Blood-brain barrier (BBB) dysfunction has been suggested to play a role in neuronal hyperexcitability underlying seizure precipitation and recurrence in symptomatic types of epilepsy (1). This concept has arisen from experimental evidence showing that BBB damage experimentally induced by bile salt application to the rat cortex results in delayed development of spontaneous and long-lasting epileptiform activity in brain tissue adjacent to the vascular damaged area (2). This alteration in neuronal network excitability is mimicked by cortical application of serum albumin, which is known to extravasate in the brain parenchyma due to BBB breakdown caused by seizures, status epilepticus or by various epileptogenic events such as trauma, stroke, infection (1,3–6). Further investigations unveiled one molecular mechanism pivotally involved in hyperexcitability induced by albumin extravasation, namely, the transforming growth factor (TGF)-β1 signaling activated by albumin in perivascular astrocytes. This signaling leads to astroglia dysfunction by decreasing Kᵢ₄.1 channels, therefore impairing K⁺-buffering capacity of astrocytes; this signaling activation also contributes to downregulation of glutamate transporter in glia leading to decreased re-uptake and consequent extracellular accumulation (7). In vivo experiments also showed that artificial opening of the BBB using mannitol decreases seizure threshold in naive and in epileptic animals (4, 8). Kinetic analysis of BBB opening during seizures suggests maximal albumin extravasation within the first 2 hours from the last experienced seizures returning to physiological conditions by 24 hours (4).

Although BBB damage is not readily detectable in human epilepsy using Gd-based imaging techniques (i.e., contrast-enhanced MRI), vasogenic edema is detected by increased T2 signal and is considered an indicator of BBB dysfunction. Moreover, postmortem analysis of epileptic tissue from drug-resistant patients with differing etiologies has clearly detected parenchymal extravasation of both albumin and IgG (1,3,5).

Recent studies suggest that blood–brain barrier (BBB) permeability contributes to epileptogenesis in symptomatic epilepsies. We have previously described angiogenesis, aberrant vascularization, and BBB alteration in drug-refractory temporal lobe epilepsy. Here, we investigated the role of vascular endothelial growth factor (VEGF) in an in vitro integrative model of vascular remodeling induced by epileptiform activity in rat organotypic hippocampal cultures. After kainate-induced seizure-like events (SLEs), we observed an overexpression of VEGF and VEGF receptor-2 (VEGFR-2) as well as receptor activation. Vascular density and branching were significantly increased, whereas zonula occludens 1 (ZO-1), a key protein of tight junctions (TJs), was downregulated. These effects were fully prevented by VEGF neutralization. Using selective inhibitors of VEGFR-2 signaling pathways, we found that phosphatidylinositol 3-kinase is involved in cell survival, protein kinase C (PKC) in vascularization, and Src in ZO-1 regulation. Recombinant VEGF reproduced the kainate-induced vascular changes. As in the kainate model, VEGFR-2 and Src were involved in ZO-1 downregulation. These results showed that VEGF/VEGFR-2 initiates the vascular remodeling induced by SLEs and pointed out the roles of PKC in vascularization and Src in TJ dysfunction, respectively. This suggests that Src pathway could be a therapeutic target for BBB protection in epilepsies.
growth factor (VEGF), a pleiotropic growth factor induced by inflammation (10, 11). Brain inflammation is a prominent feature of experimental and human epileptogenic brain tissue; it is associated with BBB damage and chiefly involves glial cells, neurons, and endothelial cells of brain microvasculature (12).

The work published by Morin-Brureau and colleagues has addressed the mechanisms of BBB damage in epilepsy using an in vitro model represented by organotypic hippocampal slice cultures obtained from neonatal rats and maintained in vitro for 2 weeks. Both microvessels and tight junctions are preserved, and the physiological interactions between the neuronal, glial, and vascular compartments are retained, therefore mimicking the in vivo condition with the exclusion of circulating blood cells or molecules.

Seizure-like activity was induced for 1 hour in this preparation by bath application of kainic acid; seizures were monitored for up to 3 hours following kainate washout by measuring dendritic field potentials in CA1. The first seizure-like event appeared within 5 minutes from kainate application followed by up to three events in 1 hour. No seizures occurred after kainate washout. This neuronal activity was responsible for sequential events studied in this slice preparation, since all these events were precluded by tetrodotoxin (TTX). Specifically, seizures induced downregulation within 2 hours of tight junction proteins such as claudin-5 and zonula occludens 1 (ZO-1), while occludin was preserved. Moreover, while claudin-5 downregulation resolved by 12 hours, ZO-1 was downregulated for at least 24 hours. In addition, concomitant VEGF mRNA upregulation was measured in tissue, with protein levels peaking at 12 hours; enhanced VEGF release in medium was detectable at 24 hours. VEGF receptor-2 was upregulated with a similar time-course and upregulation persisted at 24 hours when increased VEGF release was measured. Vascular density increase (increased number of branches) was the latest event in the observed sequela, since it was detected only at 24 hours but not before. The pivotal role of VEGF in determining BBB breakdown and vascular remodeling was suggested by the ability of VEGF inactivating antibody to preclude all these phenomena induced by seizures.

The next set of experiments was devoted to understanding which cell types were involved in VEGF synthesis and release following seizure onset and where the relevant receptors were expressed, using double-labeling immunohistochemistry. In control slices, VEGF was present in neurons and in scattered astrocytes, while VEGF-R2 was expressed by neurons and endothelial cells. After seizures, VEGF expression was predominantly induced in astrocytes and to a minor extent in neurons, and its receptor was increased in neurons and endothelial cells. This evidence highlighted the cell sources of VEGF release (astrocytes and neurons) and its target cells (neurons and brain vessels).

