excitability.

Keywords

Glymphatics; Cerebrospinal fluid; Blood–brain barrier; Epilepsy; Seizures

1. Neurovascular origin of seizures: What’s next?

There is little doubt that cerebrovascular dysfunction favors or sustains seizures (see van Vliet et al., 2007; Marchi et al., 2012b, 2014; Raabe et al., 2012; Tomkins et al., 2011 for

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review). The role of cerebrovascular damage in CNS disorders, including epilepsy, was in the past clinically accepted (Penfield, 1933) and sporadically proposed (Janigro, 1999; Marchi et al., 2007; Oby and Janigro, 2006; Seiffert et al., 2004) as a leading mechanism underlying seizure activity. It is only in the past decade that cerebrovascular research has attracted considerable attention among researchers in the field of epilepsy (Janigro and Walker, 2014). Restoring cerebrovascular integrity has also been proposed as a complementary approach to traditional antiepileptic drugs (Janigro et al., 2013a; Marchi et al., 2012a, 2011, 2012b, 2014).

In the case of human epilepsy, data are highly suggestive of loss of BBB selective permeability in focal regions from which seizures originate. In addition, data from several groups support that the epileptic BBB presents a variety of molecular signatures that are in one way or another involved in the disease. These span from expression of multiple drug resistance-related transporters (Dombrowski et al., 2001) and enzymes (Boussadia et al., 2014; Ghosh et al., 2013; Ghosh et al., 2011), to abnormal levels of GLUT-1, a glucose transporters (Cornford et al., 1998). Most of human data derive from analysis of resected tissue, where expression of an array of drug extrusion molecules has been reported (Dombrowski et al., 2001) and leakage of capillaries or vessels reported by several groups after the original observation by Cornford (Cornford and Hyman, 1999; Cornford et al., 1998). Additional evidences accumulated over the years prove that, during pathological examination, the BBB of resected brain tissue is compromised (Oby and Janigro, 2006). While the pathophysiology of a lesion occurring remotely from the location of ictal activity is not fully understood, the basis of local peri-ictal imaging findings are easier to interpret. When considering local peri-ictal imaging abnormalities hippocampal swelling, focal lesion, BBB breakdown and altered neurovascular caliber and flow are commonly observed. In general, vasogenic edema is detected by fluid-attenuated inversion recovery (FLAIR)—T2 magnetic resonance imaging (MRI) while cytotoxic edema is evaluated by diffusion weighted imaging (DWI) corresponding to a drop in apparent diffusion coefficient (ADC). Increase in ADC can correlate with the presence of vasogenic edema and BBB breakdown. Gadolinium enhanced T1 also indicates BBB damage and vasogenic edema. In brief, direct (contrast enhanced MRI) evidence for BBB damage in epileptic brain is variable while less specific sequences used to detect edema, such as FLAIR, may suggest loss of cerebrovascular integrity (Fig. 1).

Whether or not the BBB is disrupted in epilepsy was studied in animal models to reveal extravasation of contrast agent (Gd-MRI) after kainic acid-induced chronic epileptic seizures (e.g., van Vliet et al., 2014). In this case BBB disruption *follows* seizure development. Acute seizures can be in turn *caused by* sudden BBB disruption (BBBD) (Marchi et al., 2007a, 2009b, 2011, 2007b).

Generally, altered permeability (leakiness) across tight junctions and endothelial damage were considered as the main vascular culprits of abnormal neuronal activity triggered by BBBD (Janigro, 2012). The latter notion is correct and reductionist at the same time, especially considering the numerous functions exerted by the cerebrovasculature that are not related to the BBB. A flurry of recent evidence has attempted to move beyond the notion of leakiness by investigating the dynamics of neurovascular coupling and the relevance to

pathological interictal-to-ictal-transitions (Dreier, 2011; Harris et al., 2014). For example, rat seizures are remarkably associated with side-specific variation in cerebral blood flow changes during stimulation of afferents to a vibrissa cortex (Harris et al., 2014). Similarly, spreading depression-like depolarizations lead to vascular hyperperfusion in healthy tissue, but progressive damage is detected in hypoperfused brain regions (Dreier, 2011). These two examples describe the complexity of neurovascular coupling, and the direct relevance of vascular changes during ictal or interictal activity. The converse is also true, as pathological changes in endothelial cells, astrocytes or pericytes cause abnormal neuronal activity (e.g., Marchi et al., 2007a). However, the regulation of cerebral blood flow and BBB function are not the whole story: departure from physiological homeostasis is also reflected by abnormal composition and circulation of the interstitial fluid (ISF), possibly sustaining CNS diseases. A role for ISF bulk flow in the epileptic brain is herein proposed and discussed (Fig. 1).

