

## IMMUNITY AND INFLAMMATION IN EPILEPSY

# Molecular cascades that mediate the influence of inflammation on epilepsy

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### SUMMARY

Experimental evidence strongly indicates a significant role for inflammatory and immune mediators in initiation of seizures and epileptogenesis. Here we will summarize data supporting the involvement of IL-1 $\beta$ , TNF- $\alpha$  and toll-like receptor 4 in seizure generation and the process of epileptogenesis. The physiological homeostasis and control over brain immune response dep-

ends on the integrity of the blood-brain barrier, transforming growth factor (TGF)- $\beta$  signaling and leukocyte migration. To what extent targeting the immune system is successful in preventing epileptogenesis, and which signaling pathway should be beleaguered is still under intensive research.

**KEY WORDS:** Blood-brain barrier, Cytokines, Epileptogenesis, Immune.

This article examines the potential and emerging roles of inflammatory and immune mediators in both the generation of individual seizures and the process by which a previously normal brain might become epileptic (epileptogenesis), focusing on rapidly growing knowledge that supports the involvement of several specific inflammatory signaling molecules in seizures and epilepsy. Therefore, work using gene-array analysis of the hippocampus during the epileptic process identified several candidate molecules and pathways. These include perturbations of the blood–brain barrier (BBB), the cyclooxygenase (COX)-2 signaling pathway and related prostaglandins, classical cytokines and their downstream targets, as well as Toll-like receptors. To cover the rich and rapidly evolving information about these novel signaling cascades, this article is divided into two parts.

### IL-1 $\beta$ AND TNF- $\alpha$ (S. AUVIN, PARIS, FRANCE; T.Z. BARAM, IRVINE, CA, U.S.A.; R. SANKAR, LOS ANGELES, CA, U.S.A.)

Presentations focused on interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ , two proinflammatory cytokines, and their molecular and cellular actions in the context of neuronal excitability and epilepsy. These molecules are released within the brain in specific circumstances in both the developing and adult brain. The actions of these molecules, in turn, modify neuronal excitability both acutely and long term. Finally, manipulation of IL-1 $\beta$  and/or TNF- $\alpha$  levels or their downstream pathways was discussed as a means to influence neuronal excitability, seizure susceptibility, and epileptogenesis.

IL-1 $\beta$  plays a potential role in a number of clinical entities, as suggested by studies in models of febrile seizures (FS) and febrile status epilepticus (SE) (Dubé et al., 2007), pilocarpine-induced SE in immature rats (Auvin et al., 2007), and rapid kindling (Auvin et al., 2010a,b). Neuroanatomic, imaging, neurochemical, and mechanistic approaches in vivo have been employed, and effector molecules of the cytokine have been described (Viviani et al., 2003; Balosso et al., 2008; Maroso et al., 2010). The

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involvement of TNF- $\alpha$  in programming neuronal excitability acutely and long term has also been examined (Riazi et al., 2010).

New data support a role for IL-1 $\beta$  in the epileptogenesis that follows FS and febrile SE. Endogenous IL-1 $\beta$  is released in hippocampus during experimental FS, and contributes to the seizures themselves (Dubé et al., 2005), and IL-1 $\beta$  synthesis is also increased during and after febrile SE, and remains elevated up to 48 h (Dubé et al., 2010). IL-1 $\beta$  levels were also significantly higher in hippocampi of rats that became epileptic after febrile SE compared with those that did not. This evidence is consistent with, although it does not prove, the involvement of IL-1 $\beta$  in the epileptogenic process. The elevated hippocampal IL-1 $\beta$  levels are found only in FS-sustaining rats that have become epileptic as adults, and, therefore, might also be a result of IL-1 $\beta$  biosynthesis and release induced by the spontaneous seizures that these rats were experiencing. Therefore, because the precise epileptogenic role of IL-1 $\beta$  in FS-induced epilepsy is not yet resolved, future studies will aim to block IL-1 $\beta$  production and receptor signaling after the inciting FS and examine if this intervention attenuates the development of epilepsy.