Finally, the molecular mechanisms underlying BBB damage and vessel remodeling were investigated by selectively blocking three distinct phosphorylation pathways, namely, phosphatidylinositol 3 kinase (PI3K), protein kinase C (PKC), and the tyrosine kinase Src, using specific inhibitors. The results showed that inhibition of PI3K worsened kainate excitotoxicity, therefore this pathway was not further investigated. Inhibition of PKC prevented the vessel remodeling, while Src inhibition prevented ZO-1 downregulation. Src phosphorylation, indicative of its activation, was measured at 2 hours after kainate seizures, which corresponds to the peak of VEGF receptor-2 (VEGF-R2) phosphorylation, and both phenomena were precluded by inactivating anti-VEGF antibody, demonstrating the involvement of this cytokine.

To unequivocally prove that VEGF was sufficient for Src pathway activation, it was applied to slice cultures mimicking the amount released by kainate–induced seizures. VEGF application to naive slices increased its own receptor phosphorylation, Src phosphorylation, and induced downregulation of ZO-1, which was prevented by Src inhibition. Vascular remodeling was also induced by VEGF but not prevented by Src inhibition. Thus, VEGF recapitulated all the effects observed after kainate–induced seizures in the slices, showing that the Src pathway activated by VEGF regulates ZO-1 expression in brain vessels.

Three main conclusions can be drawn from this study:

1. Neuronal activity is responsible for the observed phenomena since they are all precluded by TTX application.
2. Neuronal activity releases VEGF from two sequential cell sources, first by neurons (within 2 hours after kainate application) leading to VEGF–R2 phosphorylation and activation of Src; second by astrocytes (within 12 hours after kainate application), which is likely to be implicated in the maintenance of ZO–1 downregulation still observed 24 hours following kainate seizures.
3. Vascular remodeling was a late event as compared with ZO–1 downregulation, since it was observed 24 hours after kainate application, suggesting it may be a compensatory mechanism induced in response to vessel dysfunction. The authors speculate that vessel remodeling has a neuroprotective function by improving the neurovascular coupling during seizures. Although this might be the case, it remains to be established whether or not the newly formed vessels are intact or “leaking” as suggested by previous in vivo evidence (6, 13).

If we attempt to translate these findings to the in vivo setting, we can conclude that BBB damage observed during recurring seizures in epileptic tissue is caused by intrinsic brain mechanisms that primarily involve neurons and astrocytes. Early events, such as the release of VEGF in concert with inflammatory molecules such as IL–1β, TNF–α or prostaglandins released from brain cells during seizures can activate intracellular signaling pathways in endothelial cells, leading to tight junction compromise and vessel remodeling. These are likely to be self-perpetuating mechanisms due both to the recurrence of seizures and the redundance of inflammatory cascades, as well as to the long-lasting upregulation/release of VEGF by perivascular astrocytes.

Of interest, Src activation in neurons is a downstream event induced by interleukin(IL)–1β interaction with its cognate receptor IL–1 receptor type 1 (R1), and this pathway mediates the facilitatory role in seizure of this cytokine (14). It may well be that IL–1β released from microglia and astrocytes in epileptic tissue also activates Src in endothelial cells of the BBB, which express IL–1R1 during seizures (5), thus contributing in concert with VEGF to BBB damage. This possibility is indeed supported by recent evidence that IL–1β is involved in BBB leakage,
albumin extravasation, and seizure recurrence induced by bicuculline in an in vitro guinea pig brain preparation (15).

Endogenous VEGF is upregulated by seizures in vivo and affords neuroprotection in a rat model of status epilepticus induced by pilocarpine (10); this beneficial effect appears to be mediated by activation of PI3K (as also suggested by this study in organotypic slices). The PI3K/Akt pathway was also involved in vascular permeability induced by VEGF acting on VEGF-R2 (11), thus predicting it may not be possible to exploit VEGF’s neuroprotective function without its unfavorable consequences, such as the increased vascular permeability.

The specific involvement of Src both in neuronal network hyperexcitability induced by IL-1β and in the BBB damage induced via ZO-1 downregulation by IL-1β and VEGF suggests that this signaling pathway could be a therapeutic target for preventing the deleterious effects of brain inflammation during recurrent seizure activity.

by Annamaria Vezzani, PhD

References
Instructions
The purpose of this form is to provide readers of your manuscript with information about your other interests that could influence how they receive and understand your work. Each author should submit a separate form and is responsible for the accuracy and completeness of the submitted information. The form is in four parts.

1. Identifying information.
   Enter your full name. If you are NOT the main contributing author, please check the box “no” and enter the name of the main contributing author in the space that appears. Provide the requested manuscript information.

2. The work under consideration for publication.
   This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you received, either directly or indirectly (via your institution), to enable you to complete the work. Checking “No” means that you did the work without receiving any financial support from any third party – that is, the work was supported by funds from the same institution that pays your salary and that institution did not receive third-party funds with which to pay you. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check “Yes”. Then complete the appropriate boxes to indicate the type of support and whether the payment went to you, or to your institution, or both.

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# Disclosure of Potential Conflicts of Interest

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2. **First Name** Annamaria  **Last Name** Vezzani  **Degree** PhD

3. **Are you the Main Assigned Author?** Yes  No

   If no, enter your name as co-author:

4. **Manuscript/Article Title:** Autonomous Mechanisms of Seizure-Induced BBB Dysfunction

5. **Journal Issue you are submitting for:**

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