Inflammation and epilepsy: the contribution of astrocytes
Zurolo, E.

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INFLAMMATION AND EPILEPSY: 
THE CONTRIBUTION OF ASTROCYTES

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Uitnodiging

voor het bijwonen van de openbare verdediging van mijn proefschrift

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THE CONTRIBUTION OF ASTROCYTES

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Oudezijds Voorburgwal 231
Amsterdam

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Inflammation and epilepsy: the contribution of astrocytes

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Thesis
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Inflammation and epilepsy: the contribution of astrocytes

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INFLAMMATION AND EPILEPSY:
THE CONTRIBUTION OF ASTROCYTES

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ten overstaan van een door het college voor promoties ingestelde
commissie, in het openbaar te verdedigen in de Agnietenkapel
op dinsdag 5 februari 2013, te 10:00 uur

door
Emanuele Zurolo
geboren te Castellammare di Stabia, Italië
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Faculteit der Geneeskunde
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADK</td>
<td>Adenosine Kinase</td>
</tr>
<tr>
<td>AED</td>
<td>Anti Epileptic Drug</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>CA</td>
<td>Cornus Ammonis</td>
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<tr>
<td>CB1/2R</td>
<td>Cannabinoid receptor 1/2</td>
</tr>
<tr>
<td>CFH</td>
<td>Complement Factor H</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory aminoacid transporter</td>
</tr>
<tr>
<td>ECBs</td>
<td>Endocannabinoids</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FCD</td>
<td>Focal Cortical Dysplasia</td>
</tr>
<tr>
<td>fID</td>
<td>focal Ictal Discharge</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillar Acidic Protein</td>
</tr>
<tr>
<td>GG</td>
<td>Ganglioglioma</td>
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<tr>
<td>HMEG</td>
<td>Hemimegalencephaly</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High Mobility Group Box 1</td>
</tr>
<tr>
<td>HS</td>
<td>Hippocampal Scleroses</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukine-1-beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IRAK1</td>
<td>Interleukin-1 Receptor-Associated Kinase 1</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic Acid</td>
</tr>
<tr>
<td>Kir</td>
<td>potassium inward rectifier</td>
</tr>
<tr>
<td>MCD</td>
<td>Malformation of Cortical Development</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kB</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Na+-K+-2Cl--cotransporter</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>PAs</td>
<td>Plasminogen Activators</td>
</tr>
<tr>
<td>PDS</td>
<td>Paroxysmal depolarizing shift</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol bisphosphate</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation Endproducts</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>TLE</td>
<td>Temporal Lobe Epilepsy</td>
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<tr>
<td>TLRs</td>
<td>Toll-like Receptor</td>
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<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alfa</td>
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<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
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<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor</td>
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<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase plasminogen activator receptor</td>
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Introduction
1.1 General Introduction
Glial Cells

Neurons are not the only cell type in the central nervous system (CNS). In fact they are outnumbered by different non neuronal cells, collectively called glia. This term was coined in 1859 by Rudolph Virchow to indicate the “connective tissue” surrounding the neurons (glia comes from Greek meaning glue).

With the term glia we indicate three types of cells in the CNS: oligodendrocytes, astrocytes and ependimocytes. Oligodendrocytes are the myelin producing cells, and they form a multilayer myelin sheath around the axons, contributing to their insulation and facilitating action potential conduction. Ependimocytes constitute the wall of the ventricles and play role in neurogenesis, neuronal differentiation/axonal guidance and are believed to participate in the production of cerebro spinal fluid (CSF) during development; they are involved in controlling the homeostasis in the mature brain (Bruni 1998). Astrocytes are heterogeneous cells which constitute from 20 to 50% of the volume of most brain areas and have a wide range of functions, from supporting neuronal metabolism and connectivity, to important roles in the modulation of neuronal signaling in brain patho-physiology. There is also another cell population which has a different embryonic origin called microglia: they are considered the macrophages of the brain since they mediate the immune response in the CNS. In this thesis the attention will be focused on the astrocytes, and in particular on their role in inflammation in relation to epilepsy.

Astrocytes physiology in normal brain

Astocytes are specialized glial cells and their organization in the brain is quite complex. Each astrocyte has its own separate domain and can reach more than 100 thousand synapses at once (Halassa, Fellin et al. 2007). Astrocytes are evolutionarily old, and their size increases with increased complexity of brain function (Kimelberg and Nedergaard 2010). For a long time, it was believed that they functioned as structural support cells, rather than actually contributing to network activity. Indeed astrocytes have an important role in neuronal metabolism, providing neurons with necessary nutrients from the vasculature (Nedergaard, Ransom et al. 2003). Also, astrocytes function as storage of glycogens, especially in areas of synaptic density, in order to sustain neuronal activity during hypoglycaemia (Sofroniew and Vinters 2010).

However, recently they were found to have numerous important functions that allow them to be closely involved in neuronal signalling (Perea, Navarrete et al. 2009).
Astrocyte heterogeneity

Although the heterogeneity of astrocytes was documented over a century ago, we are only beginning to understand the function of astrocytes in the past two decades. They have diverse morphologies, gene profile expression and physiological differences and their classification can be done according to different criteria. The classical distinction is between protoplasmic and fibrous astrocytes (Miller and Raff 1984) but they can be categorized according to their location, expression of different receptors or transporters. Figure 1 and 2 show the different astrocytes according to the pioneering work of Santiago Ramón y Cajal in 1913. Astrocytes can be identified in brain tissue by the expression of different markers namely glial fibrillar acidic protein (GFAP), S-100, a calcium binding protein, and glutamine synthetase (Zhang and Barres 2010). Their role is not yet completely understood but there are data supporting their role in neurogenesis, control of brain metabolism (ionic balance and neurotransmitter re-uptake) and in modulation of synaptic strength (Parpura, Heneka et al. 2012).
Astrocytes are connected to each other by gap junctions, forming a syncytium that allows ions and small molecules to diffuse in the brain parenchyma. These cells exhibit various types of membrane channels, receptors and transporters. Both K⁺ and Na⁺ channels are expressed in astrocytes, but the K⁺ channel density exceeds that of Na⁺ channels by far, presumably preventing the generation of action potentials (Jabs, Seifert et al. 2008). Astrocytes are extremely important for the buffering of extracellular potassium: they express Kir channels (K inward rectifier), in particular Kir 4.1, which are the main players in potassium buffering (Olsen and Sontheimer 2008). Kir channels are also thought to be responsible for the maintenance of the negative membrane resting potential of astrocytes (Kucheryavykh, Kucheryavykh et al. 2007) which is typically near EK (around -85mV), and displays little fluctuation in response to a wide variety of stimuli (Nedergaard, Ransom et al. 2003). One of the functions of astrocytes is to control glutamate levels in the extracellular space. The most important glutamate transporter proteins in astrocytes are EAAT1 (GLAST in rodent brain) and EAAT2 (GLT-1 in rodent brain) (EAAT: excitatory amino acid transporter). EAAT2 is the predominant glial transporter in the hippocampus (Proper, Hoogland et al. 2002). This
transporter is also expressed in neurons, though the main neuronal transporter is EAAT3 (Proper, Hoogland et al. 2002). Astrocytes also express different purinoreceptors, among which there are receptors for adenosine and ATP (P2X receptors), which have a role in short distance signaling from astrocyte to astrocyte and between astrocytes and other cell types (Verkhratsky 2010). For example, ATP released from astrocytes or neurons activates microglia through P2X receptors (Nedergaard, Rodriguez et al. 2010). An important characteristic of astrocytes is that they adjust to the network that surrounds them. This means that they often mirror the receptor expression of nearby neurons, contributing to the same signalling pathways (Kimelberg and Nedergaard 2010). Consequently, astrocytes in different regions have different properties and the class of astrocytes can be divided in many different subtypes.

**Astrocytes signal by means of intracellular Ca²⁺ elevations**

Astrocytes are often described as physiologically ‘silent’, which refers to their incapability of generating action potentials. However, they do exhibit a form of excitability through intracellular increase of Ca²⁺, in response to diverse signals from other astrocytes or neurons. Astrocyte Ca²⁺ signaling rely on intracellular source of Ca²⁺ in the Endoplasmatic Reticulum, which function as a Ca²⁺ storage. Figure 2 shows the main components of the pathway that lead to increase of intracellular Ca²⁺ concentrations. They usually involve the activation of metabotropic G-protein-coupled receptors, cleaving of phosphatidylinositol 4,5-bisphosphate (PIP2), thereby activating phospholipase C, which then produces IP3. IP3 receptors (InsP3-gated Ca²⁺ channels) on the membrane of the ER open and mediate Ca²⁺ release from the ER. These IP3 receptors are also highly sensitive for cytosolic Ca²⁺, which means that one Ca²⁺ burst initiates a fast intracellular Ca²⁺ wave that lasts beyond the initial moment of metabotropic stimulation (Nedergaard, Rodriguez et al. 2010). This signal is propagated within the cell by a complex series of events, which include Ca²⁺ buffering by mitochondria, thereby preventing desensitization of receptors to IP3 due to overexposure, and the presence of calcium binding proteins that limit the diffusion of Ca²⁺ ions within single astrocytes (Scemes and Giaume 2006). These processes allow a very specific, coordinated way of Ca²⁺ signal propagation through the cell.

Unlike most neurons, astrocytes are usually connected by gap junctions formed by connexins (Cx), mainly Cx43 and Cx30 (Jabs, Seifert et al. 2008; Giaume and Theis 2010). These gap junctions often connect adjacent processes of the same astrocyte, but also couple neighboring astrocytes at the outer borders of their separate domains (Nedergaard, Ransom et al. 2003). In this manner, neighboring astrocytes form large intercellular networks in which communication is rapid and coordinated. It is thought that the function of these intercel-
lular networks is to minimize differences in separate processes and neighboring cells, by balancing the amounts of all molecules that have a smaller molecular weight than 1000kDa (Rose and Ransom 1997). Because of fast transportation across gap junctions, excesses of ions and transmitters in the extracellular space, such as K⁺ and glutamate, can be effectively transported into the network without substantial changes of the membrane potential. Also, gap junctions are permeable to glucose, indicating that they are important for the facilitation of neuron energy supply (Nedergaard, Ransom et al. 2003). Gap junctions are also thought to contribute to propagation of Ca²⁺ waves to neighboring astrocytes, though not through direct Ca²⁺ trafficking. There are two different mechanisms described for the propagation of Ca²⁺ waves: gap junction dependent and gap junction independent. The gap-

![Principles of calcium signaling in astroglia.](image)

**Fig. 3. Principles of calcium signaling in astroglia.** [Ca²⁺]i accumulation could be caused by the entry of Ca²⁺ from the extracellular space through ionotropic receptors or store-operated channels (SOC). Plasmaemmal Ca²⁺ pumps/ATP-ases (PMCA) can extrude cytosolic Ca²⁺, while the plasmalemml sodium–calcium exchanger (NCX) can operate in both directions depending of intercellular Na⁺ concentration. An additional source of Ca²⁺ is available from the ER internal store that possesses InsP₃ receptors, which can be activated by the activity of metabotropic G-protein coupled receptors (GPCRs) and PLC. The ER store is (re)filled by the activity of the store-specific Ca²⁺-ATPase (SERCA). Cytosolic Ca²⁺ levels can be affected by a variety of cytosolic Ca²⁺-binding proteins (CBPs) and by the action of mitochondria. A negative membrane potential exists across the inner mitochondrial membrane. Mitochondrial Ca²⁺ uptake occurs through voltage-dependent anion channels (VDAC) present in the outer membrane and by the unipporter in the inner membrane as the electrochemical gradient drives Ca²⁺ into the matrix, while free Ca²⁺ exits the mitochondrial matrix through the mitochondrial Na⁺/Ca²⁺ exchanger and transient opening of the mitochondrial permeability transition pore (MPTP). From: (Verkhratsky, Rodriguez et al. 2012).
junction dependent pathway relies on direct diffusion of second messengers (such as IP3) through gap-junctions, thus triggering a Ca\(^{2+}\) response. The gap-junction independent pathway involves extracellular release of ATP, which also acts on neighboring cells as a second messenger (Scemes and Giaume 2006).

**Astrocytes-neuron communication: a new perspective**

**The tripartite synapse**

For many years, brain research has been focused on neurons. Astrocytes were labeled as supportive cells that were important for brain metabolism and homeostasis, but had no significant role in signalling. However, recent findings suggest that astrocytes, even though unable to generate action potentials, actively contribute to synaptic transmission. To introduce these results into the existing concept of synapses, the term ‘tripartite synapse’ was brought to life (Perea, Navarrete et al. 2009; Eroglu and Barres 2010).

In the tripartite synapse (figure 4), bi-directional signaling between astrocytes and neurons, shape the transmission of neuronal signals. This concept introduces a third element in the classical view of the synapse as pre-synaptic/post-synaptic terminals, exclusively neuronal: the astrocyte endfeet. The tripartite synapse was morphologically described (Perea, Navarrete et al. 2009) and functional studies demonstrate its relevance in the synaptic transmission. There are data showing that activation of Ca\(^{2+}\) signals in astrocytes, via endocannabinoid stimulation, induce synaptic potentiation in mice slices (Navarrete and Araque 2010). To be able to communicate with neurons in this way, astrocytes must have means to integrate neuronal signals and send signals of their own. Astrocytes express a wide variety of neurotransmitter receptors that, once activated, trigger intracellular Ca\(^{2+}\) elevation. Indeed, these Ca\(^{2+}\) rises have been measured in vivo as a response to different stimuli (Wang, Zhou et al. 2006). The same Ca\(^{2+}\) imaging method shows that astrocytes in the ferret visual cortex react to visual stimuli in a neuron-like discriminative manner (Schummers, Yu et al. 2008). A reasonable explanation would be that these astrocytes just reflect the level of nearby synaptic activity. However, examination of synaptically induced Ca\(^{2+}\) responses in rat hippocampal slices showed that stimulation of different pathways elicited different responses in the same astrocytes (Perea and Araque 2005).

This indicates that astrocytes process synaptic information as an integrated part of their surrounding network, a notion that is supported by the finding that astrocyte Ca\(^{2+}\) signals on their turn evoke NMDA receptor mediated slow inward currents in CA1 pyramidal neurons (Perea and Araque 2005). The exact manners in which these currents are evoked are not yet clear, but a widely accepted idea is that astrocytes release their own neurotransmitters: gliotransmitters. (Perea, Navarrete et al. 2009).
Fig. 4. Scheme of the tripartite synapse. Cartoon representing the transfer of information between neuronal elements and astrocyte at the tripartite synapse. Astrocytes respond with Ca²⁺ elevations to neurotransmitters released during synaptic activity and, in turn, control neuronal excitability and synaptic transmission through the Ca²⁺-dependent release of gliotransmitters (Gt) (Eroglu and Barres 2010).
Epilepsy and associated malformations of cortical development

Epilepsy

Epilepsy is a condition of the brain characterized by the periodic and unpredictable occurrence of seizures that occurs in about 1% of the population worldwide, with an incidence of 50 per 100,000 people/year in developed countries. Even with optimal recurrent antiepileptic drug (AED) therapy, about one third of the patients have poor seizure control and become medically refractory. The International League against Epilepsy (ILAE) has defined epilepsy as “a chronic condition of the brain characterized by an enduring propensity to generate epileptic seizures, and by neurobiological, cognitive, psychological and social consequences of this condition”. The definition of epilepsy requires the occurrence of more than one unprovoked seizures (Fisher, van Emde Boas et al. 2005). Seizures represent the clinical manifestation of epilepsies caused by both genetic and acquired factors, such as trauma, perinatal injury, postinfection lesions, and tumors. The seizure reflects a highly synchronous neuronal discharge that arises at restricted brain sites, the epileptogenic foci, and then secondarily spreads to large portion of the brain (Traub and Wong 1982; Jefferys 1990; Avoli, Mattia et al. 2002; Trevelyan, Sussillo et al. 2006). At the level of single neurons, there is a sustained neuronal depolarization resulting in a burst of action potentials, followed by a rapid repolarization and hyperpolarization. This sequence is called the paroxysmal depolarizing shift (PDS). When a large population of neurons is recruited and fires PDSs in a synchronous manner, then a seizure is triggered. The clinical manifestations of seizures depend on which brain area is involved. In 2010 ILAE revised terminology and concepts for organization of seizures and developed an international classification of seizures dividing them into two major classes: focal and generalized. Focal seizures are originating within networks limited to one hemisphere. They may be discretely localized or more widely distributed. For each seizure type, ictal onset is consistent from one seizure to another, with preferential propagation patterns that can involve the contralateral hemisphere. Generalized seizures are originating at some point within bilaterally distributed networks and can rapidly spread though the brain. Generalized seizures can be asymmetric. Status epilepticus consists of a very prolonged seizure or seizures occurring so frequently that they do not allow for full recovery of normal brain function (Berg, Berkovic et al. 2010). From an etiologic perspective (Berg, Berkovic et al. 2010), epilepsy is divided as follows: “Genetic” epilepsy: in this case epilepsy is, as best as understood, the direct result of a known or presumed genetic defect in which seizures are the core symptom of the disorder; “Structural/metabolic” epilepsy: in which there is a distinct other structural or metabolic condition or disease that has been demonstrated to be associated with an increase risk of developing epilepsy. Structural lesions include acquired disorders such as stroke, trauma and infection. “Unknown cause” epilepsy: this kind of epi-
lepsy designate that the nature of the underlying cause is as yet unknown. Structural/metabolic epilepsies are divided into generalized (generalized seizures) and localization-related disorders (focal seizures). Of particular relevance among localization-related epilepsies are those of temporal lobe origin, called Temporal Lobe Epilepsy (TLE).

Temporal Lobe Epilepsy (TLE)
TLE represents the most common partial epilepsy affecting humans. Seizures originate in one or both temporal lobes. The pathological lesion of areas of mesial temporal lobes (especially hippocampal sclerosis HS) provides a presumed anatomic basis for the origin of seizures. However, neuronal cell loss is not present in all patients affected by TLE (Dawodu and Thom 2005). Recent evidence showed that cell loss can affect extra-limbic area such as the subiculum, entorhinal cortex, and amygdale (Schwarcz and Witter 2002; Bernasconi, Bernasconi et al. 2003). In 65% of epileptic patients the etiology of TLE is either unknown or has a genetic cause; in 35% of cases, epilepsy is associated with a brain injury like febrile seizures, status epilepticus, central nervous system infection, head trauma, or birth trauma. The injury may possibly represent the trigger for the epileptogenic process that culminates in the clinical appearance of spontaneous in seizures and eventually in hippocampal sclerosis. During epileptogenesis, the brain functions are progressively altered but no seizures are present (Pitkanen and Lukasiuk 2011). About 30% of patients with TLE are refractory to the antiepileptic drugs (AED) and for appropriately chosen patients, only the surgical resection of the focus epilepticus may be curative (Cascino 2004). Several studies showed that TLE represents a progressive disorder that can also involve non-limbic areas. Neuropsychological evaluation showed that TLE patients performed worst in memory function, executive function and language and the degree of this impairment positively correlates with the duration of epilepsy (Oyegbile, Dow et al. 2004).

Malformation of cortical development (MCD) associated with epilepsy
One of the main causes of intractable epilepsy in young patients is represented by focal malformation of cortical development (MCD) (Sisodiya 2004; Krsek, Maton et al. 2008; Wong, Subar et al. 2008; Blumcke 2009). In particular, focal cortical dysplasia (FCD), cortical tubers in patients with tuberous sclerosis complex (TSC), hemimegalencephaly (HMEG), and glioneural tumors (such as ganglioma, GG) are causes of seizure resistant to the classical anti-epileptic drugs. In these cases often, to obtain a seizure control, patients require brain surgery.

(FCD) ILAE classified FCDs in three types (Blumcke, Pieper et al. 2010): FCD Type I refers to isolated forms of FCD, characterized by abnormal cortical layering compromising radial mi-
migration and maturation of neurons (Type IA) or the six-layered composition of the neocortex (type IB). Both variants present in combination correspond to FCD type IC. FCD type II refers to isolated forms of FCD, characterized by disrupted lamination of the cortex with different cytologic abnormalities. The presence of these changes in the morphology of the cells differentiates FCD type IIa which shows dysmorphic neurons without balloon cells, from FCD type IIb which shows both dysmorphic neurons and balloon cells. FCD type III associates abnormalities in cortical lamination with the presence of a principal lesion. Four subtypes can be distinguished indicating different associated lesions. FCD type IIIa, associated with hippocampal sclerosis, FCD type IIIb associated with tumors, FCD type IIIc, associated with vascular malformations and FCD type IIId associated with any other principal lesion acquired during early life (Blumcke, Pieper et al. 2010; Blumcke and Spreafico 2010).

**HMEG** is an extremely rare disease defined as a malformation of cortical development showing unilateral enlargement of a brain hemisphere associated with cytoarchitectural abnormalities. HMEG leads to delay of the development, motor deficit and severe intractable epilepsy with onset in the first months of life (Trounce, Rutter et al. 1991; Janszky, Ebner et al. 2003; Sanghvi, Rajadhyaksha et al. 2004; Sasaki, Hashimoto et al. 2005; Tinkle, Schorry et al. 2005).

**TSC** is a disorder originating from mutations in the TSC1 or TSC2 genes (Consortium 1993; van Slegtenhorst, de Hoogt et al. 1997). TSC affects several organs and patients often show dermatological, renal and neurological manifestations (Curatolo, Lo-Castro et al. 2009). Abnormalities affecting CNS include developmental delay, neurobehavioural dysfunction and severe epilepsy (Curatolo, Verdecchia et al. 2002; Bolton 2004). Histopathological examination of brain specimens reveals cortical tubers, subependymal nodules and subependymal giant cell astrocytomas (SEGAs). Over 80% of TSC patients have epilepsy that is resistant to the treatment with classical AEDs (Curatolo, Verdecchia et al. 2002; Connolly, Hendson et al. 2006).

**GGs** are defined as a rare low-grade neoplasm showing presence of dysplastic neurons and glial cells components. The incidence of GGs is only 1.3% in large brain tumor series (Blumcke and Wiestler 2002; Louis, Ohgaki et al. 2007). However, they represent the most common tumor entity in children with intractable epilepsy (Wolf and Wiestler 1993; Morris, Matkovic et al. 1998). The pathogenesis of the hyperexcitability in patients with ganglioglioma is not yet elucidated.

**Astrocytes dysfunction in epilepsy**

Gliotic scar formation is a prominent feature of human epilepsy (Chao, Humphreys et al. 1940) The presence of gliotic scar in chronic epilepsy has led many to suggest a physiological
role for glia in the disease (Pollen and Trachtenberg 1970; Harris 1975; Rapport, Ojemann et al. 1977). The following paragraphs are meant to review the common changes that astrocytes undergo during epilepsy.

**Astrogliosis is a reaction to brain damage**

Astrogliosis can be described as a reaction of astrocytes to any kind of brain injury. This response entails changes in the regulation of many specific signalling pathways and gene expression, such as an upregulation of GFAP and vimentin expression (Sofroniew and Vinters 2010), which are both intermediate filament proteins often used to indicate astrogliosis in histological examination. Based on histopathological examination of human tissue, astrogliosis can be classified in three categories ranging from mild to severe astrogliosis with glial scar formation (Sofroniew, 2009). Mild astrogliosis is often associated with mild, non-invasive injury and activation of the immune system and, although an upregulation of GFAP is seen, it does not lead to astrocyte proliferation. Proliferation does start in the second category, resulting in the extension of astrocyte processes, protruding into each others domains and disturbing the natural organization of astrocyte networks. Astrocyte proliferation is accompanied by considerable cell hypertrophy. In the last category, which contains severe astrogliosis with glial scar formation, in addition to upregulation of GFAP and other genes, cell proliferation results in complete destruction of individual astrocyte domains and the formation of dense, narrow glial scars along the borders of the injured area, which act as protective barriers against inflammatory and infectious agents (Oberheim, Tian et al. 2008; Sofroniew and Vinters 2010). These scars keep interacting with surrounding cells such as fibromeningeal and other glial cells, but the structural changes are long lasting and persist after the triggering insult has disappeared (Sofroniew 2009). The rigorous cell biological changes in reactive astrocytes probably contribute to various nervous disorders in the shape of loss of function of the damaged area. Other detrimental effects consist of interference with axonal growth and neural regeneration. However, studies with KO mice show that astrogliosis actually is neuroprotective in many ways (Sofroniew and Vinters 2010) and disruption or malfunction of the process might worsen the symptoms of different kinds of injury. Disrupting astrocyte reactivity in acute and chronically injured mice by targeting them with drugs when they start to proliferate, shows increased speed and persistence of inflammatory cells, impaired repair of the Blood Brain Barrier, increased tissue damage and neuronal loss and impaired overall recovery of function (Sofroniew 2009). Astrogliosis is found in many central nervous system disorders, like acute brain trauma or neurodegeneration (D'Ambrosio 2004; Sofroniew and Vinters 2010. In epilepsy, astrogliosis is most prominent in hippocampal sclerosis. This is the pathological hallmark of Temporal Lobe Epilepsy.
(TLE), resulting in neuronal loss and hypertrophy and proliferation of astrocytes in the damaged region, mostly the endfolium and CA1 segments (Sofroniew, 2010). The connection between severe astrogliosis and epileptic activity has been thoroughly examined in different epileptic animal models. Recent data (Oberheim, Tian et al. 2008) showed that astrocytic proliferation is in part associated with seizure activity, because it can be reduced or even prevented by medical suppression of seizure activity by valproate administration. However, this result indicates that cell proliferation is a consequence, rather than a cause of the seizure activity in this experimental setting. Also, proliferation was accompanied by neuronal changes, such as hypertrophy of apical dendrites and increased synaptic density. This suggests that both neurons and astrocytes undergo changes in epilepsy. When considering the role of astrogliosis in neuronal disorders, it is difficult to distinguish cause and effect. Since epileptic seizures damage the brain, astrogliosis can be triggered by seizure activity, but this is not necessarily the case. There are types of epilepsy that display recurring, prolonged epileptic activity, but never show any astrogliosis (Lee, Mane et al. 2007). However, reactive astrocytes are still found in the majority of epilepsy cases. Some evidence suggests that reactive astrocytes may play a functional role in causing seizure activity, particularly in relapsing forms (D'Ambrosio 2004; Sofroniew and Vinters 2010). For example, astrogliosis has been found prior to spontaneous epileptic activity in kainic acid treated rats, suggesting a causal role (Takahashi, Vargas et al. 2010). In this model, the initial Status Epilepticus caused by the KA injection is followed by a period of about 18 days with low seizure probability. After this, the animals will exhibit spontaneous, recurring seizures. Consequently, the KA model allows examination of changes in the brain that are associated with the development of recurring seizure activity after an initial insult, to which astrogliosis seems to contribute. However, in mice in which the gene coding for Adenosine Kinase (ADK) was deleted, astrogliosis does not necessarily contribute to seizure generation (Li, Lan et al. 2008). Adenosine is an endogenous anticonvulsant, and its levels are largely under control by ADK. Wild type mice developed seizures with astrogliosis and upregulation of ADK within 24 hours after Kainate Acid injections, whereas transgenic mice lacking the gene for ADK developed astrogliosis without seizure activity. Although the authors exclude a direct causing role for astrocyte reactivity alters certain mechanisms that may be epileptogenic, such as upregulation of ADK. Supporting this view data (Ortinski, Dong et al. 2010) showed that specific viral induced astrogliosis changes neuronal excitability, but did not cause seizure activity per se. However, they do find an astrogliosis induced malfunction in astrocyte glutamate metabolism resulting in impaired inhibitory signalling, suggesting an important role for astrogliosis in hyperexcitability.
Impaired K⁺ buffering in temporal lobe epilepsy

During seizure activity in vivo, the extracellular K⁺ concentration, [K⁺]₀, increases from 3 mM to a ceiling level of 10–12 mM (Heinemann and Lux 1977). This high [K⁺]₀ levels can generate epileptiform activity in acute brain slices. Astrocytes express K inward rectifier (Kir) channels that contribute to maintain the K⁺ homeostasis. Studies have shown that low concentration of Ba²⁺ (100µM) can selectively inhibit Kir4.1, the major Kir responsible for K⁺ currents (Seifert, Huttmann et al. 2009). Differences were observed in the effect of Ba²⁺ on stimulus-induced changes in [K⁺]₀ in the CA1 region of hippocampal brain slices obtained from temporal lobe epilepsy (TLE) patients with hippocampal sclerosis (HS) or without sclerosis (non-HS). In non-HS tissue, Ba²⁺ application significantly enhanced [K⁺]₀ while this effect was not observed in HS specimens suggesting impaired function of these channels in the sclerotic tissue (Kivi, Lehmann et al. 2000). The hypothesis could be confirmed with patch-clamp analyses demonstrating downregulation of Kir currents in the sclerotic human CA1 region of TLE patients (Bordey and Sontheimer 1998; Hinterkeuser, Schroder et al. 2000). Genetic downregulation of Kir4.1, the main Kir channel subunit in astrocytes (Kofuji, 2000; Neusch, Papadopoulos et al. 2006; Olsen, Higashimori et al. 2006; Seifert, Huttmann et al. 2009), profoundly reduced the ability of astrocytes to remove glutamate and K⁺ from the extracellular space, both in cell culture (Kucheryavykh, 2007) and in vivo (Djukic, Casper et al. 2007). General knockout of Kir4.1 leads to early postnatal lethality (Kofuji, Ceelen et al. 2000) while mice with astrogial deletion of the channel developed a pronounced behavioral phenotype, including seizures (Djukic, Casper et al. 2007). In addition to spatial buffering, transient K⁺ accumulations can be counterbalanced by net K⁺ uptake through Na,K–ATPase and the Na–K–Cl co-transporter NKCC1, at the cost of cell swelling due to concomitant water influx (reviewed by (Kofuji and Newman 2004)) However, whether alterations in net K⁺ uptake contribute to the enhanced [K⁺]₀ levels seen in epileptic tissue has still to be elucidated.

Astrocytes and epileptic network activity

Since astrocytes can reach tens of thousands synapses at once, they are thought to synchronize the activity of their surrounding neurons, thus contributing to the synchronized activity through the brain (Angulo, Kozlov et al. 2004; Fellin and Carmignoto 2004; Tian, Azmi et al. 2005; Jabs, Seifert et al. 2008). Fellin et al. (2004) showed that groups of neurons (most groups comprising 2 to 4 neurons, but a group of 9 neurons was also observed) show a synchronized response upon the stimulation of one astrocyte. Neurons synchronized when they were within 100µm of each other and repeats of the specific responses were also observed. In an acute model of focal ictal discharge (fID), astrocytes Ca²⁺ elevations were shown to be necessary to have synchronized neuronal activity, supporting their role in
network synchronization (Gomez-Gonzalo, Losi et al. 2010). Also the increased gap junction coupling that has been found in epilepsy and that seems to be associated with astrogliosis (Takahashi, Vargas et al. 2010) could indicate a role for Ca\(^{2+}\) waves in network activity. However, since epileptic firing activity quickly depletes cell energy stores, it is also probable that the cells are trying to maintain their energy supply by increased glucose and lactate transport through an increased number of gap junctions. A possible explanation for synchronized neuronal responses is that glutamate released by one astrocyte activates extrasynaptic NMDA receptors of the neuronal dendrites that are close enough to sense such a change in extracellular glutamate. In normal conditions, these changes are transient, as a consequence of glutamate clearance. However, in case of increased extracellular glutamate levels, as are found in epilepsy, glutamate may diffuse further in the extracellular space and thus reach more neurons at once, whose activation could initiate a similar response in other astrocytes, and so forth generating a seizure. Such a mechanism might contribute to the highly synchronized activity present in epilepsy.

Astrocytes can influence network excitability in epilepsy through different mechanisms, including a dysfunctional adenosine homeostasis, which may result from changes in ADK expression levels (Boison 2008). The ADK hypothesis of epileptogenesis (Boison 2008) is based on the observation of ADK is upregulated in reactive astrocytes in experimental models of TLE. In the last part of the thesis we evaluated the expression pattern of ADK in relation with epilepsy and epilepsy-associated tumors.

**Astrocytes and inflammation**

Recent work point out to the role of astrocytes in the inflammatory processes in the brain in particular in epilepsy. The second chapter of this thesis will give an overview on inflammatory processes that astrocytes activate under different pathological conditions.
Reference list

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1.2 Astrocyte Immune Responses in Epilepsy

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Abstract

Astrocytes, the major glial cell type of the central nervous system (CNS), are known to play a major role in the regulation of the immune/inflammatory response in several human CNS diseases. In epilepsy-associated pathologies, the presence of astrogliosis has stimulated extensive research focused on the role of reactive astrocytes in the pathophysiological processes that underlie the development of epilepsy. In brain tissue from patients with epilepsy, astrocytes undergo significant changes in their physiological properties, including the activation of inflammatory pathways. Accumulating experimental evidence suggests that proinflammatory molecules can alter glio-neuronal communications contributing to the generation of seizures and seizure-related neuronal damage. In particular, both in vitro and in vivo data point to the role of astrocytes as both major source and target of epileptogenic inflammatory signaling. In this context, understanding the astroglial inflammatory response occurring in epileptic brain tissue may provide new strategies for targeting astrocyte-mediated epileptogenesis.

This article reviews current evidence regarding the role of astrocytes in the regulation of the innate immune responses in epilepsy. Both clinical observations in drug-resistant human epilepsies and experimental findings in clinically relevant models will be discussed and elaborated, highlighting specific inflammatory pathways (such as interleukin-1β/toll-like receptor 4) that could be potential targets for antiepileptic, disease-modifying therapeutic strategies.
ASTROCYTES AS KEY PLAYERS IN BRAIN INFLAMMATION

The central nervous system (CNS) has commonly been considered an immune-privileged site in the presence of an intact blood brain barrier (BBB). However, this concept is gradually changing as a result of recent developments in the field of innate immunity supporting the role of CNS-resident cells acting as innate-immune-competent cells; [for reviews see (Amor et al. 2010; McNaull et al. 2010; Schwartz and Kipnis 2011)]. Both innate and adaptive inflammatory responses occur in the CNS. The innate immune response is a non-specific, acute defense against external agents or local injuries, and cell types chiefly involved in this response include monocytes/macrophages and microglia. The adaptive immune response is antigen-specific, and B and T lymphocytes are the key players in adaptive immunity. Communication between the innate and adaptive responses involves cell-cell interactions, as well as soluble factors such as cytokines and chemokines. In earlier studies, a considerable amount of attention was focused on the immune function of microglial cells, which are generally considered to be the “CNS-based resident macrophages” (Aloisi 2001; Graeber and Streit 1990). However, over the past decade there has been accumulating evidence supporting the central role of astrocytes in the CNS innate immune response induced by a variety of insults. Astrocytes have been shown to initiate, regulate and amplify immune-mediated mechanisms involved in different human CNS diseases, including epilepsy [for reviews see (Farina et al. 2007; Seifert et al. 2010)]. In this review we will largely focus on protoplasmic astrocytes that predominate in gray matter, and fibrous astrocytes that predominate in white matter. Astrocytes undergo complex morphological and functional changes. These changes may vary with the severity and the type of insult and a continuum of progressive morphological alterations can be observed in human brain tissue under different pathological conditions [for review see (Sofroniew and Vinters 2010)].

ASTROCYTES AS INNATE-IMMUNE-COMPETENT CELLS

Astrocytes as antigen-presenting cells

In contrast to the well-established role of microglia as antigen presenting cells (APCs) the role of astrocytes in antigen presentation is still a matter of debate. Previous studies have shown the induction of major histocompatibility (MHC) class I and II molecules on interferon-γ (IFN-γ) exposure in vitro [for reviews see (Aloisi et al. 2000a; Dong and Benveniste 2001)]. However, there are discrepancies in the literature concerning the ability of astrocytes to act as fully competent APCs (Aloisi et al. 2000a; Becher et al. 2000; Cornet et al. 2000; Cross and Ku 2000; Dong and Benveniste 2001; Girvin et al. 2002; Weber et al. 1994). Moreover, the demonstration of MHC-positive astrocytes in pathological human brain remains a controversial topic, even in inflammatory neurological disorders such as multiple sclerosis (MS) [De
Keyser et al. 2003; Gobin et al. 2001; Traugott and Raine 1985). In Rasmussen’s encephalitis (RE), a severe inflammatory epileptic encephalopathy of childhood, expression of MHC class I molecules has been reported in astrocytes (Bauer et al. 2007; Farrell et al. 1995), suggesting an MHC class I–restricted T-cell response as a possible mechanism for the occurrence of the astrocytic degeneration observed in RE (Bauer et al. 2007). However, the final effect of astrocyte-T-lymphocyte interactions is complex and depends on the type of responding T cells (Th1 or Th2 cells). Accordingly, whereas microglia may activate both Th1 and Th2 cells, astrocytes have been shown to stimulate mainly Th2 responses, providing homeostatic mechanisms which may limit brain inflammation (Aloisi et al. 1998; Aloisi et al. 2000b). The physiological role of MHC molecules (MHC class I), as well as of other immunological molecules/receptors expressed by astrocytes during brain development, is another important area for future research. Recent studies suggest that immune molecules critically modulate the development and function of the CNS [for review see (Glynn et al. 2011)].

Astrocytes as source and target of inflammatory molecules
Astrocytes represent an important source of immunologically relevant cytokines and chemokines. In vitro studies document the ability of astrocytes (particularly reactive astrocytes) to produce cytokines such as interleukin(IL) -1β, IL-6, tumor necrosis factor (TNF)-α, transforming growth factor beta (TGF)-β and chemokines, such as monocyte chemoattractant protein-1 (MCP-1; chemokine, C-C motif, ligand 2; CCL2), which are highly expressed in both experimental and human epileptogenic brain tissue [for reviews see (Aronica and Crino 2011; Vezzani et al. 2008b) Fig. 1]. The expression pattern and the role of astrocyte-derived inflammatory molecules in seizure generation and progression will be discussed in the following sections.

Astrocytes are also the target of inflammatory molecules which, through the activation of specific receptors (including pattern-recognition receptors (PRRs) and related intracellular signaling pathways), may aggravate astrogliosis and amplify the pro-epileptogenic inflammatory signaling [for reviews see (Aronica and Crino 2011; Farina et al. 2007; Sofroniew and Vinters 2010); Fig. 1]. Particular attention has recently been focused on the role of the IL-1 receptor/toll-like receptor superfamily (IL-1R/TLR) in epilepsy (Aronica and Crino 2011; Maroso et al. 2011; Vezzani et al. 2010; Vezzani et al. 2011d; Vezzani et al. 2008b). The IL-1R/TLR superfamily comprises cell surface PRRs sharing a conserved region termed the Toll/IL-1R (TIR) domain (O’Neill 2008; O’Neill and Dinarello 2000). Several TLRs are expressed in human astrocytes in vitro, including TLR2, TLR3 and TLR4 (Farina et al. 2007). However, whereas TLR3 shows consistent expression in the resting state, studies examining TLR2 and TLR4 expression in
Astrocytes have produced conflicting results, which may reflect differences in cell source and culture conditions (Crack and Bray 2007; Farina et al. 2007; Kielian 2006). Expression of IL-1R1 or TLRs in astrocytes has also been demonstrated in human brain, with low levels in resting astrocytes and upregulation in reactive astrocytes under different pathological conditions, including epilepsy [(Maroso et al. 2010; Maroso et al. 2011; Ravizza et al. 2006a; Ravizza et al. 2008a; Vezzani et al. 2011d; Zurolo et al. 2011); Fig.1].

TLRs have a key role in pathogen recognition (Kawai and Akira 2007), but in the absence of pathogens, TLR can be activated by endogenous molecules, named damage-associated molecular patterns (DAMPs), released from injured or activated cells. One of these molecules is the high mobility group box 1 (HMGB1) (Bianchi and Manfredi 2009), a ubiquitous chromatin component that can be actively secreted by immuno-competent cells in response to immune challenges (Muller et al. 2004). Both in vitro and in vivo findings suggest that astrocytes are a source of extracellular HMGB1 (Maroso et al. 2010; Zurolo et al. 2011). In particular, HMGB1 release has been shown to be induced in both rat (Hayakawa et al. 2010) and human astrocytes in culture (Zurolo et al. 2011) in response to the pro-inflammatory.

Figure 1. Astrocytes as key players in neuroinflammation in the epileptic brain. Schematic representation of the hypothetical cascade of inflammatory processes in which astrocytes are involved. Knowledge of the astrocyte immune-inflammatory function in epilepsy may create the basis for developing effective therapeutic strategies to control seizures.
cytokine IL-1β, and nuclear to cytoplasmic translocation has been observed in human and experimental epileptic tissue (Maroso et al. 2010). In addition, astrocytes have also been shown to respond to HMGB1 stimulation with induction of several inflammatory mediators (Pedrazzi et al. 2007).

Recently, a new class of regulators of the immune responses has been recognised in the form of microRNAs (miRNA), acting as post-transcriptional regulators of gene expression (Gantier 2010; Quinn and O’Neill 2011; Sonkoly et al. 2008). In particular, the miRNA-146a has been specifically associated with the regulation of TLR signaling (Cui et al. 2010; Quinn and O’Neill 2011; Sheedy and O’Neill 2008; Taganov et al. 2006). miRNA-146a is expressed in human brain, and astrocytes have been shown to be key players in the regulation of this miRNA in response to inflammatory molecules, such as IL-1β (Aronica et al. 2010; Cui et al. 2010).