Systemic administration of lipopolysaccharide (LPS) (inducing systemic and brain inflammation) enhanced epileptogenesis in postnatal day (P)14 rats exposed to lithium-pilocarpine SE (Auvin et al., 2010a). Epilepsy severity was enhanced in adult rats that were preexposed to LPS at P14, and this effect was associated with stronger reactive gliosis in CA1 as compared to epileptic animals that did not receive LPS. Auvin and colleagues found that injection of LPS at 22–24°C room temperature, although promoting long-term effects on epilepsy development, did not result either in increased body temperature or in increased duration of SE, indicating that LPS effects on epileptogenesis were due specifically to induction of inflammation. To further study the effect of inflammation on epileptogenesis in the developing brain, and to exclude the potential contribution of cell death that may be found in the lithium-pilocarpine model, Auvin et al. (2010a) employed a rapid kindling model. They found that LPS administration enhanced kindling epileptogenesis in immature rats, measured by an increased number and the duration of stage 4 seizures during kindling acquisition, and a modification of both afterdischarge threshold and its duration. Systemic administration of IL-1 receptor antagonist (ra), a competitive blocker of IL-1 $\beta$  receptor type 1, mitigated the augmentation of epileptogenesis. Future studies should investigate the underlying mechanisms by which inflammation may promote epileptogenesis.

In a lithium-pilocarpine model in P7 and P14 rats, Sankar and colleagues found little neuronal death in the hippocampus (as revealed by Fluoro-jade B and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining), a result consistent

with previously published data. However, if pups were pretreated with LPS systemically, 2 h prior to lithium-pilocarpine, considerable cell death in CA1 was seen (Auvin et al., 2007). Because both SE and LPS caused induction of a variety of brain inflammatory molecules including IL-1 $\beta$ , TNF- $\alpha$ , and COX-2, this group explored the use of inhibitors of these mediators in reducing the CA1 neuronal damage. Given before the inciting SE, IL-1ra (blocking IL-1 $\beta$  effects), minocycline (inhibiting microglia activation and generation of cytokines), or CAY10404 (a specific COX-2 inhibitor) did not reduce CA1 injury, but the combination of IL-1ra and COX-2 inhibitor reduced the neuronal damage. More importantly, seizure frequency in adult rats subjected to SE at P21 was reduced in animals pretreated for 10 days with this combination of antiinflammatory drugs, and this effect was associated with reduced mossy fiber sprouting. The clinically important question that arises from these observations concerns whether initiating the antiinflammatory treatment after the SE, as would be the case in the clinical setting, is still efficacious.

There is an important role of inflammation in contributing to FS induced by mimicking systemic infection with LPS as well as in programming long-term changes in brain excitability (Heida et al., 2005, 2009). In P14 rat pups injected with LPS, the application of a low subconvulsant dose of kainic acid at the onset of fever precipitates seizures in a fraction of animals, thus indicating that seizure threshold was reduced in these animals (Heida et al., 2004). Furthermore, the levels of IL-1 $\beta$  in the hippocampus were augmented only in the animals that exhibited seizures compared to similarly treated animals that did not have seizures. Moreover, the brain application of IL-1 $\beta$  in this model enhanced seizure frequency, whereas similar intracranial application of IL-1ra reduced or abolished these seizures (Heida & Pittman, 2005). The combination of LPS and low dose kainate in P14 rats also increased long-term excitability, as measured by decreased afterdischarge threshold and its increased duration in adulthood (Heida et al., 2005). Notably, despite these changes in hippocampal excitability, kindling development did not change in adult rats preexposed to LPS and kainate at P14 (Heida et al., 2005).

