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Blood–brain barrier breakdown-inducing astrocytic transformation: Novel targets for the prevention of epilepsy

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Summary

Epileptogenesis is common following brain insults such as trauma, ischemia and infection. However, the mechanisms underlying injury-related epileptogenesis remain unknown. Recent studies demonstrated impaired integrity of the blood–brain barrier (BBB) during epileptogenesis. Here we review accumulating experimental evidence supporting the potential involvement of primary BBB lesion in epileptogenesis. Data from animal experiments demonstrate that primary breakdown of the BBB prone animals to develop focal neocortical epilepsy that is followed by neuronal loss and impaired functions. The extravasation of albumin from the circulation into the brain neuropil was found to be sufficient for the induction of epileptogenesis. Albumin binds to transforming growth factor β receptor 2 (TGF β R2) in astrocytes and induces rapid transcriptional modifications, astrocytic transformation and dysfunction. We highlight a novel cascade of events which is initiated by increased BBB permeability, eventually leading to neuronal dysfunction, epilepsy and cell loss. We review potential mechanisms and existing experimental evidence for the important role of astrocytes and the TGF β pathway in epileptogenesis. Finally, we review evidence from human clinical data supporting the involvement of BBB lesion in epilepsy. We propose that primary vascular injury, and specifically BBB breakdown and repair, are key elements in altered interactions within the neurovascular unit and thus may serve as new therapeutic targets.

Keywords

Epileptogenesis; Blood–brain barrier; Astrocytes; Transforming growth factor β

Injury-related epileptogenesis: a potential role for blood–brain barrier disruption and serum-derived albumin

Focal epilepsy typically arises from neuronal tissue either within or adjacent to a cortical lesion (Willoughby, 2000). The focus of epileptic tissue is often located in the hippocampal formation within the temporal lobe (temporal lobe epilepsy, TLE). Neurosurgical removal of the epileptogenic area in many patients leads to control or even abolishment of seizures.

However, in light of the high rate of drug resistant focal epilepsies, and other neurological impairments following insults to the central nervous system, novel anti-epileptogenic strategies are urgently needed.

Epileptiform activity can be experimentally induced by a number of drugs which block potassium currents, augment sodium currents or impair synaptic inhibition (Opdam et al., 2002; Prince, 1969). Long-lasting or persistent focal neocortical epilepsies can be elicited by inducing developmental cortical malformations via trauma early in life (Jacobs et al., 1996), repeated electrical stimulation such as in the kindling model of epileptogenesis (McNamara, 1986) or repeated application of ictogenic agents, such as penicillin (Opdam et al., 2002; Prince and Wilder, 1967) or pentetrazole (Barkai et al., 1990), by focal application of epileptic agents (Opdam et al., 2002; Prince and Wilder, 1967), or by chronic injury to, or the deafferentation of the adult cerebral cortex (Halpern, 1972; Pitkanen and McIntosh, 2006; Prince and Tseng, 1993). The most frequently used animal models for TLE depend on the induction of status-epilepticus (SE) by systemic injection of pilocarpine or kainic acid (for review see Curia et al., 2008). In most of these animal models (similar to the situation in man), a period of days to weeks is required for the development of epileptic activity (Hoffman et al., 1994; Prince and Tseng, 1993). Typically, the transient insult is followed by a latent interval in which cellular and structural reorganization processes occur, referred to as *epileptogenesis*, which ultimately leads to chronic recurrent epileptic seizures. While the molecular, anatomical and electrophysiological activity in the epileptic brain focus have been described in great details (see for example Hoffman et al., 1994; Huguenard et al., 1996; Jacobs et al., 1999; Prince and Futamachi, 1968; Prince and Tseng, 1993; Mody and Heinemann, 1987; Prince and Gutnick, 1971), the changes that are critical to epileptogenesis, occurring following injury and before epileptic activity develops – are mostly unknown. An understanding of the molecular and physiological events during epileptogenesis is essential for the targeted development of preventive therapeutic approaches that are presently unavailable (Herman, 2002).