Another important component of the innate immune response is the complement system; this consists of a variety of soluble and surface proteins which, when activated, result in a complex cascade of processes contributing to the amplification of the inflammatory response [for review see (Bonifati and Kishore 2007)]. Reactive astrocytes are a source of complement components and also express complement-regulatory proteins, as well as complement receptors [for review see (Farina et al. 2007); Fig. 1]. Complement activation products, such as C3, regulate cytokine synthesis, and cytokines (such as IL-1β) may also induce complement factor expression in human astrocytes (Barnum and Jones 1995; Bonifati and Kishore 2007; Veerhuis et al. 1999). Astrocytes can also contribute to regulation of this inflammatory pathway by induction of inhibitory factors, such as the complement factor H (CFH) (Aronica et al. 2007; Boon et al. 2009; Griffiths et al. 2009). In addition, recent evidence suggests an extensive and complex cross-regulation between complement and the TLRs, which deserves further investigation in astrocytes (Hajishengallis and Lambris 2010).

**Astrocyte immune-inflammatory function and neurotransmitter receptors**

Signaling via neurotransmitter receptors provides an additional mechanism by which astrocytes can sense and respond to changes in the extracellular environment, influencing the inflammatory and immune response under pathological conditions associated with astrogliosis. An example is provided by the activation of astroglial G protein-coupled glutamate and purinergic receptors, the expression of which is deregulated in epileptogenic brain tissue (Byrnes et al. 2009; D’Antoni et al. 2008; Gomes et al. 2011; Hasko et al. 2005; Matute and Cavaliere 2011).

Both *in vitro* and *in vivo* studies suggest an up-regulation of group I and II metabotropic glutamate receptor (mGluR) subtypes (mGluR5 and mGluR3) in reactive astrocytes [(Aronica et
Activation of mGlur3 in human astrocytes in culture modulates the release of IL-6 in the presence of IL-1β, supporting the role of this receptor subtype in regulating the capacity of activated astrocytes to produce inflammatory cytokines (Aronica et al. 2005a).

Increasing evidence points towards a critical role of purinergic receptors in neuron–glia communication and neuroinflammation (Boison 2010; Gomes et al. 2011). Stimulation of the adenosine receptor (P1 receptor) A2B induces the release of IL-6 from astrocytes and the activation of the A3 receptor induces the synthesis of the chemokine MCP-1 [for reviews see (Abbracchio and Ceruti 2007; Gomes et al. 2011; Hasko et al. 2005)]. The P2 purinergic receptors modulate the cytokine-mediated signal transduction in human astrocytes in culture (Liu et al. 2000). Although the expression of both P2X (4,6,7) and P2Y(1,2) receptor-subtypes has been reported in cultured astrocytes, P2Y rather than P2X receptor-subtypes have been suggested as being involved in the modulation of intracellular Ca2+ (Fischer et al. 2009). In human fetal astrocytes, the blockade of P2Y receptors affects both IL-1β and TNFa signaling (Liu et al. 2000), whereas the P2X7 receptor has been implicated in the regulation of chemokine synthesis in astrocytes (Panenka et al. 2001). In addition, inflammatory molecules, such as IL-1β, may modulate the expression of adenosine kinase (ADK), providing a potential modulatory crosstalk between the astrocyte-based adenosine cycle and inflammation (Aronica et al. 2011).

Finally, it has also been suggested that cannabinoid (CB) receptors, as mediators of endocannabinoid signaling, exert an immunomodulatory function on astrocytes (Sheng et al. 2005).

**ASTROGLIAL INFLAMMATORY RESPONSE IN EPILEPSY**

Increasing evidence supports the concept of activation of innate immune responses in both experimental and human epilepsy and the critical involvement of inflammatory processes in the etiopathogenesis of seizures (Aronica and Crino 2011; Vezzani et al. 2010; Vezzani et al. 2011d). In particular, recent clinical-neuropathological and experimental observations support the notion that dysregulation of the astrocyte immune-inflammatory function (discussed above) is a common factor, which may predispose or directly contribute to the generation of seizures and to seizure-related neuronal damage in epilepsy of various etiologies (Fig. 1). Current knowledge concerning common inflammatory signaling pathways involving astrocytes that may alter neuronal excitability is discussed below.
ASTROCYTES AND INFLAMMATORY PROCESSES IN PATIENTS WITH MEDICALLY REFRACTORY EPILEPSY

Reactive astrogliosis is a pathological hallmark of various types of medically refractory focal epilepsy, including epilepsy that develops following ischemic, traumatic, or infectious brain injury (Sofroniew and Vinters 2010). Reactive astrogliosis is also the pathological hallmark of two major epilepsy-associated pathologies [hippocampal sclerosis and focal malformations of cortical development (MCD; FCD and cortical tubers in tuberous sclerosis complex TSC)]. Hippocampal sclerosis (HS) is the most common neuropathological finding in patients undergoing surgery for intractable temporal lobe epilepsy [TLE; (Wieser 2004)]. Although specimens obtained from patients undergoing surgery for intractable TLE often represent the end-stage of the pathological cascade that leads to HS, histopathological and molecular analysis of this tissue is essential to confirm the relevance and cellular sources of inflammatory molecules and related signaling. Large-scale analysis of gene expression profiles suggests a prominent upregulation of genes related to astroglial activation and innate immune/inflammatory response in human TLE [for review see (Aronica and Gorter 2007)]. This evidence, at gene expression level, has been confirmed by histopathological studies demonstrating the association between activated astrocytes and microglial cells and the induction of major proinflammatory pathways in human TLE (Aronica and Gorter 2007; Ravizza et al. 2008a; Vezzani et al. 2010).

The transcription factor nuclear factor-kappa B (NFkB) plays a central role in regulating immune and inflammatory responses, including the IL-1R/TLR signaling pathways (Oechkinghaus et al. 2011). Activation of IL-1R1-mediated signaling in cells targeted by the released IL-1β induces, via an NFkB-dependent mechanism, the transcription of other genes encoding downstream mediators of inflammation, including IL-6, TNF-α, cyclooxygenase-2 (Cox-2) or CCL2 (i.e. monocyte chemotactic protein-1) (Andjelkovic et al. 2000; Dinarello 2004; Meeuwsen et al. 2003). Crespel et al. (Crespel et al. 2002) reported NFkB over-expression in reactive astrocytes in human HS specimens. The activation of the NFkB signaling pathway in astrocytes has been confirmed by subsequent studies demonstrating prominent expression in astrocytes of both L-1β and its functional receptor, IL-1R1, providing evidence of a persistent activation of this specific inflammatory pathway in human tissue from people with chronic epilepsy (Ravizza et al. 2008a). In addition, up-regulation of astroglial Cox-2 and CCL2 has been reported in human TLE (Desjardins et al. 2003; Holtman et al. 2009; Wu et al. 2008) suggesting the activation of a complex, highly interconnected, cytokine network.

Human studies in TLE also support the involvement of the complement system. Expression of various complement components, such as C1q, C3c and C3d, has been observed in reactive astrocytes within the sclerotic hippocampus of people with TLE (Aronica et al. 2007).
Complement activation in astrocytes may regulate cytokine synthesis thus critically contributing to the propagation and persistence of the inflammatory response. The activation of the plasminogen system in astrocytes in human TLE may contribute to the regulation of the immune responses and related inflammation within the epileptic lesion (Benarroch 2007). Accordingly, in addition to neurons and microglial cells, expression of both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) has been observed in reactive astrocytes (Iyer et al. 2010). Although PAs may contribute to the activation of astrocytes (Gravanis and Tsirka 2005), astrocytes are also involved in the neutralization of tPA toxicity via an endocytic tPA receptor (Fernandez-Monreal et al. 2004). Induction of the pathways discussed above (IL-1β, complement and PA) in perivascular astrocytes suggests that the alterations of BBB permeability described in human TLE (Rigau et al. 2007; van Vliet et al. 2007) probably result from the convergence of the actions of different inflammatory molecules released from parenchymal brain cells, then acting on blood vessels. In this context, the vascular endothelial growth factor A (VEGFA)-signaling pathway may also affect the integrity of the BBB (Schoch et al. 2002). VEGFA is upregulated in reactive astrocytes (including perivascular astrocytes) in human epileptogenic tissue (HS and FCD; (Boer et al. 2008; Morin-Brureau et al. 2011; Rigau et al. 2007)), and increased VEGFA expression has recently been reported within cortical tubers of people with TSC (Parker et al. 2011).

As mentioned above, the activation of the TLR signaling pathway in astrocytes plays a key role in the regulation of the innate immune response. Interestingly overexpression of TLR4 and its endogenous ligand HMGB1 has been demonstrated in human TLE in reactive astrocytes, confirming the role of these cells as both source and target of HMGB1 (Maroso et al. 2010). The functional consequences of the astrocyte-mediated HMGB1-TRL signaling in ictogenesis and epileptogenesis in experimental models are discussed below (see Fig. 2). Reactive astrogliosis is also a major feature of focal MCD, such as FCD and cortical tubers in TSC (Blumcke et al. 2011; Blümcke et al. 2009; Orlova and Crino; Sosunov et al. 2008; Wong 2008).

The association between astrogliosis and activation of inflammatory pathways is supported by gene expression analysis of cortical tubers in TSC; high expression levels have been observed for genes encoding complement components, chemokines, MHC elements and components of the IL-1R/TLR signaling pathways (Boer et al. 2010). Activation of various proinflammatory molecules and related pathways has been confirmed at the cellular level in both TSC and FCD specimens, pointing to the central role of reactive astrocytes in the immune/inflammatory response of these developmental epileptogenic lesions as well (for review see (Aronica and Crino 2011)).
A recent study (Zurolo et al. 2011) demonstrates the activation of the TLR signaling pathway in reactive astrocytes both in FCD type and TSC specimens, as shown by overexpression of TLR4 and RAGE (receptor for advanced glycation end products) and cytoplasmic translocation of HMGB1 [as reported in human HS specimens; (Maroso et al. 2010)].

The transforming growth factor (TGF)-β mediated pathway is also upregulated in focal MCD (Boer et al. 2010; Kim et al. 2003). TGF-β is a multifunctional cytokine which, acting through its specific receptors, modulates the astrocyte immune responses and which has recently been suggested as playing a critical role in neocortical epileptogenesis acting through its specific receptors (Ivens et al. 2007).

A controversial issue is whether the dysregulation of astrocyte immune-inflammatory responses is related to the underlying cellular pathology in epileptic tissue or whether it occurs as a consequence of recurrent seizures. The observation of prenatal TLRs and HMGB1 expression in giant cells within the tuber in TSC (Aronica et al. 2008; Samadani et al. 2007; Zurolo et al. 2011) suggests that the induction of these signaling pathways could be intrinsic to the developmental lesion 

![Figure 2. Functional consequences of the production of inflammatory mediators by astrocytes.](image)

Proepileptogenic brain injuries trigger brain inflammation, involving the induction/overexpression of cytokines (e.g. IL-1β), danger signals (e.g. HMGB1) and TLRs (e.g. TLR4) in parenchymal/perivascular astrocytes. This event leads to changes in brain physiology such as neuronal hyperexcitability, BBB dysfunction and cell damage that can contribute to lower seizure threshold and trigger epileptogenesis.
familial or inflammatory state observed in focal MCD. Whether the dysregulation of astrocyte immune-inflammatory responses could contribute to progressive cognitive dysfunction in children deserves further investigation (Chew et al. 2006; Cohly and Panja 2005).

Finally, both gene expression and immunocytochemical studies suggest prominent activation of major proinflammatory pathways within the astroglial component of highly epileptogenic tumors (glioneuronal tumors, such as gangliogliomas) (Aronica et al. 2008; Samadani et al. 2007; Zurolo et al. 2011).

**ASTROCYTE AND BRAIN INFLAMMATION IN EXPERIMENTAL MODELS OF SEIZURES AND EPILEPSY**

Experimental studies provided the first evidence showing a significant contribution of reactive astrocytes to the inflammatory processes developing after seizures or induced by an epileptogenic brain injury. Acute seizures induced in rodents (following intracerebral application of kainate or bicuculline, or electrically induced status epilepticus) were shown rapidly to upregulate prototypical inflammatory cytokines in microglia and astrocytes in the brain areas where seizures originate and spread; as a consequence of this event, a downstream cascade of inflammatory mediators is transcriptionally upregulated in brain tissue similar to what has been shown in human epilepsy [(De Simoni et al. 2000; Gorter et al. 2006; Vezzani et al. 1999; Vezzani et al. 2000); reviewed by (Kulkarni and Dhir 2009; Vezzani et al. 2008a; Vezzani et al. 2011b)]. Activation of astrocytes in the absence of neuronal degeneration has been reported in a kindling model (Khurgel et al. 1995) and induction of GFAP has been observed even after a single electroconvulsive seizure (Steward et al. 1992). A detailed time-course analysis of these inflammatory processes occurring after status epilepticus in rats was instrumental in demonstrating that the mRNA of inflammatory mediators are induced within 30 minutes of seizure onset (De Simoni et al. 2000). The immunohistochemical analysis of IL-1β in status epilepticus models showed that the expression of this cytokine in microglia is time-locked to the occurrence of seizures and the extent of expression depends on the recurrence of seizures, while astrocytes appear to be involved in perpetuating inflammation even in the long-term after the initial injury (Ravizza et al., 2008a). Moreover, as in human epileptic tissue, astrocytes often express both the inflammatory mediator and the cognate cell signaling receptors, thus highlighting that these cells serve as sources and targets of inflammatory molecules (reviewed in (Maroso et al. 2011; Vezzani et al. 2011c). Experimental findings clearly show that both parenchymal and perivascular astrocytes are activated and express inflammatory molecules in epilepsy models with functional consequences on BBB function (Fig. 2) (Bauer et al. 2008; Friedman et al. 2009; Ravizza et al. 2008a; van Vliet et al. 2009; Vezzani et al. 2011c). Notably, specific anti-inflammatory molecules, such as the IL-1
receptor antagonist (IL-1ra) (De Simoni et al. 2000) or the C59 inhibitor of the complement system (Aronica et al. 2007), are induced to a limited extent by seizures or following brain injury, suggesting that the mechanisms involved in the resolution of brain inflammation are not very efficient, and possibly explaining why inflammation is detrimental for tissue excitability and cell survival.

Although induction of various inflammatory molecules has been demonstrated in astrocytes in seizure models [reviewed in (Friedman and Dingledine 2011; Vezzani et al. 2008a; Wetherington et al. 2008)], the IL-1/TLR is the first inflammatory signalling to be induced during innate immunity activation, either by a pathogen or a danger signal serving as endogenous ligand (Maroso et al. 2011; Vezzani et al. 2011c). IL-1/TLR signalling is rapidly induced by tissue injury or seizures in neurons, microglia and astrocytes resulting in transcriptional activation of inflammatory genes, and other genes potentially involved in synaptic and molecular changes underlying epileptogenesis [reviewed in (Vezzani et al. 2011c)]. Two endogenous ligands, IL-1β and HMGB1 (Fig. 2) are released by glial cells; IL-1β activates IL-1R1 and HMGB1 activates TLR4 in neurons with significant consequences for ictogenesis, mainly mediated by post-translational effects (see later).

**Functional consequences of astrocyte-mediated brain inflammation on neuronal excitability**

The activation of inflammatory pathways and the consequent release of inflammatory molecules by astrocytes alter neural network excitability via induction of various mechanisms, with either direct or indirect impact on neuronal functions. Here we focus on the IL-1R1/TLR4 signaling because of its prominent involvement in seizures and epileptogenesis [(Maroso et al. 2011; Vezzani et al. 2011c); Fig. 2].

IL-1β, by acting on IL-1R type 1, can inhibit the astrocytic reuptake of glutamate (Hu et al. 2000; Ye and Sontheimer 1996) and increases its glial release likely via induction of TNFa (Bezzi et al. 2001). These effects result in elevated extracellular glutamate levels, which in turn can promote tissue excitability. IL-1β can also increase neuronal glutamate release via the activation of inducible nitric oxide synthase in glial cells (Casamenti et al. 1999; Hewett et al. 1994). Astrocytic glutamate release may have a role in the genesis or strength of seizure-like events (Carmignoto and Fellin 2006; Tian et al. 2005).

In hippocampal neurons, IL-1R1 co-localizes with NMDA receptors, a subtype of glutamate receptor involved in the onset and spread of seizures. IL-1β potentiates NMDA receptor function in cultured hippocampal neurons (Lai et al. 2006; Viviani et al. 2003) by enhancing N-Methyl-D-aspartate (NMDA)-mediated Ca²⁺ influx via IL-1R1 dependent activation of Src kinases and subsequent NMDA receptor subunit 2 (NR2B) phosphorylation. This rapid
mechanism (within minutes) involves ceramide-mediated activation of Src kinases (Viviani et al. 2003), and contributes to seizure generation and recurrence (Balosso, 2008). IL-1β also down-regulates AMPA receptor expression and phosphorylation in hippocampal neurons in a Ca²⁺- and NMDA-dependent manner (Lai et al. 2006). Interactions of IL-1β with GABA-mediated inhibitory neurotransmission have also been reported. However, the results obtained are not consistent. Thus IL-1β can either decrease or increase GABA inhibition depending on the brain area (Alam, 2004; Wilkinson, 1993), the cytokine concentration (Wang, 2000; Zeise, 1997; Serantes, 2006) and the functional properties of the cells (Hori, 1988), highlighting a dual role of IL-1β in affecting GABAergic inhibitory system.

In the hippocampus, IL-1β affects synaptic transmission, and inhibits long-term potentiation (LTP) via activation of Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) (Bellinger et al. 1993; O’Donnell et al. 2000; Schneider et al. 1998). This cytokine can also modulate neurotransmitter release via inhibition of voltage-dependent Ca²⁺ channels, an effect that involves pertussis-sensitive G proteins and protein kinase C (PKC) (Plata-Salaman and ffrench-Mullen 1994).

TNF-a is a cytokine released from activated astrocytes and microglia, and tightly associated with IL-1β, since the two molecules reciprocally induce their respective release from glia and mutually activate their gene transcription. TNF-a has been shown to increase the mean frequency of AMPA-dependent miniature excitatory postsynaptic currents in hippocampal neurons and to decrease GABAₐ-mediated inhibitory synaptic strength. These effects are mediated by its ability to activate the recruitment of AMPA receptors lacking the GluR2 subunit at neuronal membranes (thus in a molecular conformation which favors Ca²⁺ influx into neurons) and to induce endocytosis of GABAₐ receptors (Beattie et al. 2002; Stellwagen et al. 2005).

TLR4 is the TLR subtype most extensively studied for its involvement in brain excitability. Cortical rat exposure to lipopolysaccharide (LPS), a prototypical activator of TLR4, induces rapid increases in neuronal excitability leading to seizures (Rodgers et al. 2009), which are prevented by IL-1ra, implicating a role of released IL-1β.

Regarding IL-1β, LTP and long-term depression (LTD) impairment are induced by TLR4 stimulation, compatible with cognitive deficits induced in rodents by brain inflammation (Galic et al. 2009; Harre et al. 2008; Spencer et al. 2005). These modifications in brain physiology are dependent on the release of TNF-a and IL-1β from activated glial cells (Riazi et al. 2010). Cognitive deficits are associated with specific changes in NMDA receptor subunit expression in the cortex and hippocampus, predicting modifications in CNS excitability (Galic et al. 2009; Harre et al. 2008; Spencer et al. 2005).
These fast post-translational effects of inflammatory cytokines are examples of novel pathways by which inflammatory molecules produced in diseased tissue by glia can modulate neurotransmission and contribute to hyperexcitability and associated neuropathology. In general, the amount and persistence of cytokines in brain tissue appears to be a crucial factor which determines their consequences on neuronal excitability. Another important aspect is the pattern of expression of cytokine receptors in the tissue exposed to inflammation. A typical example is represented by TNF-α, which may have inhibitory or permissive effects on seizures depending both on its brain levels and on the receptor subtypes predominantly activated (Balosso et al. 2005). Moreover, transgenic mice with low to moderate overexpression of TNF-α in astrocytes show decreased susceptibility to seizures (Balosso et al. 2005), whereas mice with high overexpression of TNF-α in astrocytes develop signs of neurologic dysfunction (Akassoglou et al. 1997; Probert et al. 1995).

**FUNCTIONAL CONSEQUENCES OF ASTROCYTE-MEDIATED BRAIN INFLAMMATION ON SEIZURES AND EPILEPTOGENESIS**

The role of cytokines released by astrocytes in seizures and epileptogenesis has been investigated at various levels, initially using genetically-modified mice with perturbed cytokine systems and subsequently by pharmacological intervention using receptor antagonists or cytokine synthesis inhibitors, or by injecting the cytokines themselves into rodent brain [reviewed in (Vezzani et al. 2008a; Vezzani et al. 2011b)].

IL-1β, TNF-α and HMGB1 are among the most studied cytokines for their permissive role in seizures. Mice with upregulation of IL-1ra in astrocytes or lacking IL-1R1 are intrinsically resistant to seizures (Vezzani et al. 2000) and the intracerebral injection of IL-1ra mediates powerful anticonvulsant effects (Vezzani et al. 2000; Vezzani et al. 2002). Because the only action of IL-1ra is to inhibit the effects of IL-1β, these data demonstrate that an endogenous increase in brain IL-1β contributes to seizures. Mice lacking caspase-1, the biosynthetic enzyme of IL-1β, and therefore unable to release the biologically active form of this cytokine, show decreased seizure susceptibility (Ravizza et al. 2006b). Pharmacological data support a proconvulsant role of IL-1β in several acute and chronic seizure models (Vezzani et al. 1999; Vezzani et al. 2000), as well as in the kindling model of epileptogenesis (Ravizza et al. 2008b). Moreover, recent data showed that inhibition of IL-1β biosynthesis in astrocytes reduces spike-and-wave discharges in rats with genetic absence epilepsy (GAERS) (Akin et al. 2011). Increase in astrocytic IL-1β in the hippocampus due to fever/hyperthermia is involved in decreasing the seizure threshold in the immature rodent (Dube et al. 2005; Heida and Pittman 2005).

Recently, we have shown that endogenous release of “danger signals” produced by stressed...
or injured neurons (i.e. HMGB1), promotes seizures by activation of neuronal TLR. Seizures, in turn, induce an additional wave of HMGB1 release from activated astrocytes and microglia, leading to a vicious positive feedback cycle of seizures and inflammation. This novel pathway may be a crucial mechanism for recurrent seizures [(Maroso et al. 2010); reviewed in (Maroso et al. 2011; Vezzani et al. 2011d); Fig. 2].

Cytokines and other inflammatory mediators have been shown to contribute to both excitotoxic and apoptotic neuronal death (Allan et al. 2005), highlighting the possibility that they contribute to seizure-mediated neuronal damage. The deleterious effects of cytokines on neuronal survival involve the production of neurotoxic compounds via autocrine or paracrine mechanisms (Allan et al. 2005; Vezzani and Baram 2007). Importantly, although cytokines can promote neurodegeneration (Fig. 2), their effects on the threshold, frequency and duration of seizures are not dependent on cell death (Vezzani et al. 2011c).

Notable examples exist of a dual role of cytokines on neuronal survival in diseased tissue (Allan et al. 2005; Bernardino et al. 2005); it has been shown the ability of cytokines to induce the synthesis of growth factors in astrocytes, to activate antioxidant pathways, manganese superoxide dismutase, or calbindin which counteracts the elevation of intracellular Ca$^{2+}$ induced by cell injuries (Allan et al. 2005), thus promoting cell repair mechanisms. In this respect, IL-1β and TNF-α can either reduce or exacerbate glutamate receptor–mediated excitotoxicity in organotypic slice cultures, depending on their extracellular concentrations, the length of time the tissue is exposed to these cytokines, and the receptor types activated by these cytokines (Bernardino et al. 2005).

Finally, the possible involvement of inflammatory mediators in epileptogenesis has been suggested by two main lines of evidence: the induction of an inflammatory state in the brain by administering proinflammatory molecules in rodents, or the use of mice that overexpress specific cytokines in astrocytes. This leads to decreased seizure threshold and induces long-term neurological deficits, particularly if applied to immature rodents, thus suggesting long-term effects of inflammation on brain functions [reviewed by (Ravizza et al. 2011; Riazi et al. 2010)]. In this context, brain inflammation has been implicated in the pathophysiology of several neuropsychiatric conditions (such as depression, memory impairments, and autism spectrum disorder) which are comorbidities of epilepsy, (Vezzani et al. 2011a).

Pharmacological interference with specific inflammatory pathways activated during epileptogenesis may reduce the severity and frequency of spontaneous seizures [reviewed in (Ravizza et al. 2011)]. Additionally, cytokines such as IL-1β and HMGB1 released in diseased tissue by parenchymal and, in particular, by perivascular astrocytes, can play a major role in BBB breakdown associated with brain inflammation in human and experimental epileptic tissue [reviewed
by (Friedman et al. 2009)]. The opening of the BBB rapidly activates the innate immune response (Cacheaux, 2002) and the accumulation of albumin in the brain because of BBB damage. Albumin triggers long-lasting hyperexcitability in surrounding tissue by impairing astrocyte capacity to buffer extracellular K⁺ and glutamate via activation of the TGF-β pathway [reviewed by (Friedman et al. 2009)]. Finally, it is increasingly recognized that pro-inflammatory molecules released by glia contribute to some of the acquired channelopathies described in epilepsy by inducing alterations in voltage- and receptor-gated ion channels via either post-translational or transcriptional mechanisms [reviewed by (Vezzani et al. 2011b; Viviani et al. 2004)].

**CONCLUDING REMARKS**

During the past decade, detailed molecular characterization of astrocytes, in particular reactive astrocytes, demonstrates that these cells are active players in the development and progression of the immune/inflammatory response that takes place in epileptic brain tissue. Both human and experimental data suggest the activation of specific proinflammatory pathways in astrocytes, which may also recruit neuronal cells and, in some cases, cells of the adaptive immune system. The identification of “harmful” pro-inflammatory pathways contributing to seizure onset and recurrence, as well as to comorbidities often associated with epilepsy, highlights the possibility of developing a therapeutic strategy targeting the astrocyte-mediated inflammatory signalings. However, we need to wait for the outcome of clinical studies before we can consider whether this approach is the right strategy. If this is so, it may not only improve control of seizures, but may also act as disease-modifying therapy in patients with epilepsy resistant to conventional antiepileptic drugs.

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AIM AND OUTLINE OF THE THESIS

In the last two decades increasing research has been focused on the role of astrocytes in brain physiology and pathology: astrocytes were shown to be actively involved in neuronal communication and homeostasis. The contribution of inflammation in epilepsy and epileptogenesis has pointed to astrocytes as key components of this condition characterized by hyperexcitability of the neural tissue. In fact astrocytes represent an important source of immunologically relevant cytokines and chemokines which have been demonstrated to increase neuronal excitability in different experimental models. As almost 30% of epileptic patients are pharmaco-resistant, understanding the role of astrocytes in the pathology could provide new targets for the development of alternative treatments for these forms of epilepsy. The general aim of this thesis was to investigate the contribution of astrocytes to epilepsy in order to gain more insight into the pathways involved in the etiopathogenesis of this disease. The specific objectives included: a) investigation of inflammatory pathways and epilepsy; b) study of mechanisms of regulation of the inflammation; and c) evaluation of the involvement of astrocytes in neuronal signaling. We used a variety of approaches, including the study of human material, animal models and in vitro systems aiming to contribute to the elucidation of mechanisms underlying untreatable epilepsy, in an attempt to find new molecular targets for therapy.

After a general introduction on the physiopathology of astrocyte in relation to epilepsy (chapter 1.1), in chapter 1.2 we reviewed current evidence regarding the role of astrocytes in the regulation of the innate immune responses in epilepsy. In chapter 2.1 we evaluated the expression and the cellular distribution of tPA and uPA in several epileptogenic pathologies. In chapter 2.2 we studied the activation of inflammatory pathways in FCD type I and II focusing on the inflammatory cell components and the induction of major proinflammatory pathways and molecules. In chapter 2.3 we investigated the expression and cellular distribution of toll-like receptors (TLRs) 2 and 4, and of the receptor for advanced glycation end products (RAGE), and their endogenous ligand high-mobility group box 1 (HMGB1), in epilepsy associated with focal malformations of cortical development. The acute effect of two proinflammatory molecules (IL-1β and HMGB1) on neuronal excitability in relation with activation of astrocytes was studied in chapter 2.4. In chapter 2.5 we examined the expression pattern of an astrocytic potassium inward rectifier channels (Kir) 4.1 in relation to epilepsy and inflammation. Further we investigated the expression and cellular distribution of miRNA-146a (miR-146a) in a rat model of TLE as well as in human TLE (chapter 3.1), in epilepsy-associated glioneuronal lesions and in cultured astrocytes (chapter 3.2). In chapter 4.1 we evaluated the expression pattern and distribution of cannabinoid receptors in nor-
mal human development as well as in patients with malformations of cortical development (MCD). Further, we focused on the expression pattern and distribution of ADK, primarily present in astrocytes, in a rat model of TLE and in human TLE (chapter 4.2) as well as in human astrocytic tumors related to epilepsy (chapter 4.3). Finally in chapter 5 we discussed the significance of our results and contextualized our findings in relation to the state of the art.
Inflammatory pathways and epilepsy
2.1 TISSUE PLASMINOGEN ACTIVATOR (tPA) AND UROKINASE PLASMINOGEN ACTIVATOR (uPA) IN HUMAN EPILEPTOGENIC PATHOLOGIES.

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Key words:
hippocampal sclerosis; focal cortical dysplasia; tuberous sclerosis; ganglioglioma; epilepsy
A growing body of evidence demonstrates the involvement of plasminogen activators (PAs) in a number of physiologic and pathologic events in the central nervous system. Induction of both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) has been observed in different experimental models of epilepsy and tPA has been implicated in the mechanisms underlying seizure activity. We investigated the expression and the cellular distribution of tPA and uPA in several epileptogenic pathologies, including hippocampal sclerosis (HS), and developmental glioneuronal lesions, such as focal cortical dysplasia (FCD), cortical tubers in patients with the tuberous sclerosis complex (TSC) and in gangliogliomas (GG), using immuno-cytochemical, western blot and real-time quantitative PCR analysis. TPA and uPA immunostaining showed increased expression within the epileptogenic lesions compared to control specimens in both glial and neuronal cells (hippocampal neurons in HS and dysplastic neurons in FCD, TSC and GG specimens). Confocal laser scanning microscopy confirmed expression of both proteins in astrocytes and microglia, as well as in microvascular endothelium. Immunoblot demonstrated also up-regulation of the uPA receptor (uPAR). Increased expression of tPA, uPA, uPAR and tissue PA inhibitor type mRNA levels was also detected by PCR analysis in different epileptogenic pathologies. Our data support the role of PA system components in different human focal epileptogenic pathologies, which may critically influence neuronal activity, inflammatory response, as well as contributing to the complex remodeling of the neuronal networks occurring in epileptogenic lesions.

The plasminogen (fibrinolytic) system comprises the inactive proenzyme, plasminogen, that can be converted to the active enzyme plasmin by two serine proteases, the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA) (Collen, 1999). TPA and uPA elicit various cellular responses, involving the activation of distinct signaling pathways. While several of these pathways have been described (Maupas-Schwalm et al., 2004; Benarroch, 2007), their interactions and the link to specific biological responses are only partly understood. Attention has been recently focused on the role of uPA receptor (uPAR) indicating that it may act as signaling receptor, also independently of uPA-mediated proteolysis (for review see Smith and Marshall, 2010). In the central nervous system (CNS), increasing evidence suggests a crucial role of the plasminogen system in a broad range of physiological and pathological processes ranging from neuronal development, cell migration and invasion, cell growth and apoptosis, immune responses, inflammation, angiogenesis and regulation of synaptic remodeling and neuronal plasticity (Seeds et al., 1999, Tsirka, 2002, Powell et al., 2003, Alfano et al., 2005, Benarroch, 2007).
TPA is widely expressed by many types of neurons in the human CNS, including the neocortical and hippocampal pyramidal neurons (Teesalu et al., 2004). Activation of the plasminogen system, involving neurons, reactive glial cells and vascular endothelium, as source of plasminogen activators, has been reported in different neurological disorders such as stroke and other forms of acute brain injury, as well as in patients with inflammatory disorders (Gveric et al., 2001, Teesalu et al., 2002, Benarroch, 2007). A complex deregulation of the plasminogen system may also been involved in neurodegenerative disorders, such as Alzheimer’s disease (Tucker et al., 2002, Fabbro and Seeds, 2009). Several experimental findings identified a role for tPA in the mechanisms underlying seizure activity (Tsirka et al., 1995, Pawlak and Strickland, 2002, Benarroch, 2007). Interestingly, induction of plasminogen activators (PAs) has been observed in different experimental models of epilepsy (Lukasiuk et al., 2003, Gorter et al., 2006, Lahtinen et al., 2006) and gene expression profile analysis of gangliogliomas (GG) revealed that both tPA and uPA represent one of the most upregulated genes in these epileptogenic lesions (Aronica et al., 2008). In rat hippocampus, tPA and uPA were both activated at one day after induction of status epilepticus and were still elevated during epileptogenesis (Gorter et al., 2006, Lahtinen et al., 2006, Gorter et al., 2007). Expression of tPA mRNA was still increased in the chronic phase in the CA3 region (Gorter et al., 2007). Recently, the cellular distribution of uPA and uPA receptor (uPAR) has been characterized in rat hippocampus during epileptogenesis (Lahtinen et al., 2006). However, whether the up-regulation of plasminogen activator proteins persists in the chronic phase in epileptic human brain is unclear. Moreover, a detailed analysis of tPA and uPA cellular expression in human epileptogenic pathologies is still lacking. Because of the functional redundancy among PAs (Carmeliet and Collen, 1995), characterization of the expression in human tissue is important for the correct interpretation of the experimental observations.

In the present study we examined the tPA and uPA tissue distribution evaluating their degree of expression and defining their cellular origin in common causes of human focal chronic refractory epilepsy.

**Materials and Methods**

**Subjects**
The human cases included in this study were obtained from the files of the departments of neuropathology of the Academic Medical Center (AMC, University of Amsterdam), the VU University Medical Center (VUMC) in Amsterdam, the University Medical Center in Utrecht (UMC) and the Neuromed Neurosurgery Center for Epilepsy, Pozzilli-Isernia, Italy. We examined a total of 35 surgical specimens, 6 hippocampal sclerosis (HS), 5 hippocampal surgical...
specimens of patients without HS (non-HS; with a focal lesion not involving the hippocampus proper), 6 focal cortical dysplasia (FCD) type IIb, 6 cortical tubers from patients with Tuberous Sclerosis Complex (TSC), 6 ganglioglioma (GG). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. The clinical characteristics derived from the patient’s medical records are summarized in Table 1. Patients underwent therapeutic surgical resection for refractory epilepsy and had predominantly medically intractable complex partial seizures.

The HS specimens include 4 cases of classical HS (grade 3, (Wyler et al., 1992), MTS type 1a (Blumcke et al., 2007)) and 2 cases of severe HS (grade IV; (Wyler et al., 1992) MTS type 1b (Blumcke et al., 2007)). For the FCD we followed the classification system proposed by Palmini et al. for grading the degree of FCD (Palmini et al., 2004). All patients with cortical tubers fulfilled the diagnostic criteria for TSC (Gomez et al., 1999). For the GG we used the revised WHO classification of tumors of the central nervous system (Louis et al., 2007). The patients undergoing epilepsy surgery predominantly had medically intractable complex partial seizures. In 6 patients (2 FCD, 2 GG and 2 TSC) a significant amount of perilesional tissue (normal-appearing cortex/white matter adjacent to the lesion) was resected as well. In addition, normal-appearing control cortex/white matter was obtained at autopsy from 6 young adult control patients (male/female: 3/3; mean age 31; range 14-35), without a history of seizures or other neurological diseases.

Table 1. Summary of clinical details of cases studied according to pathology

<table>
<thead>
<tr>
<th>Pathology type (pm or s)</th>
<th>Number of cases</th>
<th>Mean age at surgery (range/years)</th>
<th>Localization</th>
<th>Mean duration of epilepsy (range/years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS (pm)</td>
<td>6</td>
<td>26.3 (12-42)</td>
<td>Temporal</td>
<td>17 (11-32)</td>
</tr>
<tr>
<td>Non-HS (s)</td>
<td>5</td>
<td>29.5 (18-41)</td>
<td>Temporal</td>
<td>15.2 (6-22)</td>
</tr>
<tr>
<td>FCD IIb (s)</td>
<td>6</td>
<td>27.3 (14-48)</td>
<td>Temporal(4)</td>
<td>19.2 (5-25)</td>
</tr>
<tr>
<td>Cortical Tubers (TSC; s)</td>
<td>6</td>
<td>17.8 (5-35)</td>
<td>Frontal (3)</td>
<td>13.5 (2.8 – 34)</td>
</tr>
<tr>
<td>Ganglioglioma (GG; s)</td>
<td>6</td>
<td>32 (16-49)</td>
<td>Temporal(2)</td>
<td>16.1 (12-26)</td>
</tr>
<tr>
<td>Control neocortex (pm)</td>
<td>6</td>
<td>31.6 (18-35)</td>
<td>Temporal</td>
<td>–</td>
</tr>
</tbody>
</table>

HS = Hippocampal Sclerosis; FCD = Focal Cortical Dysplasia; TSC=Tuberous Sclerosis; pm: post-mortem; s: surgical specimens.
Human tissue preparation and immunocytochemistry

Formalin fixed, paraffin-embedded tissue was sectioned at 6 µm and mounted on organosilane-coated slides (SIGMA, St. Louis, MO). Specimens were processed for haematoxylin/eosin and Nissl, as well as for immunocytochemical reactions.

Glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9, DAKO; 1:400), neuronal nuclear protein (NeuN; mouse clone MAB377, IgG1; Chemicon, Temecula, CA, USA; 1:1000), neurofilament (NF, SMI311; Sternberger Monoclonals, Lutherville, MD; 1:1000), microtubule-associated protein (MAP2; mouse clone HM2; Sigma 1:100), (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400), CD68 (mouse clone PG-M1, DAKO; 1:200) and CD31 (mouse JC/70A; 1:100), were used in the routine immunocytochemical analysis of epilepsy specimens.

For the detection of tPA we use rabbit anti-human tPA (Santa Cruz Bio., CA, USA; 1:50) and a rabbit anti-human tPA [kindly provided by Dr. Lijnen, Center for Molecular and Vascular Biology, Leuven, Belgium; 1:100; (Lijnen et al., 1988)]. For the detection of uPA we used a mouse anti-human uPA (American Diagnostica Inc., Greenwich, CT, USA; 1:50) and a rabbit anti-human uPA (provided by Dr. Lijnen H.R; 1:500; (Lijnen et al., 1988)). Specificity of the staining was further confirmed by omission of the primary antibody or by its replacement with equivalent amounts of isotype-matched non-immune IgG or serum. Immunocytochemistry was carried out on paraffin-embedded tissue as previously described (Aronica et al., 2001b).

Evaluation of immunostaining

All labeled tissue sections were evaluated with respect to the presence or absence of various histopathological parameters and specific immunoreactivity (IR) for the different markers. The intensity of tPA and uPA staining was evaluated using a scale of 0-3 (0: no; 1: weak; 2: moderate; 3: strong staining). All areas of the specimen were examined and the score represents the predominant cell staining intensity found in each case for the different cell types (neurons, astrocytes, microglial cells and balloon cells). The frequency of tPA and uPA positive cells [(1) rare; (2) sparse; (3), high] was also evaluated to give information about
the relative number of positive cells within the specimen. As proposed before (Vandeputte et al., 2002, Ravizza et al., 2006), the product of these two values (intensity and frequency scores) was taken to give the overall score (total score) shown in Fig. 5 and Fig. 6. For statistical analysis of the data, SPSS for Windows was used. Data were analyzed with the nonparametric Kruskal-Wallis test, followed by a Mann-Whitney test to assess the difference between groups. Correlation between immunostainings (total score) and different clinical variables (duration of epilepsy, seizure frequency, age at surgery, age at seizure onset, epilepsy outcome) were assessed using the Spearman’s rank correlation test. The value of p < 0.05 was defined statistically significant.

**Western blot analysis**

For immunoblot analysis, freshly frozen histologically normal hippocampus (n=5), and HS (n=6) specimens, samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, Na-orthevanadate (10.4 mg/ml), 5 mM EDTA (pH 8.0), 5 mM NaF and protease inhibitor cocktail (Boehringer Mannheim, Germany). Protein content was determined using the bicinchoninic acid method (Smith et al., 1985). For electrophoresis, equal amounts of proteins (30 μg/lane) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis. Separated proteins were transferred to nitrocellulose paper for 1 h and 30 min, using a semi-dry electroblotting system (BioRad, Transblot SD, Hercules, CA, USA). Blots were incubated over night in TTBS (20 mM Tris, 150 mM NaCl, 0.1 % Tween, pH 7.5) /5% non fat dry milk, containing the primary antibody (tPA, rabbit anti-human, 1:1000, provided by Dr. Lijnen H.R; uPA mouse anti-human, 1:500, American Diagnostica Inc; uPA receptor (uPAR), 1:5000, American Diagnostica Inc., using for uPAR non-reducing conditions). After several washes in TTBS, the membranes were incubated in TTBS / 5% non fat dry milk / 1% BSA, containing the goat anti-rabbit coupled to horse radish peroxidase (1:2500; Dako, Denmark) for 1 h. After washes in TTBS, immunoreactivity was visualized using Lumi–light PLUS western blotting substrate (Roche Diagnostics, Mannheim, Germany) and digitized using a Luminescent Image Analyzer (LAS-3000, Fuji Film, Japan). Expression of β-actin (monoclonal mouse, Sigma, St. Louis, MO, 1:50.000) was used as reference protein. For statistical analysis of data, SPSS for Windows was used and data were compared using a student’s t-test; p < 0.05 was taken as level of significance.