The induction of systemic and brain inflammation in P14 rats by injection of LPS alone is sufficient to cause long-term increase in brain excitability, and this phenomenon does not appear to depend on fever, since it can occur also in experimental conditions where fever is precluded (see also Auvin et al. above). Long-term excitability is reflected in reduced seizure threshold to a variety of convulsant drugs in adulthood and in an increased number of paroxysmal discharges to bath-applied 4-amino-pyridine in hippocampal slices from adult rats (Galic et al., 2008). Interestingly, brain production of the cytokine TNF- $\alpha$  appeared to be necessary to program these long-term

changes, as intra-cerebral application of this cytokine alone mimicked the effect of peripheral LPS, and interference with its actions abrogated the long-term changes. These findings raise questions of how TNF- $\alpha$  might program excitability and the potential critical developmental windows involved (Riazi et al., 2010). Involvement of glutamate receptors in these TNF- $\alpha$ -mediated effects is suggested by the evidence of changes in glutamate receptor subunit expression in rat forebrain long term after LPS administration (Harré et al., 2008) as well as in TNF- $\alpha$  receptor subtypes knockout mice (Balosso et al., 2009), and by the ability of TNF- $\alpha$  to induce rapid changes in AMPA receptor subunit expression and function (Stellwagen et al., 2005).

An exciting new contributor to the inflammatory processes during seizures involves the activation of the signaling molecule, toll-like receptor 4 (TLR4) by an endogenous molecule, high mobility group box-1 (HMGB1); this “danger signal” is released from dying or injured neurons and by activated glia, and is involved in seizure generation and recurrence (Maroso et al., 2010). In particular, acute administration of convulsant drugs increased HMGB1 levels and activated TLR4 in brain, and HMGB1 injection resulted in accelerated onset and augmentation of seizure activity; antagonists of either TLR4 or of HMGB1 reduced both acute and chronic recurrent seizures. Interestingly, mice that lacked TLR4 were intrinsically resistant to seizures (Maroso et al., 2010). The involvement of this novel inflammatory pathway in seizure activity was further supported by evidence of enhanced expression of these molecules (TLR4 and HMGB1) in both human temporal lobe epilepsy tissue and in mouse models of chronic seizures. It is worth noting that although TLR4 is involved in pathogen recognition, and, therefore, activated during infections, the HMGB1-TLR4 axis might underlie chronic epilepsy in scenarios without frank immune activation or infection (Vezzani et al., 2011a).

This work suggests that neuronal damage arising from a proepileptogenic event or a first seizure may promote HMGB1 release and activation of TLR4, which is then perpetuated by the recurrence of epileptic activity (Maroso et al., 2010). HMGB1 release appears to be also responsible for IL-1 $\beta$  induction in glia (Ravizza T, Maroso M, Vezzani A, unpublished data), therefore, establishing a link between “danger signals” released in brain by proepileptogenic events and IL-1 $\beta$  signaling activation, which has been shown to contribute significantly to seizure activity and is also upregulated in human epilepsy (for review see Vezzani et al., 2011a). Activation of both HMGB1-TLR4 and IL-1 $\beta$ -IL-1R type 1 signaling appear to result in enhanced Src-mediated phosphorylation of the NR2B subunit of the *N*-methyl-D-aspartate (NMDA) receptors, leading to increased neuronal Ca<sup>2+</sup> influx (Viviani et al., 2003; Balosso et al., 2008; Maroso et al., 2010). This

molecular cascade of events mediates the proconvulsant actions of these cytokines.

### Future directions

Future studies will need to combine diverse experimental approaches to tease out the mechanisms by which inflammatory cytokines modulate excitability, interact with seizures, and influence the probability of developing epilepsy, and the type and severity of spontaneous seizures. In vitro and in vivo electrophysiology, the use of state-of-the-art methods to delineate epigenetic processes, including chromatin immunoprecipitation (ChIP) and transcriptome analysis, as well as pharmacologic approaches will be required. These studies should be complemented by search for as-yet undiscovered interactions of inflammatory mediators with the normal processes of circuit development and maturation. Salient biomarkers should be sought to delineate individuals at risk for inflammation-augmented epilepsy. Together, these convergent approaches should help reveal fundamental and translationally relevant mechanisms by which inflammation impacts epilepsy. These studies uncover potential new targets for pharmacotherapy directed at TLR4 or IL-1 $\beta$  (Vezzani et al., 2010; Hennessy et al., 2010; Vezzani et al., 2011b).