On the basis of clinical and animal studies, accumulating evidence support the hypothesis that primary vascular lesions and, specifically an opening of the blood–brain barrier (BBB), trigger a chain of events leading to epilepsy (Avivi et al., 2004; Ivens et al., 2007; Janigro, 1999; Marchi et al., 2007; Oby and Janigro, 2006; Pavlovsky et al., 2005; Seiffert et al., 2004; Tomkins et al., 2007, 2008; van Vliet et al., 2007). It is noteworthy that a significant and long-lasting BBB breakdown is a hallmark of cortical injury (Cervos-Navarro and Lafuente, 1991; Tomkins et al., 2008; Tomkins et al., 2001). Moreover, ultrastructural studies on human epileptic tissue show clear BBB abnormalities, including increased micropinocytosis and fewer mitochondria in endothelial cells, a thickening of the basal membrane, and the presence of abnormal tight junctions (Cornford, 1999; Cornford and Oldendorf, 1986; Kasantikul et al., 1983). Recently, a role for BBB opening in the progression of TLE was suggested based on the finding of positive immunocytochemistry to albumin following SE and a positive correlation between the extent of BBB opening and the number of seizures (van Vliet et al., 2007). While large amount of published data support a correlation between epileptogenesis, seizures and abnormal BBB, direct evidence for the involvement of BBB breakdown in epileptogenesis has been only recently given by a series of studies in which we demonstrated that in a rat model, a long-lasting opening of the BBB (using focal application of bile salts (Greenwood et al., 1991) indeed results in the delayed appearance of robust hypersynchronous epileptiform activity (Seiffert et al., 2004). Importantly, BBB disruption does not seem to induce epileptogenesis by provoking status epileptogenesis, seizures or neuronal death. Although Marchi and co-workers showed that BBB disruption can provoke seizures in pigs (Marchi et al., 2007), it may not be to the same extent in naïve rodents although it does reduce seizure threshold in epileptic animals (van Vliet et al., 2007). Either way, epileptogenesis in the BBB-disrupted brain seems to be

mediated by exposure of the brain cortex to serum albumin, mediated via its action on brain astrocytes (Ivens et al., 2007 and see below). The possible involvement of albumin in astrocytic activation and proliferation is supported by previous studies showing serum albumin inducing proliferation of fibroblasts (Tigyi et al., 1995) as well as calcium signaling and DNA synthesis in cultured astrocytes (Nadal et al., 1995). Based on their studies, Nadal and co-workers (1995) concluded that there is a specific receptor and signaling pathway for the action of albumin. While albumin is the most abundant protein in the serum, other blood-born proteins may also have a role in the epileptogenic process. For example, it has been recently shown that the serum protein, thrombin, acting through the protease-activated receptor 1 (PAR1), lowers the threshold for generating epileptic seizures in CA3 region of the hippocampus, and produces a long-lasting enhancement of the reactivity of CA1 neurons to afferent stimulation (Maggio et al., 2008) and TGF β 1 itself may also be epileptogenic (see below). Thus, it seems plausible that damage to the microvasculature during brain insults leads to the extravasation of serum born proteins, leading to the transformation of the neighboring astrocytes as a primary step in the epileptogenic process. In addition, it is notable that in many cases of epilepsy as well as in most animal models, neuronal damage is observed. In this respect it is interesting to point out that BBB breakdown has been associated with early (van Vliet et al., 2007; Rigau et al., 2007) or delayed neuronal damage (Tomkins et al., 2007). To what extent this damage contributes to epileptogenesis, a result of abnormal activity or not related, is not as yet clear.

The role of glia cells in epileptogenesis

The interactions observed between serum albumin and astrocytes, followed by astrocytic transformation and dysfunction early during epileptogenesis suggest a key role for astrocytes in the epileptogenic process. Indeed, proliferation of astrocytes is a pathological hallmark in many patients with TLE. Recent studies have implicated novel physiological roles for glia cells in the CNS, such as modulation of synaptic transmission and plasticity. Accumulating evidence show clear changes in the number, morphological and functional characteristics of astrocytes in the epileptic brain (for reviews see Binder and Steinhauser, 2006; Heinemann et al., 2000; Jabs et al., 2008; Wetherington et al., 2008). It is thus plausible that glia cells, and specifically “transformed” or “activated” glia have a functional role in neuronal hypersynchronicity and excitability, leading to the network reorganization which characterizes the epileptic brain. In addition, transformed or activated glia have also been reported to release inflammatory mediators, which can further increase BBB permeability and further augment or maintain their activated condition (Allan et al., 2005). Functional changes in astrocytic properties which could be involved in increased neuronal excitability, seizure activity and epileptogenesis include: (1) *Reduced expression of potassium inward-rectifying channels (Kir4.1) and water channels (aquaporin 4, AQP4)*: both channels are co-localized most abundantly in astrocytic endfeet and considered crucial for the regulation of the brain’s extracellular potassium ($[K^+]_o$) and water. Indeed, down-regulation of Kir4.1 and AQP4 in genetic engineered mice results in impaired $[K^+]_o$ buffering and seizures (Binder et al., 2006; Djukic et al., 2007). In the hippocampus from pilocarpine-treated epileptic rats (Gabriel et al., 1998) and in the sclerotic hippocampus from TLE patients $[K^+]_o$ buffering is impaired, due to reduced Kir4.1 channels (Kivi et al., 2000; Schroder et al., 2000). Impaired buffering of extracellular potassium will contribute to reduced firing threshold, enhanced synchronization and probably synaptic plasticity due to activity-dependent $[K^+]_o$ accumulation, consequent neuronal depolarization, increased transmitter release and activation of NMDA receptors; (2) *Reduced gap junctions*: gap junctions are functional channels between cells consisted of connexin proteins. Astrocytes are coupled via gap junctions to form large cellular networks which facilitate spatial buffering of small molecules (e.g. K^+). Connexin knockout mice indeed show a mild decrease in the spatial buffering of $[K^+]_o$ and decreased seizure threshold (Wallraff et al., 2006) although most