**RNA isolation and real-time quantitative PCR analysis (RT-PCR)**

For RNA isolation, frozen material was homogenized in Trizol LS Reagent (Invitrogen, Carlsbad, CA). After addition of 200 μg glycogen and 200 μl chloroform, the aqueous phase was
isolated using Phase Lock tubes (Eppendorf, Hamburg, Germany). The concentration and purity of RNA were determined spectrophotometrically at 260/280 nm using a nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA). Five micrograms of total RNA were reverse-transcribed into cDNA using oligo dT primers as described previously [3] using the Universal ProbeLibrary of Roche (https://www.roche-applied-science.com) on de basis of the reported cDNA sequences.

The following primers were used: TATA-binding protein (TBP; forward: caggagccaagagtgaagaac; reverse: aggaataactctgctcatactact), TPA (forward: ccggtggaatattgctggt; reverse: cccgttgaaacaccttgg); uPAR (forward: acaccaccaatgcaacga; reverse: ccccttgacgtgtaacac) and the plasminogen activator inhibitor type-1 (PAI1; forward: tccagcagtgaattcct; reverse: gcttgagacatctgcatctct). For the PCR, a master mixture was prepared on ice, containing per sample: 1 µl of cDNA, 1 µl of FastStart Reaction Mix SYBR Green (RocheApplied Science, Indianapolis, IN, USA), 0.5 µl of 10 µM primers and 1.6 µl of 25 mM MgCl₂. The final volume was adjusted with H₂O to 10 µl. Samples were run in duplicate. After the reaction mixture was loaded into a glass capillary tube, the cycling conditions were carried out as follows: initial denaturation at 95°C for 6 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 6 s and extension at 72°C for 8-12 s. The temperature transition rate was set at 20°C/s. Fluorescent product was measured by a single acquisition mode at 72°C after each cycle. Separate calibration (standard) curves for the different primers were constructed using serial dilutions of cDNA from rat/human hippocampus. The standard curve samples were included in each run. Standards were defined to contain an arbitrary starting concentration, since no primary calibrators exist. Hence, all calculated concentrations are relative to the concentration of the standard. For distinguishing specific from nonspecific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 15 s followed by a gradual increase in temperature to 95°C at a rate of 0.1°C/s, with the signal acquisition mode set continuous. Fluorescent product was measured by a single acquisition mode at 72°C after each cycle. For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 15 s followed by a gradual increase in temperature to 95°C at a rate of 2.5°C s⁻¹, with the signal acquisition mode set continuous. Quantification of data was performed described previously (Aronica et al., 2008), using the computer program LinRegPCR in which linear regression on the Log (fluorescence) per cycle number data is applied to determine the amplification efficiency per sample (Ramakers et al., 2003). The starting concentration of each specific product was divided by the amount of the reference gene TBP.
Statistical analysis
Statistical analyses were performed with SPSS for Windows (SPSS 11.5, SPSS Inc., Chicago, IL, USA) using two-tailed Student’s t-test; to assess differences between more than two groups ANOVA and a non-parametric Kruskal–Wallis test followed by Mann–Whitney U-test. P<0.05 was considered significant.

RESULTS
TPA, uPA and uPAR protein expression
Western Blot analysis was performed to quantify the total amount of tPA, uPA and uPAR in total homogenates of HS specimens, compared to non-epileptic hippocampal tissue. The level of the tPA, uPA and uPAR protein expression was also analyzed in GG and FCD specimens and compared to non-epileptic control cortex. The uPAR antibody used for Western Blot analysis did not work on paraffin-embedded tissue, therefore immunocytochemistry analysis of uPAR could not be performed.

Western blot analysis performed with tPA and uPA demonstrated a prominent band with a molecular weight of approximately 68 and 55 kDa, respectively in all tissue homogenate samples (Fig. 1A, B, D, E). Increased expression of both tPA and uPA was observed in HS, GG and FCD compared to control samples (Fig. 1A, B, D, E). Increased expression in the same pathologies compared to controls was also detected for uPAR (55 kDa, Fig. 1C, F). TPA, uPA and uPAR antibodies labeled additional bands of higher molecular weight (tPA and uPAR, >200 kDa; uPA, approximately at 140 kDa) which corresponds to SDS-resistant complexes (Fig. 1A–C, panel c).

TPA IR in control hippocampus and in hippocampal sclerosis
To evaluate the changes in expression and the cellular localization of tPA in the hippocampus of patients with temporal lobe epilepsy (TLE), immunocytochemical analysis was performed in surgical specimens of patients with HS and in surgical and autopsy specimens of histologically normal hippocampus. Two different anti-human tPA antibodies were used (see material and methods), with similar results; Fig. 2 shows the immunostaining performed with anti-uPA polyclonal antibody (Lijnen et al., 1988).

In agreement with previous observations in human tissue (Teesalu et al., 2004) in histologically normal hippocampus (autopsy and non-sclerotic hippocampus, non-HS) tPA IR was mainly observed in neuronal cells, including pyramidal neurons of the different CA subfields, granule cells of the dentate gyrus (DG) and hilar neurons (Fig. 2 A, D, J). As previously reported, neurons displayed a punctate cytoplasmic pattern of staining (Teesalu et al., 2004). Resting glial cells did not show detectable tPA IR and low expression was observed in...
endothelial cells of control hippocampal blood vessels (Fig. 2 and Fig. 6 A and B).

In specimens from patients with HS increased expression of tPA was observed within the different hippocampal sub-fields (Fig. 2 B-C, E-F, H-I, K-L). Strong tPA IR was detected in pyramidal neurons of CA1 and CA3 (Fig. 2 B-C, E-F), as well as in and in the granule cells of the dentate gyrus (Fig. 2 H-I). In regions of prominent gliosis, reactive glial cells showed strong tPA IR (Fig. 2 B-C, E-F, H-I, K-L; Fig. 6 A). In addition, increased expression of tPA IR was detected in the large majority of blood vessels within the hippocampus (Fig. 2 B-C, E-F, H-I, K-L and Fig. 6 B).

Figure 1. Western blot analysis of tPA, uPA and uPAR (A–C): Representative immunoblots (a) and densitometric analysis (b) of total homogenates from control autopsy hippocampus (Ctr), and hippocampal sclerosis (HS) specimens. (D–F): Representative immunoblots (a) and densitometric analysis (b) of total homogenates from control autopsy cortex (Ctr), ganglioglioma (GG) and focal cortical dysplasia (FCD). Additional higher molecular weight bands detected with tPA, uPA and uPAR antibodies are shown in total homogenates of HS (tPA and uPAR, >200 kDa; uPA, 140 kDa; A–C, panel c). Densitometric analysis: values (optical density units, O.D.) are mean±SEM five controls (autopsy cortex and hippocampus) and six HS, six GG, five FCD relative to the optical density of β-actin; * P<0.05.
Figure 2. Distribution of tPA immunoreactivity in the hippocampus of control and TLE patients with hippocampal sclerosis. Sections are counterstained with Hematoxylin. (A, D, G, J): Control autopsy hippocampus, showing neuronal immunoreactivity (IR) in the pyramidal neurons of CA3 (A), CA1 (D), granule cell layer and hilus of the dentate gyrus (DG, (G); hilus, (J)). (B, C), (E, F), (H, I), (K, L): Hippocampal sclerosis (HS), showing increased expression within the different hippocampal sub-regions (CA3, (B, C); CA1, (E, F); DG, (H, I), hilus, (K, L)). Expression is observed in both pyramidal neurons and granule cells of the DG, as well as in reactive glial cells (arrow-heads in C, F, I, L) and in blood vessels (arrows in C, F, K and L). Insert a in (H) shows immunoreactivity in the DG obtained with mouse anti-human tPA; insert b in (H) shows absence of immunoreactivity after preadsorption with tPA. Insert in (B) shows co-localization (yellow) of tPA (red) with NeuN (green) in a pyramidal neuron. Insert (a) in (K) shows co-localization (yellow) of tPA (red) with GFAP (green) in reactive astrocytes; Insert (b) in (K) shows co-localization (yellow) of tPA (red) with HLA-DR (green) in cells of the microglia/macrophage lineage; Insert (c) in (K) shows co-localization (yellow) of tPA (red) with CD31 (green) in microvascular endothelium. Scale bar in (B). (A, B), (D, E), (G, H) and (J, K): 200 μm. (C, F, I, L): 60 μm. Granule cells layer (gcl); dentate gyrus (DG). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
Double labelling experiments (performed with glial, neuronal and endothelial markers) confirmed the tPA expression in neurons (insert in Fig. 2 B), GFAP-positive reactive astrocytes, HLA-DR-positive cells of the microglia/macrophage lineage and in endothelial cells (inserts in Fig. 2 K).

The percentage of glial cells positive for tPA and co-expressing GFAP was quantified in CA1, CA3 and hilar region in five HS specimens (84±5, CA1; 86±6, CA3; 97±3, hilus); the percentage of neuronal cells positive for tPA and co-expressing NeuN was quantified in CA1 and CA3 (98±3, CA1; 97±4, CA3).

**TPA IR in control cortex and focal developmental lesions**

To evaluate the occurrence of changes in expression and cellular localization of tPA in focal malformations of cortical development (MCD), we studied tPA expression patterns in surgical specimens of patients with FCD (type IIB), cortical tubers (TSC), GG and in surgical and autopsy specimens of histologically normal cortex. Fig. 3 shows the immunostaining
performed with anti-uPA polyclonal antibody (Lijnen et al., 1988).

Low tPA staining was observed within the normal cortex (Fig. 3A-B). The staining was localized in pyramidal neurons; resting glial cells did not show tPA IR and low expression was observed in blood vessels (and Fig. 7A-B). Histologically normal perilesional cortex displayed a pattern of IR similar to that observed in control autopsy cortex.

In FCD and TSC cases (Fig. 3 C-E, FCD; Fig. 3 F, TSC), intense tPA IR was observed throughout the dysplastic cortex. Staining was detected in dysplastic neurons, balloon and giant cells, reactive glial cells and in blood vessels (Fig. 3 C-F and Fig. 7 A-B). Both the neuronal and glial components, as well as the blood vessels of GG displayed tPA IR (Fig. 3 G and Fig. 7 A-B). Double labelling experiments (performed with glial, neuronal and endothelial markers in FCD, TSC and GG) confirmed the tPA expression in neurons, GFAP-positive cells, in endothelial cells (inserts in Fig. 3), as well as in HLA-DR-positive cells of the microglia/macrophage lineage (not shown). The percentage of glial cells positive for tPA and co-expressing GFAP (89±5) and neuronal cells co-expressing NeuN (96±4) was quantified in five FCD specimens.

UPA IR in control hippocampus and in hippocampal sclerosis

To evaluate the changes in expression and the cellular localization of uPA in the hippocampus of patients with temporal lobe epilepsy (TLE), immunocytochemical analysis was performed in surgical specimens of patients with HS and in surgical and autopsy specimens of histologically normal hippocampus. Two different anti-human tPA antibodies were used (see material and methods), with similar results; Fig. 4 shows the immunostaining performed with anti-uPA polyclonal antibody (Lijnen et al., 1988).

In agreement with previous observations in control rat hippocampus (Lahtinen et al., 2006), low uPA IR was detected in histologically normal human hippocampus (autopsy and non-sclerotic hippocampus, non-HS). The intensity of staining was light in CA1-3 pyramidal cells, as well as in granule cells of the DG and hilar neurons (Fig. 4A, D, G, J). Resting glial cells did not show detectable uPA IR and low expression was observed occasionally in endothelial cells (Fig. 4 and Fig. 6 C and D).

In specimens from patients with HS increased expression of uPA was observed within the different hippocampal sub-fields (Fig. 4 B-C, E-F, H-I, K-L). Strong uPA IR was detected in pyramidal neurons of CA1 and CA3 (Fig. 4 B-C, E-F), as well as in the granule cells of the dentate gyrus (Fig. 4 H-I). In regions of prominent gliosis, reactive glial cells showed strong uPA IR (Fig. 4 L; Fig. 6 C). In addition, increased expression of uPA IR was detected in the large majority of blood vessels within the hippocampus (Fig. 4 K and Fig. 6 D).

Double labelling experiments (performed with glial, neuronal and endothelial markers) confirmed the uPA expression in neurons, GFAP-positive reactive astrocytes, HLA-DR-positive
Figure 4. Distribution of tPA immunoreactivity in control-cortex and malformations of cortical development. Sections are counterstained with Hematoxylin. Panels (A, B): tPA immunoreactivity (IR) in control autopsy cortex (A) and white matter (B) showing low expression in pyramidal neurons (A, insert in A) and in blood vessels (arrow in B), but no detectable glial labeling. Panels (C–E): tPA IR in focal cortical dysplasia (FCD) showing strong IR within the dysplastic cortex. High magnification photographs (D and E) show expression in dysplastic neurons (arrow-head in D) and in blood vessels (arrows in D), as well as in balloon cells (arrows in E). Insert in (D): co-localization (yellow) of tPA (red) with CD31 (green) in microvascular endothelium. Panel (F): tPA IR in cortical tuber of Tuberous Sclerosis Complex (TSC) showing strong expression within the dysplastic cortex. Insert (a) in (F): expression in a giant cell; insert (b) in (F): expression in dysplastic neurons (arrow-head) and in blood vessels (arrow). Insert (c) in (F): co-localization (yellow) of tPA (red) with NeuN (green) in a dysplastic neuron. Insert (d) in (F): co-localization (yellow) of tPA (red) with GFAP (green) in a reactive astrocyte. Panel (G): tPA IR in ganglioglioma (GG) showing expression in dysplastic neurons (insert a), blood vessels (arrows and insert b). Insert (c): co-localization (yellow) of tPA (red) with GFAP (green) in tumor astrocytes. Scale bar in B. (A, C, F): 400 μm. (B, E): 80 μm. (D): 30 μm; (G): 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
Cells of the microglia/macrophage lineage and in endothelial cells (inserts in Fig. 4 K). The percentage of glial cells positive for uPA and co-expressing GFAP was quantified in CA1, CA3 and hilar region in five HS specimens (74±2, CA1; 73±5, CA3; 79±4, hilus); the percentage of neuronal cells positive for uPA and co-expressing NeuN was quantified in CA1 and CA3 (97±3, CA1; 98±2, CA3).

uPA IR in control cortex and focal developmental lesions
To evaluate the occurrence of changes in expression and cellular localization of uPA in focal malformations of cortical development, we studied uPA expression patterns in surgical specimens of patients with FCD (type II B), cortical tubers (TSC), GG and in surgical and autopsy specimens of histologically normal cortex. Fig. 5 shows the immunostaining performed with anti-uPA polyclonal antibody (Lijnen et al., 1988).

Fig. 5. Evaluation of tPA and uPA immunoreactivity (IR) in FCD, TSC and GG. Distribution of tPA and uPA IR scores (total score; see for details Methods section) in different cell types of normal control autopsy cortex (Ctx), focal cortical dysplasia (FCD), cortical tubers in patients with Tubero Sclerosis Complex (TSC) and in gangliogliomas (GG). (A, B): tPA; (C, D): uPA; (A) and (C): astrocytes and microglia. (B) and (D): neurons, blood vessels and balloon/giant cells (BCs/GCs).
Figure 6. Distribution of uPA immunoreactivity in the hippocampus of control and TLE patients with hippocampal sclerosis. Sections are counterstained with Hematoxylin. (A, D, G, J): Control autopsy hippocampus, showing weak neuronal immunoreactivity (IR) in the pyramidal neurons of CA3 (A), CA1 (D), granule cell layer and hilus of the dentate gyrus (DG, (G); hilus, (J)). Insert in (D): high magnification showing weak neuronal and endothelial IR in CA1. (B, C), (E, F), (H, I), (K, L): Hippocampal sclerosis (HS), showing increased expression within the different hippocampal sub-regions (CA3, (B, C); CA1, (E, F); DG, (H, I); hilus, (K, L)). Arrows in (K) show IR in blood vessels; high magnification in (L) shows expression in perivascular glial cells (arrows). Insert (a) in (H) shows immunoreactivity in the DG obtained with mouse anti-human uPA; insert (b) in (H) shows absence of immunoreactivity after preadsorption with uPA. Inserts in (B) and (E): co-localization (yellow) of uPA (red) with NeuN (green) in a pyramidal neurons. Insert (a) in (K) shows co-localization (yellow) of uPA (red) with GFAP (green) in a reactive astrocyte; Insert (b) in (K) shows co-localization (yellow) of uPA (red) with HLA-DR (green) in cells of the microglia/macrophage lineage; Insert (c) in (K) shows co-localization (yellow) of uPA (red) with CD31 (green) in microvascular endothelium. Scale bar in B. (A, B), (D, E), (G, H) and (J, K): 200 μm. (C, F, I): 120 μm. (L): 60 μm. Granule cell layer (gcl); dentate gyrus (DG). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
Figure 7. Distribution of uPA immunoreactivity in control-cortex and malformations of cortical development. Sections are counterstained with Hematoxylin. Panels (A, B): uPA immunoreactivity (IR) in control autopsy cortex (A) and white matter (B) showing low expression in pyramidal neurons (A, insert in A) and no detectable expression in blood vessels (arrow in B) or in glial cells. Panels (C–E): uPA IR in focal cortical dysplasia (FCD) showing strong IR within the dysplastic cortex. High magnification photographs (D and E) show expression in dysplastic neurons (D) and in balloon cells (E). Insert (a) in (C): co-localization (yellow) of uPA (red) with NeuN (green) in a dysplastic neuron. Insert (b) in (C): co-localization (yellow) of uPA (red) with GFAP (green) in reactive astrocytes. Insert (c) in (C): co-localization (yellow) of uPA (red) with HLA-DR (green) in cells of the microglia/macrophage lineage. Panel (F): uPA IR in cortical tuber of Tuberous Sclerosis Complex (TSC) showing strong expression within the dysplastic cortex in dysplastic neurons (arrow-head) and in blood vessels (arrows). Insert (a) shows expression in giant cells. Insert (b) in (F): co-localization (yellow) of uPA (red) with CD31 (green) in microvascular endothelium. Panel (G): uPA IR in ganglioglioma (GG) showing expression in dysplastic neurons (arrow-heads), blood vessels (arrows) and in tumor astrocytes (insert). Scale bar in (B). (A, C): 400 μm. (B): 80 μm. (D, E): 30 μm; (F, G): 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
Very low uPA staining was observed within the normal cortex (Fig. 5A-B). Occasionally scattered lightly labeled neurons were detected, but resting glial cells and blood vessels did not display detectable uPA IR (Fig. 5A-B and Fig. 7 C-D). Histologically normal perilesional cortex displayed a pattern of IR similar to that observed in control autopsy cortex.

In FCD and TSC cases (Fig. 5 C-E, FCD; Fig. 5 F, TSC), intense uPA IR was observed throughout the dysplastic cortex. Staining was detected in dysplastic neurons, balloon and giant cells, reactive glial cells and in blood vessels (Fig. 5 C-F and Fig. 7 C-D). Both the neuronal and glial components, as well as the blood vessels of GG displayed uPA IR (Fig. 5 G and Fig. 7 C-D). Double labelling experiments (performed with glial, neuronal and endothelial markers in FCD, TSC and GG) confirmed the uPA expression in neurons and GFAP-positive cells, in endothelial cells, as well as in HLA-DR-positive cells of the microglia/macrophage lineage and (inserts in Fig. 5). The percentage of glial cells positive for uPA and co-expressing GFAP (77±6) and neuronal cells co-expressing NeuN (82±4) was quantified in five FCD specimens.

Quantitative analysis of tPA, uPA, uPAR and PAI 1 mRNA expression

Real-time quantitative PCR analysis was used to evaluate the level of tPA, uPA, uPAR and PAI1 in specimens of patients with HS, GG and FCD. The expression of tPA mRNA was significantly up-regulated in GG (Fig. 8 A), whereas uPA, uPAR and PAI1 were up-regulated in all the three pathologies analysed (HS, GG and FCD; Fig. 8 B-D).

Figure 8. RT-PCR of tPA, uPA uPAR and plasminogen activator inhibitor type 1 (PAI1) in epileptogenic pathologies. Expression levels were determined in duplicate, corrected for the expression levels of TBP and normalized to control expression levels. Expression levels in hippocampal sclerosis (HS; n=6) were compared to levels in autopsy control hippocampal specimens (n=5); expression levels in ganglioglioma (GG; n=6) and focal cortical dysplasia (FCD; n=6) were compared to levels in autopsy control cortical specimens (n=5) (A): tPA mRNA levels were significantly increased in GG specimens. uPA (B), uPAR (C) and PAI1 (D) mRNA levels were significantly increased in HS, FCD and GG. The error bars represent SEM; statistical significance: * P<0.05 compared with control.
**DISCUSSION**

Data obtained in different experimental models suggest a critical role for the plasminogen system in the development of spontaneous seizures leading to a chronic epileptic condition (Pawlak and Strickland, 2002, Lukasiuk et al., 2003, Gorter et al., 2006, Lahtinen et al., 2006). Several mechanisms are possibly involved, including a direct regulation of neuronal excitability (Tsirka et al., 1995, Benarroch, 2007). In addition, PAs may act in concert contributing to the disturbance of the blood-brain barrier (BBB) and inflammation (Lo et al., 2002, Del Rosso et al., 2008), which both represent critical components of the pathological changes underlying chronic refractory epilepsy (Vezzani and Granata, 2005, Oby and Janigro, 2006). The present study was designed to test the hypothesis that increased expression of tPA and uPA can be detected in common causes of human focal chronic refractory epilepsy. Therefore, specimens of hippocampal sclerosis and MCD obtained from patients undergoing epilepsy surgery for chronic epilepsy were examined with western blot analysis and with immunocytochemistry. We further assessed the cellular distribution of tPA and uPA proteins, to define the source and potential pharmacological targets of modulation of the plasminogen system in these epileptogenic human pathologies.

According to previous observations in control rat and human hippocampus, among the two plasminogen activators, the tPA protein is highly enriched in human CNS, particularly in the hippocampus (Teesalu et al., 2004, Lahtinen et al., 2006). The expression of tPA (and to a lesser extent of uPA) in control tissue was mainly present in neuronal cells and, occasionally, in endothelial cells, whereas the resting glia did not express detectable levels of both proteins. This observation is in agreement with the neuronal distribution of tPA and uPA mRNA (Dent et al., 1993, Masos and Miskin, 1996, Teesalu et al., 2004). This cellular distribution supports the role of PAs in the regulation of synaptic activity under physiological conditions also in human brain (Tsirka, 2002, Benarroch, 2007).

**Hippocampal sclerosis and plasminogen activators**

HS, also known as Ammon’s horn sclerosis, is the most common neuropathological finding in patients undergoing surgery for medically intractable TLE and is histopathologically characterized by selective neuronal cell loss and gliosis in CA1 and endfolium (Wieser and Epilepsy, 2004). Material obtained from patients undergoing surgery for TLE with HS represents the end-stage of a long and complex process which can be in part reproduced in animal models (for review see (Loscher, 2002). The present study shows increased expression of both tPA, uPA and uPAR proteins in HS humans specimens, confirming the prominent and persistent activation of the plasminogen system reported in the animal models of TLE (Lukasiuk et al., 2003, Gorter et al., 2006, Lahtinen et al., 2006). The hippocampal up-regu-
lation of the cell surface receptor of uPA (uPAR) observed with western analysis, could not be further evaluated at the cellular level, since the antibodies tested were not suitable for immunocytochemistry on paraffin-embedded specimens. Increased expression of both uPA and uPAR mRNA was, however, detected by RT-PCR analysis. However, a recent report in the post-status epilepticus (SE) model of TLE shows both astroglial and neuronal expression in the rat hippocampus, suggesting the critical role of uPA/uPAR pathway in epileptogenic tissue remodeling (Lahtinen et al., 2009, 2010).

Although both uPA mRNA and uPA protein are up-regulated in epileptogenic tissue, the different expression levels observed could reflect differences in sensitivity between the two methods or be related to post-transcriptional regulation. For instance, a recent study shows weak or no significant correlations between uPA mRNA and protein in breast cancer samples, suggesting posttranscriptional regulation (Biermann et al., 2008). A more recent study confirmed this possibility, demonstrating a microRNA mediated post-transcriptional regulation of uPA through miR-193b (acting as a negative regulator for uPA at the post-transcriptional level; (Li et al., 2009)).

Analysis of the cellular distribution was performed for tPA and uPA and shows prominent expression of both proteins in neuronal cells within the different hippocampal subfields. This is consistent with previous experimental observations demonstrating that limbic seizures trigger neuronal expression of tPA and uPA (Tsirka et al., 1995, Lahtinen et al., 2006). The presence of a prominent pool of PAs in neurons implies its acute release under pathological conditions associated with increased neuronal activity. Accordingly, a rapid release has been demonstrated for both tPA and uPA and may critically contribute to increase the amount of PAs in neuronal tissue (Pittman et al., 1989, Gualandris et al., 1996). One crucial mechanism by which tPA can increase neuronal excitability consists of its ability to enhance NMDA receptor signaling by cleavage of NMDA receptor subunit 1, which constitutes a direct substrate of tPA, (Tsirka et al., 1995, Benarroch, 2007). Neuronal PA expression was observed within the different hippocampal sub-fields, including the granule cells of the dentate gyrus. Interestingly, PAs have been shown to modulate neurite outgrowth and to be involved in the reorganization of granule cell axons (mossy fiber sprouting) which is a prominent feature in human TLE (Pittman et al., 1989, Wu et al., 2000, Tsirka, 2002).

In addition to neurons, tPA and uPA were expressed in reactive glial cells, including both astrocytes and activated microglial cells. Regulation of PAs expression has been shown in vitro by different molecules that stimulate glial cell activation/proliferation and are produced within injured human brain tissue (Rogister et al., 1988, Tranque et al., 1992, Faber-Elman et al., 1995, Falcone et al., 1995, Gravanis and Tsirka, 2005, Nakajima et al., 2005). Our data provide the first evidence that astrocytic and microglial expression of PA proteins is present.
within the sclerotic hippocampus in human TLE. This observation indicates that glial cells within the epileptogenic tissue may represent an important source of PAs. Furthermore, glial PAs may also contribute the activation of glial cells (Gravanis and Tsirka, 2005). tPA has been proposed as key regulator of microglia at the site of injury, promoting additional tissue damage (Siao et al., 2003, Gravanis and Tsirka, 2005). In contrast, astrocytes have been suggested to be involved in the neutralization of tPA toxicity, through the uptake of the tPA/PAI1 complex via an endocytic tPA receptor, the lipoprotein receptor-related protein (LRP) (Fernandez-Monreal et al., 2004). Increased expression of PAI1 mRNA has been observed in both experimental and human TLE ((Gorter et al., 2006) and present results). Whether the tPA expression in astrocytes indicates sequestration in the form of an inactive complex with PAI1 in vivo remains to be explored.

Expression of PAs was also increased in blood vessels within the sclerotic hippocampus, indicating that the vascular endothelium represents an additional source of endogenous PAs within the human epileptogenic tissue. Exposure of endothelial cells to angiogenic and/or pro-inflammatory mediators differentially regulates the PA expression in vitro (Gerritsen et al., 1993, Niedbala, 1993, Mandriota et al., 1995, Larsson et al., 2008). Recently, an increase in uPA and uPAR expressing blood vessels has been reported in rat hippocampus after SE induction (Lahtinen et al., 2006, Lahtinen et al., 2009, in press). The plasminogen system has been shown to play a critical role in angiogenesis (Pepper et al., 1996, Brodsky et al., 2001, Rakic et al., 2003), as well as in the regulation of the BBB permeability (Yepes et al., 2003). Interestingly, alterations of the BBB permeability and angiogenesis have been recently observed in both human and experimental TLE with a positive correlation between the increased vascular permeability and the occurrence of spontaneous seizures in chronic epileptic rats (Rigau et al., 2007, van Vliet et al., 2007, Ravizza et al., 2008). In addition, release of PAs by brain endothelial cells has been suggested to critically regulate monocyte diapedesis through the BBB (Reijerkerk et al., 2008). Thus PAs could indirectly play a pro-epileptogenic role and contribute to the persistent inflammatory reactions observed in human and experimental TLE (Fabene et al., 2008, Ravizza et al., 2008, Vezzani et al., 2008).

Developmental lesions and plasminogen activators

Focal developmental lesions, including FCD, TSC cortical tubers and glioneuronal tumors (i.e. gangliogliomas) represent another frequent finding in patients with medically intractable TLE (Blumcke et al., 2002, Thom, 2004). These lesions share a number of clinical and pathological features and have been recently included among the malformations of cortical development (MCD) characterized by active proliferation and abnormal cell types (Barkovich et al., 2005).
Our large-scale gene expression profile analysis recently demonstrated that both tPA and uPA are strongly up-regulated within GG (Aronica et al., 2008). Increased expression was also observed for annexin II, a membrane protein which has been identified as binding partner of tPA, possibly involved in the tPA-dependent microglial activation (Siao and Tsirka, 2002).

In the present study we confirmed the activation of the plasminogen system in GG, showing increased expression of both tPA and uPA proteins and mRNAs. Increased tPA and uPA protein expression was also detected in FCD. RT-PCR analysis showed up-regulation of uPA and uPAR in both GG and FCD specimens. Similarly to HS specimens, PA IR was observed in both neuronal and glial cells of MCD cases. Dysplastic neurons in FCD and TSC specimens, as well as neuronal cells of GG specimens displayed strong uPA and tPA expression, indicating that neurons are a major source of PAs also in developmental lesions. The presence of tPA within these highly epileptogenic lesions may exacerbate neuronal excitability, increasing NMDA receptor signaling. Interestingly, both dysplastic neurons and neuronal cells in GG specimens contain high levels of NMDAR subunit proteins (Ying et al., 1999, Aronica et al., 2001a, c). The prominent neuronal expression of PAs in MCD is also interesting considering the increasing evidence that indicates a crucial role for the plasminogen system during brain development ([Zhang et al., 2005] and [Royer-Zemmour et al., 2008]). Results obtained using mice lacking the tPA gene indicate a role for tPA in facilitating neuronal migration (Seeds et al., 1999), whereas selective alteration of the distribution of cortical interneurons have been reported in mice lacking the uPAR ([Powell et al., 2003] and [Eagleson et al., 2005]). Thus, the induction of PA proteins, producing changes in extracellular matrix and activation of matrix metalloproteinases ([Irigoyen et al., 1999] and [Dityatev and Fellin, 2008]) could contribute to the abnormal morphology of dysplastic cells and their abnormal positioning within the dysplastic cortex. Moreover, increasing evidence indicates a critical role for uPAR signaling, independently of uPA-mediated proteolysis (Smith and Marshall, 2010). Recently new uPA ligands have been detected, such as the SRPX2 (Sushi-Repeat Protein, X-linked 2). Interestingly, a mutation in SRPX2 is associated with Rolandic epilepsy and bilateral perisylvian polymicrogyria and leads to a gain of affinity of SRPX2 with uPAR (Royer-Zemmour et al., 2008). In addition, considerable evidence indicates that integrins, a major family of extracellular matrix (ECM) receptors (such as β1 and β3 integrins) represent the most important transmembrane receptors associated with uPAR signalling (reviewed in (Smith and Marshall, 2010)). Several genes associated with cell adhesion, including integrins, are up-regulated in cortical tubers (Boer et al., 2009).

Expression of tPA and uPA was also observed in balloon cells in FCD and giant cells in TSC specimens. The presence of PAs in balloon/giant cells may reflect the immature phenotype
of these cells ([Cepeda et al., 2003], [Alonso-Nanclares et al., 2005], [Cepeda et al., 2005] and [Kalderon et al., 1990]). Furthermore, analysis of tPA and uPA cellular distribution in MCD demonstrated both astroglial and microglial cells IR, supporting the contribution of these cells to the amount of PAs that is present in these lesions, as well as the potential role of PAs in glial activation (Gravanis and Tsirka, 2005). The large majority of reactive astrocytes in FCD and TSC, as well as tumor astrocytes in GG, expressed tPA and uPA. Expression of PAs was also increased in blood vessels within the different developmental lesions studied. Interestingly, alterations of the BBB permeability and prominent inflammatory reactions have also been observed in focal MCD ([Aronica et al., 2005], [Aronica et al., 2007], [Boer et al., 2006], [Boer et al., 2008] and [Ravizza et al., 2006]).

Concerning the expression patterns of PAs in glial tumors, increased levels of uPA have been observed in high-grade gliomas, whereas the data on the expression of tPA are still controversial (for review see; Levicar et al., 2003). In particular attention has been focused on their role in tumor invasion and angiogenesis in high grade tumors ([Yamamoto et al., 1994] and [Levicar et al., 2003]). Our observations indicate that induction of the plasminogen system may also occur in low-grade glioneuronal tumors, such as GG. Although a rapid induction of PAs can be triggered in experimental epilepsy models ([Lukasiuk et al., 2003], [Gorter et al., 2006] and [Lahtinen et al., 2006]), seizures alone cannot account for the increased PA expression in these developmental lesions since the analysis of the histologically normal perilesional cortex showed a pattern of neuronal IR similar to that observed in control cortex. However, recent evidence suggests that molecular and functional alterations in the perilesional region, may also contribute to the epileptogenicity of focal MCD (Wong, 2008). Thus, evaluation of a larger number of samples, including also areas of electrocortico-graphically defined perilesional epileptogenic regions, is required to exclude a possible occurrence of perilesional tPA and uPA changes.

Acknowledgments
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2.2 EVALUATION OF THE INNATE AND ADAPTIVE IMMUNITY IN TYPE I AND TYPE II FOCAL CORTICAL DYSPLASIAS

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SUMMARY

Purpose: Induction of inflammatory pathways has been reported in epileptic patients with focal malformations of cortical development. In the present study we examined the innate and adaptive immune responses in focal cortical dysplasia (FCD) with different histopathologic and pathogenetic features.

Methods: The inflammatory cell components and the induction of major proinflammatory pathways and molecules [complement pathway, interleukin (IL)-1β, and chemokine monocyte chemotactic protein-1 (MCP1)] was investigated in surgical specimens of sporadic type IA and type IIB FCD using immunocytochemical methods.

Results: FCD II but not FCD I cases exhibit activation of the mammalian target of rapamycin (mTOR) cascade with strong neuronal expression of the phosphorylated isoform of S6 protein. Microglia reactivity was increased in all lesions (FCD I and II) compared to control tissue; however, the number of HLA-DR–positive cells was significantly higher in FCD II than in FCD I. In FCD II specimens we also observed perivascular and parenchymal T lymphocytes (CD3+), with a predominance of CD8+ T-cytotoxic/suppressor lymphocytes, as well as a few dendritic cells. Expression of components of the complement cascade, IL-1β, and MCP1 was prominent in FCD II cases.

Discussion: Our findings indicate a prominent activation of both innate and adaptive immunity, with involvement of different inflammatory pathways in FCD II cases, supporting the possible involvement of inflammation in the epileptogenesis of these lesions, as well as the notion that FCD II is pathologically distinct from FCD I.
INTRODUCTION

Focal cortical dysplasias (FCD) represent sporadic architectural and cytoarchitectural malformations of the cerebral cortex, usually confined to a single hemispheric lobe, which are recognized causes of chronic medically intractable epilepsy in children and young adults (Thom, 2004, Fauser et al., 2006, Najm et al., 2007, Blümcke et al., 2009). According to the current histopathological classification system, FCD have been classified into Type I, characterized by cortical dyslamination, and Type II, characterized by additional cytoarchitectural abnormalities, i.e. the presence of dysmorphic neurons and balloon cells (Palmini et al., 2004). The molecular pathogenesis of FCD type I and type II remains to be clearly elucidated. However, recent molecular-genetic and histopathological studies indicate the involvement of the phosphatidyl-inositol 3-kinase (PI3K)-mTOR (mammalian target of rapamycin) pathway in FCD type II (Becker et al., 2006, Schick et al., 2007). In contrast, activation of the mTOR pathway has not been reported in FCD type I. The cellular mechanism(s) underlying the epileptogenicity of FCD has also not been fully elucidated. Previous studies support the role of developmental alterations of the balance between excitation and inhibition (Wong, 2008). In particular, impairment of the GABAergic system has been suggested as a possible mechanism for the epileptogenicity of both type I and II FCD (Sprefico et al., 2000, Garbelli et al., 2006). Recent evidence strongly suggest the involvement of inflammatory processes in the etiopathogenesis of seizures (Vezzani and Granata, 2005, Vezzani et al., 2008). Activation of cells of the microglia/macrophage lineage and induction of different inflammatory pathways have been described in epileptogenic tissue from temporal lobe epilepsy with hippocampal sclerosis (Ravizza et al., 2008b), as well as in malformations of cortical development (MCD), including FCD type II (Aronica et al., 2005, Boer et al., 2006, Ravizza et al., 2006, Aronica et al., 2007, Boer et al., 2008). Whether these inflammatory changes represent an intrinsic feature of FCD or they are triggered by chronic seizure activity is still unclear. In addition the involvement of the mTOR pathway in the regulation of the inflammatory response in malformations associated with activation of this pathway cannot be excluded (Weinstein et al., 2000, Potter et al., 2001, Lim et al., 2003).

The aim of the present study was to determine whether components of innate and adaptive immunity were present and similarly distributed in brain tissue from patients with FCD type I and FCD type II and chronic medically intractable epilepsy. In addition, the expression and cellular distribution of major pro-inflammatory pathways and molecules known to be up-regulated in MCD associated with activation of the mTOR pathway were also analyzed.
MATERIALS AND METHODS

Subjects
The cases included in this study were obtained from the archives of the departments of neuropathology of the Academic Medical Center (University of Amsterdam), the University Medical Center in Utrecht, and the VU University Medical Center (VUMC) in Amsterdam. Twenty-seven brain tissue specimens, removed from patients undergoing surgery for intractable epilepsy, were examined. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All cases were reviewed independently by two neuropathologists, and the diagnosis was confirmed according to the system recently proposed by Palmini et al. for grading the degree of FCD (Palmini et al., 2004). Table S1 summarizes the clinical findings of epilepsy patients and controls. None of the patients with FCD fulfilled the diagnostic criteria for tuberous sclerosis complex (TSC). Table S2 summarizes the neuropathologic findings of FCD specimens and the standard stains used.

All patients underwent presurgical evaluation with phase I investigations consisting of noninvasive tests, history, medical, neurological and neuropsychological assessment, structural neuroimaging, and extensive interictal and ictal electroencephalography (EEG) studies with video monitoring. In phase II, an intracarotid sodium amytal test (Wada test), interictal positron emission tomography (PET), (inter)ictal single photon emission computed tomography (SPECT), and functional magnetic resonance imaging (fMRI) were performed on indication (van Veelen et al., 1990). Patients who underwent implantation of strip and/or grid electrodes for chronic subdural invasive monitoring before resection were excluded from this study. Patients had complex partial seizures (CPS), and all patients had daily seizures, which were resistant to maximum doses of antiepileptic drugs. Seizure duration represents the interval in years from age at seizure onset and age at surgery. The postoperative seizure outcome was classified according to Engel (1993).

Normal-appearing control cortex was obtained at autopsy from six age-matched patients without history of seizures or other neurologic diseases. Autopsied brain tissues from patients with neuroinflammatory pathologies [viral encephalitis and multiple sclerosis (MS)] were also examined (as positive controls). All autopsies were performed within 12 h after death. Histologically normal temporal neocortex (without evidence of significant neuronal loss, gliosis or malformation; epilepsy controls) from four patients undergoing extensive surgical resection of the mesial structures for the treatment of medically intractable complex partial epilepsy was also used for immunocytochemical analysis. This material represents good control tissue, since it is exposed to similar seizure activity, duration of epilepsy, and fixation protocol, and is also useful for investigating whether seizure activity itself triggers the inflammatory response.
Tissue preparation
One representative paraffin block per case (containing the complete lesion or the largest part of the lesion resected at surgery) was sectioned, stained, and assessed. Formalin fixed, paraffin-embedded tissue was sectioned at 6 μm and mounted on precoated glass slides (Star Frost, Waldemar Knittel GmbH, Barunschweig, Germany). Sections of all specimens were processed for hematoxylin and eosin (H&E), Luxol fast blue (LFB), and Nissl stains as well as for immunocytochemical stainings for a number of neuronal and glial markers as described in subsequent text.

Antibody characterization and immunocytochemistry
Antibodies (Abs) specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4,000), vimentin (mouse clone V9; DAKO; 1:1,000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemi-Con, Temecula, CA, U.S.A.; 1:2,000), neurofilament (SMI31; Sternberger Monoclonals, Lutherville, MD, U.S.A.; 1:1,000), cleaved caspase-3 (rabbit polyclonal, Cell Signaling Technology, Beverly, MA, U.S.A.; 1:100), and phospho-S6 ribosomal protein (Ser235/236; pS6, rabbit polyclonal, Cell Signaling Technology; 1:50) were used in the routine immunocytochemical analysis of FCD cortical specimens to document the presence of a heterogeneous population of cells, apoptosis, and activation of the mTOR pathway (Baybis et al., 2004).

For the detection of the inflammatory cells and proinflammatory pathways the following Abs were used: anti-human leukocyte antigen (HLA)-DP, DQ, DR (HLA-DR; mouse clone CR3/43; DAKO, Glostrup, Denmark; 1:400), anti-CD68 (mouse monoclonal, clone PG-M1; DAKO; 1:200, monocytes, macrophages, microglia), anti-CD3 (mouse monoclonal, clone F7.2.38; DAKO; 1:200, T lymphocytes), anti-CD4 (mouse monoclonal, clone 4B12; Neomarkers; 1:100, helper/inducer T-lymphocyte subset), anti-CD8 (mouse monoclonal, clone C8/144B; DAKO; 1:100; cytotoxic/suppressor T-lymphocyte subset), anti-CD20 (mouse monoclonal, clone L26; DAKO; 1:400, B lymphocytes), DC-SIGN (CD209; monoclonal mouse, BD Pharmaningen, San Diego, CA, U.S.A.), MCP1 (MCP1/CCL2; monoclonal mouse, R&D Systems, Minneapolis, MN, U.S.A.; 1:10), interleukin (IL)-1β [goat polyclonal, sc-1250, Santa Cruz Bio., CA, U.S.A.; 1:70, (Ravizza et al., 2006)], anti-C1q and anti-C3d [rabbit polyclonal, DAKO, Glostrup, Denmark; C1q, 1:100; C3c, C3d, 1:200, (Aronica et al., 2007)].