## OTHER INFLAMMATORY MECHANISMS: BLOOD-BRAIN BARRIER AND COX-2 (J. GORTER, AMSTERDAM, THE NETHERLANDS; R. DINGLELINE, ATLANTA, GA, U.S.A.; A. FRIEDMAN, BEER-SHEVA, ISRAEL; P. FABENE, VERONA, ITALY)

Epilepsy has traditionally been considered mainly a neuronal disease, with less attention to nonneuronal cells until recently, when growing evidence suggests that astrocytes, microglia, blood-derived leukocytes, and BBB breakdown are involved in the pathogenesis of epilepsy. BBB dysfunction via transforming growth factor (TGF)- $\beta$  induction, the recruitment of leukocytes into the brain, induction of COX-2, and EP2 receptor activation may all be key mediators for the brain immune response associated with epileptogenesis. Interestingly, there appears to be close and bidirectional interactions between the tripod composed of BBB opening and innate and peripheral immune response, and seizures.

There is clinical and experimental evidence for BBB dysfunction following SE and in relation to seizures and epilepsy (for review see Friedman et al., 2009). Recent evidence indicates that leak of serum proteins (specifically albumin) through a dysfunctional BBB may be a key event in initiating specific signaling cascades within different elements of the neurovascular unit. Specifically, astroglia

transformation and the associated robust upregulation of inflammatory cytokines have been shown to occur in association with opening of the BBB and prior to the appearance of seizures. Jointly, these events are temporally associated with enhanced neuronal excitability, excitatory–inhibitory imbalance, and altered synaptic plasticity, establishing hypersynchronicity and epileptiform activity within the local network. Finally, the TGF- $\beta$  signaling pathway has been identified as potentially involved in epileptic activity evolving in a model of BBB opening (Cacheaux et al., 2009). Proteins of the TGF- $\beta$  family are pleiotropic cytokines that play a pivotal role in intercellular communication, cell growth, embryogenesis, differentiation, morphogenesis, wound healing, immune response, and apoptosis. In the brain, TGF- $\beta$  is considered an injury-related cytokine that is upregulated in different forms of brain injury. Upon BBB opening, the TGF- $\beta$  signaling pathway is activated, probably mediated by the binding of serum albumin to brain TGF- $\beta$  receptor 2 (Cacheaux et al., 2009). Furthermore, preliminary evidence was presented showing that blocking TGF- $\beta$  signaling prevents the transcriptome profiles activated in astrocytes during epileptogenesis, specifically preventing brain inflammation, and critically reducing the occurrence of spontaneous seizures. TGF- $\beta$  signaling thus emerges as a potential promising signaling pathway in those epilepsy cases where BBB dysfunction plays a key role in their development.

Studies of nonneural alterations leading to epileptogenesis in the pilocarpine model of epilepsy have recently indicated that seizures can induce leukocyte–endothelial interactions (Fabene et al., 2008; Kleen & Holmes, 2008; Ransohoff, 2009), BBB leakage (Oby & Janigro, 2006; Janigro, 2007), and angiogenesis characterized by a poor barrier function (Rigau et al., 2007; Marcon et al., 2009). The role of other nonneural cells, such as astrocytes, as critical signaling elements that contribute to the induction of excitotoxicity following pilocarpine-induced SE has been also demonstrated (Ding et al., 2007). Leukocyte trafficking mechanisms, as well as leukocyte migration through the brain endothelium, induce BBB damage contributing to epileptogenesis (Fabene et al., 2008; Kim et al., 2009). Using an animal model of meningitis (Kim et al., 2009), it was shown that leukocyte migration through the brain endothelium contributes to BBB breakdown and causes severe seizures. In addition, status epilepticus lead to chronic expression of VCAM-1, the ligand for VLA-4 integrin, and enhanced leukocyte rolling and arrest in brain vessels mediated by the leukocyte mucin P-selectin glycoprotein ligand-1. These mechanisms potentially contribute to increased BBB permeability and consequent neuroinflammatory response. In support of these data, a recent study showed that epileptiform activity induced in isolated guinea pig brain is able to rapidly induce expression of adhesion molecules on brain endo-