2. Paths of brain fluid circulation

Appropriate ISF circulation and clearance is a prerequisite for normal brain physiology. Contrary to the periphery, brain lacks a lymphatic drainage system that collects the interstitial fluid exiting the capillaries due to Starling forces and protein extravasation. This is accompanied by net water loss driven by the pressure differential between vascular and oncotic pressures, the latter being lower. While part of the ISF is reabsorbed into the venous bed, a quote is drained into the lymphatic circulation, constituting the lymph (Levick and Michel, 2010). Interstitial fluid recycling and clearance is fundamental to keep volemia, xenobiotic or waste compounds, and immune cells in check. In the brain, the absence of a bona fide lymphatic system is compensated by cerebrospinal and interstitial fluid (CSF and ISF) circulation. The CSF is produced by the choroid plexus circulating through the cerebral ventricles and arriving in the sub-arachnoid space (SAS) where it is reabsorbed into the systemic circulation or reaching lymphatics at the cranial nerve level. A portion of CSF in the SAS could percolate into the parenchyma along penetrating vessels (Virchow-Robin space) and perivascular space of capillaries (Begley et al., 2000; Johanson et al., 2008). The movement of CSF could be driven by arterial pressure or ISF could be directly produced by capillary secretion (Iliff et al., 2013b). At the blood–brain barrier, endothelial cells and astrocytes express a battery of polarized ion channels (endothelium) and aquaporins (astrocytes; e.g., AQP-4) controlling ion osmolarity and driving water fluxes. Water passively follows ionic gradients, generating ISF. Direct arterial secretion leads to clean ISF, localized at the brain parenchyma, directly bathing the neurons. According to a recent hypothesis, CSF is not formed primarily by the choroid plexuses, nor it is circulated to be absorbed (Oreskovic and Klarica, 2011). The alternative hypothesis states that CSF appears and disappears throughout the entire CSF system, depending on the hydrostatic and osmotic forces between the CSF, ISF and blood capillaries. Osmotic and hydrostatic forces are crucial to the regulation of ISF–CSF volume. In terms of the capacity of fluid exchange, the cerebral capillaries are the dominant location, and the choroid plexuses are a less relevant place for this process.

Regardless of its origins, the ISF moves along the perivascular space driven by the hydrostatic pressure (Abbott, 2004; Begley et al., 2000; Davson and Segal, 1995) or arterial pulsation (Iliff et al., 2013b) and then re-absorbed at the venular level (Fig. 2). Controversy

has existed all along the development of these hypotheses, mainly due to inconsistent results and technical challenges (see for review Johanson et al., 2008). The extent and directionality (inward or outward) of the ISF bulk flow, extent of CSF–ISF communication and the nature of ISF driving force have been questioned (Abbott, 2004; Johanson et al., 2008; Oreskovic and Klarica, 2011). Recently the term *glymphatics* was used to identify the AQ-4-dependent CSF–ISF exchange (Iliff et al., 2013b).

3. A role for abnormal ISF circulation in seizure disorders?

As ISF flow is an accepted route of parenchymal clearance, interfering with ISF may exacerbate disease pathophysiology. Recent evidence support a role in amyloid β (A β) clearance in Alzheimer's disease (Iliff and Nedergaard, 2013; Kress et al., 2014), as A β clearance requires AQ-4 expression and proper CSF–ISF exchange. Suppressing CSF–ISF exchange after traumatic brain injury affected the appearance of brain biomarkers in blood, supporting a role of CSF–ISF communication in clearance functions (Plog et al., 2015).