Immunocytochemistry was carried out as described previously (Aronica et al., 2003). Single-label immunocytochemistry was performed using the Powervision kit (Immunologic, Duiven, The Netherlands) and 3,3-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin. Sections incubated without the primary Ab, with preimmune serum, or with the primary Ab (for IL-1β and MCP1) and an excess of the antigenic peptide...
were essentially blank. A similar pattern of immunoreactivity was observed in surgical and autopsy control specimens included in this study. For double-label immunocytochemistry with DC-SIGN (IgG2b) and CD3 (IgG1), we used secondary Ig subtype specific Abs and as chromogens 3-amino-9-ethyl carbazole (AEC, Sigma, St. Louis, MO, U.S.A.) and Fast Blue B salt (Sigma). For double-labeling with HLA-DR and pS6 (as well as for caspase-3 with GFAP, HLA-DR, or NeuN, not shown), we used, as secondary antibodies, Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor 488 anti-mouse IgG (1:100; Molecular Probes, Leiden, The Netherlands). Sections were mounted with Vectorshield containing DAPI (targeting DNA in the cell nucleus; blue emission) and analyzed by means of a laser scanning confocal microscope (Leica TCS SP2; Wetzlar, Germany).

RESULTS
Case material and histological features
The clinical features of the cases included in this study are summarized in Table 1. All patients had a history of chronic pharmaco-resistant epilepsy. Post-operatively, 13 patients (76%) in this cohort were completely seizure free. Age at surgery, seizure duration and seizure frequency were not statistically different between patients with FCD I and FCD II in this cohort. In this study we excluded patients with mild degree of cortical dysplasia (mild malformation of cortical development; (Palmini et al., 2004). The FCD cases included had all the previously described histopathological features of Type I A or Type II B FCD (Palmini et al., 2004). FCD IA is characterized by cortical dyslamination and often presence of ectopic white matter neurons; the Type II B is characterized by additional cytoarchitectural abnormalities, including the presence of dysmorphic neurons and balloon cells (Fig. 1). We only included

<table>
<thead>
<tr>
<th>Pathology type</th>
<th>Number of cases</th>
<th>Gender (M/F)</th>
<th>Mean age at surgery (range/years)</th>
<th>Localization</th>
<th>Mean duration of epilepsy (range/years)</th>
</tr>
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<tbody>
<tr>
<td>FCD I</td>
<td>8</td>
<td>4/4</td>
<td>27.8 (19-39)</td>
<td>5 Temporal</td>
<td>7.7 (6-13)</td>
</tr>
<tr>
<td>FCD II</td>
<td>9</td>
<td>5/4</td>
<td>26.2 (16-36)</td>
<td>3 Frontal</td>
<td>8 (6-14)</td>
</tr>
<tr>
<td>Epilepsy control (no MCD)</td>
<td>4</td>
<td>2/2</td>
<td>29.5 (25-31)</td>
<td>Temporal</td>
<td>8.1 (7-13)</td>
</tr>
<tr>
<td>Controls (no epilepsy)</td>
<td>8</td>
<td>3/4</td>
<td>30.1 (20-41)</td>
<td>Temporal</td>
<td>~</td>
</tr>
</tbody>
</table>

FCD = Focal Cortical Dysplasia.
cases of FCD which were isolated and not associated with a primary lesion (i.e. tumor or hippocampal sclerosis). As described previously (Baybis et al., 2004, Miyata et al., 2004, Ljungberg et al., 2006, Schick et al., 2007a, Schick et al., 2007b), both dysplastic neurons and balloon cells in FCD type II displayed enhanced S6 protein phosphorylation (Fig. 1 D). In contrast, phospho-S6 protein expression was not detected in FCD type I cases, similarly to epilepsy (no MCD) or normal control specimens (Fig. 1 B).

Figure 1. Histopathological features of focal cortical dysplasia type I (FCD I) and FCD II: distribution of cells of the microglia/macrophage lineage. (A, C) NeuN staining showing the disorganization of the neuronal component within the dysplastic cortex in FCD I (A) and FCD II (C). (B, D) Phospho-S6 (pS6) staining showing absence of immunoreactivity (IR) in FCD I (B), but several pS6-positive cells in FCD II (D), including dysplastic neurons and balloon cells (insert in D). (E–H) HLA-DR staining. (E, F) FCD I showing moderate presence of HLA-DR–positive cells in both cortex (CTX; E) and white matter (Wm; F) regions; insert in E shows positive perivascular cells. (G, H) FCD II showing strong presence of HLA-DR–positive cells in both cortex (G) and white matter (H); inserts in G and H show high magnification photographs of HLA-DR–positive cells surrounding dysplastic neurons (insert a in G) and balloons cells (insert a in H). Insert b in H: merged image showing HLA-DR–positive microglial cells (green) surrounding a pS6-positive balloon cell (red). Scale bar in F: A–C, E, G–H: 200 μm; B–D: 120 μm; F: 80 μm.
Microglial reactivity in FCD type I and type II

As previously reported (Boer et al., 2006) areas of reactive microglia were often encountered in specimens of patients with FCD type II, throughout the dysplastic region (Fig. 1G-H). Cells strongly positive for HLA-DR and with the morphology of activated microglial cells (showing bushy morphology with dense and short stout processes) were often clustered around dysplastic neurons (Fig. 1G) and around balloon cells (Fig. 1H). In contrast, little microglial activation was observed in specimens of patients with FCD type I. HLA-DR IR was often restricted to the perivascular space, sparse reactive microglial cells were mainly observed within the subcortical white matter (Fig. 1E-F).

The number of HLA-DR immunoreactive cells was quantified in four categories of neocortex examined in this study, including FCD type I, FCD type II and control specimens (histologically normal temporal neocortex of patients with and without history of chronic epilepsy; Fig. 4A). Statistical analysis applied to the mean number of positive cells across three distinct 1 mm² areas indicated increased microglial reactivity in epilepsy specimens compared to control non-epileptic tissue. The number of HLA-DR positive cells was found to be significantly higher in FCD type II compared to FCD type I and epilepsy surgical specimens (Fig. 4A). In contrast, no significant differences were observed between FCD type I and epilepsy surgical specimens. In this cohort (including cases matched in terms of age, duration of epilepsy and preoperative seizure frequency) no significant correlation was found between the number of HLA-DR-positive cells clinical variables such as age at surgery, age at seizure onset, duration of epilepsy and seizure outcome after surgery.

T-lymphocyte Infiltration in FCD type I and type II

Lymphocytes were detected within the dysplastic regions, with more prominent presence in FCD type II specimens (Fig. 2; Fig. 4B). The lymphocyte population was represented by T lymphocytes (as shown by immunocytochemical analysis performed with anti-CD3 antibody; Fig. 2A-F) and was predominantly composed of CD8-positive cells (Fig. 2G-H). T lymphocytes were detected around blood vessels, but also in the tissue parenchyma in FCD type II specimens, and were often clustered around balloon cells (Fig. 2D-H). CD4-positive cells and B lymphocytes were not detected (data not shown). The number of CD3 immunoreactive cells in FCD and control specimens was quantified and the mean number of positive cells across three distinct 1 mm² areas was found to be significantly higher in FCD specimens than in control non-epileptic tissue (Fig. 4B). The number of CD3 positive cells was found to be significantly higher in FCD type II compared to FCD type I and epilepsy surgical specimens (Fig. 4B). In contrast, no significant differences were observed between FCD type I and epilepsy surgical specimens. Differences between control non-epileptic (autopsy) and epilepsy
surgical specimens were not observed. No significant correlation was found between the number of CD3-positive cells and the amount of microglial reactivity in FCD type I and II specimens (analysis was performed in serial sections stained with CD3 and HLA-DR). No significant correlation was found between the number of CD3-positive cells and the different clinical variables in the different patient groups.

Figure 2. Distribution T lymphocytes in FCD I and FCD II. (A–F) CD3 staining. (A–C) Focal cortical dysplasia type I (FCD I) with few CD3 positive cells in cortex (CTX; arrow) and white matter (Wm; arrows). (D–F) FCD II with several CD3 positive cells in cortex (CTX; D–E, arrows) and white matter (Wm; F); arrows in E show lymphocytes surrounding a balloon cell (asterisk) and a dysmorphic neuron (arrowhead); insert in F shows perivascular CD3 positive infiltrates in Wm. (G, H) CD8-positive T lymphocytes in FCD II. (G) Perivascular CD8 positive cells. (H) CD8 T lymphocytes around balloon cells (asterisks). Scale bar in H: A, B: 40 μm; C: 50 μm. E D–F: 80 μm. G, H: 20 μm.
Figure 3. Distribution of dendritic cells in focal cortical dysplasia type I (FCD I) and FCD II. (A–C) DC-SIGN staining in control tissue. (A) Tonsil; (B) choroid, plexus; (C) cortex (CTX) with DC-SIGN–positive cells in meninges. (D–F) FCD I with DC-SIGN–positive cells in the leptomeninges, but absence of positive cells in cortex (E) and white matter (F). (G, H) FCD II showing perivascular DC-SIGN–positive cells and occasionally few cells with parenchymal location (insert in G). I and insert in H: CD83 staining showing perivascular positive cells. (J) Perivascular distribution of DC-SIGN (red) and CD3 (blue) positive cells in FCD II. Scale bar in J: A: 25 μm; B: 30 μm; C: 40 μm; D–F: 80 μm; G–I: 20 μm; J: 40 μm.
**Dendritic cells in FCD type I and type II**

Dendritic cells (DCs) were identified using DC-SIGN (CD209, a marker for both immature and mature DCs). DC-SIGN stains dendritiform cells in T-cell zones of lymph node (Fig. 3A), in the choroid plexus (Fig. 3B), and a few cells in the leptomeninges of control cortical specimens (Fig. 3C). No DC-SIGN IR was detected in the gray and white matter of the large majority of control and FCD I specimens (Fig. 3D–F). Differences between FCD I, control nonepileptic (autopsy), and epilepsy surgical specimens were not observed (Fig. 4C). However, in FCD II specimens, few DC-SIGN–positive cells, with an irregular and ramified morphology were detected around blood vessels (Figs. 3G–H and 4C). No DC-SIGN IR was detected on vascular endothelia. We did not observe parenchymal deposits of DC-SIGN IR. The intrameningeal DC-SIGN cell distribution (with scattered positive cells) did not differ between the different patient groups. The presence of mature DCs in the FCD specimens was also investigated using an anti-CD83 Ab (Serafini et al., 2006; Cudrici et al., 2007). Only in FCD II, a few CD83-positive cells were present around blood vessels. No CD83-positive cells were detected in the tissue parenchyma. DC-SIGN–positive cells were consistently and abundantly detected in the meninges, as well as around intraparenchymal blood vessels, and in tissue specimens from patients with MS or viral encephalitis. To determine a possible morphologic correlation between the presence of DCs and T-cell infiltrates, we used double staining for DC-SIGN and CD3. Indeed many cells situated close to DC-SIGN–positive cells were positive for CD3 (Fig. 3J).

![Figure 4. Evaluation of cells of the microglia/macrophage lineage and T lymphocytes and dendritic cells in focal cortical dysplasia type I (FCD I) and FCD II.](image)

(A) Graph showing HLA-DR cell counting in control cortex, epilepsy control (no malformation of cortical development, MCD), FCD I, and FCD II. (B) Graph showing CD3 cell counting in control cortex, epilepsy control, FCD I, and FCD II. (C) Graph showing DC-SIGN cell counting in control cortex, epilepsy control, FCD I, and FCD II (*p < 0.05).
Figure 5. Distribution of interleukin (IL)-1β and monocyte chemotactic protein-1 (MCP1) in focal cortical dysplasia type I (FCD I) and FCD II. (A, B and E, F) FCD I. A and B (IL-1β staining) showing absence of glial or neuronal immunoreactivity (IR) in both cortex (CTX, A) and white matter (Wm, B). E and F (MCP1 staining) showing very light neuronal IR in cortex (E), without detectable glial IR in both cortex and white matter (F). (C, D and G, H) FCD II. C and D (IL-1β staining) and G, H (MCP1 staining) showing strong IR within cortex (C and G) and white matter (D and H). Arrows in C and G indicate positive dysplastic neurons; arrowheads in C and G indicate positive glial cells; arrows in D and H indicate positive balloon cells. Insert in D shows positive astrocytes. Scale bar in H: B, C, E, F, G, H: 80 μm; A and D: 150 μm.
Complement activation in FCD type I and type II
In agreement with our previous report (Aronica et al., 2007; Boer et al., 2008), both neurons and resting glial cells in control nonepileptic (autopsy) specimens did not express detectable levels of any of the two (C1q and C3d) complement components examined. Occasionally, C1q and C3d IR was detected in a few glial cells and blood vessels in epileptic surgical control and FCD I specimens, but not in neurons (Fig. S1A–D). In contrast, moderate to strong C1q and C3d IR was detected within all FCD II specimens examined (Figs. S1E–J and 2A,B). Expression of the two complement components was observed in glial cells, neurons, and in balloon cells (Fig. S1G,J).

IL-1β immunoreactivity in FCD type I and type II
In agreement with previous reports (Kadhim et al., 2003; Ravizza et al., 2006; Boer et al., 2008), both neurons and resting glial cells in control nonepileptic (autopsy) specimens did not express detectable levels of IL-1β (Fig. S2C). Occasionally, IL-1β IR was detected in a few glial cells in epileptic surgical control and FCD I specimens, but not in neurons (Figs. 5A,B and S2C). In contrast, moderate to strong IL-1β IR was detected in all the FCD II specimens (Figs. 5C,D and S2C). Expression of IL-1β was observed in glial cells, neurons, and giant cells (Fig. 5D).

MCP-1 immunoreactivity in in FCD type I and type II
Light neuronal MCP1 IR was observed in both control and FCD I specimens; resting glial cells in controls and in the large majority of FCD I cases did not express detectable levels of MCP1 IR (Figs. 5E–F and S2D). In contrast, moderate to strong MCP1 IR was detected in all FCD II specimens (Figs. 5G,H and S2D). Expression of MCP1 was observed in glial cells, neurons, and giant cells (Fig. 5H).

DISCUSSION
We examined the features and the severity of the inflammatory response occurring in tissue from patients with FCD and chronic medically intractable epilepsy. Several observations indicate that activation of inflammatory processes is a common feature of various experimental models of seizures and in human epileptic disorders with different etiologies, but without a primary inflammatory pathogenesis (Vezzani & Granata, 2005; Ravizza et al., 2008). Activation of both innate and adaptive immune response has been recently reported in focal MCDs (Aronica et al., 2005, 2007; Boer et al., 2006, 2008; Ravizza et al., 2006).
In this study, we confirmed the occurrence of complex inflammatory changes, involving both the innate and the adaptive immune response in FCD specimens, and we demonstrate that the severity of these changes is greater in FCD II than in specimens from patients with FCD I (despite the absence of significant differences in seizure frequency and duration). These results indicate that activation of inflammatory processes is not simply an effect of seizure activity, although the presence of activated microglia in temporal cortex from patients with epilepsy (without malformations) does not exclude that this could be triggered by chronic seizure activity. However, the prominent activation of microglia observed within the FCD II specimens and the close association of activated immune cells with abnormal cell types, such as dysplastic neurons and balloon cells (displaying mTOR activation) suggests a diverse nature of activation in this type of pathology. In the present study we observed aberrant phosphorylation of S6 ribosomal protein (a known marker of mTOR activation) only in the FCD II specimens, suggesting a pathogenetic distinction between the two types. Interestingly, the mTOR pathway does not only play a role in regulating cell growth and size, but also in the innate and adaptive immune response (Lim et al., 2003; Schmitz et al., 2008; Weichhart & Saemann, 2009). One could speculate that mTOR activation within the cellular components of FCD II also contributes to the inflammatory response. Activated microglial cells are often observed around pS6 positive cells (dysplastic neurons and balloon cells; present results and Boer et al., 2008). However, further investigation is required to ascertain this possibility. Interestingly, the mTOR inhibitor rapamycin is an effective immunosuppressive agent (Thomson et al., 2009) and has been recently shown to suppress seizures or even prevent the development of epilepsy in a mouse model of TSC (Zeng et al., 2008), and reduction in seizure frequency has been reported in a young patient with TSC (Muncy & Butler, 2009). Because our study includes only FCD IIB specimens, the issue of whether the induction of the inflammatory response (associated with mTOR activation) occurs also in FCD IIA still needs to be clarified. In addition, because we only analyzed the lesion, we cannot exclude that activation of proinflammatory pathways may also occur in the perilesional cortex. Accordingly, recent observations indicate increased expression of adhesion and inflammatory factors in the perilesional cortex in TSC compared to autopsy control specimens (Boer et al., 2009).

Although activated microglial cells are the major components of the inflammatory cell population in FCD, some degree of activation of components of the adaptive immunity is also observed. The presence of T lymphocytes (CD8+, T-cytotoxic/suppressor immunophenotype) was greater in FCD II specimens than in FCD I (despite the absence of differences in seizure duration). In addition, only in FCD II cases both perivascular and intraparenchymal
lymphocytes were detected and lymphocytes were often clustered around balloon cells. Inflammatory perivascular lymphocytic infiltrates are not uncommon in the leptomeninges and are occasionally present in the upper cortical layers of patients undergoing implantation of subdural electrodes for chronic monitoring before resection (Stephan et al., 2001; Rhodes et al., 2007). However, the patients included in this study did not undergo invasive monitoring before surgery and did not show signs of leptomeningitis. Moreover, the lymphocytic infiltrates were often located in deeper cortical layers or in the white matter. As expected, the T-cell response was less severe in FCD cases than in cases of MS or viral encephalitis (present results; Serafini et al., 2006). A recent study (Wirenfeldt et al., 2009) reports that microglial activation is increased in patients with active Rasmussen’s encephalitis (RE), compared with cases of FCD and TSC; however, the entity of the lymphocytic infiltrates in RE compared to FCD was not evaluated. The factors that trigger these T-lymphocyte infiltrations in FCD are not known. Alterations in blood–brain barrier permeability, resulting from seizure activity and/or induction of inflammatory mediators by activated microglia, may facilitate their entry. Few T-lymphocytes were found in tissue of patients with temporal lobe epilepsy (TLE) and hippocampal sclerosis, or in experimental models of TLE, indicating that not all types of epileptogenic causes promote accumulation of lymphocytes.

Recently attention has been focused on the role of the antigen-presenting DCs in the initiation of the adaptive immunity (Reis e Sousa, 2006). These cells normally reside in the leptomeninges and choroid plexus. However, under different inflammatory conditions, perivascular and intraparenchymal DCs can be detected in the brain (Serafini et al., 2006; Cudrici et al., 2007). A subarachnoid mixed infiltrate of DCs has been reported in a patient with MCD and chronic epileptic encephalopathy (Rhodes et al., 2007). In the present study we found DCs around intraparenchymal blood vessels only in FCD II specimens. These DCs express the maturation marker CD83 and are associated with perivascular T-lymphocytes. Although they represent a numerically minor population of immune cells compared to the number of DCs in cases of viral encephalitis or MS (Serafini et al., 2006; Cudrici et al., 2007), they deserve attention in view of their ability to drive chronic inflammation interacting with T cells and regulating chemokine production (Tang & Cyster, 1999; Penna et al., 2002). Interestingly, function of DCs is also under control of the mTOR signaling pathway (Thomson et al., 2009), which is deregulated in FCD II.

The complexity of the inflammatory response induced in FCD II is supported by the observed activation of both complement and IL-1β signaling pathways. Prominent glial and neuronal expression of key complement components, C1q and C3d, was detected within the dysplas-
tic region of FCD II specimens. In particular the presence of the biologically active fragments of C3d indicates that the activation of the complement cascade has reached a point that may support a sustained inflammatory process [for review see (Lucas et al., 2006)]. Persistent activation of the complement cascade has been reported recently in both experimental and human temporal lobe epilepsy (Aronica et al., 2007) as well as in TSC-associated cerebral lesions (Boer et al., 2008). As recently reported (Boer et al., 2007), FCD type II lesions displayed also prominent glial and neuronal expression of the proinflammatory and proepileptogenic cytokine IL-1β (Vezzani & Granata, 2005; Vezzani et al., 2008). In contrast, significant activation of complement and IL-1β pathway was not observed in FCD I, despite similar duration of epilepsy. Notwithstanding the fact that both pathways are known to be rapidly upregulated following seizures (Vezzani & Granata, 2005; Aronica & Gorter, 2007), this indicates that seizures alone cannot account for the increased expression in FCD II lesions.

In agreement with the prominent microglial reactivity observed in FCD II specimens, we also detected increased expression of MCP1 (or CCL2), which was prominently expressed in reactive glial cells, dysplastic neurons, and balloon cells. MCP1 is a chemokine that regulates migration and infiltration of monocytes/macrophages and microglial activity, and is involved in the regulation of blood–brain barrier permeability (Stamatovic et al., 2005). Prominent induction of MCP1 has been reported in both experimental and human TLE (Gorter et al., 2006; Wu et al., 2008), as well as in TSC-lesions (data not shown). MCP1 may play a role in both neurodegenerative and regenerative processes (Kalehua et al., 2004).

Our data clearly distinguish FCD I from FCD II, supporting the critical role of sustained inflammatory reaction in FCD II, with activation of both the innate and the adaptive immune response and involvement of complement, as well as, IL-1β and MCP-1 signaling pathways.

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2.3 Activation of TLR, RAGE and HMGB1 signaling in malformations of cortical development

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ABSTRACT

Recent evidence in experimental models of seizures and in temporal lobe epilepsy support an important role of high-mobility group box 1 and toll-like receptor 4 signalling in the mechanisms of hyperexcitability leading to the development and perpetuation of seizures. In this study, we investigated the expression and cellular distribution of toll-like receptors 2 and 4, and of the receptor for advanced glycation end products, and their endogenous ligand high-mobility group box 1, in epilepsy associated with focal malformations of cortical development. Immunohistochemistry showed increased expression of toll-like receptors 2 and 4 and receptor for advanced glycation end products in reactive glial cells in focal cortical dysplasia, cortical tubers from patients with the tuberous sclerosis complex and in gangliogliomas. Toll-like receptor 2 was predominantly detected in cells of the microglia/macrophage lineage and in balloon cells in focal cortical dysplasia, and giant cells in tuberous sclerosis complex. The toll-like receptor 4 and receptor for advanced glycation end products were expressed in astrocytes, as well as in dysplastic neurons. Real-time quantitative polymerase chain reaction confirmed the increased receptors messenger RNA level in all pathological series. These receptors were not detected in control cortex specimens. In control cortex, high-mobility group box 1 was ubiquitously detected in nuclei of glial and neuronal cells. In pathological specimens, protein staining was instead detected in the cytoplasm of reactive astrocytes or in tumour astrocytes, as well as in activated microglia, predictive of its release from glial cells. In vitro experiments in human astrocyte cultures showed that nuclear to cytoplasmic translocation of high-mobility group box 1 was induced by interleukin-1β. Our findings provide novel evidence of intrinsic activation of these pro-inflammatory signalling pathways in focal malformations of cortical development, which could contribute to the high epileptogenicity of these developmental lesions.
INTRODUCTION

Increasing evidence indicates that activation of inflammatory processes in the brain is a common feature of various epileptic disorders. Activation of cells of the microglia/macrophage lineage and astrocytes, associated with concomitant induction of various inflammatory pathways, and expression of inflammatory mediators in neurons, have been observed in epileptic human tissue, including focal malformations of cortical development (MCD), which represent a major cause of paediatric epilepsy (Aronica et al., 2005a, 2007; Boer et al., 2006, 2008; Ravizza and Vezzani, 2006; Ravizza et al., 2006, 2008; Iyer et al., 2010a). Similar to the human condition, a prominent and long-lasting increase in inflammatory mediators has also been described in epileptogenic areas in different experimental models of seizures and epilepsy (Vezzani and Granata, 2005; Aronica and Gorter, 2007; Vezzani et al., 2008).

Experimental studies provide evidence that induction of specific pro-inflammatory pathways in forebrain mediates proconvulsant effects, and their pharmacological modulation represents a potential strategy to reduce seizure activity [reviewed in Vezzani et al. (2008, 2010)]. Particular attention has recently focused on the role of toll-like receptor (TLR) signalling pathways in epilepsy (Rodgers et al., 2009; Maroso et al., 2010; Riazi et al., 2010). TLRs play a key role in pathogen recognition (Kawai and Akira, 2007) as they bind various molecules of microbial origin, called pathogen-associated molecular patterns, and trigger inflammation by inducing the transcription of genes encoding cytokines, including interleukin-1β (IL-1β). Increasing evidence indicates that, in the absence of pathogens, TLR signalling can be activated by molecules released by injured tissue, namely damage-associated molecular patterns, which include high-mobility group box 1 (HMGB1) (Bianchi and Manfredi, 2009). This is almost an ubiquitous chromatin component that is passively released by necrotic cells, retained by cells undergoing apoptosis and actively secreted by cells following immune challenges or various kind of biological stress (Muller et al., 2004). Nuclear HMGB1 regulates transcription of different set of genes, including proinflammatory genes (Pedrazzi et al., 2007; Mouri et al., 2008; Bianchi and Manfredi, 2009); secreted HMGB1 can bind the receptor for advanced glycation end products (RAGE) (Scaffidi et al., 2002) and TLR2 and TLR4 (Park et al., 2004).

Increased expression of genes and proteins involved in the TLR pathways has been detected in epileptogenic focal lesions, such as focal cortical dysplasia, gangliogliomas and cortical tubers of patients with tuberous sclerosis complex (Aronica et al., 2008; Boer et al., 2009). More recent studies in animal models of acute and chronic seizures have demonstrated that HMGB1, via activation of TLR4, plays a role in generating and perpetuating seizures (Maroso et al., 2010). The same work reported evidence of activation of HMGB1–TLR4 axis in human temporal lobe epilepsy. HMGB1 proconvulsant effects are mediated by a neuronal signal-
ling involving tyrosine phosphorylation of the NR2B regulatory subunit of the N-Methyl-d-aspartic acid receptor complex that controls Ca2+ influx (Maroso et al., 2010). The role of HMGB1 binding to RAGE in seizures has not yet been addressed.

The present study was designed to test the hypothesis that activation of HMGB1–TLR4 axis, originally described in temporal lobe epilepsy (Maroso et al., 2010), also occurs in focal epileptogenic developmental lesions, and may additionally involve TLR2 and RAGE, possibly representing a common mechanism of epileptogenesis. A detailed analysis of the cellular expression of HMGB1 and its cognate receptors allowed us to identify both the cellular source of HMGB1 and its targets in epileptogenic tissue.

MATERIAL AND METHODS

Subjects
A total of 18 surgical specimens were examined: six focal cortical dysplasia type IIB, six cortical tubers from patients with tuberous sclerosis complex and six gangliogliomas. The cases included in this study were obtained from the departments of Neuropathology of the Academic Medical Centre (University of Amsterdam) in Amsterdam, the University Medical Centre in Utrecht and the Netherlands. The clinical characteristics derived from the patient’s medical records are summarized in Table 1. Patients underwent therapeutic surgical resection for refractory epilepsy and had, predominantly, medically intractable complex partial seizures. All of the patients included in our series did not have apparent seizure activity in the 24h before surgery. Patients who underwent implantation of strip and/or grid electrodes for chronic subdural invasive monitoring before resection were excluded from the study. For the grading of the degree of focal cortical dysplasia specimens, we followed the classification system proposed by Palmini et al. (2004). All patients with cortical tubers fulfilled the diagnostic criteria for tuberous sclerosis complex (Gomez et al., 1999). For the gangliogliomas, we used the revised WHO classification of tumours of the CNS (Louis et al., 2007). In five patients (one focal cortical dysplasia, one tuberous sclerosis complex and three gangliogliomas), a significant amount of perilesional tissue (normal-appearing cortex/white matter adjacent to the lesion) was resected. Peritumoural tissue (cortex/white matter

Table 1. Summary of clinical details of cases studied according to pathology

<table>
<thead>
<tr>
<th>Pathology type</th>
<th>Number of cases</th>
<th>Mean age (yrs) at surgery (mean ± SD)</th>
<th>Mean age (yrs) at seizure onset (mean ± SD)</th>
<th>Localization</th>
<th>Mean duration (yrs) of epilepsy (mean ± SD)</th>
<th>Seizure frequency (months)</th>
<th>Postoperative epilepsy: Engel’s class</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCD IIB</td>
<td>6</td>
<td>26.2 ± 11.7</td>
<td>7.2 ± 5</td>
<td>Temporal (3)</td>
<td>10.1 ± 7.3</td>
<td>&lt;10 (33%); &gt;20 (67%)</td>
<td>I (67%); ii (33%)</td>
</tr>
<tr>
<td>Cortical Tuber (TSC)</td>
<td>8</td>
<td>17.8 ± 10.1</td>
<td>4.5 ± 2</td>
<td>Frontal (3)</td>
<td>13.5 ± 10.9</td>
<td>&lt;10 (33%); &gt;20 (67%)</td>
<td>I (50%); ii (50%)</td>
</tr>
<tr>
<td>Ganglioglioma (GG)</td>
<td>8</td>
<td>32.0 ± 10.5</td>
<td>15.8 ± 4.4</td>
<td>Temporal</td>
<td>10.1 ± 2.1</td>
<td>&lt;10 (67%); &gt;20 (33%)</td>
<td>I (63%); ii (17%)</td>
</tr>
</tbody>
</table>

FCD = Focal Cortical Dysplasia; TSC = Tuberous Sclerosis; GBM = glioblastoma multiforme
adjacent to the lesion with reactive changes, such as astrogliosis and microglia activation, but not tumour cells) of three patients with brain tumours (two astrocytomas and one lymphoma), but without refractory epilepsy, was also analysed. We also included brain tissue from an autopsy case of tuberous sclerosis complex (34th gestational week, obtained from medically induced abortion with appropriate maternal consent for brain autopsy; kindly provided by Dr M. Sinico, Service d’anatomie pathologique, CHI de Créteil, Creteil, France). In addition, normal-appearing control cortex/white matter was obtained at autopsy from six young adult control patients (male/female 3/3; mean age 30.8; range 14–35 years), without a history of seizures or other neurological diseases. All autopsies were performed within 12 h after death. Informed consent was obtained for the use of brain tissue. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki.

**Real-time quantitative PCR analysis**

Real-time quantitative polymerase chain reaction analysis was performed using RNA prepared from freshly frozen histologically normal human cortex ($n = 5$; autopsy specimens) and specimens of patients with MCD (focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas; $n = 5$ in each series). All the specimens used for the polymerase chain reaction analysis were carefully inspected by microscopy prior to messenger RNA extraction using both histological and immunocytochemical stainings [haematoxylin and eosin, luxol-PAS, glial fibrillary acidic protein (GFAP), neuronal nuclear protein (NeuN)] to confirm that the lesion was present in the sample and attention was taken to provide for RNA isolation equal grey/white matter tissue components. The concentration and purity of RNA (isolated with the TRizol® LS Reagent) were determined spectrophotometrically at 260/280 nm with a nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA). Five micrograms of total RNA were reverse-transcribed into complementary DNA with oligo dT primers. The reverse transcription was performed in 50 µl reactions. Oligo dT primers (5 nmol) were annealed to 5 µg total RNA in a total volume of 25 µl by incubation at 72°C for 10 min and cooled to 4°C. Reverse transcription was performed by the addition of 25 µl RT-mix, containing: First Strand Buffer (Invitrogen-Life Technologies), 2 mM dNTPs (Pharmacia, Germany), 30 U RNase inhibitor (Roche Applied Science, Indianapolis, IN, USA) and 400 U M-MLV reverse transcriptase (Invitrogen—Life Technologies, The Netherlands). The total reaction mix (50 µl) was incubated at 37°C for 60 min, heated to 95°C for 10 min and stored at −20°C until use. Polymerase chain reaction primers (Eurogentec, Belgium) were designed with the Universal ProbeLibrary from Roche (https://www.roche-applied-science.com) on the basis of the reported complementary DNA sequences. The following primers were used: TLR2 (forward: tgatgtgcctcattctcattc; reverse: cgcagctctcagatttaccc), TLR4 (forward: aatccctgaggcatatgagg;
reverse: aaactctggatggggtttc), RAGE (forward: aggaccagggaacctacg; reverse: cctgatcctc-
cacagagc), HMGB1 (forward: aagcacccagatgcttcagt; reverse: tccgcttttgccatatcttc), TATA box-
binding protein (forward: caggagccaagagtgaagaac; reverse: aggaaataactctggctcataactact) and hypoxanthine phosphoribosyl transferase (forward: tggcgtcgtcgtgattagtgatg; reverse: tgaatccagcagtgtaa). For each polymerase chain reaction, a mastermix was prepared
on ice, containing per sample: 1 µl complementary DNA, 2.5 µl of FastStart Reaction Mix SYBR Green I (Roche Applied Science, Indianapolis, IN, USA), 0.4 µM of both reverse and forward primers. The final volume was adjusted to 5 µl with H2O (polymerase chain reaction grade). The LightCycler® 480 Real-Time PCR System (Roche Applied Science) was used with a 384-multiwell plate format. The cycling conditions were carried out as follows: initial dena-
turation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing
at 55–60°C for 5 s and extension at 72°C for 10 s. Fluorescent product was measured by a sin-
gle acquisition mode at 72°C after each cycle. For distinguishing specific from non-specific
products and primer dimers, a melting curve was obtained after amplification by holding
the temperature at 65°C for 15 s followed by a gradual increase in temperature to 95°C at
a rate of 2.5°C/s, with the signal acquisition mode set to continuous. Quantification of data
was performed using the computer programme LinRegPCR in which linear regression on
the Log(fluorescence) per cycle number data was applied to determine the amplification
efficiency per sample (Ramakers et al., 2003). The mean efficiency per primer set and the
individual cycle threshold (Ct) values were then used to estimate the starting concentration
per sample (Karlen et al., 2007). The concentration of each specific product was divided by
the concentration of reference genes (TATA box-binding protein and hypoxanthine phospho-
ribosyl transferase) and this ratio was compared between patient and control groups.

**Tissue preparation for in situ hybridization and immunocytochemistry**
The tissue was fixed in 10 % buffered formalin (J.T. Baker, Deventer, The Netherlands) (au-
topsy tissue, for 2 weeks; surgical specimens, for 24 hours). In all cases a representative
formalin-fixed, paraffin-embedded tissue block was studied, selecting large resection speci-
mens containing normal cortex adjacent to abnormal cortex for comparison, as an internal
control. Paraffin-embedded tissue was sectioned at 6 µm, and mounted on pre-coated glass
slides (StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany) and
two slices in each paraffin block were used for in situ hybridization and immunocytochemi-
cal staining as described below.

**In situ hybridization**
In situ hybridization for human TLR2 and TLR4 was performed using a 5’ fluorescein labeled
19mer antisense oligonucleotide containing Locked Nucleic Acid and 2′OME RNA moieties (TLR2: 5′FAM-1TmAm GlCm UmCl TmGmUl AmGm AITmCmUlGmAmAlG; TLR4: 5′FAM-lTmUm CIUm Um UIAm Cm UIAm GmCITm CmAlTmUmCIC; capitals indicate LNA, lower case indicates 2′OME RNA). The oligo’s were synthesized by Ribotask ApS, Odense, Denmark. The hybridizations were done at 59 °C on 6 µm sections of paraffin embedded materials described previously (Budde et al., 2008). The hybridization signal was detected using a rabbit polyclonal anti-fluorescein/oregon green antibody (A21253, Molecular probes, Invitrogen) and a horse radish peroxidase (HRP) labeled goat anti rabbit polyclonal antibody (P0448 Dako, Glostrup, Denmark) as secondary antibody. Signal was detected with chromogen 3-amino-9-ethyl carbazole (AEC, Sigma, St. Louis, USA).

**Immunocytochemistry**

Glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9, DAKO; 1:400), neuronal nuclear protein (NeuN; mouse clone MAB377, IgG1; Chemicon, Temecula, CA, USA; 1:1000), neurofilament (NF, SMI311; Sternberger Monoclonals, Lutherville, MD; 1:1000), microtubule-associated protein (MAP2; mouse clone HM2; Sigma 1:100), (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400), CD68 (mouse clone PG-M1, DAKO; 1:200) and CD31 (mouse JC/70A; 1:100), were used in the routine immunocytochemical analysis of epilepsy specimens.

For the detection of TLR4 we used a rabbit polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA, sc10741; 1:20;(Maroso et al., 2010)), for TLR2 a polyclonal goat (R & D systems, Abingdon UK; 1:100), for RAGE goat anti-RAGE antibody (1:200, AGE 001; Biologo, Kronshagen, Germany), and for HMGB1 rabbit polyclonal antibody (Pharmigen, San Diego, CA, USA; 1:100;(Maroso et al., 2010); Abcam Cambridge, UK,) and HMGB1 monoclonal mouse (1:100; HMGBiotech srl, kindly provided by Dr. M.E. Bianchi, department of Genetics and Cell Biology, San Raffaele University, Italy). Immunocytochemistry was carried out on paraffin-embedded tissue as previously described (Aronica et al., 2001a). Single-label immunocytochemistry was developed using the Powervision kit (Immunologic, Duiven, The Netherlands). 3,3-Diaminobenzidine (Sigma, St. Louis, USA) was used as chromogen. Sections were counterstained with haematoxylin. To detect differences in labeling related to technical variables such as tissue fixation, we also tested the antibodies in specimens of selected regions (temporal cortex/ hippocampus) collected at autopsy and immediately fixed in formalin for 24 hours (same fixation time used for the surgical specimens). No differences in the immunoreactivity pattern were observed. To evaluate the specificity of the staining the following control experiments were performed on paraffin-embedded specimens: (1) omission of the primary antibody; (2) substitution of the primary antibody with a rabbit
pre-immune or non-immune IgG or a monoclonal mouse IgG of irrelevant specificity; and
(3) preadsorption of polyclonal antibodies using an excess of antigen, or using the specific
immunogen peptide for the anti-human TLR4. These control experiments resulted in the
absence of staining Paraffin-embedded human specimens of multiple sclerosis were used as
positive controls for immunocytochemical staining.
For double-labelling studies, sections, after incubation with primary antibodies, were in-
cubated for 2h at RT with with Alexa Fluor® 568 and Alexa Fluor® 488 (anti-rabbit IgG or
anti-mouse IgG; 1:200; Molecular probes, Eugene, USA). Sections were analyzed by means
of a laser scanning confocal microscope Leica SP2 (Leica Microsystems, Wetzlar, Germany)
equipped with an argon-ion laser.

Evaluation of immunostaining
All labelled tissue sections were evaluated by two independent observers blind to the iden-
tity of the specimens, for the presence or absence of various histopathological parameters
and specific immunoreactivity for the different markers, resulting in an average x% concord-
ance between respective results. Two representative sections per case were stained and
assessed with the TLR, RAGE and HMGB1 antibodies. The intensity of TLR4, TLR2 and RAGE
immunoreactive staining was evaluated as previously described (Iyer et al., 2010b; Ravizza
et al., 2006) using a semi-quantitative scale ranging from 0 to 3 (0: -, no; 1: ±, weak;
2: +, moderate; 3: ++, strong immunoreactivity). All areas of the specimen were examined
and the score represents the predominant cell staining intensity found in each case. The
approximate proportion of cells showing TLR and RAGE immunoreactivity [(1) single to 10
%; (2) 11-50 %; (3) > 50 %] was also scored to give information about the relative number
(‘frequency’ score) of positive cells within the MCD specimens. As proposed before (Aronica
et al., 2005a; Iyer et al., 2010b), the product of these two values (intensity and frequency
scores) was taken to give the overall score (immunoreactivity total score), shown in Fig. 3
and Fig 5 (I-J). Neuronal cell bodies were differentiated from glia and glia-neuronal balloon
cells and giant cells based on morphology. Balloon/giant cells have eccentric nuclei, bal-
looned cytoplasm and did not exhibit clear axonal or dendritic processes.
In the FCD and TSC cases, quantitative analysis was carried out for the numbers of TLR2,
TLR4 and RAGE immunoreactive balloon/giant cells cells, as previously described (Martin-
ian et al., 2009). An identical region in the white matter, beneath the region of dysplasia
or tuber, was outlined at low magnification (x 2.5 objective) on adjacent sections stained
with the different anti-antibodies. One section was randomly selected and the mean area
for quantitative analysis was 5.95 mm². All balloon cells within this region were counted
systematically at high magnification (x 40 objective) as positive immunoreactivity (including
strong or intermediate intensity of labeling) or negative. The percentage of labeled balloon cells (or giant cells) was calculated based on the total number of balloon cells (or giant cells). Quantitative analysis was also performed for HMGB1 and the numbers of positive cells were quantified as previously described (Maroso et al., 2010). Briefly, two representative adjacent non-overlapping fields of the pathological tissue (FCD, TSC, GG and control cortex) were captured (magnification 40x; total area of each field: 171,600 μm²) and digitized using a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA; MRC1024). We counted the total number of GFAP- and HLA-DR (human leukocyte antigen system- DP, DQ, DR) - positive cells, and those showing nuclear or extra-nuclear HMGB1 staining. We counted only activated HLA-DR cells expressing HMGB1 since the morphology of resting or weakly activated microglia (small cell bodies with extensive ramifications) did not allow an accurate counting.