thelium (Librizzi et al., 2007), suggesting that each seizure may induce proinflammatory mediators able to activate endothelial cells, which in turn may favor the generation of other seizures. Indeed, recent evidence shows that bicuculline-induced seizures in isolated guinea pig brain trigger IL-1 $\beta$  biosynthesis and release in parenchymal and perivascular astrocytes, and concomitant BBB breakdown, thus highlighting a crucial role of proinflammatory cytokines produced by glia in BBB damage (Librizzi et al., 2010). In support of the results obtained from animal models of seizures and epilepsy, it has been shown that BBB disruption by intraarterial injection of mannitol in human patients with cerebral lymphoma induces focal motor seizures (Marchi et al., 2007). Vascular alterations and lymphocyte accumulation into the brain parenchyma were documented in a subgroup of pediatric patients with refractory epilepsy (Hildebrandt et al., 2008). Increased number of leukocytes was observed in brain parenchyma of patients with refractory epilepsy independent of the disease etiology (Fabene et al., 2008). Other studies reported the presence of macrophages but not of B or T cells in epileptogenic brain tissue from patients with temporal lobe epilepsy (Ravizza et al., 2008). In summary, leukocyte–endothelium interactions and eventually subsequent leukocyte recruitment in the brain parenchyma has been proposed to represent a possible player in the epileptogenic cascade.

Microarray analysis has shown that inflammation and the immune response are among the most activated processes during the acute, latent, and chronic phases of epilepsy that develops after electrically induced SE in the adult rat (Gorter et al., 2006). SE leads to upregulation of proinflammatory molecules including COX-2, which has been implicated in the epileptogenic process and in seizures (Kulkarni & Dhir, 2009). For example, recent studies show that COX-2 can regulate expression of P-glycoprotein (P-gp) during epileptogenesis and epilepsy (Bauer et al., 2008). P-gp could cause pharmacoresistance by reducing brain entry of antiepileptic drugs such as phenytoin (PHT). COX-2 is an enzyme that is induced by seizure activity and that converts arachidonic acid into prostaglandins such as PGE<sub>2</sub>. Gorter and colleagues have investigated the effects of COX-2 inhibition on epileptogenesis, spontaneous seizures, and PHT treatment in a rat model for TLE. In the first study, the selective COX-2 inhibitor, SC58236, was orally administered daily starting 4 h *after* electrically induced SE for 1 week during the latent period. Seizures were monitored using EEG/video until 35 days after SE. Cell death and microglia activation were investigated using immunocytochemistry. In the second study, a 3-day pretreatment with SC58236 that was started 24 h *before* electrically induced SE. In the third study, chronic epileptic rats were treated with SC58236 for 14 days, followed by 1 week treatment with a combination of SC58236 and PHT. Seizure activity was



monitored continuously by EEG. SC58236 treatment for 1 week during the latent period effectively reduced PGE<sub>2</sub> production but did not modify seizure development or the extent of cell death or microglia activation in the hippocampus. SC58236 treatment for 3 days before SE did not affect its duration, but led to an increased number of rats that died during the first 2 weeks thereafter. COX-2 inhibition during the chronic period led to an increased number of seizures during the second week of treatment in 50% of the rats. PHT treatment together with COX-2 inhibition reduced spontaneous seizures significantly for only 2 days, whereas PHT alone was ineffective. These results indicate that COX-2 inhibition *after* induction of SE does not affect epileptogenesis in the electrical stimulation model. Indeed, COX-2 inhibition initiated before SE is induced, or during the chronic epileptic phase, led to adverse effects in this epilepsy model. Despite a temporal reduction in seizure frequency with PHT and SC58236 combination treatment, COX-2 blockade does not seem to be a suitable approach for antiepileptic therapy (Holtman et al., 2010). Targeting of PGE<sub>2</sub> subtype receptors could be a more promising approach (Pekcec et al., 2009).