The possibility exists that an abnormal ISF circulation could trigger seizure activity or perpetuates epilepsy. A transient damage occurring at large penetrating or at the parenchymal capillaries could affect the CSF–ISF flow (Fig. 1). Rupture of superficial vessel wall could further interfere with the CSF penetration into the deeper parenchymal regions from the Virchow-Robin space, altering microvascular ISF. The latter scenario assumes that sub-arachnoid CSF is the main source of ISF. However, if ISF derives from blood–brain barrier secretion, only a microvascular damage occurring in the parenchyma will affect ISF circulation. The dysfunction may extend beyond the time window of BBB leakage, when serum proteins extravasate or altered ionic balance persists over-time in the perivascular compartment (Fig. 3). Initial insults occur during concussion or TBI, infection or ischemic stroke, all determinants of seizures and associated with an initial loss of BBB integrity (de Vries et al., 2012). Topographic correspondence between BBB damage and abnormal EEG activity suggests a role for a vascular lesion in the pathogenesis of post-traumatic epilepsy (Korn et al., 2005). Seizure occurrence is delayed, resulting from metabolic unbalance, edema and an inflammatory response (Tomkins et al., 2011). Long-term pathological changes, when installed, may modify the ISF flow, reducing clearance mechanisms and favoring the accumulations of toxic molecules (Figs. 1 and 2). This mechanism may apply to the epileptic brain where repetitive transitions to abnormal neuronal activity could affect ISF flow, sustaining a pathological vicious cycle.

Phosphorylated tau (PT) protein expression levels and patterns in epileptic brain resections are comparable to those observed in samples from patients diagnosed *post mortem* with cerebral traumatic encephalopathy (CTE) (Fig. 4 and unpublished observations). Thus, both CTE and epileptic brain samples displayed perivascular staining for PT which was also present in neuronal cell bodies and neurites. Given that both CTE and refractory seizures are associated with PT deposition, which pathophysiological events do they have in common possibly resulting in an abnormal deposition of PT? A “blood–brain barrier hypothesis” whereby tau accumulates in brain due to leakage is unlikely (Fig. 5A). The axonal injury hypothesis is also not supported by our results since TBI or head injury were not prerequisites of PT accumulation. We postulate that an abnormal clearance of PT is the

potential mechanism of its accumulation (Fig. 5B). According to this hypothesis, regardless of the causes initiating the phosphorylation of tau and its release from microtubules, extracellular levels of tau are cleared by the “glymphatic” system as showed for β -amyloid (Iliff et al., 2013a, 2013b). According to this mechanism, the presence of elevated levels of tau reflect poor clearance of waste from the brain. This is supported by several observations: (1) There was no co-localization between the extravasation markers albumin or IgGs and perivascular phosphorylated tau; (2) The perivascular accumulation of phosphorylated tau was found around larger (diameter greater than 100 μ m, Fig. 4) vessels with a poorly delineated vascular wall, or venules, as predicted by the glymphatic hypothesis; (3) Perivascular tau did not extend radially from the vessel, as predicted by extravasation, but rather followed extracellular, longitudinal, paravascular tracks; and (4) Unlike IgGs and albumin (Janigro, 2012), phosphorylated tau brain concentrations exceed serum levels, which makes a vascular origin of brain phosphorylated tau highly unlikely. These results show evidence of tau accumulation in human epileptic brain due to poor clearance, as predicted by animal models (Iliff et al., 2014).

4. Visualization of the blood–brain barrier and parenchymal diffusion during seizures

Imaging the blood–brain barrier and interstitial or perivascular space is challenging. Available tools include microangiography (Cavaglia et al., 2001), 2-photon microscopy (Iliff et al., 2013b), magnetic resonance imaging (MRI) and serum markers (Bargerstock et al., 2014; Janigro et al., 2013b; Kanner et al., 2003; Marchi et al., 2012b). Fluorescent Dextran or Albumin has been used to visualize regional cerebrovascular morphology, damage and the pattern of interstitial protein accumulation (Boussadia et al., 2014; Cavaglia et al., 2001; Fan et al., 2008; Hallene et al., 2006). Fluorescent microangiography in combination with fluorescent markers is useful to elucidate differential parenchymal penetration and interstitial diffusion depending on molecular weight or size. Fig. 6 shows an example where a cock-tail of FITC-Albumin and Evans Blue was injected in the external carotid artery following blood–brain barrier opening. Abnormal electrographic activity followed a successful BBB opening (Fieschi et al., 1980; Marchi et al., 2007a). Evans Blue binds to all serum proteins independently from their molecular weight, with a percent of dye remaining unbound. Evans Blue rapidly diffuses into the parenchymal as compared to FITC-Albumin that accumulates in microvessels or is present in the immediate perivascular space (Fig. 6). Protein diffusion across a leaky BBB alters perivascular osmolarity, creating a localized driving force for water movement and possibility affecting ISF circulation or CSF–ISF communication (Fig. 3). However, the molar contribution of protein to brain or serum osmolarity is quite low, and it appears that increased neuronal firing and the downstream ionic cascade that follows is a potentiating factor for the establishment of edema around the vessel (Janigro and Walker, 2014; Marchi et al., 2014).