**Cell cultures**

For cell culture experiments (astrocytes-enriched human cultures), fetal brain tissue (22–23 weeks of gestation) was obtained from spontaneous or medically induced abortions with appropriate maternal written consent for brain autopsy. Resected tissue samples were collected in Dulbecco’s modified Eagle’s medium (DMEM)/HAM F10 (1:1) medium (Gibco, Grand Island, NJ). Cell isolation was performed as previously described (Aronica et al., 2003; Aronica et al., 2005c). Briefly, after removal of meninges and blood vessels, tissue was dissociated by incubation at 37 °C for 20 min in a Hank’s balanced salt solution containing 2.5 mg/ml trypsin (Sigma, St. Louis, MO, USA) and 0.1 mg/ml bovine pancreatic Dnase I (Boehringer Mannheim, Germany). Tissue was triturated and washed with DMEM/HAM F10 medium, supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin and 10% fetal calf serum (FCS). Cell suspension (containing ~ 0.5 g wet weight tissue/10 ml culture medium) was passed through a 70μm cell sieve (Becton Dickinson, USA) and plated into poly-L-lysine (PLL; 15 μg/ml, Sigma) pre-coated 25 cm² flasks (Falcon, Lincoln Park, NJ) and maintained in a 5% CO₂ incubator at 37°C. After 48 h the culture medium was replaced by fresh medium and cultures were subsequently fed twice a week. Cultures reached confluence after 2-3 weeks. Secondary astrocyte cultures were established by trypsinizing confluent cultures and sub-planting onto PLL-precoated 25 cm² flasks (2 X 10⁴ cell/ml; for western blot analysis or for the generation of serial passages) and simultaneously into PLL- precoated 12 mm coverslips (Sigma) in 24-well plates (Falcon; 2 X 10⁴ cell/well; for immunocytochemistry). More than 98% of the cells in primary culture, as well as in the successive 12 passages were strongly immunoreactive for the astrocytic marker GFAP. In the present study astrocytes were used for immunocytochemical analyses at passage 3-4. Human recombinant (r)IL-1 β (Peprotech, NJ, USA; 10 ng/ml) was applied and maintained in the medium for 24 h before harvesting.
them for immunocytochemistry. As previously shown (Aronica et al., 2005c), the viability of human astrocytes in culture was not influenced by the treatments. The astrocytoma cell line U373 was obtained from the American Type Culture Collection (Rockville, MD, USA); cells were cultured in (DMEM)/HAM F10 (1:1) supplemented with 50 units/ml penicillin, 50 µg /ml streptomycin and 10 % FCS. IL-1 β (10 ng/ml) was applied and maintained in the serum free medium for 24 h before harvesting them for western blot analysis.

**Preparation of Cellular Extracts and western blot analysis**

Twenty-four hours after IL-1β (10 ng/ml) treatment, medium was collected and glial cells were washed twice with cold phosphate buffered saline. Nuclear and cytoplasmic extracts were prepared as previously described (Hayakawa et al., 2010). Briefly, samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, Na-orthovanadate (10.4 mg/ml), 5 mM EDTA (pH 8.0), 5 mM NaF and protease inhibitor cocktail (Boehringer Mannheim, Germany) by incubating on ice for 15 min. The homogenates were centrifuged at 13000 rpm for 15 min and the supernatant was treated as the cytoplasmic/membrane fraction and the pellet was used as the nuclear fraction. Protein content was determined by the bicinchoninic acid method (Smith et al., 1985). Western blot analysis was performed, as previously described (Aronica et al., 2005b). For electrophoresis, equal amounts of proteins (50 µg/lane) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoretic analysis. Separated proteins were transferred to nitrocellulose paper for 15–30 min at 10 V, using a semi-dry electroblotting system (BioRad, Transblot SD, Hercules, CA, USA). Blots were incubated overnight in Tris buffered saline with Tween (TBST; 20 mM Tris, 150 mM NaCl, 0.1% Tween, pH 7.5)/5% non-fat dry milk, containing the primary antibody (HMGB1 rabbit polyclonal antibody, 1:1000). After several washes in TBST, the membranes were incubated in TBST/5% non-fat dry milk/1% bovine serum albumin, containing the goat anti-rabbit coupled to horseradish peroxidase (1:2500; Dako, Denmark) for 1 h. After washes in TBST, immunoreactivity was visualized with ECL PLUS western blotting detection reagent (GE Healthcare Europe, Diegen, Belgium). Expression of β-actin (monoclonal mouse, Sigma, St Louis, MO, 1:50.000) was used as loading control.

**Statistical analysis**

Statistical analyses were performed with SPSS for Windows (SPSS 11.5, SPSS Inc., Chicago, IL, USA) using two-tailed Student’s t-test and to assess differences between more than two groups ANOVA and a non-parametric Kruskal–Wallis test followed by Mann–Whitney U-test. P < 0.05 was considered significant.
RESULTS

TLR2

mRNA expression. TLR2 mRNA expression was studied using qPCR in MCD: an average 3- to 4-fold increase in mRNA expression was observed in FCD, TSC and GG as compared to control cortex (p<0.05, vs autopsy specimens; Fig. 1 A). In situ hybridization was additionally performed to study the cellular distribution of TLR2 mRNA in FCD cases, which confirmed the protein expression pattern (see below). TLR2 mRNA was detected in balloon cells and in glial cells of FCD specimens (Fig. 1 C), but was not detected in neurons or in resting glial cells in control cortex (both autopsy material and the perilesional surgical cortex; not shown).

Immunoreactivity. In human control cortical autopsy specimens, as well as in the normal-appearing cortex adjacent to the lesions (not shown), TLR2 immunoreactivity was not detected either in neurons or in glial cells in both cortex and white matter throughout all cortical layers (Fig.2 A,B; Fig. 3 A,C).

In FCD, TSC and GG the dysplastic neuronal cells were not labeled with TLR2 (Fig.2 C, H, inset b in J and Fig. 3 A). In contrast, 54.16 ± 11% of balloon cells in FCD and 51.3 ± 14% of giant cells in TSC showed TLR2 staining (Fig. 2 D-G, H-I; see immunoreactive score (I.S.) in Fig. 3 B). TLR2 immunoreactivity was also observed in cells exhibiting glial morphology (Fig. 2 C, D, J; Fig. 3 C).

Double labeling demonstrated TLR2 expression in cells of the microglial/macrophages lineage in all series (HLA-DR positive cells; Fig. 2 K-N), but not in neuronal cells (NeuN positive cells; not shown). Reactive astrocytes and endothelial cells within the dysplastic cortex only occasionally displayed TLR2 immunoreactivity (not shown).

TLR4

mRNA expression. An average 4-fold increase in TLR4 messenger RNA expression was observed by quantitative polymerase chain reaction in focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas as compared with control cortex (P<0.05 versus autopsy specimens; Fig. 1B). In situ hybridization analysis was additionally performed to study the cellular distribution of TLR4 messenger RNA in focal cortical dysplasia cases, which confirmed the protein expression pattern (see below). In focal cortical dysplasia, TLR4 messenger RNA was detected in neurons and in glial cells (Fig. 1D) but not in neurons or in resting glial cells in control cortex (both autopsy material and the perilesional surgical cortex; data not shown).

Immunoreactivity. In human control cortical autopsy specimens, TLR4 immunoreactivity was not detected in neurons throughout all cortical layers (Fig. 4A); only in one case, faint immunoreactivity was observed in few pyramidal neurons. Glial staining was not observed...
in both cortex and white matter (Fig. 4B and D). In focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas, strong TLR4 expression was observed in a large number of dysplastic neuronal cells (62.8 ± 5.5 in focal cortical dysplasia, 61.7 ± 16 in tuberous sclerosis complex and 57.8 ± 18 in gangliogliomas; Figs 4C, F, H and 3A). In contrast, only 1.6 ± 0.9% of balloon cells in focal cortical dysplasia and 1.3 ± 0.5% of giant cells in tuberous sclerosis complex showed TLR4 staining (Figs 4D–I and 3B). TLR4 immunoreactivity was also detected in cells exhibiting glial morphology (Figs 4E, H and 3D). Double labelling demonstrated TLR4 expression in neuronal cells and in GFAP-positive astrocytes (Fig. 4I–K). Activated microglial cells within the dysplastic cortex were occasionally TLR4-positive [Fig. 4K (inset a)]. As compared with the staining in normal brain, peritumoural tissue, with evidence of astrogliosis

Figure 1. TLR2 and TLR4 and RAGE messenger RNA expression in control and focal malformations of cortical development. Real-time polymerase chain reaction. (A, B and E) Expression levels were determined in duplicate, corrected for the expression levels of TATA box-binding protein and hypoxanthine phosphoribosyl transferase. Expression levels in control cortex (n = 5), focal cortical dysplasia (FCD, n = 5), cortical tubers of tuberous sclerosis complex (TSC, n = 5) and ganglioglioma (GG, n = 5). TLR2 (A), TLR4 (B) and RAGE (E) messenger RNA levels were significantly increased in epilepsy specimens as compared with controls. There were no significant differences in TLR2 and TLR4 between focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas. The error bars represent SEM; *P < 0.05. In situ hybridization analysis of TLR2 (C) and TLR4 (D) expression. (C) Expression is observed in cells with glial morphology (arrows) and balloon cells (inset). (D) Expression is observed in neurons (arrows) and glial cells (arrow-head and inset). Sections are counterstained with haematoxylin. Scale bar: C = 40 μm; D = 80 μm.
and microglia activation, from patients without history of epilepsy, showed low or undetectable immunoreactivity for both TLR2 and TLR4 (Fig. 7A–C). As compared with the staining in normal brain, peritumoural tissue, with evidence of astrogliosis and microglia activation, from patients without history of epilepsy, showed low or undetectable immunoreactivity for both TLR2 and TLR4 (Fig. 7A–E). HMGB1 immunoreactivity was similar to control cortex, showing only detectable nuclear staining (Fig. 7E, inset).

RAGE

mRNA expression. An average 2- to 3-fold increase in RAGE messenger RNA expression was found using quantitative polymerase chain reaction in focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas as compared with control cortex (P < 0.05 versus autopsy specimens, Fig. 1E).

Immunoreactivity. In human control cortical autopsy specimens, variable RAGE immunoreactivity was detected in neurons throughout all cortical layers (Fig. 5A, arrows and inset a; inset b shows absence of neuronal immunoreactivity after pre-absorption); only low or undetectable staining was observed in glial cells (Fig. 5B and J). In focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas RAGE immunostaining was observed in a large number of dysplastic neuronal cells (74.6 ± 6.3 in focal cortical dysplasia, 73.4 ± 13 in tuberous sclerosis complex and 77.9 ± 16 in gangliogliomas) (Fig. 5C, E, G and I); 67.12 ± 13% of balloon cells in focal cortical dysplasia and 61.7 ± 9% of giant cells in tuberous sclerosis complex showed RAGE staining. RAGE immunoreactivity was also detected in cells exhibiting glial morphology (Fig. 5C–H and J) phenotypically identified by double labelling as GFAP-positive cells (Fig. 5H) and activated microglial cells (Fig. 5H, inset).

Figure 2. Distribution of TLR2 immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining for TLR2 in the control cortex (A, Ctx) and white matter (B, Wm) showing undetectable expression in neurons and in glial cells. (C–G) Representative photomicrographs of immunohistochemical staining for TLR2 in focal cortical dysplasia (FCD) specimens. (C) Positive glial cells (with the morphology of microglial cells; arrows) within the dysplastic cortex; arrowhead in C indicates a TLR2-negative dysmorphic neuron; insets show high magnification of positive cells around a blood vessel (a) and in the vicinity of a dysmorphic neuron (b, arrowhead). (D) Positive glial cells within the white matter (arrows; high magnification in inset); arrowheads indicate positive balloon cells. (E–G) Strong TLR2 immunoreactivity in balloon cells (arrows in E, high magnification in F and G). (H–J) Representative photomicrographs of TLR2 immunoreactivity in cortical tubers of tuberous sclerosis complex (TSC) showing positive giant cells (asterisks in H, arrows in I), glial cells (arrow in H) and a negative dysmorphic neuron (arrowhead in H). (J) TLR2 immunoreactivity in ganglioglioma (GG) showing different positive glial cells (arrows and inset a); inset b shows a negative dysplastic neuron (arrowhead). Sections were counterstained with haematoxylin. (K–N) Focal cortical dysplasia. (K) HLA-DR (green) positive microglial cells; (L) TLR2 (red) expression in a balloon cell (arrowhead) and microglial cells (arrows); (M) merged image showing colocalization in microglial cells (yellow; nucleus, blue, DAPI). (N) Merged image showing colocalization of TLR2 (red) with (HLA-DR, green) in cells of the microglia/macrophage lineage in ganglioglioma. Scale bar: A and B: 80 µm; C, D, H–N: 40 µm; E: 160 µm; F and G: 20 µm.
HMGB1

mRNA expression. No statistically significant differences were detected using quantitative polymerase chain reaction in focal cortical dysplasia, tuberous sclerosis complex as compared with control cortex (P > 0.05 versus autopsy specimens, not shown). Immunoreactivity. In human control cortical autopsy specimens, HMGB1 immunoreactivity was detected in nuclei of both neurons (Fig. 6A; arrows) and glial cells (Fig. 6A; arrowheads). In focal cortical dysplasia, tuberous sclerosis complex and ganglioglioma (GG), neuronal cells, balloon and giant cells displayed exclusively nuclear HMGB1 staining, whereas cytoplasmic staining was substantially increased in glial cells (Fig. 6C–K; see quantification in Q and R). Double labelling confirmed the HMGB1 expression in neuronal cells, and in both astrocytes and activated microglial cells (Fig. 6L–P).

No significant correlation was found between the increased cytoplasmic HMGB1 staining in glia or TLR2, TLR4 and RAGE staining in tissue specimens, and the seizure frequency before surgery, or postoperative seizure outcome (data not shown). The pattern of immunoreactivity observed for HMGB1, TLR2 and TLR4, and RAGE in control autopsy specimens was similar to that observed in normal appearing cortex adjacent to the lesions (data not shown).
Figure 4. Distribution of TLR4 immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining for TLR4 in the control cortex (A, Ctx) and white matter (B, Wm) showing undetectable expression in large majority of neurons (arrows) and in glial cells (arrowheads). (C–G) Representative photomicrographs of immunohistochemical staining for TLR4 in focal cortical dysplasia (FCD) specimens. (C) Positive neuronal cells within the dysplastic cortex; inset: a positive dysmorphic neuron (arrow; arrowhead, positive glial cell). (D) Balloon cells (arrows) with faint TLR4 immunoreactivity; faint immunoreactivity is occasionally detected in a few balloon cells (inset). (E) A negative balloon cell (arrow), surrounded by positive glial cells (arrowheads). (F and G) Representative photomicrographs of TLR4 immunoreactivity in cortical tubers of tuberous sclerosis complex with neuronal (arrows in F) and glial immunoreactivity (inset in F), whereas the large majority of giant cells are negative (arrow in G). (H) TLR4 immunoreactivity in ganglioglioma (GG) showing different positive glial [arrowheads and neuronal cells (inset)]. Sections were counterstained with haematoxylin. (I–K) GFAP (I, green), TLR4 (J, red) and merged image (K) showing colocalization in focal cortical dysplasia. (K) Merged images showing TLR4 positive cells (red) in focal cortical dysplasia, but lack of colocalization with HLA-DR (green); occasionally expression is observed in HLA-DR positive cells (inset a); inset b shows colocalization with the neuronal marker NeuN. Scale bar: A, B and D: 80 µm; C: 160 µm; E: 20 µm; F–H, K–L: 40 µm.
As compared with the staining in normal brain, peritumoural tissue with evidence of astrogliosis and microglia activation from patients without history of epilepsy showed low or undetectable immunoreactivity for both TLR2 and TLR4; the HMGB1 immunoreactivity was similar to control cortex, showing only detectable nuclear staining (Fig. 7A–E). A pattern of immunoreactivity similar to control was also observed for RAGE (data not shown). In contrast, increased expression of TLR2, TLR4 and HMGB1 was observed within the tuber, compared with non-tuberal cortex, in a case of tuberous sclerosis complex (34th gestational week, before the development of seizures; Fig. 7F–J).

**Cell cultures**

Astrocyte-enriched human cell cultures and glioma cells were exposed to IL-1β to study whether this cytokine, which is prominently expressed in MCD (Ravizza et al., 2006; Aronica et al., 2008; Boer et al., 2008), affects the cellular localization of HMGB1, as suggested by previous evidence in rat astrocytes (Hayakawa et al., 2010) (Fig. 7). In unchallenged astrocytes, HMGB1 was localized only in the nuclei (Fig. 8A and C), whereas after IL-1β exposure, HMGB1 signal was also localized in the cytoplasm (Fig. 7B and E), indicating nuclear to cytoplasmic translocation, as detected in surgical specimens, and predictive of its subsequent release (Muller et al., 2004; Hayakawa et al., 2010). Western blot analysis demonstrated that IL-1β increased the expression of HMGB1 in the cytoplasmic fraction of glial cells and showed that the response to this cytokine involved an active release of HMGB1 into the culture media (Fig. 8E).

Figure 5. Distribution of RAGE immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining of RAGE in the control cortex (A, Ctx) and white matter (B, Wm) showing moderate expression in some pyramidal neurons (arrows; inset in A; inset b shows absence of neuronal immunoreactivity after pre-absorption) and low or undetectable expression in glial cells (arrows in B and inset). (C and D) Representative photomicrographs of immunohistochemical staining for RAGE in focal cortical dysplasia (FCD) specimens. (C) A positive dysmorphic neuron (arrow) and positive glial cells (arrowheads). (D) Balloon cells (arrows) with RAGE immunoreactivity; (E) neuronal (arrows) and glial immunoreactivity (arrowheads and inset) within the dysplastic cortex; (E and F) Representative photomicrographs of RAGE immunoreactivity in cortical tubers of tuberous sclerosis complex (TSC). (F) RAGE immunoreactivity within the white matter with positive balloon cells (arrows and inset); arrowheads indicate positive blood vessels. (G) RAGE immunoreactivity in ganglioglioma (GG) showing different positive neuronal cells (arrows); arrowheads indicate positive blood vessels. Inset (a) in G shows a positive astrocyte; inset (b) in G shows colocalization with GFAP (merged image). (H) Merged images showing colocalization of RAGE (red) with (GFAP, green) in both reactive astrocytes and balloon cells in focal cortical dysplasia; inset in H shows colocalization with HLA-DR. Sections (single labelling) were counterstained with haematoxylin; DAPI stains the nucleus in the confocal images. (I and J) Plots depicting the cellular distribution of RAGE in controls, focal cortical dysplasia, cortical tubers of tuberous sclerosis complex and ganglioglioma (GG) in neurons and balloon/giant cells (BCs/GCs; I) and in glial cells (J). The immunoreactivity score represents the total score, which was taken as the product of the intensity score and the frequency score (for details refer to the Materials and methods section). Scale bar: A, B and G: 80 µm; C, D and H: 40 µm; E: 70 µm; F: 160 µm.
Figure 6. High-mobility group box 1 (HMGB1) immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining for HMGB1 in the control cortex (A, Ctx) and white matter (B, Wm) showing nuclear immunoreactivity in pyramidal neurons (arrows and inset in A) and in glial cells (arrowheads in A and B; inset in B). (C–F) Representative photomicrographs of immunohistochemical staining of HMGB1 in focal cortical dysplasia (FCD) specimens. (C and D) Nuclear expression is observed in both neuronal and glial cells. Insets (a and b) in C show high magnifications of dysmorphic neurons with nuclear immunoreactivity; arrows in C (inset a) and in D show glial cells with both nuclear and cytoplasmic staining. (D) High magnification of an immunoreactive glial cells and glial processes. (E) Nuclear immunoreactivity in a balloon cell. (F) Nuclear and cytoplasmic staining in perivascular glial cells (arrows). (G–J) Representative photomicrographs of HMGB1 immunoreactivity in cortical tubers of tuberous sclerosis complex (TSC). (G) Dysmorphic neurons with nuclear immunoreactivity (arrows) and glial cells with both nuclear and cytoplasmic staining (arrowheads). (H and I) HMGB1 immunoreactive glial cells and processes (arrowheads) within the white matter, with occasionally few negative dysmorphic neurons (arrow in H). (J) Giant cell showing only nuclear staining. (K) HMGB1 immunoreactivity in ganglioglioma (GG) showing neurons with nuclear immunoreactivity (arrows) and glial cells with both nuclear and cytoplasmic staining (arrowheads). Inset (a) shows glial cells with prominent cytoplasmic immunoreactivity; inset (b) shows high magnification of a positive neuron and a glial cell with immunoreactive processes. Sections were counterstained with haematoxylin. (L–N) Merged images showing colocalization of HMGB1 (red) with NeuN (green; L), GFAP (green; M), HLA-DR (green; N) in focal cortical dysplasia. (O and P) Merged images showing colocalization of HMGB1 (red) with GFAP (green; O), and HLA-DR (green; P) in gangliogliomas. (Q and R) Quantification bar chart of HMGB1-positive cells (astrocytes in Q and microglia/macrophages in R) in control, focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas specimens. Extraneuronal staining: *P<0.05 versus control, one-way ANOVA followed by Tukey’s test. nd = not detectable. Scale bar: A, B and K: 160 µm; C, E, H, I, L, N and P: 80 µm; G and J: 20 µm; D, F, M and O: 40 µm.

Figure 8. Relocation of nuclear HMGB1 in human astrocytes and glioma cells induced by IL-1β. (A and B) Confocal laser scanning microscopic images showing merged signal of HMGB1 (red) and DAPI (blue) in cultured human astrocytes and glioma cells (C and D) untreated (A and C) and treated (B and D) for 24 h with 10 ng/ml IL-1β. (A and C) HMGB1 was located only in the nuclei of glial cells. (B and D) After exposure to IL-1β, HMGB1 signal was observed also in the cytoplasm. (E) Western blot analysis in glioma cells showed that the expression levels of HMGB1 were increased in the cytosolic fraction 24 h after IL-1β stimulation (evidence of translocation) and increased HMGB1 level was also detected in culture medium (evidence of active release). Scale bar: A, D and F: 15 µm; B: 20 µm.
Recent data obtained in different experimental models of acute and chronic seizures, identified the crucial role played by the activation of HMGB1–TLR4 signalling in the hippocampus in the generation and recurrence of seizures (Maroso et al., 2010). This evidence is corroborated by findings of increased levels of HMGB1 and TLR4 in surgical specimens from patients with temporal lobe epilepsy and hippocampal sclerosis, supporting the involvement of the HMGB1–TLR4 axis in human epilepsy (Maroso et al., 2010).

In the present study, we demonstrate the intralesional overexpression and cellular distribution of HMGB1 and its cognate receptors TLR2, TLR4 and RAGE in focal cortical dysplasia, tuberous sclerosis complex and ganglioglioma specimens from patients with medically intractable epilepsy. These findings provide evidence of a chronic inflammatory state involving these novel pathways in human epileptic developmental lesions.

**DISCUSSION**

Recent data obtained in different experimental models of acute and chronic seizures, identified the crucial role played by the activation of HMGB1–TLR4 signalling in the hippocampus in the generation and recurrence of seizures (Maroso et al., 2010). This evidence is corroborated by findings of increased levels of HMGB1 and TLR4 in surgical specimens from patients with temporal lobe epilepsy and hippocampal sclerosis, supporting the involvement of the HMGB1–TLR4 axis in human epilepsy (Maroso et al., 2010).

In the present study, we demonstrate the intralesional overexpression and cellular distribution of HMGB1 and its cognate receptors TLR2, TLR4 and RAGE in focal cortical dysplasia, tuberous sclerosis complex and ganglioglioma specimens from patients with medically intractable epilepsy. These findings provide evidence of a chronic inflammatory state involving these novel pathways in human epileptic developmental lesions.

**Differential cellular distribution of TLRs and RAGE in MCD**

**TLR2 and TLR4.** These receptors play a critical role in determining the pathological outcomes in several neurological disorders, including autoimmune diseases, neurodegeneration, trauma, stroke and more recently, epilepsy (Bsibsi et al., 2002; Aravalli et al., 2007; Crack and Bray, 2007; Andersson et al., 2008; Drexler and Foxwell, 2010; Maroso et al., 2010).

In the present study, we provide the first evidence of cell-specific upregulation of both messenger RNA and protein levels of TLR2 and TLR4 in different MCD. In histologically normal cortex (autopsy and surgical tissue samples), both receptors could be detected by real time-polymerase chain reaction while messenger RNAs and respective proteins were below detection levels in neuronal and resting glial cells as assessed by in situ hybridization and immunohistochemistry. In agreement, the expression of TLR2 and TLR4 messenger RNA has been shown to be generally low in brain, as compared with other tissues (Nishimura and Naito, 2005).

Immunocytochemical analysis showed a differential cellular distribution of TLR2 and TLR4; TLR2 protein was consistently and predominantly expressed in activated microglia within...
the different epileptogenic lesions, in agreement with previous studies in microglial cell cultures [for review see Kielian (2006)]. Notably, TLR2 has been suggested to play a role in mediating microglia activation (Babcock et al., 2006; Kielian, 2006; Aravalli et al., 2007; Mallard et al., 2009). Moreover this receptor is involved in the induction of inflammatory pathways (O’Neill et al., 2009) and thus it may contribute to the increase in inflammatory mediators in microglia previously described in epileptogenic human tissue (Aronica et al., 2005a, 2007; Boer et al., 2006, 2008; Ravizza et al., 2006, 2008).

In contrast to its commonly recognized microglial expression, the expression of TLR2 in astrocytes is more controversial [for review see Kielian (2006)]. Accordingly, we have been able to detect sporadic expression of TLR2 in reactive astrocytes, which were abundantly present in the epileptic lesions examined. However, we report expression of TLR2 in balloon and giant cells, but not in dysplastic neurons, in higher proportion as compared with TLR4. Since balloon and giant cells represent a significant source of proinflammatory molecules (Ravizza et al., 2006; Boer et al., 2008), the evaluation of the TLR2 function in these cell types deserves further investigation, with a recently described culture system (Yasin et al., 2010).

TLR4, differently from TLR2, showed a prominent expression in both reactive astrocytes and neuronal cells. Studies examining TLR4 expression in astrocytes in vitro have produced conflicting results [reviewed in Crack and Bray (2007)]; in some studies astrogial expression of TLR4 could not be demonstrated (Farina et al., 2005; Kielian, 2006) whereas other studies have shown a constitutive expression of TLR4 in astrocytes and its upregulation following cell activation (Bsibsi et al., 2002; Bowman et al., 2003; Carpentier et al., 2005). These discrepancies may reflect differences in cell source and culture conditions (Kielian, 2006); alternatively, lack of TLR4 induction may be ascribed to insufficient release of pro-inflammatory cytokines (such as IL-1β) or damage-associated molecular patterns (such as HMGB1) by the injured/activated cells in some of these studies. Accordingly, TLR4 expression in astrocytes has been observed in experimental models of seizures associated with release of IL-1β and HMGB1 by neurons and astrocytes (Maroso et al., 2010).

TLR4 expression in dysplastic neuronal cells may critically affect neural homeostasis [for reviews see Crack and Bray (2007); Mallard et al. (2009)]. The activation of this receptor may indirectly enhance neuronal Ca2+ influx through the phosphorylation of NMDA-NR2B receptors, supporting neuronal hyperexcitability (Maroso et al., 2010), and thus playing a role in the epileptogenicity of focal MCD. Notably, dysplastic neurons in focal cortical dysplasia and gangliogliomas specimens express high levels of NR1 and NR2A/B subunit proteins (Ying et al., 1999; Aronica et al., 2001b, c), and NR2A/B distribution correlates with in situ epileptogenicity in patients with focal cortical dysplasia (Najm et al., 2000; Moddel et al., 2001).
Although a rapid induction of TLR4 has been shown in different experimental models of lesional and non-lesional seizures (Maroso et al., 2010), seizures alone may not account for changes in its neuronal and glial expression in MCD since perilesional tissue, although exposed to seizures, did not show significant upregulation of TLR4. Therefore, the lesion per se, or the concomitant presence of the lesion and the epileptic activity, is likely to play a role in modulating the TLR system in these developmental disorders.

**Receptor for advanced glycation end products**

In human control brain, RAGE expression has been found in neurons and glial cells, and its expression is increased in activated astrocytes and microglial cells in tissue of patients with neurodegenerative disorders, such as Alzheimer's and Huntington's disease (Lue et al., 2005; Ma and Nicholson, 2004; Sasaki et al., 2001).

In our study, prominent RAGE expression was observed in both glial and neuronal cells in different MCD. RAGE was consistently expressed in activated astrocytes and microglial cells, as well as in dysplastic neurons and balloon/giant cells in FCD and TSC.

RAGE is a receptor that binds different molecules, including HMGB1 and members of the S100 protein family (Sims et al., 2010). Interestingly, overexpression of both HMGB1 and S100B has been observed in human TLE, and our study showed changes in HMGB1 in MCD (see below) (Griffin et al., 1995; Maroso et al., 2010). While HMGB1-TLR4 interaction has been studied in experimental model of seizures, the role of the HMGB1-RAGE axis, and possibly S100 ligands, in epilepsy awaits further investigation (Dyck et al., 2002; Sakatani et al., 2007; Sakatani et al., 2008; Sims et al., 2010).

**Cellular distribution of HMGB1 in control cortex and MCD**

HMGB1 is a nearly ubiquitous structural nonhistone chromatin protein involved in the regulation of transcription of a set of inflammatory genes (Bianchi and Manfredi, 2007; Mouri et al., 2008; Pedrazzi et al., 2007). However, HMGB1 can be released from cells upon its cytoplasmatic translocation under immune/inflammatory challenges or injurious conditions (Bianchi and Manfredi, 2009; Czura et al., 2001). Extracellular HMGB1 acts as a “danger signal” to orchestrate an homeostatic defensive response in challenged tissues; however, its pro-inflammatory properties, acquired upon its cellular release and consequent TLR/RAGE stimulation, appear to contribute to the pathogenesis of various inflammatory and CNS diseases (Bianchi, 2009; Bianchi and Manfredi, 2007; Hreggvidsdottir et al., 2009).

In histologically normal temporal cortex (autopsy and surgical tissue samples), HMGB1 is expressed in nuclei of neuronal and glial cells. Accordingly, a similar pattern of expression has been reported in human control hippocampus (Maroso et al., 2010).
In MCD, HMGB1 remained predominantly nuclear in dysplastic neurons and balloon/giant cells, suggesting that it may contribute to regulate inflammatory gene transcription in these cells (Bianchi and Manfredi, 2009; Mouri et al., 2008; Pedrazzi et al., 2007; Ravizza et al., 2006). Differently, we observed a cytoplasmic translocation of HMGB1 in reactive astrocytes in FCD and TSC, in tumor astrocytes in GG and in activated microglia in all MCD cases. A cytoplasmic translocation in activated glia has been recently reported also in experimental models of seizures and in hippocampal tissue from TLE patients (Maroso et al., 2010). These findings indicate that glial cells are a major source of extracellular HMGB1 in epileptic pathologies.

We show that IL-1β induces the relocation of nuclear HMGB1 to the cytoplasm and release in human cultured astroglial cells, as previously reported in rat cultured astrocytes (Hayakawa et al., 2010). Since IL-1β is up-regulated in epileptogenic tissue from MCD and TLE (Vezzani et al., 2008), this pro-inflammatory cytokine may play a pivotal role in inducing HMGB1 release from glia in human epilepsy. Since microglia and astrocytes have been shown to respond to HMGB1 stimulation with production of several inflammatory mediators (Andersson et al., 2008; Kim et al., 2006; Pedrazzi et al., 2007), these cells are likely to provide a positive feedback loop that amplifies the inflammatory response in epilepsy-associated MCD.

HMGB1 has been suggested to promote stem cell migration and differentiation (Chavakis et al., 2007; Huttunen et al., 2002; Palumbo et al., 2004), raising the possibility that HMGB1 release within the epileptogenic lesions interferes with neuronal migration and differentiation during brain development (Flores-Sarnat et al., 2003; Lamparello et al., 2007; Yasin et al., 2010).

Although the activation of HMGB1–TLR/RAGE pathways has been shown in several neurological disorders associated with gliosis (Bsibsi et al., 2002; Aravalli et al., 2007; Crack and Bray, 2007; Andersson et al., 2008; Drexler and Foxwell, 2010), this phenomenon is not simply the result of glial activation, since non-epileptic perilesional tissue with reactive gliosis show immunoreactivity patterns similar to control tissue. Seizures alone may not be the only determinants of the observed effects since perilesional tissue with normal morphology, but probably exposed to similar seizure activity, did not show detectable changes in TLR/HMGB1 expression as compared with control tissue. Moreover, prenatal TLRs and HMGB1 expression was found in giant cells within the tuber in tuberous sclerosis complex. It is likely, therefore, that the induction of these signalling pathways is intrinsic to the developmental lesion per se, and the concomitant occurrence of seizures could contribute to perpetuate this activation. Interestingly, the mTOR (mammalian target of rapamycin) pathway is concomitantly activated in these lesions (Iyer et al., 2010b), and this signalling plays a role both
in regulating cell growth and size, as well as in activating immune/inflammatory responses (Lim et al., 2003; Schmitz et al., 2008; Weichhart and Saemann, 2009). This study, together with our former functional and pharmacological evidence obtained in experimental models of temporal lobe epilepsy (Maroso et al., 2010), supports the role of HMGB1–TLR/RAGE pathways in the mechanisms underlying the intrinsic high epileptogenicity of focal developmental glioneuronal lesions. In the absence of genuine models of MCD (Wong, 2009), we believe that the HMGB1-mediated proconvulsant mechanism recently described in temporal lobe epilepsy models, and concordant with data in temporal lobe epilepsy human tissue, would reasonably support that the same mechanism is likely to be operative in MCD epileptogenic areas. Thus, pharmacological modulation of the HMGB1–TLR/RAGE axis with receptor antagonists or inactivating antibodies (Yang et al., 2002; Hennessy et al., 2010) may represent a potential novel antiepileptic strategy in MCD. The evidence we provide here using human epileptogenic surgical tissue offers instrumental information to guide future studies into the possibility of interfering with inflammatory mechanisms in humans to prevent or alleviate seizures associated with developmental lesions [ClinicalTrials.gov Study of VX-765 in Subjects with Treatment-resistant Partial Epilepsy [online], http://clinicaltrials.gov/ct2/show/NCT01048255 (2010)].

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2.4 The inflammatory molecules IL-1β and HMGB1 can rapidly enhance focal seizure generation in rat entorhinal cortex.

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ABSTRACT

Purpose
Inflammatory signalling pathways involving Interleukin-1 β (IL-1β) and high-mobility group B1 (HMGB1) are recognized to increase the susceptibility to epileptic seizures. In the present study, we investigated the role of these proinflammatory molecules in the generation of focal seizure-like discharge in slice preparations from the entorhinal cortex (EC).

Methods
Seizure like-discharges were evoked by either slice perfusion with low Mg2+ and picrotoxin or local stimulation with NMDA applied in the presence of the proconvulsant 4-amino-pyridine (4-AP). The effects of local applications with IL-1β or HMGB1 were evaluated by monitoring seizure discharge generation through laser scanning microscope imaging of the Ca2+ signal from tens of neurons and astrocytes.

Key findings
We revealed that upon local IL-1β or HMGB1 applications a single NMDA pulse, per se ineffective, could evoke a focal ictal discharge (fID). HMGB1 enhanced the direct response of neurons to NMDA stimulation, but only after the neuronal network experienced a sustained epileptiform activity.

Significance
These findings demonstrate that both IL-1β and HMGB1 can rapidly lower focal ID threshold and raise the possibility that targeting these inflammatory pathways may represent an effective therapeutic strategy to prevent seizures.
INTRODUCTION

Epilepsy is a neurological disorder characterized by recurring, unprovoked seizures. It affects about 1% of the population worldwide and often requires lifelong medication (Hauser, Annegers et al. 1993). About 30% of epileptic patients are considered drug resistant as they do not respond to the currently available anti-epileptic drugs (AEDs) (Perucca, French et al. 2007). Increasing evidence supports the involvement of inflammatory and immune processes in the etiopathogenesis of seizures (Vezzani and Granata 2005). Inflammation induced by brain-damaging events such as trauma, stroke, infection, febrile seizures and status epilepticus is associated with acute symptomatic seizures and a high risk of developing epilepsy (Pitkanen and Sutula 2002; Bartfai, Sanchez-Alavez et al. 2007). In particular, high levels of proinflammatory cytokines [e.g. interleukin (IL)-1beta, tumour necrosis factor (TNF)-alpha], danger signals [high-mobility group box (HMGB)1, S100 beta] and downstream inflammatory mediators (e.g. prostaglandins, the complement system) have been measured in epileptogenic tissue from patients affected by epilepsy of various aetiologies (Vezzani, French et al. 2011). The major contributors to the synthesis of these inflammatory mediators are brain-resident cells such as activated microglia, astrocytes and neurons.

Recently, two proinflammatory molecules were found to be proconvulsant in animal models of temporal lobe epilepsy (TLE): IL-1b and HMGB1. These molecules, applied before the induction of experimental TLE, were able to increase the time spent in seizures and reduce the onset time of the first seizure. The effects of HMGB1 and IL-1β seem to be very similar, especially because both are blocked by ifenprodil (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010), a selective antagonist of NR2B-containing NMDA receptors (Yu, Askalan et al. 1997).

We tested here the hypothesis that IL-1b and HMGB1 act as proconvulsant in two different models of focal seizure-like discharges in rat entorhinal cortex (EC) slices (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). To evoke focal seizure-like, ictal discharges (IDs) we used either slice perfusion with low Mg2+ / picrotoxin or pressure pulses applied to an N-methyl-D-aspartate (NMDA)-containing glass pipette to stimulate an episode of hyperactivity in a small number of layer V–VI neurons, in the presence of the proconvulsant 4-AP and low Mg2+. Fast laser scanning microscope imaging of Ca2+ signals from tens of neurons revealed that local applications with either IL-1b or HMGB1 rapidly favor the generation of an epileptogenic site for focal ID initiation.
MATERIALS AND METHODS

Brain slices and dye loading

Transverse cortico-hippocampal slices were prepared from postnatal day 14–18 Wistar rats and loaded with OGB1-AM (excited at 488 nm) or Rhod-2 (excited at 543 nm), respectively, as previously described (Losi, Cammarota et al. 2010). Briefly, brain was removed and transferred to ice-cold cutting solution containing (in mM): NaCl, 120; KCl, 3.2; KH₂PO₄, 1; NaHCO₃, 26; MgCl₂, 2; CaCl₂, 1; glucose, 10; Na-pyruvate, 2; and ascorbic acid, 0.6; at pH 7.4 (with 5% CO₂/95% O₂). Coronal slices were obtained by cutting with a Leica vibratome VT1000S in the presence of the ionotropic glutamate receptor inhibitor kynurenic acid (2 mM). Slices were recovered for 15 min at 37°C and then loaded with the Ca²⁺-sensitive dye OGB1-AM (Invitrogen) for 60 min at 37°C. Loading was performed in the cutting solution containing sulfinpyrazone (200 µM), pluronic (0.12%), and kynurenic acid (1 mM). After loading, slices were recovered and kept at room temperature in the presence of 200 µM sulfinpyrazone. All experiments were performed at 33–35°C.

Ca²⁺ imaging

Brain slices were visualized with a TCS-SP2-RS or a TCS-SP5-RS confocal microscope (Leica) equipped with a 20× objective (NA, 1.0) and a CCD camera for differential interference contrast images. Time frame acquisitions from 314 ms to 1.24 s (with one to six line averaging) were used. No background subtraction or other manipulations were applied to digitized Ca²⁺ signal images that are reported as raw data. Neurons and astrocytes were distinguished on the basis of the distinct kinetics of their Ca²⁺ response to a stimulation with high K⁺ extracellular solution (40 mM) obtained by isosmotic replacement of Na⁺ with K⁺, applied at the end of the recording session in the presence of 1 µM TTX. Due to the lack of voltage-dependent Ca²⁺ channels in astrocytes, the Ca²⁺ elevation in these cells upon high K⁺ stimulation occurs with a delay of several seconds with respect to the response in neurons, and appears to be mediated by glutamate release from depolarizing neurons. The onset of the slow Ca²⁺ elevation in astrocytes was determined on the basis of a threshold criterion. The onset was identified by the change in ΔF/F₀ that should be more than two standard deviations over the average baseline and remained above this value in the successive frames for at least 2 s (two to six frames, depending on the frame acquisition rate).
Drugs
TTX (1 µM, Sigma-Aldrich, Milan, Italy), 4-AP (100 µM, Ascent Scientific Avonmouth, Bristol, U.K.) were bath applied. NMDA (1 mM; Sigma-Aldrich, Milan, Italy), IL-1β (1 µg/mL R&D System Minneapolis, USA), and HMGB1 (1 µM, HMGBiotech, Milano, Italy) were pressure applied through a glass-pipette by a PDES Picospritzer (NPI, Tamm, Germany).

Data analysis
The Ca²⁺ signal is reported as ΔF/F₀, where F₀ is the baseline fluorescence. Data are shown as mean ± standard error of the mean (S.E.M.). Unless stated otherwise, the Student t-test was used, with p values ≤0.05 taken as statistically significant.