buffering capacity is maintained. It is yet to be shown if and to what extent gap junctions are reduced in the human epileptic brain. (3) *Impaired glutamate metabolism*: glia cells (especially the GluR cells) express glutamatergic receptors, can release glutamate and are most important for the uptake and metabolism of glutamate. In the hippocampal slice preparation from healthy mice, astrocytic glutamate release has been implicated to contribute to a slow, TTX resistant, NMDA sensitive neuronal inward current which may represent the typical “paroxysmal depolarization shift” (PDS) underlying the inter-ictal burst (Tian et al., 2005). It is not clear, however, to what extent such release contributes to epileptogenesis or to the propagating seizure activity in the epileptic brain. There is no direct evidence for increased glutamate release from transformed astrocytes in chronic epileptic tissue. However, one potential mechanism is the up-regulation of TNF α which (together with microglial activation) is a prominent modulator for glutamate release (Bezzi et al., 2001). Transformed astrocytes may also affect extracellular glutamate levels by decreased uptake and metabolism. Astrocytes express two specific glutamate transporters, the EAAT1 (AKA GLAST in rodents) and the EAAT2 (GLT-1) which, based on genetic deletion experiments, underlie most glutamate uptake in the brain (Tanaka et al., 1997). There is strong data to support the conclusion that significant impairment of astrocytic glutamate transporters is associated with the development of seizure; however, whether glutamate transporters are down-regulated in reactive astrocytes in the epileptic tissue is controversial, with reports demonstrating reduced, normal and increased levels (reviewed by Wetherington et al., 2008). Astrocytes are also responsible for the conversion of the transported glutamate into glutamine by glutamine synthetase. Glutamine, in turn is transported back into neurons where it is converted back to glutamate by mitochondrial glutaminase. Sclerotic brain tissue from TLE patients does show around 40% reduction in the levels of glutamine synthetase in astrocytes (Eid et al., 2004) and glutamine synthetase inhibitors (80–90% inhibition) cause seizure in experimental animals (Eid et al., 2008). (4) Increased release of inflammatory mediators by transformed astrocytes: astrocytes can produce many pro- and anti-inflammatory molecules, and these can be pro- and anti-epileptogenic. Accumulating evidence, mainly contributing by the group of Vezzani support the role of IL-1 β in reducing seizure threshold and epileptogenesis in the pilocarpine SE model of epilepsy (Ravizza et al., 2008; Vezzani and Baram, 2007). Seizures, in turn, rapidly induce production of both IL-1 β and IL-1Ra, a natural antagonist of IL-1 β (De Simoni et al., 2000). The role of transforming growth factor β (TGF β) will be discussed separately below. Recent reports suggest that other inflammatory mediators (e.g. cyclooxygenase-2) which are released from astrocytes may directly affect synaptic signaling (Yang et al., 2008), plasticity (Cowley et al., 2008) and perhaps epileptogenesis (Cole-Edwards and Bazan, 2005; Zhang et al., 2008). It is still not yet clear to what extent the different inflammatory mediators contribute to epileptogenesis and seizure generation, what is the role of activated microglia and what are the detailed interactions between the different cells and neurons; however, recent studies strongly suggest a role for transformed astrocytes in the early inflammatory events occurring during epileptogenesis.