The following should be considered when performing fluorescent microangiography: (i) high molecular weight fluorescent tracers (>30–50 kDa) are suitable for a detailed evaluation of cerebrovascular structures, independently from anatomy (deep cortical layers, hippocampus); (ii) severe BBB damage allows extravasation of high molecular weight

(MW) markers (Albumin or Dextran); (iii) Evans Blue easily accesses the perivascular and interstitial space owing its non-specific protein binding and low molecular weight; (iv) Evans Blue can be quantified by spectroscopy or fluorescent microscopy since it emits under UV in the red spectrum; (v) a drawback of the use of Evans blue is the potential for false positive (e.g., non-BBBD related parenchymal extravasation); (vi) combinations of dyes and MW tracers is technically advantageous, allowing a qualitative and quantitative analysis of differential protein blood-to-brain diffusion; (vii) the route (peripheral circulation vs. CNS) and the modality (acute injection vs. infusion) of fluorescent dye administration are significant. CNS injections have been extensively used to assess CSF production and parenchymal fluid diffusion at the sub-arachnoid, parenchymal, intra-ventricular (Abbott, 2004) and *cisterna magna* (Iliff et al., 2013b). Intra-arterial (carotid artery), intra-venous (tail) or intra-cardiac injections all allow cerebrovascular mapping. The choice of peripheral access point depends on the size of the animal used (difficulty of intra-arterial access in small rodents) and the time interval to be studied. The net passage of dye into the parenchymal space is visualized and quantitatively determined performing post-perfusion with saline, eliminating the intravascular quote of dye.

Recent advancement in 2-photon microscopy (2-PM) has allowed visualizing the cerebrovasculature in experimental disease conditions, including epilepsy (Fabene et al., 2008). The use of 2-PM in cerebrovascular research was extensively reviewed by Shih et al. (2012a). This technique has several advantages compared to *ex-vivo* microangiography, with the main caveat of anatomical limitation associated with the cranial window preparation (100–150 μm cortical depth). The latter can be overcome by surgically removing cortical areas and directly visualizing the hippocampus. However, brain inflammation is intrinsic to the preparation, introducing a main confounding factor. Most 2-PM surgeries are terminal, also limiting the time-window for manipulation. The latter drawback have been recently overcome using a thinned-skull transcranial 2-PM (Shih et al., 2012b). This technique permits survival and repetitive imaging sessions within a defined cortical region of interest. Thinned-skull preparation is devoid of inflammatory changes. Intravenous fluorescent microangiography at each imaging session allows the visualization of microvascular structures.

The use of MRI in experimental models of epilepsy has progressively increased, allowing for longitudinal studies and whole brain imaging within the same animal. This has been reviewed including the application of post-acquisition T2- and T1-weighted imaging in animal models (Grohn and Pitkanen, 2007; Nodde-Ekane et al., 2010). Available radiological findings support BBB damage during seizures. Recently, intravenous Gd^{++} infusions in rat were used to longitudinally assess BBB permeability changes during epileptogenesis (van Vliet et al., 2014). While most studies have focused on BBB damage, a radiological indication of altered ISF circulation in the epileptic brain is lacking. The latter is challenging as dissecting cerebrovascular integrity and edema (cytotoxic or vasogenic) overtime is not an easy task. Combining T1- Gd^{++} and T2-FLAIR (or DWI) during seizure progressions could represent an approach to determine BBB permeability and subsequent changes in parenchymal water. Available evidence indicates a focal BBB damage after *status epilepticus* (SE) in rodents (van Vliet et al., 2007, 2014). Others have shown that MRI changes induced by SE are reversible and secondary T2—changes were

reported a few weeks after SE. Although indirect and rather speculative, we propose changes in water content and movement (e.g., FLAIR) affecting ISF circulation and CSF–ISF communication. The net results may be failure of interstitial clearance from toxic seizure by-products or serum molecules and immune cells reaching the epileptic brain.