RESULTS
IL-1β and HMGB1 favour ictal discharge generation
Picrotoxin/zero-Mg²⁺ entorhinal cortex slice model.
The change in the cytosolic Ca²⁺ signal is a useful tool to study seizure-like, ictal discharges (IDs) in neuronal ensembles since it reflects faithfully the action potential bursts that characterize the epileptic discharges in individual neurons (Aoki, Tajima et al. 2008; Carmignoto and Gomez-Gonzalo 2010). To start to investigate a possible role of inflammatory agents such as IL-1β and HMGB1 in epileptogenesis, we loaded entorhinal cortex (EC) slice preparations from young rats with the Ca²⁺ indicator Oregon Green BAPTA1 (OGB1) and we evoked epileptiform activities by slice perfusion with the GABA A receptor antagonist picrotoxin in low extracellular Mg²⁺. We found that after the onset of the picrotoxin/low Mg²⁺ perfusion, the first ID occurred with a significantly shorter latency in slices pretreated with IL-1β (applied locally for 15 minutes through an IL-1β-containing glass pipette; see methods) with respect to slices treated with saline applications (Fig. 1B). Furthermore, ID frequency was also increased in IL-1β-treated with respect to saline-treated slices. Differently from IL-1β, HMGB1 affected neither the ID duration nor the latency of the first ID. However, the ID frequency was significantly increased (Fig. 1B).

Focal seizure model.
We next asked whether IL-1β and HMGB1 can also affect the generation of a focal ID. To this aim, we used our recently developed entorhinal cortex (EC) slice model in which focal IDs were reproducibly generated at a restricted site by perfusing the slice with 100 mM 4-aminopyridine (4-AP) and 0.5 mM Mg²⁺, and stimulating a small number of neurons with pressure pulses applied to an NMDA-containing glass pipette. As previously reported (Losi, 2010 #9474; Gomez-Gonzalo, 2010 #9032), in control, saline treated slices, a double, but not
a single, NMDA pulse triggered a focal ID. The DIC (left) and fluorescence (right) images in Figure 2A show a representative field in EC layer V-VI, the NMDA- and the IL-1b-containing pipettes. As illustrated by the difference images (Fig. 2B-D) generated by subtracting the fluorescence image captured at basal conditions to that obtained after the NMDA stimulation, a single NMDA pulse induced only a transient Ca\(^{2+}\) rise in a limited number of neurons close to the pipette tip, an area that we defined as the focal area (Field A; Fig. 2B). In contrast, a double NMDA pulse stimulation evoked a stronger activation of Field A neurons as well as Ca\(^{2+}\) elevations in these and the surrounding neurons with the typical pattern of an ID (Fig. 2E,F). The ID evoked by a double NMDA pulse was highly reproducible and only one out of fifty single NMDA pulse performed in 16 slices generated an ID within 45 minutes of 4-AP perfusion. We found that if a single NMDA stimulation, per se ineffective, was preceded by IL-1b or HMGB1 applications, a focal ID was evoked in 45 of 90 and 17 of 31 single pulse stimulations, respectively, suggesting that the cytokines can lower the threshold for ID generation (Fig. 2G).

Fig. 1. – IL-1b and HMGB1 effects on ID generation in the 0 Mg\(^{2+}/\)Picrotoxin model. A, Ca\(^{2+}\) changes in representative neurons from a rat EC slices perfused with low Mg\(^{2+}/\)Picrotoxin in presence of saline (upper trace), IL-1b (middle trace) and HMGB1 (lower trace) pulse applications. The pre-treatment with IL-1b reduce the latency time for the first ID, its duration and the ID frequency. HMGB1 application doesn’t influence the first ID, but increases ictal frequency. B, Mean latency and duration of the first ID and ictal and interictal frequency (right) in saline-treated (white bars, n = 11), IL1b-treated (black bars, n = 8) and HMGB1 treated (grey bars, n = 9) slices. *p ≤ 0.05, **p ≤ 0.01
Fig. 2 - IL-1β and HMGB1 local applications enhance focal ID generation. (A) DIC (left) and fluorescence (right) images of a cortical region from an EC slice showing the NMDA and the IL-1β pipettes. B, C) Difference images of the neuronal Ca²⁺ increase upon a single ineffective NMDA pulse (B), a double (C) NMDA pulse that successfully evoked a focal ID, and (D) a single NMDA pulse that after IL-1β also evoked a focal ID. E) Ca²⁺ changes in representative neurons of field A (nA) and field B (nB) upon a single, a double NMDA pulses and a single NMDA pulse applied after IL-1β (left) or HMGB1 (right). F) Quantitative evaluation of successful single NMDA pulses in saline-treated (50 pulses, n= 16 experiments) IL-1β- (90 single NMDA pulses, n=26 experiments) and HMGB1 (31 pulses, n=10 experiments).

Fig. 3 – A focal ID can initiate at the site of IL-1β local applications. A) Schematic view and neuronal Ca²⁺ changes during a focal ID evoked by a double NMDA pulse. The focal ID arose in field A neurons (nA), close to the NMDA pipette, and then propagate to neurons in B (nB). B) In the same slice, after IL-1β applications (red spot in the inset), a focal ID was evoked by a single NMDA pulse. The Ca²⁺ change that marked the onset of the focal ID occurred first in neurons from the site of IL-1β applications and then it propagated to neurons from the site of the single NMDA pulse with a delay of about 20 s. Similar results were observed in 4 out of 26 IDs.
In a few IL-1b experiments (4 out of 26) we also noted that the ID was not generated, as usually, at the original site of NMDA stimulation, but rather at the site where IL-1b was applied (Fig. 3). According to the Ca\(^{2+}\) signal change in these experiments, the focal ID initiated, indeed, in neurons from the IL-1b site and then it spread to neurons from the NMDA stimulation site (Fig. 3B).

**The responsiveness of neurons to NMDA pulse increases after IL-1b and HMGB1 local applications**

We then asked whether cytokines could lower the threshold for focal ID generation by enhancing the response of neurons to NMDA stimulation. We measured the number of activated neurons and astrocytes as well as the amplitude of the Ca\(^{2+}\) change in these cells in response to a single NMDA pulse that was preceded by either saline or IL-1b (or HMGB1).

![Image](image.png)

Fig 4 --The Ca\(^{2+}\) elevations in neurons upon the single NMDA pulse stimulation was amplified following IL-1b and HMGB1 applications. A, representative Ca\(^{2+}\) changes in neurons and astrocytes in field A evoked by a single NMDA pulse in the absence (left traces) and presence (right traces) of IL1b. B, Bar histograms reporting the significant increase in the responsiveness of neurons and astrocytes to a single NMDA pulse applied after IL-1b (black bars, n = 12 slices, 614 neurons and 356 astrocytes, * p < 0.05; ** p < 0.01) or HMGB1 (gray bars, n = 6 slices, 321 neurons and 115 astrocytes).
applications. Since in this latter case the single NMDA stimulation induced a focal ID, we restricted the analysis to the initial phase of the response to NMDA, i.e., the time interval between the NMDA pulse and the Ca\(^{2+}\) rise in neurons surrounding the focus that marked the ID onset (Fig. 4A) (Gomez-Gonzalo, 2010 #9032; Losi, 2010 #9474). As reported in the bar histograms of Fig. 4B, both Ca\(^{2+}\) elevation amplitude (dF/F0) and the number of neurons and astrocytes responsive to a single NMDA pulse were increased significantly after IL-1b and HMGB1 applications.

**IL-1b and HMGB1 action depends on synaptic transmission**

To clarify whether IL-1b and HMGB1 lowered the ID threshold by either affecting synaptic transmission or enhancing the direct response of neurons to NMDA, we performed experiments in the presence of 4 mM tetrodotoxin (TTX). In these experiments the amplitude of the Ca\(^{2+}\) change and the number of neurons activated by five successive single NMDA pulses (applied every two minutes) were measured before and after saline, IL-1b or HMGB1 pulses (applied every 20 s). We found that when synaptic transmission was blocked by TTX, both IL-1b and HMGB1 failed to enhance the NMDA-mediated Ca\(^{2+}\) response of neurons, suggesting that to lower the ID threshold the two cytokines does not act directly on the NMDA receptor activation. However, if TTX was applied to a brain slice that already experienced repetitive IDs, HMGB1, but not IL-1b, increased the responsiveness of neurons to a single NMDA pulse, in terms of both of Ca\(^{2+}\) elevation amplitude and number of activated neurons.

![Fig. 5](image-url) **IL-1b and HMGB1 enhanced NMDA-mediated Ca\(^{2+}\) activation of neurons through two distinct mechanisms.** A,B) Mean Ca\(^{2+}\) change amplitude (A) and responsive neurons (B) after IL1b (black bars; n = 9, 375 neurons), or HMGB1 (gray bars; n = 5, 367 neurons) with the respect to controls (dashed line) in EC slices that did not experienced any IDs (-IDs) or experienced a sustained epileptic activity (+IDs). * P < 0.05. The white bars expressed the values obtained after saline applications (n = 3, 289 neurons) expressed with respect to the values obtained in control untreated slices.
The role of inflammation in epilepsy and epileptogenesis is supported by different observations, in experimental models and in human temporal lobe epilepsy (TLE) (reviewed by (Vezzani and Friedman 2011)). Astrocytes represent a major source of pro-inflammatory molecules in the brain: they can secrete and sense a large variety of cytokines and chemokines and therefore actively contribute to the inflammatory status of the brain (Aronica, Ravizza et al. 2012).

The focal ictal discharge is an episode of synchronous activity of neurons which is largely influenced by Ca\(^{2+}\) activity in the astrocytes (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). In the present study 2 different models of focal seizures in acute brain slices were used to assess the role of inflammation in triggering epileptiform activity, especially with respect to the involvement of IL-1β and HMGB1. In the first model, EC slices are perfused with a solution containing 0 Mg\(^{2+}\) and Picrotoxin, which leads to spontaneous epileptic activity arising from unpredictable foci (Demir, Haberly et al. 1998). In the second model EC slices are exposed to 0,5mM Mg and 100µM 4AP before receiving focal NMDA application which triggers the ictal discharge (ID) activity (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). The latter model offers the unique opportunity to repetitively evoke an ID from the same restricted site, representing a powerful approach to analyze the contribution of different molecules to the onset of the ictal activity. Here we report that in both models of focal seizures (0 Mg\(^{2+}\)/picrotoxin and 0,5 Mg\(^{2+}\)/4AP/NMDA) IL-1β and HMGB1 were shown to increase the excitability of the network.

**IL-1β and HMGB1 enhance ictal discharge generation**

We show here that IL-1β and HMGB1 increase the excitability of neurons in EC slices. This fast proconvulsant effect as has been recently reported in an in vivo mouse model in which IL-1β and HMGB1 were effective in enhancing the seizure activity through a mechanism that involves the phosphorylation of the NMDA receptor subunit NR2B (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010). Other data obtained from cultured hippocampal neurons support the role of IL-1β in the modulation of activity of NMDA channels by their phosphorylation by Src kinases (Viviani, Bartesaghi et al. 2003). Although we could not show any increase in neuronal calcium response to NMDA after IL-1β application, we can not exclude that phosphorylation is occurring. Interestingly, in our experimental model the IL-1β application not only the reduced the threshold of fID generation, but also shifted the onset of the ictal activity from the NMDA application site to the IL-1β application site. This observation suggests that the cells that had a direct contact with IL-1β are more susceptible to ex-
citation. Since we are investigating an inflammatory pathway, on a cautionary note we also have to consider the inflammatory component triggered by the procedure used to prepare the acute slices. It is known that microglia gets quickly activated during this procedure and that could contribute to the inflammatory status of the slices. Moreover microglia actively participates in the modulation of excitatory neurotransmission by recruiting astrocytes via ATP release (Pascual, Ben Achour et al. 2012).

Astrocytes $\text{Ca}^{2+}$ elevation

Astrocytes have been showed to have a pivotal role in the generation of focal ictal discharge (fID) in rat EC slices: they contribute to the neuronal synchronization by signaling back to the neurons in a $\text{Ca}^{2+}$ dependent (Gomez-Gonzalo, Losi et al. 2010). As recently shown (Gomez-Gonzalo, Losi et al. 2010), blocking the $\text{Ca}^{2+}$ signaling of the astrocytes in a focal seizure model in slices increases the threshold of fID, whereas enhancing $\text{Ca}^{2+}$ signaling decreases it, facilitating the onset of fID. In the present study, we saw a strong increase in $\text{Ca}^{2+}$ in astrocytes after the application of NMDA in HMGB1 pretreated slices. Moreover blocking the synaptic transmission with TTX prevented $\text{Ca}^{2+}$ elevation in astrocytes. This evidence suggests that the $\text{Ca}^{2+}$ elevation in astrocytes depends on the synaptic activity and that the proconvulsant effects of IL1β and HMGB1 may reflect a regulation of the neuron-glia communication. Indeed astrocytes have been shown to express a large variety of glutamate receptors, metabotropic an ionotropic (Schipke, Ohlemeyer et al. 2001; Lalo, Pankratov et al. 2006; Verkhratsky and Kirchhoff 2007; D'Antoni, Berretta et al. 2008; Lundborg, Westerlund et al. 2011). Furthermore, increasing number of studies have showed the role of the astrocytic calcium signaling in glia-neuron communication (for rew.: {Zorec, 2012 #580}). The release of gliotransmitters, such as glutamate, ATP or D-serine, can modulate signaling among neurons, leading to synaptic modulation (Yang, Ge et al. 2003; Perea and Araque 2007). Here we propose that the inflammatory molecules IL-1β and HMGB1 increase the excitability of the network, leading to astrocytes $\text{Ca}^{2+}$ elevation which in turn causes the synchronization of more neurons leading to a decreased threshold for fID.

**HMGB1 is pro-ictogenic only after the tissue had experienced a fID**

In our experiments HMGB1 was effective in decreasing the threshold of the fID only after the tissue had experienced one or more episodes of fID. The reason for this has to be searched in the physiological modifications that occur following the fID. The presence of extranuclear staining for HMGB1 in astrocytes and occasionally in neurons after repetitive fID episodes (fig. 6) suggests a translocation of this molecule from the nucleus to the cytoplasm and a possible release in the extracellular space. In a previous study, we showed that
cultured astrocytes can indeed release HMGB1 following IL-1β stimulation (Zurolo, Iyer et al. 2011). Astrocytes, microglia and neurons (expressing TLR4) may respond to HMGB1 stimulation with production of several pro-epileptogenic inflammatory inflammatory mediators (Andersson, Ronnback et al. 2005; Kim, Choi et al. 2006; Pedrazzi, Patrone et al. 2007) providing a positive feedback loop that amplifies neuronal excitability.

If we consider the acute slices as inflammation primed tissue on account of the slice preparation procedure, it could be speculated that the inflammatory receptors/pathways are somehow already receptive to a stimulatory trigger. Under these conditions, a focal ictal discharge episode could induce translocation of HMGB1. Moreover, its primary receptor, TLR4 could be recruited following an ictal discharge episode. It should be noted that since the time scale of our experiments precludes de novo synthesis of this receptor, an alternative mechanism could involve the accelerated mobilization of pre-synthesised receptor from the Golgi to the membrane (see (Saitoh and Miyake 2009; McGettrick and O'Neill 2010).

**Significance:** In the present study we showed that IL-1β and HMGB1 induced calcium increase in astrocytes that could promote increased synchronization of the neurons. This enables the neurons to reach the threshold of onset of fID with a less intense stimulus—These findings demonstrate that both IL-1β and HMGB1 can rapidly lower fID threshold and raise the possibility that targeting these inflammatory pathways may represent an effective therapeutic strategy to prevent seizures.

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2.5 Regulation of Kir4.1 expression in astrocytes and astrocytic tumors: a role for interleukin-1 β

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Abstract

Objective: Decreased expression of inwardly rectifying potassium (Kir) channels in astrocytes and glioma cells may contribute to impaired K+ buffering and increased propensity for seizures. Here, we evaluated the effect of inflammatory molecules, such as interleukin-1β (IL-1β) on Kir4.1 mRNA and protein expression.

Methods: We investigated Kir4.1 (Kcnj10) and IL-1β mRNA expression in the temporal cortex in a rat model of temporal lobe epilepsy 24 hours and 1 week after induction of status epilepticus (SE), using real-time PCR and western blot analysis. The U373 glioblastoma cell line and human fetal astrocytes were used to study the regulation of Kir4.1 expression in response to pro-inflammatory cytokines. Expression of Kir4.1 protein was also evaluated by means of immunohistochemistry in surgical specimens of patients with astrocytic tumors (n= 64), comparing the expression in tumor patients with (n= 38) and without epilepsy (n= 26).

Results: Twenty-four hours after onset of SE, Kir4.1 mRNA and protein were significantly down-regulated in temporal cortex of epileptic rats. This decrease in expression was followed by a return to control level at 1 week after SE. The transient down-regulation of Kir4.1 corresponded to the time of prominent upregulation of IL-1β mRNA. Expression of Kir4.1 mRNA and protein in glial cells in culture was down-regulated after exposure to IL-1β.
Evaluation of Kir4.1 in tumor specimens showed a significantly lower Kir4.1 expression in the specimens of patients with epilepsy compared to patients without epilepsy. This paralleled the increased presence of activated microglial cells, as well as the increased expression of IL-1β.

Conclusions: Taken together, these findings indicate that alterations in expression of Kir4.1 occurring in epilepsy-associated lesions are possibly influenced by the local inflammatory environment and in particular by the inflammatory cytokine IL-1β.

INTRODUCTION
Astrocytes, the major glial cell type of the central nervous system (CNS), are known to play a major role in normal brain signaling and their dysfunction has been shown to be critically involved in the pathogenesis of several human CNS disorders, including epilepsy [for reviews see [1, 2]. One of the most important physiological functions of astrocytes is their ability to control ionic homeostasis, in particular the extracellular concentration of potassium, which influences neuronal excitability. The inwardly rectifying potassium (Kir) channel 4.1 has been identified as a key player among the potassium channels expressed in astrocytes responsible for spatial buffering [3, 4]. Conditional knock-out of Kir4.1 has been shown to lead to inhibition of potassium and glutamate uptake, hyperexcitability and seizures [5, 6]. Mutations in the human Kir4.1 gene, KCNJ10, are associated with epilepsy [7] and a compromised glial potassium spatial buffering has been suggested to underlie the epilepsy phenotype [8]. In addition, alterations in expression, localization, and function of Kir4.1, have been reported in astrocytes in a number of neurological disorders, including temporal lobe epilepsy (TLE) and malignant gliomas (for review see [9]). In tissue specimens obtained from patients with epilepsy, both electrophysiological and molecular studies suggest that impaired potassium buffering and enhanced seizure susceptibility may result from reduced expression of Kir4.1 channels [10-14]. In the fluid percussion injury model in rat, a chronic dysfunction of Kir channels (with depletion of Kir4.1 immunoreactivity in processes of neocortical astrocytes) in the epileptic focus has been reported [15]. In case of astrocytic tumors, which are often associated with seizure development, mislocalization and/or redistribution of Kir4.1, as well as changes in the expression related to the malignancy grade, have been reported [9, 16-18]. In addition, attention has been focussed on the role of Kir channels as critical regulators of cell division, suggesting that a loss of functional Kir4.1 may underlie the re-entry of glial cells into the cell cycle supporting gliosis and tumor development [9]. Although these observations support an important role for astrocytic Kir4.1, it remains still unclear whether the changes in Kir4.1 expression represent the cause or the consequence of epilepsy and the mechanism underlying the regulation of the expression of Kir4.1 is still matter of dis-
cussion. It has been shown that albumin uptake into astrocytes, mediated by transforming growth factor (TGF)-β receptors produces a downregulation of Kir4.1 in these cells [19]. In spinal cord injury, the downregulation of Kir4.1 has been suggested to be dependent on the nuclear estrogen receptor signaling [20]. Moreover, recently, Kir4.1 expression has been suggested to be influenced by changes in the extracellular environment of inflammatory cytokines, such as interleukin-1β (IL-1β) [21]. Interestingly, increasing evidence supports the notion that dysregulation of the astrocyte immune-inflammatory function is a common factor predisposing or directly contributing to the generation of seizures in epilepsy of various etiologies [1, 22, 23].

Our major aim was to investigate the potential effect of inflammatory molecules, such as IL-1β on Kir4.1 expression using both a glioblastoma cell line and human astrocytes in culture. The recently anti-inflammatory effects of the antiepileptic drug (AED) levetiracetam reported recently in vivo and in vitro [24, 25], prompted us to evaluate the effect of this AED on Kir4.1 expression in cultures exposed to IL-1β. In addition, in order to detect changes in Kir4.1 expression and/or localization in tumor astrocytes and their relationship to IL-1β expression and to the tumor epileptogenicity, astrocytic tumors with and without epilepsy were studied.

**MATERIALS AND METHODS**

**Experimental animals.** Adult male Sprague Dawley rats (Harlan CPB laboratories, Zeist, The Netherlands) weighing 300-500 grams were used in this study which was approved by the University Animal Welfare committee. The rats were housed individually in a controlled environment (21±1°C; humidity 60%; lights on 08:00 AM - 8:00 PM; food and water available ad libitum).

**Electrode implantation and seizure induction.** In order to record hippocampal EEG, a pair of insulated stainless steel electrodes (70 µm wire diameter, tips were 80 µm apart) were implanted into the left dentate gyrus (DG) under electrophysiological control as previously described [26]. A pair of stimulation electrodes was implanted in the angular bundle. Rats underwent tetanic stimulation (50 Hz) of the hippocampus in the form of a succession of trains of pulses every 13 seconds. Each train had a duration of 10 seconds and consisted of biphasic pulses (pulse duration 0.5 ms, maximal intensity 500 µA). Stimulation was stopped when the rats displayed sustained forelimb clonus and salivation for minutes, which usually occurred within 1 hour. However, stimulation never lasted longer than 90 minutes. Differential EEG signals were amplified (10x) via a FET transistor that connected the headset to a differential amplifier (20x; CyberAmp, Axon Instruments, Burlingame, CA, USA), filtered (1-60 Hz), and digitized by a computer. A seizure detection program (Harmonie, Stellate Sys-
tems, Montreal, Canada) sampled the incoming signal at a frequency of 200 Hz per channel. EEG recordings were monitored also visually and screened for seizure activity. Behavior was observed during electrical stimulation and several hours thereafter. Immediately after termination of the stimulation, periodic epileptiform discharges (PEDs) occurred at a frequency of 1-2 Hz and they were accompanied by behavioral and EEG seizures (status epilepticus).

**Rat tissue preparation for RNA isolation and western blot analysis.** After decapitation, the brain was removed and dissected and the temporal cortex was cut out of the slices under a dissection microscope. Rats were decapitated in the acute phase (one day after SE, n= 6) and in the latent period (1 week after SE, n= 6; the rats in this group did not exhibit spontaneous seizures). Age matched rats that were implanted but not stimulated except for field potential recordings, were also included (n= 6). All material was frozen on dry ice and stored at -80 °C until use. For western blot analysis frozen samples of control (n=5), 1 day post-SE (n=5) and 1 week after SE (n=5) were homogenized in lysis buffer (as described below) and protein content was determined using the bicinchoninic acid method [27].

**Cell cultures.** For experiments with astrocytes-enriched human cultures, fetal brain tissue (15–23 weeks of gestation) was obtained from spontaneous or medically induced abortions with appropriate maternal written consent for brain autopsy. Resected tissue samples were collected in Dulbecco’s modified Eagle’s medium (DMEM)/HAM F10 (1:1) medium (Gibco, Life Technologies), supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin and 10% fetal calf serum (FCS). Cell isolation was performed as previously described described [28, 29]. Briefly, after removal of meninges and blood vessels, tissue was minced and dissociated by incubation at 37 °C for 20 min in a Hank’s balanced salt solution containing 2.5 mg/ml trypsin (Sigma, St. Louis, MO, USA) and 0.1 mg/ml bovine pancreatic Dnase I (Boehringer Mannheim, Germany). Tissue was triturated and washed with DMEM/HAM F10 medium, supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin and 10% FCS. Cell suspension (containing ~ 0.5 g wet weight tissue/10 ml culture medium) was passed through a 70µm cell sieve (Becton Dickinson, USA) and plated into 25 cm² flasks (Falcon, Lincoln Park, NJ) and maintained in a 5% CO2 incubator at 37°C. After 48 h the culture medium was replaced by fresh medium and cultures were subsequently fed twice a week. Cultures reached confluence after 2-3 weeks. Secondary astrocyte cultures were established by trypsinizing confluent cultures and sub-plating into 6 and 24-well plates (Costar; 0.5 X 106 cell/well in a 6-well plate for western blot analysis or 0.1 X 106 cell/well in a 24-well plate for RNA isolation and PCR) and simultaneously into 12 mm coverslips (Sigma) in 24-well plates (Costar; 2 X 104 cell/well; for immunocytochemistry). More than 98% of the cells in primary culture, as well as in the successive 12 passages were strongly immunoreactive for the astrocytic marker GFAP and S100β. In the present study astrocytes were used for immunocy-
Tochemical analyses at passage 3-4. The astrocytoma cell line U373 was obtained from the American Type Culture Collection (Rockville, MD, USA); cells were cultured in (DMEM)/HAM F10 (1:1) supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin and 10 % FCS.

**Treatment of cell cultures.** Human recombinant (r)IL-1β (Peprotech, NJ, USA; 10 ng/ml) was applied and maintained for 24 h before harvesting the cells for RNA isolation, western blot analysis or for immunocytochemistry. In some experiments different time periods of IL-1 exposure (ranging from 10 min to 48 h) were used and rIL-6 (10ng/ml; Strathmann Biotec A.G., Hamburg, Germany), tumor necrosis factor α (TNFα; 1ng/ml; Peprotech, NJ, USA) and high mobility group box 1 (HMGB1; 40nM; HMGBiotech S.r.l., Milan, Italy) alone or together with IL-1β were applied and maintained in the medium for 24 h before harvesting the cells for RNA isolation. Human IL-1receptor antagonist (IL-1Ra; 1 µg/ml; Peprotech, NJ, USA) was used to neutralize IL-1β activity (applied 1 h before IL-1β). As previously shown [29] the viability of human astrocytes in culture was not influenced by the treatments. In other experiments, cells exposed to IL-1β (for 24 h) were extensively washed with phosphate-buffered saline (PBS) and incubated up to 48 hours in culture medium, before harvesting them for western blot analysis.

**Preparation of Cellular Extracts.** Cells were harvested at 24 h after treatment and washed twice with cold PBS. The samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, Na orthovanadate (10.4 mg/ml), 5 mM EDTA (pH 8.0), 5 mM NaF and protease inhibitor cocktail (Boehringer Mannheim, Germany) by incubating on ice for 15 minutes. The homogenates were centrifuged at 14000 rpm for 10 mins and the supernatant was used for further analysis.

**Western blot analysis.** Western blot analysis was performed, as previously described [30]. For electrophoresis, equal amounts of proteins (15-20µg/lane) were separated on a 10% sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) gel. Separated proteins were transferred to nitrocellulose paper for 90 min at 100V, using a wet electroblotting system (BioRad, Hercules, CA, USA). Membranes were blocked for 1 hour in 5% non fat dry milk in Tris-buffered saline-Tween (TBST) (20 mM Tris, 150 mM NaCl, 0.1 % Tween 20, pH 7.5). The blots were incubated overnight with the primary antibody (Kir4.1 rabbit polyclonal antibody 1:1000 in 5% milk solution, Alomone Labs, Jerusalem, Israel).

After several washes in TBST, the membranes were incubated in TBST / 5% non fat dry milk, containing the goat anti-rabbit or rabbit anti-mouse coupled to horse radish peroxidase (1:2500; Dako, Denmark) for 1h. After washes in TBST, immunoreactivity was visualized using ECL PLUS western blotting detection reagent (GE Healthcare Europe, Diegen, Belgium). Expression of β-actin (monoclonal mouse, Sigma, St. Louis, MO, 1:50000) or β-tubulin (monoclonal mouse, Sigma, St Louis, MO, 1:30000) were used as loading control. For the
quantification of the blots the band intensities were measured densitometrically using the Scion Image for Windows (beta 4.02) image-analysis software. A ratio of the band intensity of the protein of interest to that of the reference protein was used to normalize expression.

**RNA isolation and Real-time quantitative PCR analysis (qPCR).** For RNA isolation, 800 µl of Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA) was added to 0.1 – 0.5 x 10^6 cells. After addition of 200 µg glycogen and 200 µl chloroform, the aqueous phase was isolated using Phase Lock tubes (5 Prime GmBH, Hamburg, Germany). RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and dissolved in water. The concentration and purity of RNA were determined at 260/280 nm using a nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Five micrograms of total RNA were reverse-transcribed into cDNA using oligo dT primers. Five nmol oligo dT primers were annealed to 5 µg total RNA in a total volume of 25 µl, by incubation at 72 °C for 10 min, and cooled to 4°C. Reverse transcription was performed by the addition of 25 µl RT-mix, containing: First Strand Buffer (Invitrogen-Life Technologies), 2 mM dNTPs (Pharmacia, Germany), 30 U RNAse inhibitor (Roche Applied Science, Indianapolis, IN, USA) and 400 U M-MLV reverse transcriptase (Invitrogen - Life Technologies, The Netherlands). The total reaction mix (50 µl) was incubated at 37 °C for 60 min, heated to 95 °C for 10 min and stored at -20°C until use.

PCR primers (Eurogentec, Belgium) were designed using the Universal Probe Library of Roche (https://www.roche-applied-science.com) on the basis of the reported mRNA sequences. For the rat we used: Kir4.1/Kcnj10 (forward: gtgacaggcaactgcttca and reverse: gggctatcagaggtgtctc), IL-1β (forward: gcatccagctacgaatctcc; reverse: gaaccagcatcttcctcagc and GAPDH; (forward: ATGACTCTACCCAGGCAAG; reverse: TACTCAGACCAGATCACC). For the human cell cultures we used: Kir4.1 (forward: acctcggacccaagatgac; reverse: gatcatggcgcaggctag), IL-1ß (forward: gatcatggcgcaggcctag; reverse: gatcatggcgcaggcctag), elongation factor 1-alpha (EF1α; forward: atccacctttgggtcgcttt; reverse: cgccacagtctgctctcatcata), and hypoxanthine phosphoribosyl transferase (HPRT; forward: tcggtctgctgattagtagt; reverse: ttagctcagaggtagc). For each PCR, a mastermix was prepared on ice, containing per sample: 1 µl cDNA, 2.5 µl of FastStart Reaction Mix SYBR Green I (Roche Applied Science, Indianapolis, IN, USA), 0.4 µM of both reverse and forward primers. The final volume was adjusted to 5 µl with H₂O (PCR grade). The LightCycler® 480 Real-Time PCR System (Roche-applied-science) was used with a 384-multiwell plate format. The cycling conditions were carried out as follows: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 55-60°C for 5 s and extension at 72°C for 10 s. The fluorescent product was measured by a single acquisition mode at 72°C after each cycle. For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after ampli-
Quantification of data was performed using the computer program LinReg PCR in which linear regression on the Log(fluorescence) per cycle number data is applied to determine the amplification efficiency per sample [31]. The starting concentration of each specific product was divided by the starting concentration of reference genes (GAPDH, for rat material; HPRT and EF1a for the cell cultures) and this ratio was compared between patient/control groups.

**Human material.** The human cases included in this study were obtained from the files of the departments of neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and the VU University Medical Center (VUmc), both situated in Amsterdam and both tertiary referral centres for brain tumor patients in the Netherlands. We examined immunocytochemically 73 surgical specimens of brain tumor patients with astrocytic tumors (6 WHO grade II astrocytoma; 12 WHO grade III astrocytoma; 55 glioblastoma multiforme, GBM; Table 1). Normal-appearing control cortex/white matter was obtained at autopsy from 8 adult control patients without a history of seizures or other neurological diseases. All autopsies were performed within 12 hours after death. Cortical samples (cortex/white matter adjacent to the lesion with reactive changes, such as astrogliosis, but not tumor cells) of 5 patients with non-glial brain tumors (2 meningiomas, 1 metastasis of carcinoma and 1 lymphoma) and without refractory epilepsy were also analyzed (control cortex/surgical, table 1). Frozen tissue from histologically normal cortex (n=2) and GBM (n=4) samples was used for western blot analysis and total RNA prepared from normal cortex (n=6) and GBM (n=8; 4 with epilepsy and 4 without epilepsy) was used for qPCR.

A chart review was conducted of all patients. Epilepsy was defined as the experience of one or more seizures and data regarding seizure frequency and seizure type were obtained from patient histories. We collected additional data including age, gender, tumor location, and epilepsy duration. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. Two neuropathologists reviewed all cases independently and the diagnosis was confirmed according to the revised WHO classification of tumors of the central nervous system [32].

**Tissue preparation for immunocytochemistry.** Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 5 µm, mounted on precoated glass slides (Star Frost, Waldemar Knittel GmbH, Brunschweig, Germany) and used for immunohistochemical staining as described below.

**Antibodies.** Antibodies specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000; monoclonal mouse; DAKO; 1:50), vimentin (mouse...
clone V9; DAKO; 1:1000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:2000), synaptophysin (mouse clone Sy38; DAKO; 1:200; rabbit anti-synaptophysin; DAKO; 1:200), Ki67 (mouse clone MIB-1; DAKO; 1:200), (HLA)-DP, DQ, DR (HLA-DR; mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400), MAP2 (mouse clone HM2; Sigma 1:100) and p53 (Clone DO-7 + BP53-12; Neomarkers; 1:2000), were used in the routine immunohistochemical analysis of glial tumors. For the detection of Kir4.1, we used a polyclonal rabbit antibody (Alomone Labs, Ltd, Jerusalem, Israel; 1:100); for the detection of IL-1β a polyclonal goat antibody (sc-1250, Santa Cruz Bio., CA, USA; 1:70;[33]) and for the detection of HMGB1, we used a polyclonal rabbit antibody (Pharmingen, San Diego, CA, USA; 1:100; [34]; Abcam Cambridge, UK).

**Immunohistochemistry.** Paraffin-embedded sections were deparaffinized, re-hydrated, and incubated for 20 min in 0.3% H₂O₂ diluted in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed by incubation for 10 min at 121 °C in citrate buffer (0.01 M, pH 6.0), sections were washed with phosphate-buffered saline (PBS) and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK).

<table>
<thead>
<tr>
<th>Table 1. Clinical and histopathological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m/f)</td>
</tr>
<tr>
<td>Gender (m/f)</td>
</tr>
<tr>
<td>Age Yrs ¹</td>
</tr>
<tr>
<td>Location</td>
</tr>
<tr>
<td>Frontal</td>
</tr>
<tr>
<td>Temporal</td>
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<tr>
<td>Parietal</td>
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<tr>
<td>Occipital</td>
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<tr>
<td>Thalamus</td>
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<tr>
<td>Parietooccipital</td>
</tr>
<tr>
<td>Temporoparietal</td>
</tr>
<tr>
<td>Temporoparietal</td>
</tr>
<tr>
<td>Frontotemporal</td>
</tr>
<tr>
<td>Frontoparietal</td>
</tr>
<tr>
<td>Epilepsy</td>
</tr>
<tr>
<td>Duration epilepsy (months) ¹</td>
</tr>
</tbody>
</table>

¹mean (range); A II: Astrocytoma WHO grade II; A III: Astrocytoma WHO grade III; GBM: Glioblastoma multiforme.
Coverslips with adherent cells (U373 or fetal astrocytes) were rinsed in PBS (pH 7.4) and fixed for 15 min in 4% paraformaldehyde in PBS. After rinsing, cultures were incubated in PBS containing 10% normal goat serum for 15 minutes prior to the incubation with the primary antibodies.

Sections were incubated with the primary antibodies overnight at 4 °C. Hereafter, sections were washed in PBS and the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3’-diaminobenzidine (DAB; Sigma) was used to develop the colour reaction. Sections were counterstained with haematoxylin, dehydrated and coverslipped. Sections incubated without the primary antibody were essentially blank. To test the specificity of the antibody, western blot analysis of the total homogenates of human histologically normal cortex (n=2) and GBM (n=4) samples was performed, as described above.

The number of available frozen tumor samples from patient with and without epilepsy was too small to perform meaningful statistical comparisons in subgroups and to assess whether Kir4.1 expression is more directly dependent on presence or absence of seizures or tumor type by western blot analysis.

**Evaluation of immunostaining.** Semi-quantitative evaluation of immunoreactivity (IR) in tumor specimens was performed as previously [35] using a semi-quantitative scale ranging from 0 to 3 (0: -, no; 1, weak; 2: +, moderate; 3: ++, strong IR). Two representative sections per case were stained and assessed with the Kir4.1 and IL-1β antibodies. The intensity score represents the predominant staining intensity found in each specimen as averaged from the selected fields and the different sections per group. The evaluation of the IR in tumor specimens was performed in the center of the lesion, the infiltration zone was disregarded. The sections were evaluated by two independent observers blind to clinical data. In case of disagreement independent reevaluation was performed by both observers to define the final score. The approximate proportion of cells showing IR (0, < 1%; 1, single to 25%; 2, 6-50%; 3, 51-75%; and 4, >75%) was also scored to give information about the relative number (‘frequency’ score) of positive cells tumor specimens. As proposed before [36, 37], the product of these two values (intensity and frequency scores) was taken to give the overall score (immunoreactivity total score; IR score), shown Table 2 and 3. We also evaluated the IR score of HLA-DR (markers of microglia activation) in tumor tissue of patients with or without epilepsy and quantitative analysis was performed for HMGB1 in these two patient groups ([34]; supplementary material).

In cell cultures (U373 and fetal astrocytes) quantitative analysis was carried out for the number of Kir4.1 immunoreactive cells. All cells were counted systematically at high magnification (x40 objective; using an ocular grid and counting 1,000 cells from two separate experiments) as positive IR (including strong or intermediate intensity of labeling) or negative.
percentage of labeled Kir4.1 was calculated based on the total number of cells.

**Statistical analysis.** Statistical analysis was performed with SPSS 15.0 and Prism® (Graph Pad Software, Inc.) software for Windows. To assess differences between groups, a non-parametric Kruskal–Wallis test, followed by the Mann-Whitney-U test were performed. Correlations between Kir4.1 immunostaining and different variables (histopathological diagnosis, epilepsy, the use of levetiracetam and IL-1β immunoreactivity) were assessed with the Mann-Whitney-U test and the Spearman’s rank correlation test. The value of $P < 0.05$ was defined as statistically significant. Multiple testing was corrected by the Bonferroni correction.

**Table 2. Kir4.1 and IL-1b immunoreactivity in astrocytic tumors**

<table>
<thead>
<tr>
<th>IR score</th>
<th>A II (n=6)</th>
<th>A III (n=12)</th>
<th>GBM (n=55)</th>
<th>Control cortex/autopsy (n=8)</th>
<th>Control cortex/surgical (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir4.1</td>
<td>3.6 ± 0.85*</td>
<td>7.18 ± 0.41</td>
<td>5.5 ± 0.30</td>
<td>7.13 ± 0.36</td>
<td>7.03 ± 0.26</td>
</tr>
<tr>
<td>IL-1b</td>
<td>2.46 ± 0.67*</td>
<td>3.9 ± 0.9*</td>
<td>3.75 ± 0.45*</td>
<td>0</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>5.4 ± 0.77*</td>
<td>5.7 ± 0.80*</td>
<td>5.83 ± 0.35*</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

A II: Astrocytoma WHO grade II; A III: Astrocytoma WHO III; GBM: Glioblastoma multiforme. Values represent the average immunoreactive score (IR) ± SEM. *P< 0.05 (compared to control cortex, both autopsy and surgical samples). Kir4.1 IR score for AIII > Kir4.1 score for AII (p< 0.05).

**Table 3. Kir4.1 and IL-1b immunoreactivity in patients with/without epilepsy and with/without levetiracetam use**

<table>
<thead>
<tr>
<th>IR score</th>
<th>With epilepsy (n=42)</th>
<th>Without epilepsy (n=31)</th>
<th>With levetiracetam (n=14)</th>
<th>Without levetiracetam (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir4.1</td>
<td>4.9 ± 0.36*</td>
<td>6.5 ± 0.37</td>
<td>6.8 ± 0.62**</td>
<td>4.5 ± 0.37</td>
</tr>
<tr>
<td>IL-1b</td>
<td>5.5 ± 0.49*</td>
<td>1.3 ± 0.17</td>
<td>3.9 ± 1.14**</td>
<td>6.3 ± 0.46</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>5.8 ± 0.31*</td>
<td>3.6 ± 0.36</td>
<td>4.28 ± 0.15</td>
<td>5.2 ± 0.40</td>
</tr>
</tbody>
</table>

Values represent the mean immunoreactive score (IR) ± SEM. *P <0.05: significant difference compared to patients without epilepsy; ** P <0.05: significant difference compared to patients without levetiracetam use.
RESULTS
Kir4.1 and IL-1β expression in rat temporal cortex after induction of SE

To determine the temporal-spatial expression of Kir4.1 expression we performed qPCR in tissue samples of control rats and rats that were sacrificed at different time points after SE (1 day and 1 week post-SE). Kir4.1 expression significantly decreased at 24 h post-SE and returned at control levels at 1 week after the onset of SE (Fig. 1A). Western blot analysis of total homogenates of rat temporal cortex revealed a band at molecular weight of approximately 40 kDa which showed a significant decrease at 24h post-SE as compare to controls (Fig. 1 C-D). The transient prominent decrease of Kir4.1 mRNA expression following SE prompted us to evaluate whether this decrease might be related to an increased level of cytokines, such as IL-1β. Prominent IL-1β upregulation was indeed observed 24 h post-SE (Fig. 1 B).