Most of the prominent risk factors for developing epilepsy (e.g., traumatic brain injury, stroke, brain infection, brain tumors, prolonged febrile seizures, SE) are associated with a strong inflammatory response, including the rapid induction of COX-2 in selected forebrain neurons after prolonged seizures. To study the role of seizure-triggered COX-2 induction, Dingledine and colleagues have developed mice in which COX-2 is ablated from the principal forebrain neurons (e.g., hippocampal pyramidal and dentate granule neurons) beginning around P15; COX-2 expression is normal in the rest of the brain and in the periphery. They have also developed novel selective modulators of a key PGE<sub>2</sub> receptor, that is, the EP2 receptor. Whereas the seizure threshold of global COX-2 knockout mice is reduced (Toscano et al., 2008), precluding meaningful interpretation of post-SE phenotypes, ablation of COX-2 from the principal forebrain neurons had no effect on seizure onset or intensity in the pilocarpine model. Therefore, this mouse is appropriate for studies of the consequences of COX-2 signaling after SE. In contrast to the results obtained by global inhibition of COX-2 pharmacologically (see above), selective ablation of COX-2 limited to principal forebrain neurons was neuroprotective in the hippocampus, and blunted the broad, mostly glial-mediated, neuroinflammatory reaction as judged by evaluating a panel of cytokines. Delayed mortality in the week after SE was also reduced in this mouse. These results point to a key role for neuron-derived COX-2 in some of the deleterious consequences of seizures.

COX-2 activation by seizures results in the enzymatic synthesis of up to five prostanoids (PGE<sub>2</sub>, PGF<sub>2a</sub>, PGD<sub>2</sub>,

prostacyclin, thromboxane), which in turn activate up to nine or more G-protein-coupled prostanoid receptors. COX-2 inhibitors can be beneficial or harmful in various models of brain injury, PGE<sub>2</sub> is prominently produced in hippocampus after SE, and activation of the EP2 receptor for PGE<sub>2</sub> has been shown to be neuroprotective in models of cerebral ischemia (McCullough et al., 2004). The group of Dingledine (Jiang et al., 2010), therefore, set out to develop selective pharmacologic modulators of EP2 to explore its role after SE. Using ultra-high throughput screens and medicinal chemistry they have succeeded in creating both allosteric potentiators and competitive inhibitors of EP2. EP2 is a G $\alpha$ s-coupled receptor that stimulates adenylate cyclase when activated by PGE<sub>2</sub>, resulting in elevation of cytoplasmic cAMP level. The EP2 allosteric potentiators show low cellular toxicity and are neuroprotective against NMDA-induced excitotoxicity on cultured hippocampal neurons (Jiang et al., 2010). The situation is likely to be complicated, however, because EP2 activation also seems to promote microglial activation (Shie et al., 2005; Quan Y, Jiang J, Ganesh T, Du Y, Dingledine R, unpublished data). Low solubility has so far thwarted attempts to test the allosteric potentiators *in vivo*.

### Future directions

Several open questions require additional studies; the most salient ones include the following: Are any of these proinflammatory pathways essential to the generation of an epileptic network? Do they induce irreversible changes in network properties or connectivity? Which prostanoid receptors exacerbate the neurodegeneration caused by pilocarpine? Does neuronal COX-2 induction influence BBB opening?

Structural lesions and subsequent inflammatory events have until now only been casually associated. Spontaneous recurrent seizures can occur in rats with seemingly preserved hippocampal (and extrahippocampal) morphology. Furthermore, inhibition of cytokines actions on modulation of leukocyte-endothelium interaction can reduce seizures. More integrative studies based on the different inflammatory pathways involved are needed, and it will be essential going forward to examine the role of specific pathways in multiple animal models.

## ADDITIONAL CONTRIBUTORS

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## DISCLOSURE

The authors have no conflicts of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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