5. Seizure pharmacology and altered ISF flow

Seizure pharmacology depends on drug permeability across the blood–brain barrier and mechanisms of drug biotransformation. As previously proposed, changes in capillary ISF secretion or in CSF–ISF communication could affect local concentration of therapeutics. The latter hypothesis has a two-fold value: (i) in the brain parenchyma, the reduced ISF flow along the perivascular space will impede drug distribution. As proposed by us (Marchi et al., 2009a), hydrophilic and lipophilic molecules will be differently affected by changes of brain water content. As water movement is driven by changing protein gradients, serum proteins entering the brain during BBB damage will also affect brain drug availability, as for lipophilic anti-epileptic drugs; (ii) impaired CSF–ISF communications may reduce drug brain clearance, favoring side effects. These considerations may have a direct impact not only on systemically administered drugs but when attempting direct convection-enhanced brain delivery (CED). The latter is intended to by-pass the BBB, delivering molecules directly in the brain parenchyma using stereotactically implanted microcatheters. It is reasonable to assume that altered ISF flow will impact CED drugs during their diffusion away from the catheter.

6. Final remarks

A growing body of experimental and clinical data show an etiological and therapeutic significance of cerebrovascular dysfunction in epilepsy. This demands innovative experimental and clinical tools which extend our observations beyond the neurocentric view that permeated epilepsy research. BBB damage and increased vascular permeability, together with pathological changes in ISF and ISF–CNS flow or circulation may be synergistically involved in the pathogenesis of neurological diseases.

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HIGHLIGHTS

- Impaired CSF–ISF circulation may contribute to seizure activity.
- BBB damage may contribute to ISF abnormalities and seizures.
- Changes in CSF–ISF could impact drug brain biodistribution in the epileptic brain.

Fig. 1.

Interictal to ictal transition and cyclic cerebrovascular permeability. (A) At each seizure event, passage of serum solutes into the brain and changes of interstitial fluid ionic composition drive water accumulation in the brain. (B) The net result may include modifications of the bulk flow, pathological CSF–ISF communication or brain fluid clearance.

Factors that may affect clearance of ISF in epileptic brain

- Altered CBF
- Increased intracranial pressure/decreased tissue volume
- Altered homeostasis, edema

Fig. 2.

Interstitial solute clearance from the brain. Arterial pulsation or hydrostatic pressure moves ISF along the para-arterial route, whereas para-venous route cleans ISF.

Fig. 3. Altered homeostasis leads to different types of edema. Note that water movements are a consequence of increased brain osmolarity due to either increased concentration of ions (BBB leakage) or loss of metabolic substrates (anoxia/ischemia).

Fig. 4. Perivascular accumulation of phosphorylated tau protein in epileptic brain. AT8 staining for phospho-tau in Mesial Temporal Lobe (51 year old female) or, right panel, Lateral Temporal Lobe (48 years old male). Bars indicate 0.3 mm.

Fig. 5.

Source of abnormally deposited phosphorylated-tau on peri-vascular region. (A) The “BBB hypothesis” of tau accumulation in the perivascular space is refuted by the fact that the gradient for tau across the BBB is *from* the brain to the blood. (B) Impaired paravenous drainage pathway is a more likely mechanism of interstitial-tau accumulation.

Fig. 6. Dual-color cerebrovascular microangiography. Dissimilar MW fluorescent tracers can be used to differentially assess vascular permeability and diffusion of serum proteins into the interstitial space. Evans Blue (indicated by red fluorescence) diffuses into the brain owing its low molecular weight or a specific protein binding. Conversely, FITC-Albumin (50 kDa) is present luminally in microvessels and in the immediate perivascular space, concomitant to

dye colocalization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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