Figure 1. Kir4.1 and IL-1β expression in rat temporal cortex after status epilepticus (SE)
(A) and (B): Quantitative real-time PCR. mRNA expression levels of Kir4.1 (A) and IL-1β (B) in the temporal cortex of control rats (n= 6; Con), rats at 1 week (n= 6) and 3 months (n= 6) after SE. Data represent the target gene expression normalized to the reference genes. The error bars represent SEM and * represents a p-value < 0.05. (C) and (D): western blot analysis of Kir4.1. (C): Representative immunoblot of total homogenates from temporal cortex of controls and post SE (24h and 1 week) rats. (D): Densitometric analysis: values (optical density units, O.D.) are mean ± SEM, (control, n=5; 24 h post SE, n=5 and 1 week post-SE, n=5), relative to the optical density of β-tubulin; *, p < 0.05, compared to controls.
Regulation of Kir4.1 expression by IL-1β in human glial cells in culture

To address the question of whether IL-1β was involved in the modulation of Kir4.1 expression we used both human fetal astrocytes and the U373 glioblastoma cell line in culture. qPCR demonstrated that exposure to IL-1β consistently decreased Kir4.1 expression in both cell types (Fig. 2 A and B). The effect of IL-1β was blocked by the IL-1β RA, a naturally occurring antagonist of the IL-1 receptor ([38]; Fig. 2 C). IL-1β significantly decreased Kir4.1 mRNA levels already 30 min after exposure to IL-1β (not shown) and its effect was maximal at 24 h. The down-regulation of Kir4.1 mRNA could be partially reverted when IL-1β was removed and cultures were incubated for 48 hours in culture medium [Kir4.1 expression (relative to control of 100%): 24 h IL-1β: 13.8 % ± 2.0; 48 h after washout: 51.3 % ± 2.7]. In contrast, under our culture conditions we did not observe significant changes in the expression levels of Kir4.1 after exposure to IL-6 (10ng/ml), TNFα (1 ng/ml) or HMGB1 (40nM); cytokine

Figure 2. Kir4.1 expression in U373 glioblastoma cell line and in cultured human astrocytes after exposure to IL-1β.

(A-C): Quantitative real-time PCR. Expression levels of Kir4.1 mRNA 24 hours after exposure to IL-1β (10 ng/ml) in U373 glioblastoma cell line (A) and in cultured human astrocytes (B). (C): Expression levels of Kir4.1mRNA 24 hours after exposure to IL-1β in U373 cell line in the presence or absence of the IL-1β receptor antagonist (IL-1ra; 1 µg/ml) or levetiracetam (LEV; 10 µg/ml). Data are expressed relative to the levels observed in unstimulated cells (untreated controls, Con) and are mean ± SEM from two separate experiments performed in triplicate (*p<0.05; ***p < 0.0001 compared to control).

(D-E). Western blot analysis of Kir4.1. Representative immunoblot of total homogenates from U373 glioblastoma cell line (D) and from human fetal astrocytes (E) untreated and treated for 24 h with 10 ng/ml IL-1β, in the presence or absence of the IL-1 β receptor antagonist (IL-1ra; 1 µg/ml). Densitometric analysis: values (optical density units, O.D.) are mean ± SEM, relative to the optical density of β-actin; *, p < 0.05, compared to controls.
treatments, including IL-1β, did not influence the expression of Kir2.1, 2.3 and 3.1 mRNA (not shown). NFκB inhibition (by BMS-345541, 20μM) effectively blocked both interleukin-6 (IL-6) and cyclo-oxygenase (COX)-2 induction by IL-1β, but did not affect the IL-1β-induced Kir4.1 mRNA downregulation in the same culture, suggesting an NFκB-independent mechanism (supplementary Fig. 1).

Figure 3. Kir4.1 (IR) in U373 glioblastoma cell line and in cultured human astrocytes after exposure to IL-1β.
(A-D): representative photomicrographs showing Kir4.1 IR in glioma cells (U373; A-B) and cultured human astrocytes (C-D), untreated (A and C; Con) and treated (B and D) for 24 h with 10 ng/ml IL-1β; high magnifications are shown in the inserts; in U373 cells Kir4.1 was also detected in the nuclei of glial cells (insert in A and B). Scale bar in A: A and B: 80 μm; C-D: 40 μm.
(E-F): percentage of Kir4.1 positivity in glioma cells (U373; E) and cultured human astrocytes (F) untreated and treated for 24 h with 10 ng/ml IL-1β. *, p < 0.05, compared to untreated controls (Con).
The recently described anti-inflammatory property of the AED levetiracetam [24, 25] prompted us to evaluate its effect on IL-1β–induced Kir4.1 down-regulation. Exposure to levetiracetam did not affect IL-1β induced Kir4.1 downregulation. However levetiracetam treatment (for 24 h) significantly increased Kir4.1 mRNA compared to untreated cells (Fig. 2C). A similar effect was observed 48 h after exposure to levetiracetam (not shown).

Western blot analysis confirmed the downregulation of Kir4.1 induced by IL-1β in U373 cells (Fig. 2D) and fetal astrocytes (Fig. 2E) at the protein level. No significant differences (at 24 h) in Kir4.1 protein levels were detected in either cell culture after treatment with levetiracetam (in the presence or absence of IL-1β; not shown).

In both astrocytes-enriched human cell cultures and glioma cells incubated with IL-1β, immunocytochemistry demonstrated a lower percentage of Kir4.1-labeled cells (Fig. 3 A-D) as compared to controls (Fig. 3 A-D). In U373 cells (but not in fetal astrocytes), we also observed nuclear IR (Fig. 3 A-B) in addition to cytoplasmic Kir4.1 IR.

**Kir4.1 and IL-1β expression in human astrocytic tumors**

**Patients.** Table 1 summarizes the clinical and histopathological characteristics of the patients and control cases. Thirty eight of the 73 tumor patients had epilepsy. The majority of the patients had secondary generalized seizures, followed by simple partial seizures. All 42 patients with epilepsy used antiepileptic drugs, 14 of them used levetiracetam before operation.

**Kir4.1 immunoreactivity.** In control tissue we did not detect obvious differences in the distribution of Kir4.1 between surgical and autopsy cortical specimens. Kir4.1 IR was detected around blood vessels (Fig. 4A) as previously reported [16, 17] and occasionally in the cytoplasm of astroglial cells. Astrocytoma WHO grade II and III, as well as GBM displayed mainly cytoplasmic staining in tumor cells (Fig. 4 and 6). The expression at perivascular endfeet membranes was less prominent and occasionally nuclear expression was observed in astrocytoma grade III and GBM (not shown). The IR score was significantly lower in astrocytoma grade II compared to control cortex, as well as astrocytoma grade III (table 2). GBM showed variable Kir4.1 expression and the IR score was not significantly different compared to the other tumor subtypes (table 2; Fig. 4 E-F). The variable Kir4.1 expression in GBM is also reflected by western blot analysis of total homogenates (Fig. 4 G). However, in this retrospective study, the number of frozen specimens available was too small to perform statistical comparisons in subgroups.

**Kir 4.1 expression and epilepsy.** The expression and distribution of Kir 4.1 IR was compared in tumor tissue of patients with astrocytoma WHO grade II, WHO grade III and GBM with or without epilepsy. A significantly lower Kir 4.1 expression was found in tumor tissue of
Figure 4. Expression of Kir4.1 immunoreactivity (IR) in glial tumor from patients with and without epilepsy.

(A): control white matter showing Kir4.1 IR in processes of perivascular astrocytes. (B): astrocytoma grade II (AII); (C-F): representative photomicrographs of Kir4.1 IR in astrocytoma grade III (A III; C-D) and glioblastoma multiforme (GBM; E-F) with (C and E) and without epilepsy (D and F); inserts: high magnifications, showing cytoplasmic staining, with weak IR in epilepsy associated tumors. Sections were counterstained with hematoxylin. Scale bars: A: 40 μm; B-F: 160 μm.

(F): Representative immunoblot of total homogenates from control cortex and GBM (with and without epilepsy) that revealed a band at a molecular weight of approximately 40 kDa.
patients with epilepsy (Figure 4B, C, E; table 3). qPCR demonstrated lower Kir 4.1 mRNA expression in GBM with epilepsy compared to GBM without epilepsy (supplementary Fig. 2A). The number of astrocytomas grade II and grade III with and without epilepsy was too small to perform a meaningful statistical comparison between these subgroups so that we could not assess whether Kir 4.1 expression is dependent on the presence of seizures or tumor type.

Figure 5. Expression of IL-1β immunoreactivity (IR) in glial tumors from patients with and without epilepsy.
Representative photomicrographs of IL-1β IR in control white matter (A), astrocytoma grade II (All; B, with epilepsy), astrocytoma grade III (A III; A-D) and glioblastoma multiforme (GBM; E-F) with (C and E) and without epilepsy (D and F); inserts: high magnifications, showing strong IR in epilepsy associated tumors. Sections were counterstained with hematoxylin. A-F: scale bar in A: 80 μm.
**IL-1β immunoreactivity.** As previously reported [16, 17, 33], IL-1b was under detection level in both surgical and autopsy cortical specimens of healthy controls (Fig. 5 A). Expression of IL-1b was detected in the different tumor subtypes in tumor cells (Fig. 5 B-F). The IR score for each tumor and control tissue is summarized in table 2. The IR score was significantly higher in astrocytoma grade II, III, as well as GBM compared to control cortex. No significant differences were detected between tumor subtypes (table 2). Astrocytoma grade II, III, as well as GBM displayed also higher IR score for HLA-DR compared to controls (table 2).

**IL-1β expression and epilepsy.** A significantly higher IL-1b expression was observed in tumor tissue of patients with epilepsy (Figure 5 B, C, E; table 3). qPCR demonstrated higher IL-1b mRNA expression in GBM with epilepsy compared to GBM without epilepsy (supplementary Fig. 2B). Tumor tissue of patients with epilepsy displayed also a higher IR score for HLA-DR compared to controls (table 3; supplementary Fig. 3). We also evaluated the cellular localization of HMGB1, a nuclear protein that can also act as an extracellular signal of inflammation [39-41]. We observed increased cytoplasmic translocation of HMGB1 IR in tumor tissue of patients with epilepsy compared to patients without epilepsy; supplementary Fig. 3).

**Figure 6.** Expression of Kir4.1 and IL-1β immunoreactivity (IR) in glial tumors from patients with epilepsy, with and without levetiracetam use.
Representative photomicrographs of Kir4.1 (A-B; high magnifications in inserts) and IL-1β (C-D) IR in glioblastoma multiforme (GBM) without (A and C) and with (B and D) levetiracetam treatment (LEV); Sections were counterstained with hematoxylin. A-D: scale bar in D: 80 μm.
The number of astrocytomas grade II and grade III with and without epilepsy was too small to perform a meaningful statistical comparison in these subgroups. The Spearman’s rank correlation test was applied to evaluate the relationship between Kir4.1 and IL-1b IR. A weak but significant negative correlation was observed between the Kir4.1 and the IL-1b IR score ($r = -0.3663; P < 0.05$). No significant correlations were found between Kir4.1 or IL-1b IR and clinical variables such as age at surgery, age at seizure onset and duration of epilepsy.

**Kir 4.1 expression in levetiracetam treated patients.** The expression of Kir4.1 was evaluated in relation to AED regimens, in particular to levetiracetam treatment in patients with epilepsy. A significantly higher Kir4.1 IR was observed in the patients treated with levetiracetam compared to the patients who were not treated with this AED (table 3; Fig. 6 B). In contrast a lower expression of IL-1b (table 3; Fig. 6 D) was observed in levetiracetam treated patients, whereas no differences were observed for HLA-DR. In addition, the seizure free interval was evaluated in levetiracetam treated patients to assess whether Kir4.1 expression was associated with the presence of seizures and whether it was influenced by levetiracetam treatment. Of the 14 patients with epilepsy who were treated with levetiracetam, 6 patients were seizure free, 6 patients were not and in 1 patient no data regarding seizure free interval was available. Kir4.1 expression was not correlated with seizure free interval in levetiracetam treated patients.

**DISCUSSION**

The present study we investigated the effect of the proinflammatory molecule IL-1β on the expression of Kir4.1, a major K⁺ inward rectifying channel in astrocytes. In addition, the expression pattern of Kir4.1 in primary human glial tumors and its relationship to seizure activity and inflammation was studied. The following observations were made: (1) in a rat model of TLE, Kir4.1 mRNA and protein were significantly down-regulated in temporal cortex 24h after onset of SE; this down-regulation corresponded to the time of prominent upregulation of IL-1β. (2) IL-1β treatment reduced the expression of Kir4.1 mRNA and protein in both a glioma cell line and human astrocytes in culture. (3) Kir4.1 expression was lower in tumors with epilepsy compared to tumors without epilepsy. (4) Astrocytic tumors with epilepsy displayed higher IL-1β IR compared to tumors without epilepsy (5) Among the patients with epilepsy, a significantly higher Kir4.1 IR was detected in the patients treated with levetiracetam compared to the patients who did not use this antiepileptic drug. The significance of these findings in relation to epileptogenesis in astrocytic tumors is discussed below.
Down-regulation of Kir4.1 mRNA after induction of SE parallels the increased IL-1β expression.

Impaired potassium buffering and enhanced seizure susceptibility have been suggested to result from reduced expression of Kir4.1 channel in TLE ([11-14, 42]; for review see [2]). A previous micro-array study in the electrical post-SE rat model showed that several potassium channel genes, including Kir channels were found to be down-regulated 24 hrs after induction of SE in the CA3 region of the hippocampus [43]. The present study confirmed the down-regulation of Kir4.1 mRNA at 24h post SE in the temporal cortex. However, this decrease in expression (both mRNA and protein) recovered to control levels after the latent period. A recent study suggests a role for inflammatory cytokines, such as IL-1β, in the regulation of the expression of Kir4.1 [21]. Interestingly, experimentally induced seizures in rodents trigger a rapid up-regulation of IL-1β and its receptor ([43, 44]; for review see [1, 22]). IL-1β is among the best-characterized early-response inflammatory cytokines and a key mediator in the response of the brain to various forms of CNS injury (for review see [45-47]. Accordingly, in the present study, it was observed that IL-1β peaked in the temporal cortex at 1 day after SE, which corresponds to the time point of prominent reduction of Kir4.1 expression. A decrease of functional Kir channels has been shown in other pathologies associated with activation of the inflammatory response, including amyotrophic lateral sclerosis and retinopathies ([48-50]); for review see [9]). These observations suggest a role for IL-1β in the regulation of Kir4.1 mRNA expression, which was further investigated in vitro, using glial cells in culture.

IL-1β treatment down-regulated Kir4.1 expression human glial cells.

Both U373 glioblastoma cells and human fetal astrocytes in culture expressed Kir4.1 mRNA and protein. Immunocytochemical analysis showed cytoplasmic expression of Kir4.1 in human astrocytes, whereas both cytoplasmic and nuclear expression was observed in glioma cells. This is in agreement with previous studies reporting a mislocalization of Kir channels to the nucleus in glioma cell lines [18]. In the present study, IL-1β treatment significantly decreased Kir4.1 mRNA levels in both the U373 glioma cell line and fetal astrocytes in culture. This effect (already observed at 30 min and maximal at 24 h after exposure to IL-1 β) could explain the suppression of Kir4.1 mRNA expression observed after seizure-induced release of this cytokine in vivo. Under our experimental conditions, the effect of downregulation of Kir4.1 expression observed with IL1-b treatment could not be reproduced by other pro-inflammatory cytokines, such as IL-6 and TNFa or the toll-like receptor 4 agonist, HMGB1. The observation that IL-1Ra inhibited the effect of IL-1b on suppression of Kir4.1 is consistent with the fact that IL-1b signals through the type I IL-1 receptor. IL-1Ra is a naturally oc-
curring antagonist of the IL-1 receptor [38], which is also regulated in response to different forms of CNS insult (for review see [1, 22, 51]). Thus, it is tempting to speculate that differential expression of inhibitory components of the IL-1 system and in particular local changes in the IL-1Ra/IL-1b ratio in brain, may critically contribute to the regulation of Kir4.1 expression under both physiological and pathological conditions. Moreover, the effect of IL-1b was partially reversible, with Kir4.1 levels showing partial recovery 48 h after removal of the cytokine. These observations suggest that the expression of Kir4.1 mRNA could be critically influenced by local dynamic changes in the level of IL-1b in the extracellular environment.

Recently, anti-inflammatory effects have been reported for levetiracetam [24, 25] an AED frequently used to treat partial onset seizures, also in patients with brain tumors (for review see [52]). In particular, treatment with this AED in neonatal rat astrocytes that were co-cultured with activated microglia or treated with IL-1β has been shown to restore impaired astrocyte membrane resting potentials via modification of inward and outward rectifier currents [25]. These studies prompted us to evaluate the effect of levetiracetam on IL-1β-induced Kir4.1 down-regulation observed in human astrocytes and glioma cells. Under our experimental condition, levetiracetam was not able to counteract the downregulatory effect of IL-1β on Kir4.1 mRNA. It could be conceived that this lack of effect of levetiracetam on IL-1b treated cells is related to the dose of the cytokine used. However, in the absence of IL-1b, levetiracetam positively regulated Kir4.1 mRNA expression. The potential effect of a chronic exposure to levetiracetam on IL-1b and Kir4.1 protein expression was further investigated in surgical astrocytic tumor specimens from patients treated with levetiracetam.

**Differential expression of Kir4.1 and IL-1b in astrocytic tumors.**

Immunocytochemical analysis showed variable Kir4.1 expression in astrocytic tumors with mainly cytoplasmic staining in tumor cells. Decrease of IR in glial processes and particularly in perivascular astrocyte endfeet was observed in both low- and high-grade gliomas, whereas nuclear expression was detected only occasionally in high-grade gliomas. Thus, the localization in the nucleus observed in glioma cell lines in culture ([18]; present study) does not represent a consistent feature of human primary glial tumor. Accordingly, nuclear localization has not been reported in other studies analyzing the expression pattern of Kir4.1 in surgical specimens of both low- and high-grade astrocytomas [16, 17]. However, in agreement with our observations, Warth and colleagues [16] reported a redistribution of Kir4.1 in astrocytomas (with reduced perivascular astrocyte endfeet), suggesting a compromised buffering capacity of glial tumor cells. In our study the IR score was significantly lower in astrocytoma grade II compared to astrocytomas grade III, whereas no differences were observed compared to GBM. Tan and colleagues [17] investigated the expression of
Kir4.1 mRNA and protein in astrocytic tumors and reported higher expression in high-grade astrocytic tumors compared to low-grade tumors. They suggested that activation of Kir4.1 produced intracellular alkalinization and could promote proliferation and inhibit apoptosis in the tumors [17]. In contrast, other studies suggest that the function of Kir4.1 channel is correlated with an exit from the cell cycle (for review see [9]). Thus the consequences of alterations in Kir4.1 expression on the proliferation of astrocytic tumors are still unclear and remain to be further explored. Moreover, in these previous studies [16, 17] no information concerning the presence/absence of epilepsy or about the AED treatment in epileptic patients was available and considered in the evaluation of the correlation between Kir4.1 expression and pathologic tumor grade.

In the present study, we evaluated the expression of Kir4.1 and IL-1β in patients in relation to the presence or absence of epilepsy. We found a significantly lower Kir4.1 expression in tumor tissue of patients with epilepsy, which paralleled the increased expression of IL-1β. The IL-1β-mediated down-regulation of Kir4.1 expression could represent an additional mechanism contributing to the pro-epileptogenic effect of this cytokine. In our study we found a significant higher IL-1β expression in tumor tissue of patients with epilepsy and a significant (although weak) negative correlation with the expression levels of Kir4.1. Interestingly, a recent study shows that minocycline treatment in the retina of diabetic rats, increases Kir4.1 levels and this effect is associated with a decrease of the levels of IL-1β [21].

Cytokine production, including also IL-1β, has been previously reported in human astrocytoma cell lines and surgical specimens of astrocytic tumors ([53-56] for review see [57]). We confirmed IL-1β expression in tumor cells, of both low- and high-grade astrocytomas, in agreement with the notion that astroglial cells represent a main source of brain IL-1β [1, 58]. Accordingly, high expression of IL-1β has also been reported in tumor astrocytes in ganglioglioma, which represent a well-known cause of chronic intractable epilepsy [59]. The expression of IL-1β in tumor astrocytes may be involved in enhancing neuronal excitability in the peritumoral region (for reviews see [1, 58]). A cytokine-mediated inhibition of glutamate reuptake by astrocytes may lead to increased extracellular glutamate concentrations [60, 61]. Additionally, IL-1β has been shown to increase nitric oxide production and cortical glutamate release [62]. Furthermore, IL-1β may also regulate gamma-aminobutyric acid (GABA)-mediated Cl⁻ fluxes (possibly reducing inhibitory transmission) and molecular and functional interactions between IL-1β and N-methyl-D-aspartate (NMDA) receptors have been recently reported (for reviews see [1, 58]). Substantial experimental evidence supports the proconvulsant role of IL-1β (for review see [1, 58, 63]). Thus, production of IL-1β by tumor astrocytes may (through different mechanisms) contribute to the epileptogenicity of glial tumors (for review see [52]). Interestingly, the higher expression of IL-1β in tumors
associated with epilepsy was linked with increased presence of activated microglial cells, as well as with the cytoplasmic translocation of HMGB1, which may contribute to amplify the inflammatory response via a signaling pathway involving the TLR4 [34]. Relocation of the nuclear protein to the cytoplasm has been shown to be induced in rat [64] and human cultured astrocytes and glioma cells by IL-1b [65]. In addition, a potential role for HMGB1 has been suggested in promoting growth and migration of human glioblastoma cells [66]. Since rapid changes in IL-1β and Kir4.1 expression are induced by seizures in experimental models (present results; for review see [1, 58, 63]), we cannot exclude that seizure activity may contribute to their level of expression. No significant correlation was found between Kir4.1 (or IL-1 1β) IR and duration of epilepsy in our cohort. However since our study does not focus on long-term epilepsy-associated tumors (LEATs; [67]), future investigations on a large cohort of LEATs are necessary to address the relationship between Kir4.1 expression and/or function and duration and/or severity of epilepsy.

As discussed above, anti-inflammatory effects have been reported for levetiracetam [24, 25]. In particular, treatment with this AED (but not with valproic acid) has been shown to reduce reactive gliosis and expression levels of IL-1b in the hippocampus and the piriform cortex in chronic epileptic rats [24]. These observations prompted us to evaluate the expression levels of both IL-1β and Kir4.1 in relation to AED treatment, in particular the exposure to levetiracetam. Among the patients with epilepsy who were treated with levetiracetam, we found significantly higher expression levels of Kir4.1, and, conversely, lower expression of IL-1b, compared to patients who did not use this AED. These observations, together with previous experimental findings [24] suggest that an anti-inflammatory effect (targeting the IL-1b system) could, at least in part, contribute to the anti-epileptic effect of levetiracetam. We acknowledge limitations to the interpretation of these results, since the cohort was relatively small to assess whether Kir 4.1 expression is directly dependent on the presence or absence of seizures or on tumor type. Further quantitative analysis and *in vitro* electrophysiological studies are needed to confirm these findings and to establish their functional significance. However, this report underscores the complexity of Kir4.1 alterations in astrocytes and astrocytic tumor cells, and the potential contribution of the local inflammatory environment, involving in particular the pro-inflammatory cytokine IL-1β, in regulating the expression of Kir4.1 in epilepsy-associated lesions. Whether this mechanism could play a role in other neurological disorders (multiple sclerosis, neurodegenerative disorders) characterized by high levels of IL-1β and dysfunction of Kir4.1 [50, 68-70] deserves further investigation.
AUTHORS’ CONTRIBUTIONS
The experiments in the rat model of temporal lobe epilepsy were performed by JG and EvV. Cell culture experiments were performed by EZ, AI. Immunohistochemistry, qPCR, western blot and analysis of the data were performed by EZ, MG, AI, JA. EZ, MG, JG and AI helped EA in drafting and preparing the manuscript for submission. The overall experimental design was conceived and supervised by EA, JG, JR and JH. MG, JR and JH helped in the selection and collection of brain tissues. We are grateful to Caterina Carbonell for technical assistance.

Competing interests
The authors declare that they have no competing interests.

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Regulation of inflammation in astrocytes: the role of miR 146a
3.1 Expression pattern of miR-146a in experimental and human temporal lobe epilepsy

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Abstract.  
Increasing evidence supports the involvement of inflammatory and immune processes in temporal lobe epilepsy (TLE). MicroRNAs (miRNA) represent small regulatory RNA molecules that have been shown to act as negative regulators of gene expression controlling different biological processes, including immune-system homeostasis and function. We investigated the expression and cellular distribution of miRNA-146a (miR-146a) in a rat model of TLE as well as in human TLE. miR-146a analysis in rat hippocampus was performed by polymerase chain reaction and immunocytochemistry at 1 week and 3–4 months after induction of status epilepticus (SE). Prominent upregulation of miR-146a activation was evident at 1 week after SE and persisted in the chronic phase. The miR-146a expression was confirmed to be present in reactive astrocytes. In human TLE with hippocampal sclerosis, increased astroglial expression of miR-146a was observed mainly in regions where neuronal cell loss and reactive gliosis occurred. The increased and persistent expression of miR-146a in reactive astrocytes supports the possible involvement of miRNAs in the modulation of the astroglial inflammatory response occurring in TLE and provides a target for future studies aimed at developing strategies against pro-epileptogenic inflammatory signalling.

Introduction

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Temporal lobe epilepsy (TLE) is a common and often medically intractable neurological disorder. TLE is often associated with hippocampal sclerosis (HS) which is histopathologically characterized by selective neuronal cell loss, gliosis and synaptic reorganization (Thom 2004, Wieser and Epilepsy 2004). Increasing evidence highlights the activation of inflammatory pathways in TLE and suggests that a persistent upregulation of inflammatory gene expression may contribute to the etiopathogenesis of TLE (Vezzani and Granata 2005, Vezzani, et al. 2008).

MicroRNAs (miRNA) represent an evolutionarily conserved class of endogenous ~22-nucleotide non-coding RNAs that act as small regulatory molecules involved in posttranscriptional gene repression (Cao et al., 2006; Tsai & Yu, 2009). Several miRNAs have been found in the human brain, and they are found to play a crucial role in a wide range of biological processes, including the regulation of the innate and adaptive immune response (Pedersen & David, 2008; Sonkoly et al., 2008; Pauley et al., 2009). Unique miRNA expression profiles have been recently reported in injured rat hippocampus after ischaemic stroke, intracerebral haemorrhage and kainic acid-induced acute seizures (Liu et al., 2009). In addition to the brain, miRNAs are also reported to be regulated in blood, suggesting the possible use of blood miRNAs as biomarkers for brain injury (Liu et al., 2009).

Attention has been focused on miRNA-146a (miR-146a), which can be induced by different pro-inflammatory stimuli, such as interleukin (IL)-1β and tumour necrosis factor alpha (TNF-α; Taganov et al., 2006; Sheedy & O’Neill, 2008), and is upregulated in various human pathologies associated with activation of inflammatory responses (Lukiw et al., 2008; Nakasa et al., 2008; Pauley et al., 2008; Sonkoly et al., 2008). Furthermore, miR-146a has been shown to critically modulate innate immunity through regulation of Toll-like receptor (TLR) signalling and cytokine responses (Taganov et al., 2006; Pedersen & David, 2008; Sheedy & O’Neill, 2008). Thus, miRNA, such as miR-146a, may represent a potentially interesting tool for therapeutic intervention in pathological conditions where inflammatory processes are key players in the disease biology. In order to understand the regulation and function of miR-146a in epilepsy, we investigated the dynamics of miR-146a expression during epileptogenesis in a rat model of TLE, as well as the expression and cellular distribution in hippocampal specimens of patients with TLE with HS.
Material and methods

Experimental animals

Adult male Sprague Dawley rats (Harlan CPB laboratories, Zeist, The Netherlands) weighing 300-500 grams were used in this study which was approved by the University Animal Welfare committee. The rats were housed individually in a controlled environment (21±1°C; humidity 60%; lights on 08:00 AM - 8:00 PM; food and water available ad libitum).

Electrode implantation and seizure induction

Rats were anaesthetized with an intramuscular injection of ketamine (57 mg/kg; Alfasan, Cuyk, The Netherlands) and xylazine (9 mg/kg; Bayer AG, Germany), and placed in a stereotactic apparatus. In order to record hippocampal electroencephalograms (EEG), a pair of insulated stainless steel electrodes (70 μm wire diameter, tips were 80 μm apart) were implanted into the left dentate gyrus (DG) under electrophysiological control as previously described (Gorter et al., 2001). A pair of stimulation electrodes was implanted in the angular bundle. Rats underwent tetanic stimulation (50 Hz) of the hippocampus in the form of a succession of trains of pulses every 13 s. Each train had a duration of 10 s and consisted of biphasic pulses (pulse duration 0.5 ms, maximal intensity 500 μA). Stimulation was stopped when the rats displayed sustained forelimb clonus and salivation for minutes, which usually occurred within 1 h. However, stimulation never lasted longer than 90 min. Differential EEG signals were amplified (10 ×) via a FET transistor that connected the headset to a differential amplifier (20 ×; CyberAmp, Axon Instruments, Burlingame, CA, USA), filtered (1–60 Hz), and digitized by a computer. A seizure detection program (Harmonie, Stellate Systems, Montreal, Canada) sampled the incoming signal at a frequency of 200 Hz per channel. EEG recordings were also monitored visually and screened for seizure activity. Behaviour was observed during electrical stimulation and several hours thereafter. Immediately after termination of the stimulation, periodic epileptiform discharges occurred at a frequency of 1–2 Hz and they were accompanied by behavioural and EEG seizures (SE; status epilepticus). Most rats were monitored continuously from the cessation of SE to the time of death (24 h–1 week). The chronic epileptic group (3–4 months after SE) was monitored during and shortly after SE, and during 3–5 days before death in order to determine the frequency of spontaneous seizures. Sham-operated control rats were handled and recorded identically, but did not receive electrical stimulation. None of these rats needed to be reimplanted. Chronic epileptic rats had frequent daily seizures (range, 5–12). The time between the last spontaneous seizure and the time the animals were killed was < 5 h. The experimental protocols followed the European Communities Council directive 86/609/EEC and the Dutch Experiments on Animals Act (1997), and were approved by the Dutch Animal Welfare Committee (DEC).
Rat tissue preparation for RNA isolation

After decapitation, the hippocampus was removed and sliced into smaller parts (200-300 µm). CA3 region was dissected from the slices under a dissection microscope. All material was frozen on dry ice and stored at -80 °C until use. Rats were decapitated in the acute phase (one day after SE, n=3), in the latent period (1 week after SE, n=6; the rats in this group did not exhibit spontaneous seizures) and in the chronic epileptic phase (3-4 months after SE, n=3; only rats that exhibited daily seizures were included in this group). Rats that did not develop SE during stimulation (non-SE rats) were included as controls and were sacrificed 3-4 months after stimulation (n=3).

Rat tissue preparation for immunocytochemistry and in situ hybridization

Rats were disconnected from the EEG recording set-up and deeply anaesthetized with pentobarbital (Nembutal, intraperitoneally, 60 mg/kg). For immunocytochemistry, the animals were perfused through the ascending aorta with 300 mL of 0.37% Na2S solution, followed by 300 mL 4% paraformaldehyde in 0.1 m phosphate buffer, pH 7.4. Thereafter, the brains were removed, incubated for 72 h in 0.3 m EDTA, pH 6.7 (Merck, Amsterdam, The Netherlands) and paraffin embedded. Paraffin-embedded tissue was sectioned at 6 µm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel GmbH, Brunschweig, Germany) and used for in situ hybridizations and immunocytochemistry. Horizontal sections were analysed at a mid-level of the brain (5300–6100 µm below cortex surface). In situ hybridization was performed on two adjacent serial hippocampal sections from each group (control, n = 6; 24 h, n = 4; 1 week, n = 6; 3–4 months, n = 6). Two additional serial slices were used for the double-staining, combining in situ hybridization with immunocytochemistry (in the same slices) with different antibodies, as described below.

Human material

The human cases included in this study were obtained from the files of the Department of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and the VU University Medical Center (VUMC). Ten patients underwent resection of the hippocampus for medically intractable TLE. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All samples were obtained and used in a manner compliant with the Declaration of Helsinki. Two neuropathologists reviewed all cases independently. In six cases a pathological diagnosis of HS (without extra-hippocampal pathology) was made. The HS specimens include four cases of classical HS (grade 3, mesial temporal sclerosis type 1a) and two cases of severe HS (grade IV; mesial temporal sclerosis type 1b; Wyler et al., 1992; Blumcke et al., 2007). Four non-HS cases, in which a focal lesion (ganglioglioma not involving the hippocampus proper) was identified,
were also included to provide a comparison group to HS cases. Control hippocampal tissue was obtained at autopsy from five patients without history of seizures or other neurological diseases. Brain tissue from a patient with viral encephalitis was also used for in situ hybridization (as positive control for miR-146a expression). All autopsies were performed within 12 h after death. Table 1 summarizes the clinical features of TLE and control cases. Hippocampus from control patients (n = 5) and surgical hippocampal tissue block from patients with HS (n = 6) was snap frozen in liquid nitrogen and stored at −80°C until further use [RNA isolation for reverse transcriptase polymerase chain reaction (RT-PCR)]. Additional hippocampal tissue from the same patients and from four non-HS cases was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 μm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel GmbH, Brunswick, Germany) and organosilane-coated slides (SIGMA, St Louis, MO, USA), and two slices were used for in situ hybridizations and immunocytochemistry. Two additional slices were used for the double-staining, combining in situ hybridization with immunocytochemistry (in the same slices) with different antibodies, as described below. Additional immunocytochemistry (single-labelling) was performed for complement factor H (CFH) in both control and HS hippocampal tissue.

**RNA isolation**

For RNA isolation, frozen material was homogenized in Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA). After addition of 200 μg glycogen and 200 μL chloroform, the aqueous phase was isolated using Phase Lock tubes (Eppendorf, Hamburg, Germany). RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and dissolved in water. The concentration and purity of RNA were determined at 260/280 nm using a nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA).

**Real-time quantitative PCR analysis (qPCR).** cDNA was generated using Taqman MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions. miRNA (miR-146a and the U6B small nuclear RNA gene, rnu6b) expression was analysed using Taqman microRNA assays (Applied Biosystems), which were run on a Roche Lightcycler 480 (Roche Applied Science, Basel, Switzerland) according to the instructions of the manufacturer. Data analysis was performed with the software provided by the manufacturer.

**Statistical analysis**

Statistical analyses were performed with spss for Windows (spss 11.5, SPSS, Chicago, IL,
USA) using two-tailed Student’s t-test, and to assess differences between more than two groups a non-parametric Kruskal–Wallis test followed by Mann–Whitney U-test were used. A value of P < 0.05 was considered significant.

**In situ hybridization.** In situ hybridization for miR-146a was performed using a 5’ fluorescein-labelled 19mer antisense oligonucleotide containing locked nucleic acid and 2’OME RNA moieties (FAM – AacCcaTggAauTcaGuuCucA, capitals indicate LNA, lower case indicates 2’OME RNA). The oligonucleotides were synthesized by Ribotask ApS, Odense, Denmark. The hybridizations were done on 6-μm sections of paraffin-embedded materials described previously (Budde et al., 2008). The hybridization signal was detected using a rabbit polyclonal anti-fluorescein/Oregon green antibody (A21253, Molecular Probes, Invitrogen) and a horseradish peroxidase-labelled goat anti-rabbit polyclonal antibody (P0448 Dako, Glostrup Denmark) as secondary antibody. Signal was detected with chromogens 3-amino-9-ethyl carbazole (St Louis, MO, USA) or Vector NovaRed (Vector Laboratories, Burlingame, CA, USA), and the nuclei were stained with haematoxylin. Slides were sealed with glycerol-gelatin (St Louis, MO, USA). As control for non-specific binding, other similarly modified oligonucleotides were used. These probes were specific for other human transcripts (miR-338, MIMAT0004701; miR-218, MIMAT0000275; miR-204, MIMAT0000265; miR-134, MIMAT0000447). These oligonucleotides showed different staining patterns (no expression in glial cells). Additionally negative control assays were performed without probes and without

<table>
<thead>
<tr>
<th>Total or mean (Range or percentage)</th>
<th>Male/female</th>
<th>HS (n = 6)</th>
<th>Non-HS (n = 4)</th>
<th>Control (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at surgery (Mohaupt, et al.)</td>
<td>31.5 (19-50)</td>
<td>35.1 (21-43)</td>
<td>44.3 (26 –52)</td>
<td></td>
</tr>
<tr>
<td>Seizure type</td>
<td>CPS (100%); SGS (17%)</td>
<td>CPS (100%); SGS (17%)</td>
<td>NA</td>
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<tr>
<td>Seizure frequency (CPS/months)</td>
<td>10.6 (3-50)</td>
<td>10.1 (3-30)</td>
<td>NA</td>
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<tr>
<td>Duration of epilepsy (Mohaupt, Karas, Babiychuk, Sanchez-Freire, Monastyrskaya, Iyer, Hoppeler, Breil and Draeger)</td>
<td>11.8 (9 – 20)</td>
<td>12.8 (9 – 18)</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

HS, hippocampal sclerosis; CPS, complex partial seizures; SGS, secondary generalized seizures; HS, hippocampal sclerosis; NA: not applicable.
primary antibody (sections were blank). For the double-staining, combining immunocytochemistry with in situ hybridization, sections were first processed for immunocytochemistry as previously described (Aronica et al., 2001a, 2003) with glial fibrillary acidic protein (GFAP; polyclonal rabbit; DAKO, Glostrup, Denmark; 1 : 4000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1 : 2000), HLA-DR [anti-human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43); DAKO, Glostrup, Denmark; 1 : 400], CFH (polyclonal goat; Quidel, San Diego, CA, USA; 1 : 100) or the biotinylated lectin Ricinus Communis Agglutinin I (RCA 120; Vector Laboratories, Burlingame, CA, USA; 1 : 500, for the visualization of microglial cells on rat tissue), using Fast Blue B salt (St Louis, MO, USA) or Vector Blue substrate (Vector Laboratories) as chromogen. After washing, sections were processed for in situ hybridization as described above. Images were captured with an Olympus microscope (BX41, Tokyo, Japan) equipped with a digital camera (DFC500, Leica Microsystems-Switzerland, Heerbrugg, Switzerland).

To analyse the percentage of double-labelled cells positive for miR-146a and GFAP, or for the microglia marker (HLA-DR, human; lectin, rat), digital photomicrographs were obtained from five hippocampal samples. Images of three representative fields (CA3 and DG) per section were collected (Leica DM5000B). Images were analysed with a Nuance VIS-FL Multispectral Imaging System (Cambridge Research Instrumentation, Woburn, MA, USA). Spectra were acquired from 460–660 nm at 10-nm intervals, and Nuance software (version 2.4) was used for analysis, as previously described (Boer et al., 2008; van der Loos, 2008). The total number of cells stained with miR-146a and GFAP (or HLA-DR or lectin), as well as the number of cells double-labelled, were counted visually and percentages were calculated (expressed as mean ± SEM) of cells co-expressing miR-146a and GFAP (or HLA-DR or lectin) in two regions

Figure 1. Quantitative real-time PCR of miR-146a expression in rat and human hippocampus
(A) Fold change (compared with expression in control rats, n = 6) in CA3 at 24 h (n = 4), 1 week (n = 6) and 3–4 months (n = 6) after status epilepticus (SE) or after stimulation (non-SE; n = 4). (B) Expression levels of miR-146a in human (autopsy) control hippocampus (n = 5) and human hippocampal sclerosis specimens (HS; n = 6). In both rat and human tissue the miR-146a expression was normalized to that of the U6B small nuclear RNA gene (rnu6b). The error bars represent SEM; statistical significance: *P < 0.05 compared with control.
of prominent gliosis (CA3 and DG of rat, at 1 week post-SE, and of human hippocampus). Sections incubated without the primary Ab or with pre-immune serum were blank, and when processed for in situ hybridization showed only the in situ hybridization signal.

**RESULTS**

**miR-146a expression by real time qPCR**

miR-146a expression was studied using qPCR in both rat and human hippocampal tissue. miR-146a expression in rat CA3 region was significantly increased at 1 week (latent phase) and 3–4 months (chronic phase) post-SE, compared with non-SE values (Fig. 1A). Increased miR-146a expression was also observed in human TLE HS specimens compared with control hippocampus (Fig. 1B). In both rat and human tissue the miR-146a expression was normalized to that of the U6B small nuclear RNA gene (rnu6b).