To summarize, experimental evidence unequivocally support the notion that transformed (activated) astrocytes are prominent in the epileptic brain, and that these astrocytes bear the properties which reduce seizure threshold. However, it is important to distinguish between seizure generation and epileptogenicity – which carries a more complex and chronic changes in the network. To what extent astrocytes do have a role in such network changes directly? Hints for such a role come from experiments showing that in the SE-induced model of epilepsy, transformation of astrocytes starts within hours to days following SE, during the latent period of epileptogenesis (Shapiro et al., 2008). Similar observations that astrocytic transformation, reduced Kir4.1 expression and [K⁺]_o buffering precede the development of seizure activity have also been documented in the BBB disruption or albumin-exposure models (Ivens et al., 2007), strengthening the argument that transformation of astrocytes

may not only “reflect” an hyperexcitable network, but rather contribute to the epileptogenesis process itself. The possible involvement of BBB breakdown and albumin in signaling astrocytic transformation is supported by studies showing calcium signaling and DNA synthesis in cultured astrocytes (Nadal et al., 1995). The observations that the action of serum albumin to induce astrocytic transformation is mediated via TGF β receptor II point to the involvement of TGF β pathway in epileptogenesis following insult.

The TGF β pathway and epileptogenesis

TGF β s are pleiotropic cytokines that play a pivotal role in intercellular communication (for review see Massague, 2000; Shi and Massague, 2003), and their signaling pathways are frequently involved in cell growth, embryogenesis, differentiation, morphogenesis, wound healing, immune response, and apoptosis in a wide variety of cells (Blobe et al., 2000; Flanders et al., 1991; Gold and Parekh, 1999). TGF β signaling is mediated mainly by two serine threonine kinase receptors, TGF β RI and TGF β RII, which activate an intracellular signaling system, such as the Smad protein complex and the p38 mitogen-activated protein kinase (MAPK) pathway. In the former, TGF β signaling is followed by translocation of the phosphorylated Smad2/3 complex to the nucleus regulating transcriptional responses. In response to brain injury TGF β 1 is strongly expressed in immune cells (and probably activated microglia) within and in the vicinity of the wound. TGF β 1 has been shown in cell cultures strongly inhibit the proliferation of astrocytes (Lindholm et al., 1992) and to mediate neuro-protection (Brionne et al., 2003; Prehn et al., 1993b; Ruocco et al., 1999). The TGF β 1-induced neuroprotection was shown to be mediated through upregulation of the transcription factor NF κ -B, which induce up-regulation of the antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL (Kim et al., 1998; Zhu et al., 2004). This cascade, contrasting documented TGF β actions in different cell types, was resolved in a recent paper (Konig et al., 2005), demonstrating that TGF β 1 activates two distinct TGF β type I receptors and signal transduction pathways in neurons: the canonical activin-like kinase 5 (ALK5)/Smad2/3 pathway and a novel, ALK1/Smad1/5-regulated pathway. ALK1 expression is up-regulated in neurons in response to injury, and the signaling through ALK1 mediates the activation of the antiapoptotic NF κ -B pathway. ALK5 was demonstrated as the predominant TGF β signaling cascade in astrocytes.

Indeed, the past decade has seen an explosion of information regarding the expression and action (sometime contradictory) of cytokines in the brain. Accumulating evidence unequivocally indicates that TGF β is synthesized in many disease conditions (for reviews see Szelenyi, 2001; Vitkovic et al., 2001): TGF β 1 expression is up-regulated in the brains of individuals suffering from multiple sclerosis, AIDS, Alzheimer’s disease, stroke, tumors, or trauma. TGF β has also been shown to be elevated in the cerebro-spinal fluid (CSF) of some patients following brain injury (Phillips et al., 2006), to be produced in neurons after ischemia (Zhu et al., 2000) and to be involved in pericyte-induced upregulation of BBB function (Dohgu et al., 2005). Experiments in Smad3 null mice showing reduced glial scarring after cortical stab wound injury (Wang et al., 2007) further support the role of TGF β as an injury-related cytokine. Indeed, while many researchers consider TGF β 1 to be a “protective” cytokine (Brionne et al., 2003; McNeill et al., 1994; Prehn et al., 1993a; Zhu et al., 2002), it has also been found to exacerbate excitotoxicity (Mesples et al., 2005; Prehn and Kriegstein, 1994); thus, its action seems to depend on the experimental conditions (for review see also Vivien and Ali, 2006).

Is TGF β associated with epileptogenesis? The potential involvement of TGF β in epileptogenesis is supported by animal experiments showing TGF β up-regulation in neurons from amygdala-kindled rats (Plata-Salaman et al., 2000). Aronica et al. (2000) showed TGF β expression in astrocytes from the hippocampus of SE-experienced rats and suggested that

TGF β represents “a novel mechanism for modulation of glial function and for changes in glial–neuronal communication in the course of epileptogenesis”. However, direct evidence for the role of TGF β in cortical dysfunction and epileptogenesis is scarce. Evidence from BBB-disrupted animals showing that TGF β R may be involved in albumin-induced epileptogenesis (Ivens et al., 2007) is supportive for a role for the TGF β pathway, but this has yet to be shown directly. Interestingly, TGF β was shown to cause a rapid down-regulation of K⁺ inward-rectifying (K_{IR}) channels in reactive astrocytes (Perillan et al., 2002), suggesting a similar transformation as observed in the epileptic tissue. The accumulating results point to the TGF β pathway as a novel target for the prevention of epileptogenesis. Future research would be required to explore which of the TGF β -related pathways, if any, is critical to the epileptogenic process and to what extent, and under which conditions, it could serve as new target for anti-epileptic treatment.

Detection of BBB damage in the human epileptic brain

Is there any evidence in human studies for the involvement of pathology at the BBB and epileptogenesis? As mentioned above, pathological and immunohistochemical studies in human epileptic tissue consistently demonstrated structural evidence for abnormal BBB and serum albumin within the neuropil and cellular elements as functional evidence for abnormal vessels permeability for large hydrophilic molecules. These data call for the development of strategies to detect BBB permeability changes for diagnostic needs (i.e. to identify the epileptic region prior to surgery), but also for targeting population at risk to develop epilepsy. A diagnostic tool for measuring BBB permeability should give reliable, objective and quantitative information with high spatial resolution. Qualitative evaluation of BBB disruption in humans is available using imaging modalities (magnetic resonance imaging [MRI], computerized tomography [CT], and single photon emission CT [SPECT]) following the peripheral administration of non-permeable contrast agents. Although MRI imaging, due to its high spatial resolution, is considered the best available method for studying anatomical lesions, it is regarded as relatively insensitive for detecting small changes in contrast agent accumulation (as compared with SPECT, Volkow et al., 1997). A quantitative evaluation of BBB permeability functioning in patients may be obtained using analysis of the cerebro-spinal fluid for serum proteins or brain constituents (e.g. S100 β) in the peripheral blood (Marchi et al., 2003). However, these methods do not offer spatial information, are invasive, may give false positive results in the presence of intracerebral hemorrhage and S100 levels may depend on the extent of injury or activation of brain astrocytes (Kanner et al., 2003). Few quantitative methods for evaluating BBB permeability using dynamic contrast enhance imaging (e.g. Tofts et al., 1999 and see also review by Zaharchuk, 2007) have been developed, most often applied to relatively small number of patients with brain tumors. We have recently further developed and implemented similar quantitative methods on a cohort of patients with post-traumatic epilepsy (PTE). Our data, while collected retrospectively, clearly show that in about 56% ($n = 14$) patients with PTE, BBB breakdown could be detected. Interestingly, in most of these patients ($n = 13$) there was a close correlation between the BBB lesion and the suspected epileptic focus, based on inter-ictal EEG and the use of source localization methods. Moreover, the spatial extent of cortical dysfunction as measured by quantitative EEG analyses correlated with the size of the BBB-disrupted region, but not with that of the anatomical lesion (usually post-traumatic hemorrhagic contusion), and patients with PTE were more likely to show abnormal BBB permeability, and in larger cortical areas, compared to non-epileptic controls following traumatic brain injury.

The data from experimental animals and human clinical studies indicate that studying mechanisms underlying epileptogenesis and epileptic seizures must consider variety of interactions within the “neurovascular units”, and that significant changes occur in the

vascular system, astrocytes and microglia cells which contribute significantly to the pathogenesis of the disease (Fig. 1). Recent advances in imaging indicate that identification and quantification of such events are in hand and call for large-scale prospective studies to explore the role of BBB breakdown in the epileptogenic process. New developments in imaging BBB permeability using new tracers (Stoll et al., 2008) and molecular imaging (Stoll and Bendszus, 2008) may add valuable information for the time resolution and extent of BBB permeability changes, the role of astrocytes, inflammation and specific molecular pathways in human epileptogenesis, thus allowing a better design of anti-epileptogenic and anti-epileptic treatments for specific populations.

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Figure 1.

A schematic illustration of BBB breakdown-induced brain response: within hours following BBB breakdown albumin diffuses into the brain's extracellular space, binds to astrocytic TGF β receptors and induces transcriptional change resulting in the transformation of astrocytes. Potential candidate changes are listed and include down-regulation of Kir4.1 and Glutamate transporters, connexins and glutamine synthase with up-regulation of pro-inflammatory cytokines (see text for details). The change in astrocytic functions leads within days to weeks to activity-dependent increase in neuronal excitability, hypersynchronicity and pathological plasticity which may eventually lead (within weeks–months) to excitotoxicity and damage.