**miR-146a expression by in situ hybridization in rat hippocampus**

To determine the temporal–spatial expression and cellular distribution of miR-146a, we performed in situ hybridization using LNA- and 2’OMe RNA-modified oligonucleotides in tissue samples of control rats and rats that were killed at different time points after SE (1 day, 1 week and 3–4 months post-SE). In control hippocampus miR-146a was confined to neuronal cells, including pyramidal cells of CA1 and CA3 regions, as well as granule cells and hilar neurons of the DG (Fig. 2A, C, E and G). No detectable staining was observed in resting glial cells. At 1 day post-SE, miR-146a showed a similar pattern as control hippocampus, with predominant neuronal staining; occasionally expression was observed in cells with glial appearance in the areas of neuronal damage (CA1, CA3, hilus; not shown). At 1 week post-SE (Fig. 2B, D, F and H–J), prominent upregulation of miR-146a expression was detected within the different hippocampal regions in glial cells. Strong and diffuse glial miR-146a expression was particularly observed in the inner molecular layer of the DG and in the hilar region (Fig. 2I). Pyramidal neurons of CA1 and CA3 regions and granule cells of DG also displayed strong miR-146a expression. In the chronic phase (3–4 months post-SE) the hippocampus showed a pattern similar to that observed at 1 week post-SE, with both neuronal and glial expression, which was mainly localized in regions of prominent gliosis, such as the hilar region (Fig. 2J). Co-localization studies indicated that miR-146a was induced in glial cells in this region and that expression was confined to astrocytes, whereas no detectable expression was observed in lectin-positive cells of the microglial/macrophage lineage (Fig. 2J and inserts a/b). The percentage of cells positive for miR-146a and co-expressing GFAP was quantified in both CA3 and DG at 1 week post SE (76 ± 2, CA3; 70 ± 4, DG). No co-localization with lectin was observed in both regions.
Figure 2. In situ hybridization analysis of miR-146a expression in hippocampal tissue of control rats and after induction of SE. (A, C, E, G) Control hippocampus showing neuronal miR-146a expression in the different hippocampal subfields, including pyramidal neurons of CA3 (C; insert in C) and CA1 (E; insert in E) regions, granule cells of the dentate gyrus (DG; G) and hilar neurons (G; insert in G: high-magnification of hilar neurons). gcl, granule cell layer. (B, D, F, H) Hippocampus 1 week post-SE showing increased miR-146a expression within the different hippocampal regions, including CA3 (D; insert in D: high-magnification of CA3), CA1 (F; insert in F: high-magnification of CA1) and DG (H). miR-146a expression was observed in both neuronal and glial cells; arrows in D, F (and insert in D) indicate positive pyramidal neurons of CA3 and CA1; positive glial cells (arrowheads in D, F and insert in D) were particularly abundant in regions of prominent gliosis (CA1, CA3 and the inner molecular layer, iml, of the DG). Insert in (H) shows a high-magnification of positive glial cells in the DG (iml). (I and J) In situ hybridization analysis of miR-146a expression in the hilar region of the hippocampus 1 week (I) and 3–4 months post-SE (J) showing increased expression in glial cells (arrows; insert in I). Sections are counterstained with haematoxylin. Inserts in (J): in situ hybridization and immunohistochemistry analysis showing in (a) absence of miR-146a (red) expression in lectin-positive microglial cells (blue) and in (b) colocalization with the astroglial marker glial fibrillary acidic protein (GFAP; purple) in astrocytes. Scale bars: 1250 μm (A and B); 70 μm (C–J); 35 μm (inserts in C, D, F and G); 20 μm (insert in E); 17 μm (inserts in H and I); 11 μm (insert in J). LT, long-term, 3–4 months after SE.
Figure 3. In situ hybridization of miR-146a expression in the hippocampus of control and patients with TLE with hippocampal sclerosis (HS). (A and C) Control hippocampus showing miR-146a expression in pyramidal neurons of CA1 (A), in granule cells of the dentate gyrus (DG; C) and hilar neurons (C and insert in C). gcl, granule cell layer. (B, D–F) HS showing increased miR-146a expression in CA1 (B) and DG (D–F). (B) miR-146a expression is observed in residual pyramidal neurons (arrows in B, CA1), as well as in glial cells (arrowheads). (D) Increased miR-146a expression is observed in the inner molecular layer (iml) of the DG. (E) High-magnification of DG with positive neuronal (gcl) and glial cells (iml; arrows). (F) High-magnification of the hilar region of the DG showing positive glial cells (arrows; insert). (G–J) Double in situ hybridization and immunohistochemistry analysis in HS, with microglial (human leukocyte antigen (HLA)-DR, G–H) and astroglial markers (glial fibrillary acidic protein (GFAP), I), and with complement factor H (CFH, J). (G) miR-146a expression in residual CA3 pyramidal neurons (red), but absence of expression in HLA-DR-positive microglial cells (blue). (H) (hilus) miR-146a expression in a glial cell (red; HLA-DR-negative), but the absence of expression in a HLA-DR-positive microglial cell (blue). (I) Co-localization (purple) of miR-146a in a GFAP-positive astrocyte. (J) miR-146a expression (red) in glial cells expressing CFH (blue). Scale bars: 80 μm (A and B, and E and F); 120 μm (C and D); 40 μm (G); 30 μm (H); 15 μm (I); 20 μm (J); 40 μm (insert in F).
miR-146a expression by in situ hybridization in hippocampal sclerosis

The cellular distribution of miR-146a in human hippocampus was investigated using in situ hybridization. Differences in the expression level, as well as in the cell-specific distribution, were found in specimens from patients with HS (Fig. 3). In control hippocampus, we observed miR-146a expression in neuronal cells, including pyramidal cells of CA1 and CA3 regions, as well as granule cells and hilar neurons of the DG (Fig. 3A, C and E). No detectable staining was observed in resting glial cells. In all the HS specimens examined, miR-146a expression was increased in the different subfields of the hippocampus; abundant miR-146a-positive glial cells with typical astroglia morphology were observed in the areas of prominent gliosis (Fig. 3B, D and F). Pyramidal neurons of CA1–CA4 regions and granule cells of DG also displayed miR-146a expression. Non-sclerotic hippocampus (non-HS) displayed a pattern of expression similar to that observed in control autopsy hippocampus. Double-labelling confirmed miR-146a expression in GFAP-positive reactive astrocytes, whereas no detectable expression was observed in HLA-DR-positive cells of the microglial/macrophage lineage (Fig. 3G–I). The percentage of cells positive for miR-146a and co-expressing GFAP was quantified in both CA3 and DG in HS specimens (76 ± 5, CA3; 78 ± 5, DG). No co-localization was observed with HLA-DR in both regions.

Similar cellular distribution with miR-146a expression, confined to neurons and reactive astrocytes, was also observed in tissue specimens from a patient with viral encephalitis and prominent gliosis (not shown). Because upregulation of miR-146a has been shown to be associated with a downregulation of CFH in Alzheimer’s disease (AD) brain tissue (Lukiw et al., 2008), CFH expression was evaluated with double-labelling in miR-146a-positive cells. CFH was expressed in miR-146a-positive cells with glial morphology (Fig. 3J). In control hippocampus only neuronal expression was observed (not shown).

DISCUSSION

The miR-146a has been recently indentified as a potentially endogenous regulator of TLR and cytokine receptor signalling, suggesting a link between miRNAs and human inflammatory diseases (Taganov et al., 2006; Pedersen & David, 2008; Sheedy & O’Neill, 2008; Otaegui et al., 2009). An upregulation of miR-146a has also been shown in human AD brain, suggesting that the misregulation of specific miRNAs could contribute to the inflammatory pathology observed in AD brain (Lukiw et al., 2008). Until now, however, the expression of miR-146a at the cellular level in both rat and human hippocampus has not been previously assessed. The present study, which reveals that miR-146a is highly expressed in the hippocampus, is the first to focus on the cellular distribution of miRNA in a rat model of TLE, as well as in hippocampal tissue from patients with TLE.
miR-146a expression during epileptogenesis in the rat.

We detected an upregulation of miR-146a during epileptogenesis and in the chronic epileptic phase in the rat hippocampus of the TLE model. The results of both qPCR and in situ hybridization analyses indicated a prominent expression at 1 week after SE, which corresponds to the time of maximal astroglial and microglial activation and upregulation of several other genes involved in the immune response (Aronica et al., 2000, 2001b; Hendriksen et al., 2001; Gorter et al., 2006). miR-146a was still significantly upregulated in the chronic phase. In situ hybridization analysis of miR-146a in rat hippocampus showed expression in both neuronal and glial cells. Double-labelling experiments showed miR-146 expression in astrocytes. Previous experimental evidence in rodent models of seizures has demonstrated that reactive glial cells express high levels of pro-inflammatory cytokines, such as IL-1β and TNF-α (for review, see Vezzani et al., 2008). The analysis of the spatio-temporal expression of IL-1β in TLE models indicates that astrocytes mostly represent a source of this cytokine during epileptogenesis, as well as in the chronic epileptic tissue, (Vezzani et al., 2008). Because IL-1β represents a major pro-inflammatory cytokine involved in the induction of miR-146a (Taganov et al., 2006; Nakasa et al., 2008; Sheedy & O’Neill, 2008), it is possible that expression of miR-146a in astrocytes may represent an attempt to modulate the inflammatory response triggered by this cytokine. Accordingly, recent studies identify miR-146a as a key regulator in a feedback system whereby induction of nuclear factor kappa-B (NFkB) through a myeloid differentiation factor 88 (MyD88)-dependent pathway may upregulate the miR-146a, which in turn could downregulate the levels of two key adapter molecules, IL-1RI-associated protein kinases-1 (IRAK1) and -2, and TNF receptor-associated factor 6 (TRAF6) downstream of TLR and cytokine receptors, reducing the activity of this inflammatory pathway (Taganov et al., 2006; Hou et al., 2009). These observations are particularly interesting considering the known proconvulsant action of IL-1β mediated by the IL-1 receptor type 1, as well as the recently reported role of TLR-signalling pathways in epilepsy (Vezzani et al., 2008; Maroso et al., 2009), and suggest that miR-146a induction could function in fine-tuning the response to cytokines in TLE during epileptogenesis.

miR-146a expression in TLE patients with HS.

The upregulation of miR-146a observed in the chronic epileptic phase in the post-SE model of TLE was confirmed in human HS specimens of patients undergoing surgery for pharmacologically refractory TLE. In situ hybridization analysis of miR-146a in human control hippocampus and HS specimens demonstrated expression in neuronal cells. In contrast (as observed in the post-SE rat hippocampus), the expression in glial cells was detected only in tissue of patients with HS, particularly in regions with prominent gliosis. Expression of
the miR-146a was observed in neurons and in reactive astrocytes in HS tissue. Neurons constitute an additional source of pro-inflammatory cytokines (including IL-1β), potentially contributing to the inflammatory pathology observed in TLE (Ravizza et al., 2008). Thus, the neuronal expression of miR-146a may also represent an attempt to regulate this inflammatory pathway. A physiological mechanism of defence against activation of inflammatory pathways during epileptogenesis is represented by induction of inhibitory factors, such as CFH (Boon et al., 2009), an important repressor of inflammatory signalling. This factor inhibits excessive activation of the complement cascade, which is prominently activated in both experimental and human TLE (Aronica et al., 2007). Interestingly, CFH has been identified as a target of miR-146a. For instance, in AD brains, upregulation of miR-146a has been linked to downregulation of CFH (Lukiw et al., 2008). Thus, the possible repression of the bioavailability of inhibitory molecules, such as CFH, has to be taken into account with respect to the interpretation of the ultimate effects of changes in the expression of miR-146a. However, CFH gene expression has been shown to be induced during epileptogenesis in the post-SE model (Aronica et al., 2007). In addition, expression of CFH protein was observed in miR-146a-positive glial cells in the chronic epileptic phase in HS specimens. In conclusion, our observations demonstrate an upregulation of miR-146a with prominent expression in astrocytes during epileptogenesis in a rat model of TLE as well as in human TLE. Understanding the role of miR-146a epilepsy-associated pathologies may be relevant for the development of new therapeutic strategies whereby glial function is targeted. Whether a misregulation of specific miRNAs, such as miR-146a, could contribute to epileptogenesis remains to be explored. Overexpression and loss of function studies in vitro, as well as in animal models, will help to further identify the exact role of miR-146a in the modulation of the inflammatory response and associated pathogenic signalling in epilepsy.

ACKNOWLEDGEMENTS
We are grateful to J.T. van Heteren for her technical help. This work has been supported by National Epilepsy Funds, NEF 09-05 (E.A.), NEF07-19 (J.A.G.); EU FP7 project NeuroGlia, Grant Agreement N° 202167.

REFERENCES


Abstract

Purpose: Increasing evidence supports the involvement of microRNAs (miRNA) in the regulation of inflammation in human neurological disorders. In the present study we investigated the role of miR-146a, a key regulator of the innate immune response, in the modulation of astrocyte-mediated inflammation.

Methods: Using Taqman PCR and in situ hybridization, we studied the expression of miR-146a in epilepsy-associated glioneuronal lesions (ganglioglioma, GG; focal cortical dysplasia, FCD type IIb), which are characterized by prominent activation of the innate immune response. In addition, cultured human astrocytes were used to study the regulation of miR-146a expression in response to proinflammatory cytokines. Furthermore, we used qPCR and western blot to evaluate the effects of overexpression (with miR-146a mimic) or knockdown (with antisense miR-146a) of miR-146a on IL-1β signaling. Downstream signaling molecules (IRAK-1, IRAK-2 and TRAF-6) in the IL-1β pathway, as well as the expression of IL-6 and COX-2 were evaluated by western blot and ELISA. Release of HMGB1 and several cytokines, (such as IL-6, TNF-α) was evaluated using western blot and a human magnetic multiplex cytokine assay on a Luminex® 100™/200™ platform, respectively.

Results: Increased expression of miR-146a was observed in both GG and FCD specimens by Taqman PCR. In situ hybridization confirmed the increased expression in reactive astrocytes.
in FCD type IIb. MiR-146a expression in human glial cell cultures was strongly induced by IL-1β and blocked by IL-1β receptor antagonist. Modulation of miR-146a expression by transfection of astrocytes with anti-miR146a or mimic, regulated the mRNA expression levels of downstream targets of miR-146a (IRAK-1, IRAK-2 and TRAF-6) and the expression of IRAK-1 protein. In addition, the expression of IL-6 and COX-2 upon IL-1β stimulation was suppressed by increased levels of miR-146a and increased by the reduction of miR-146a. Modulation of miR-146a expression affected also the release of several cytokines such as IL-6, TNF-α.

**Conclusion:** In response to inflammatory cues miR-146a is induced as a negative-feedback regulator of the astrocyte-mediated inflammatory response. This supports an important role of miR-146a in human neuroinflammation associated neurological disorders and suggests that this miR may represent a novel target for therapeutic strategies.

**INTRODUCTION**

During the last decade, both experimental and clinical studies have demonstrated the relevance of inflammation in the pathophysiology of epilepsy (for reviews see (Aronica and Crino 2011; Vezzani et al. 2011a; Vezzani et al. 2008). Neuropathological examination of surgical epilepsy specimens provides evidence of a complex and sustained inflammatory phenomenon with production of pro-inflammatory molecules in patients with temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS). Activation of cells of the microglia/macrophage lineage and astrocytes, and concomitant induction of various inflammatory pathways, also have been observed in focal malformations of cortical development (MCD; such as focal cortical dysplasia FCD), which represent major causes of pediatric epilepsy (Blümcke et al. 2009). Both the innate and the adaptive immune responses are activated in these lesions ((Boer et al. 2010); reviewed in (Aronica and Crino 2011)). In particular, both in vitro and in vivo data point to the role of astrocytes as a major source and/or targets of pro-epileptogenic inflammatory signaling, such as the interleukin(IL)-1β and Toll-like receptor (TLR) signaling pathways (Aronica and Crino 2011; Maroso et al. 2011; Vezzani et al. 2011b; Vezzani et al. 2008; Zurolo et al. 2011).

An increasing body of literature supports the critical role of miRNAs in post-transcriptional gene regulation in several biological processes of the central nervous system (CNS), as well as in the pathogenesis of different disorders (including developmental, neurodegenerative, vascular and neuroinflammatory disorders), and in oncogenesis (Li et al. 2010; Sethi and Lukiw 2009; Urbich et al. 2008). Various miRNAs are also considered to represent a new class of mediators of inflammation (Gantier 2010; Quinn and O’Neill 2011; Rusca and Monticelli 2011; Sonkoly et al. 2008). In particular, miRNA-146a has been associated with the regulation of Toll-like and interleukin-1 receptors (TIRs) signaling (Cui et al. 2010; Quinn and
Interestingly, this miRNA has been shown to be upregulated in experimental models of epilepsy, as well as in human TLE (Aronica et al. 2010; Song et al. 2011). miRNA-146a is expressed in human brain in astrocytes which may be key targets in the regulation of this miRNA in response to inflammatory molecules (Aronica et al. 2010; Aronica et al. 2011; Cui et al. 2010).

In the present study we evaluated the role of miRNA in human astrocytes. We first assessed the expression levels of miR-146a in epilepsy-associated glioneuronal lesions (ganglioglioma, GG; focal cortical dysplasia, FCD type IIb) characterized by prominent activation of the innate immune response in astroglial cells (Aronica and Crino 2011; Blumcke and Spreafico 2011; Iyer et al. 2010a; Thom et al. 2012) and analyzed the cellular distribution in FCD type IIb. Furthermore, we investigated the regulation of miR-146a in response to inflammatory molecules in cultured human glial cells (U373 glioblastoma cell line and human astrocytes cell cultures) and further evaluated its role in regulating TLR/IL-1R-IRAK-NF-κB signaling particularly in astroglia.

**Materials and Methods**

**Human material**

The cases included in this study were obtained from the archives of the departments of neuropathology of the Academic Medical Center (University of Amsterdam) and the University Medical Center in Utrecht. A total of 6 brain tissue specimens, removed from patients undergoing surgery for intractable epilepsy, were examined. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All cases were reviewed independently by two neuropathologists and the diagnosis was confirmed according to the international consensus classification system recently proposed for grading FCD (Blumcke and Spreafico 2011). For the GG we used the revised WHO classification of tumors of the central nervous system (Louis et al. 2007). Table 1 summarizes the clinical findings of epilepsy patients and controls. None of the FCD patients fulfilled the diagnostic criteria for tuberous sclerosis complex (TSC). All patients underwent presurgical evaluation with investigations consisting of non-invasive tests, including history, medical, neurological and neuropsychological assessment, structural neuroimaging and extensive interictal and ictal EEG studies with video monitoring. Patients who underwent implantation of strip and/or grid electrodes for chronic subdural invasive monitoring before resection were excluded from this study. Patients had complex partial seizures (CPS) and all patients had daily seizures, which were resistant to maximal doses of anti-epileptic drugs. Seizure duration represents the interval in years from age at seizure onset to age at surgery. Normal-appearing control cortex was obtained at autopsy from 6 age-matched patients without history of...
seizures or other neurological diseases. Autopsied brain tissues from patients with neuroinflammatory pathologies (viral encephalitis) were also examined (as positive controls). All autopsies were performed within 12 hours after death. Furthermore, we also used histologically normal temporal neocortex from patients undergoing extensive surgical resection of the mesial structures for the treatment of medically intractable complex partial epilepsy to control for a possible effect of recurrent seizures, antiepileptic drugs or post mortem interval on the expression of miR146a.

### Table 1. Summary of clinical findings of epilepsy patients and controls.

<table>
<thead>
<tr>
<th>Pathology type</th>
<th>Number of cases</th>
<th>Gender (M/F)</th>
<th>Mean age at surgery (years/range)</th>
<th>Localization</th>
<th>Mean duration of epilepsy (years/range)</th>
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<tr>
<td>FCD IIb</td>
<td>6</td>
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<td>25.5 (21-33)</td>
<td>3 Temporal, 3 Frontal</td>
<td>11.1 (6-17)</td>
</tr>
<tr>
<td>GG</td>
<td>5</td>
<td>3/2</td>
<td>29.5 (16-34)</td>
<td>Temporal</td>
<td>10.1 (3-16)</td>
</tr>
<tr>
<td>Controls (no epilepsy/autopsy)</td>
<td>6</td>
<td>3/3</td>
<td>40.3 (25-52)</td>
<td>Temporal</td>
<td>-</td>
</tr>
<tr>
<td>Controls (no FCD/surgical)</td>
<td>3</td>
<td>3/0</td>
<td>33.0 (22-36)</td>
<td>Temporal</td>
<td>13.0 (10-18)</td>
</tr>
</tbody>
</table>

FCD = Focal Cortical Dysplasia. GG: ganglioglioma; M = male; F = female.

### Tissue preparation

Brain tissue from control patients and surgical tissue block from patients with epilepsy was snap frozen in liquid nitrogen and stored at −80°C until further use (RNA isolation for RT-PCR). Additional tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraaffin-embedded tissue was sectioned at 6 µm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel GmbH, Braunschweig, Germany) and two sections were used for in situ hybridizations and immunocytochemistry. Two additional sections were used for the double staining, combining in situ hybridization with immunocytochemistry (in the same sections) with different antibodies, as described below. One representative paraffin block per case (containing the complete lesion or the largest part of the lesion resected at surgery) were sectioned, stained and assessed. Sections of all specimens were processed for haematoxylin eosin (HE), luxol fast blue (LFB) and Nissl stains, as well as for immunocytochemical stainings for a number of neuronal and glial markers to confirm the diagnosis of FCD IIb.
In situ hybridization

In situ hybridization for miR-146a was performed using a 5’ fluorescein labeled 19mer antisense oligonucleotide containing Locked Nucleic Acid and 2’OME RNA moieties (FAM - Aac-CcaTggAauTcaGuuCucA, capitals indicate LNA, lower case indicates 2’OME RNA). The oligo’s were synthesized by Ribotask ApS, Odense, Denmark. The hybridizations were done on 6 µm sections of paraffin embedded materials described previously (Budde et al. 2008). The hybridization signal was detected using a rabbit polyclonal anti-fluorescein/Oregon green antibody (A21253, Molecular probes, Invitrogen) and a horse radish peroxidase labeled goat anti-rabbit polyclonal antibody (P0448 Dako, Glostrup Denmark) as secondary antibody. Signal was detected with chromogens 3-amino-9-ethyl carbazole (St. Louis, MO, USA) or Vector NovaRed (Vector Laboratories, Burlingame, CA, USA) and the nuclei were stained with haematoxylin. Slides were sealed with glycerol-gelatin (St. Louis, MO, USA). As control for non-specific binding, other similarly modified oligonucleotides were used. These probes were specific for other human transcripts (miR-338, MIMAT0004701; miR-218 MIMAT0000275; miR-204, MIMAT0000265; miR-134, MIMAT0000447). These oligonucleotides showed other staining patterns (no expression in glial cells). Sections without probes were blank. For the double staining, combining immunocytochemistry with in situ hybridization, sections were first processed for immunocytochemistry as previously described (Iyer et al. 2010a) with GFAP (glial fibrillary acidic protein; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000) or HLA-DR (anti-human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark; 1:400) using Fast Blue B salt (St. Louis, MO, USA) or Vector Blue substrate (Vector Laboratories, Burlingame, CA, USA) as chromogen. After washing, sections were processed for in situ hybridization as described above. Images were captured with an Olympus microscope (BX41, Tokyo, Japan) equipped with a digital camera (DFC500, Leica Microsystems-Switzerland Ltd., Heerbrugg, Switzerland).

RNA isolation

For RNA isolation, 800 µl Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA) was added to 0.1 – 0.5 x 10⁶ cells. After addition of 200 µg glycogen and 200 µl chloroform, the aqueous phase was isolated using Phase Lock tubes (5 Prime GmBH, Hamburg, Germany). RNA was precipitated with isopropyl alcohol, washed with 75% ethanol and dissolved in water. The concentration and purity of RNA were determined at 260/280 nm using a nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Real-time quantitative PCR analysis (qPCR)

Five micrograms of total RNA were reverse-transcribed into cDNA using oligo dT primers.
Five nmol oligo dT primers were annealed to 5 µg total RNA in a total volume of 25 µl, by incubation at 72 °C for 10 min, and cooled to 4°C. Reverse transcription was performed by the addition of 25 µl RT-mix, containing: First Strand Buffer (Invitrogen-Life Technologies), 2 mM dNTPs (Pharmacia, Germany), 30 U RNAse inhibitor (Roche Applied Science, Indianapolis, IN, USA) and 400 U M-MLV reverse transcriptase (Invitrogen - Life Technologies, The Netherlands). The total reaction mix (50 µl) was incubated at 37 °C for 60 min, heated to 95 °C for 10 min and stored at -20°C until use.

PCR primers (Eurogentec, Belgium) were designed using the Universal Probe Library of Roche (https://www.roche-applied-science.com) on the basis of the reported mRNA sequences. For the human cell cultures we used the following primers: IRAK1 (forward: gcccagaggtacatcaaga; reverse: ccgcacctgccctgcttc), IRAK2 (forward: cctccctgaggccctgtg; reverse: tggatctcatacttgccacagaa), TRAF6 (forward: tggcattacgagaagctca; reverse: tgggttacgtgactgcat:), IL-6 (forward: tccagcctgcggaagagaga; reverse: ttcagcacatcctggaagagga), cyclooxygenase-2 (COX-2; forward: gaattggggtgatgagcagtt; reverse: gcacactcatgatattcactg), elongation factor 1-alpha (EF1a; foward: atccactttttggtagttcc; reverse: ccccaactctgtcctctctcatc), and hypoxanthine phosphoribosyl transferase (HPRT; forward: tggctctgcgggtattttct; reverse: tgggttacgtgactgcat:). For each PCR, a mastermix was prepared on ice, containing per sample: 1 µl cDNA, 2.5 µl of FastStart Reaction Mix SYBR Green I (Roche Applied Science, Indianapolis, IN, USA), 0.4 µM of both reverse and forward primers. The final volume was adjusted to 5 µl with H₂O (PCR grade). The LightCycler® 480 Real-Time PCR System (Roche-applied-science) was used with a 384-multiwell plate format. The cycling conditions were carried out as follows: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 55-60°C for 5 s and extension at 72°C for 10 s. The fluorescent product was measured by a single acquisition mode at 72°C after each cycle. For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 15 s followed by a gradual increase in temperature to 95°C at a rate of 2.5°C s⁻¹, with the signal acquisition mode set to continuous. Quantification of data was performed using the computer program LinRegPCR in which linear regression on the Log(fluorescence) per cycle number data is applied to determine the amplification efficiency per sample (Ramakers et al. 2003). The starting concentration of each specific product was divided by the starting concentration of reference genes (EF1a and HPRT) and this ratio was compared between patient/control groups.

miRNA (miR-146a and the U6B small nuclear RNA gene, rnu6b) expression was analyzed using Taqman microRNA assays (Applied Biosystems, Foster City, CA). cDNA was generated using Taqman MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions and the PCRs were run on a Roche Lightcycler 480 (Roche...
Applied Science, Basel, Switzerland), according to the instructions of the manufacturer. Data analysis was performed with the software provided by the manufacturer.

Cell cultures

For astrocytes-enriched human cell cultures, fetal brain tissue (15–23 weeks of gestation) was obtained from spontaneous or medically induced abortions with appropriate maternal written consent for brain autopsy. Resected tissue samples were collected in Dulbecco’s modified Eagle’s medium (DMEM)/HAM F10 (1:1) medium (Gibco, Life Technologies), supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin and 10% fetal calf serum (FCS). Cell isolation was performed as previously described (Aronica et al. 2003; Aronica et al. 2005c). Briefly, after removal of meninges and blood vessels, tissue was minced and dissociated by incubation at 37 °C for 20 min in a Hank’s balanced salt solution containing 2.5 mg/ml trypsin (Sigma, St. Louis, MO, USA) and 0.1 mg/ml bovine pancreatic Dnase I (Boehringer Mannheim, Germany). Tissue was triturated and washed with DMEM/HAM F10 medium, supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin and 10% FCS. Cell suspension (containing ~ 0.5 g wet weight tissue/10 ml culture medium) was passed through a 70µm cell sieve (Becton Dickinson, USA) and plated into poly-L-lysine (PLL; 15 µg/ml, Sigma) pre-coated 25 cm² flasks (Falcon, Lincoln Park, NJ) and maintained in a 5% CO₂ incubator at 37°C. After 48 h the culture medium was replaced by fresh medium and cultures were subsequently refreshed twice a week. Cultures reached confluence after 2-3 weeks. Secondary astrocyte cultures were established by trypsinizing confluent cultures and sub-plating onto PLL-precoated 6 and 24-well plates (Costar; 0.5 X 10⁶ cell/well in a 6-well plate for western blot analysis or 0.1 X 10⁶ cell/well in a 24-well plate for RNA isolation and PCR) and simultaneously into PLL- precoated 12 mm coverslips (Sigma) in 24-well plates (Costar; 2 X 10⁴ cell/well; for immunocytochemistry). More than 98% of the cells in primary culture, as well as in the successive 12 passages were strongly immunoreactive for the astrocytic marker GFAP and S100β. In the present study astrocytes were used for immunocytochemical analyses at passage 3-4.

The astrocytoma cell line U373 was obtained from the American Type Culture Collection (Rockville, MD, USA); cells were cultured in (DMEM)/HAM F10 (1:1) supplemented with 50 units/ml penicillin, 50 µg /ml streptomycin and 10 % FCS.

Treatment of cell cultures

Human recombinant (r)IL-1β (Peprotech, NJ, USA; 10 ng/ml) was applied and maintained for different time periods (from 10 min to 48 h) before harvesting the cells for RNA isolation or western blot analysis. Medium was collected to perform enzyme-linked immunosorbent assay (ELISA). In some experiments lipo-polsaccharide (LPS; 100ng/ml; Sigma, St. Louis, USA),
rIL-6 (10ng/ml; Strathmann Biotec A.G., Hamburg, Germany), tumor necrosis factor α (TNFα; 1ng/ml; Peprotech, NJ, USA) and high mobility group box 1 (HMGB1; 40nM; HMGBiotech S.r.l., Milan, Italy) alone or together with IL-1β were applied and maintained in the medium for 24 before harvesting the cells for RNA isolation. In some experiments cells stimulated with LPS were treated with LPS-RS (a TLR4 antagonist from the photosynthetic bacterium *Rhodobacter sphaeroides*; Invivogen, Toulouse, France; 10 μg/ml), applied 1 h before LPS. Human IL-1 receptor antagonist (IL-1Ra; 1 μg/ml; Peprotech, NJ, USA) was used to neutralize IL-1β activity (applied 1 h before IL-1β). As previously shown (Aronica et al. 2005c), the viability of human astrocytes in culture was not influenced by the treatments.

**Transfection of cells**

Cells in 12-well plates were transfected either with anti-miR146a LNA (FAM – AacCcaTg-gAauTcaGuuCucA; Ribotask ApS, Odense, Denmark) or scrambled LNA (FAM™ dye-labeled Anti-miR™ Negative Control #1; Applied Biosystems, Carlsbad, CA, USA) in blocking experiments or with miR146a precursor (pre-mir146a; mir146 mimic, Applied Biosystems, Carlsbad, CA, USA) or scrambled pre-mir (FAM™ dye-labeled Pre-miR™ Negative Control #1; Applied Biosystems, Carlsbad, CA, USA) in overexpression experiments using Lipofectamine (Invitrogen, USA) according to manufacturer’s instructions. Briefly, 5 ml lipofectamine was added to 200 ml of serum free medium (DMEM + F10) per condition for 5 minutes followed by incubation with anti-miR146a LNA or pre-miR146a at a final concentration of 50nM. The volume was adjusted to 1 ml with medium containing 10% FCS and added to the cells. Transfection efficiency was found to be 76% by flow cytometry. After 24 hours the IL-1β was added to the wells for 24 hours after which the cells were harvested for RNA or protein isolation.

**Preparation of Cellular Extracts and western blot analysis**

Cells were harvested at 24 h after treatments and/or transfection. Medium was collected (for the detection of cytokine and HMGB1 release) and glial cells were washed twice with cold PBS. The samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, Na orthovanadate (10.4 mg/ml), 5 mM EDTA (pH 8.0), 5 mM NaF and protease inhibitor cocktail (Boehringer Mannheim, Germany) by incubating on ice for 15 minutes. The homogenates were centrifuged at 14000 rpm for 10 mins and the supernatant was used for further analysis. Protein content was determined using the bicinechinonic acid method (Smith et al. 1985). Western blot analysis was performed, as previously described (Aronica et al. 2005a). For electrophoresis, equal amounts of proteins (15-20μg/lane) were separated on a 10% sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) gel. Separated proteins were transferred to nitrocellulose paper for
90 min at 100V, using a wet electroblotting system (BioRad, Hercules, CA, USA). Blots were blocked for 1 hour in 5% non fat dry milk in Tris-buffered saline-Tween (TBST) (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5; for IRAK1 and IRAK2) or in 5% bovine serum albumin (BSA) in TBST (for TRAF6). The blots were incubated overnight with the primary antibody (in 5% milk solution: 1:1000 IRAK1 rabbit polyclonal antibody; 1:500 IRAK2 rabbit polyclonal antibody; 1:500 in 5% BSA solution TRAF6 mouse monoclonal, (all from Santa Cruz, CA, USA); COX-2 (1:2500, goat anti-human; Cayman Chemical Company, Ann Arbor, MI, USA); HMGB1 (rabbit polyclonal antibody 1:1000; Pharmingen, San Diego, CA, USA). After several washes in TBST, the membranes were incubated in TBST / 5% non fat dry milk, containing the goat anti-rabbit or rabbit anti-mouse coupled to horse radish peroxidase (1:2500; Dako, Denmark) for 1h. After washes in TBST, immunoreactivity was visualized using ECL PLUS western blotting detection reagent (GE Healthcare Europe, Diegen, Belgium). Expression of β-actin (monoclonal mouse, Sigma, St. Louis, MO, 1:50000) and β-tubulin (1:30000, monoclonal mouse, Sigma, St. Louis, MO, USA) was used as loading control. For the quantification of the blots the band intensities were measured densitometrically Scion Image for Windows (beta 4.02) image-analysis software. A ratio of the integrated band density (IntDen) of the protein of interest to the IntDen of the reference protein was used to normalize band intensities.

**Detection of cytokine release**

Evaluation of levels of 16 cytokines/chemokines/inflammatory molecules [IL-1b, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-18, interferon (IFN)-a2, IFN-g, IP-10 (CXCL10), macrophage inflammatory protein-1b (MIP-1b), G-CSF (granulocyte-colony stimulating factor), Rantes, Tumor necrosis factor (TNF)-a, and VEGF] in the medium of treated cultures was carried out with the human magnetic 10-plex panel (Invitrogen, Camarillo, CA, USA) to which extra cytokine beads were added according to the manufacturer’s protocol. The plate was read on a Luminox® 100™/200™ platform. Data were analyzed and statistics calculated with GraphPad Prism 4 software (San Diego, CA, USA).

Levels of MCP-1 were measured using a kit from Ebioscience (Vienna, Austria) according to manufacturer’s instructions. Briefly, flat bottomed ELISA microtiter plates (Costar, Cambridge, MA, USA) were coated overnight with the capture antibody in coating buffer. The plates were washed, preblocked with assay diluent (provided with the kit and incubated with the culture supernatants diluted 1:50 assay diluent or standard antigen dilutions in triplicate. The plates were washed and incubated with biotinylated detection antibody followed by horse radish peroxidase labelled streptavidin. Tetramethyl benzidine (TMB) (Sigma Chemical Co, USA) was used as the colour substrate, and the reaction was stopped with an
equal volume of 1M H₂SO₄. The absorbance was read in a Titertek Multiskan microplate reader (Germany) at 450 nm with a reference wavelength of 570 nm.

**Statistical analysis**
Statistical analyses were performed with Graphpad Prism® software (Graphpad software Inc., La Jolla, CA, USA) using two-tailed Student’s t-test or, for multiple groups, a non-parametric Kruskal–Wallis test followed by the Dunn’s post hoc test to assess differences between groups. P < 0.05 was considered significant.

**RESULTS**

**miR-146a expression in GG and FCD type IIb**

miR-146a expression was studied using qPCR in control human cerebral cortex and in GG and FCD type IIb samples. Expression of miR-146a was significantly increased in GG and FCD specimens compared to control cortex (Fig. 1A). There were no significant differences in miR-146a expression between autopsy and surgical control samples (Figure S1). The cellular distribution of miR-146a in control cortex and FCD type IIb specimens was investigated using in situ hybridization (Fig. 1 B–E). miR-146a was undetectable in glial cells in control grey and white matter specimens (Fig. 1 B–C). In FCD specimens miR-146a expression was detected in glial cells with typical astroglia morphology, particularly in the areas of prominent gliosis (Fig. 1 D). Balloon cells displayed also miR-146a expression (Fig. 1 E). Double labeling confirmed miR-146a expression in GFAP-positive reactive astrocytes (Fig. 1 D, inserts), whereas no detectable expression was observed in HLA-DR positive cells of the microglial/macrophage lineage (not shown). miR-146a expression in reactive astrocytes was also observed in tissue specimens from patients with viral encephalitis and in tumor astrocytes in GG specimens (not shown).

**Regulation of mir146a expression by IL-1β in human glial cells in culture**

In the present study we used both U373 glioblastoma cell line and human fetal astrocytes in culture to examine the effect of IL-1β and other inflammatory molecules on the expression levels of miR-146a. qPCR demonstrated that exposure to IL-1β consistently increased the miR-146a expression in both cells types (Fig. 2 and 3). The effect of IL-1β was blocked by the IL-1β ra, a naturally occurring antagonist of the IL-1 receptor ([36]; Fig. 2 A and Fig. 3 A). Induction of miR-146a (although to a lower extent compared to IL-1β) was also observed with LPS and blocked by LPS-RS (Fig. 2 A). In contrast, under our culture conditions we did not observe increased miR-146a levels after exposure to IL-6 (10 ng/ml), TNFα (1 ng/ml;) or HMGB1 (40 nM; Fig. 2 B and Fig. 3 B). IL-1β induced upregulation of miR-146a at 0.1 ng/ml and its effect
Figure 1. miR-146a expression in GG and FCD type IIb.
(A) Quantitative real-time PCR of miR-146a in control cortex (n = 6; autopsy), FCD type IIb (n = 6) and GG (n = 5) specimens. miR-146a expression was normalized to that of the U6B small nuclear RNA gene (rnu6b). The error bars represent SEM; statistical significance: *P<0.05 compared to control. (B–E) In situ hybridization of miR-146a expression in control (B–C) and FCD type IIb (D–E) specimens. miR-146a was expressed at low levels in neurons and undetectable in glial cells in control grey (B) and white matter (C) specimens. Panels D: FCD type IIb tissue showing miR-146a expression in glial cells (arrows; insert a); inserts (b,c) in D show colocalization (purple) of miR-146a (red) in GFAP (blue) positive astrocytes. Panel E: FCD type IIb tissue showing miR-146a expression in balloon cells (arrows). Scale bar in B: 80 µm. C–E: 40 µm.
was maximal with doses ranging from 1 to 50 ng/ml (Fig. 2 C and Fig. 3 C); increased miR-146a levels were detected 16 hours after exposure to IL-1β (Fig. 2 D and Fig. 3 D).

Regulation of mir146a expression by transfection with anti-miR-146a LNA or miR-146a mimic

After transfection with 50 nM LNA-antimiR-146a into U373 glioblastoma cell line (Fig. 4 A) and human fetal astrocytes in culture (not shown) for 24 hours, qPCR revealed significant reduction of miR-146a. On the other hand significant overexpression of miR-146a was seen in U373 glioblastoma cell line (Fig. 4 B) and human fetal astrocytes in culture (not shown) after transfection with miR-146a mimic (pre-miR-146a) for 24 hours at concentrations ranging from 1 to 50 nM. Upon stimulation with 10 ng/ml IL-1β for 24 hours lower miR-146a levels were observed in cells transfected with LNA-anti miR-146a (but not with the LNA-control) and higher levels in cells transfected with miR-146a mimic (but not by the mimic-control) compared to non-transfected cells (Fig. 4 C and D).

Figure 2. miR-146a expression levels in U373 glioblastoma cell line after exposure to IL-1β.

Quantitative real-time PCR of miR-146a expression in U373 cells in culture. (A) Expression levels of miR-146a 24 hours after exposure to IL-1β (10 ng/ml) or LPS (100 ng/ml) in the presence or absence of the IL-1β receptor antagonist (IL-1ra; 1 µg/ml) or LPS-RS (10 µg/ml) respectively. (B) Expression levels of miR-146a 24 hours after exposure to IL-1β (10 ng/ml), TNFα (1 ng/ml), IL-6 (10 ng/ml), HMGB1 (40 nM alone or in the presence of IL-1β). (C) Expression levels of miR-146a 24 hours after exposure to 0.1, 1, 10 or 50 ng/ml of IL-1β. (D) Expression levels of miR-146a in U373 cells incubated for different durations (10, 30, 60 min and 6, 16, 24, 48) hours in the presence of IL-1β (10 ng/ml). Data are expressed relative to the levels observed in unstimulated cells and are mean ± SEM from two separate experiments performed in triplicate (*p<0.05 compared to control).
Effects of miR-146a inhibition or overexpression on its downstream targets IRAK-1, IRAK-2 and TRAF-6

Transfection with LNA-anti miR-146a or miR-146a mimic modulated the mRNA expression levels of downstream targets of mir-146a, such as IRAK-1, IRAK-2 and TRAF-6 after exposure to IL-1β in both U373 (Fig. 5 A-C) and fetal astrocytes (Fig. 5 D, IRAK-1; IRAK2 and TRAF-6: data not shown). In particular, a consistent downregulation of all 3 targets was observed under conditions in which miR-146a was overexpressed. Increased mRNA levels of IRAK-1, IRAK-2 and TRAF-6 were observed after transfection with LNA-anti miR-146a (Fig. 5 B-C, U373; Fig. 5 D, IRAK-1, fetal astrocytes). Western blot analysis showed the downregulation of IRAK-1 protein induced by IL-1β in both U373 (Fig. 5 E) and fetal astrocytes (not shown). This effect was enhanced by transfection with miR-146a mimic. In contrast LNA-anti miR-146a transfection resulted in the restoration of IRAK1 protein to control level. No significant differences in IRAK-2 and TRAF-6 protein were observed in either cell culture.
Figure 4. miR-146a expression levels after transfection with anti-miR-146a LNA or miR-146a mimic (pre-miR146a).
Quantitative real-time PCR of miR-146a. (A) miR-146a expression after transfection with LNA-antimiR-146a (50 nM) in U373 cells; insert shows in green transfected cells (B) miR-146a expression after transfection of miR-146a mimic (pre-mir-146a, 1, 25 and 50 nM). (C) miR-146a expression 24 hours after exposure to IL-1β in U373 cells transfected with LNA-antimiR-146a (50 nM) or miR-146a mimic (pre-mir-146a, 50 nM). (D) miR-146a expression 24 hours after exposure to IL-1β in cultured human astrocytes transfected with LNA-antimiR-146a (50 nM) or miR-146a mimic (pre-mir-146a, 50 nM). Data are expressed relative to the levels in unstimulated cells and are mean ± SEM from two separate experiments performed in triplicate (*p<0.05 compared to control; **p<0.05 LNA or mimic transfected cells stimulated with IL-1β, compared to IL-1β alone).

Figure 6 (right page). Effect of anti-miR-146a LNA or miR-146a mimic upon IL-1β-induced IL-6 and COX-2 mRNA.
Quantitative real-time PCR of IL-6 (A–B) and Cox-2 (C–D). (A–B) IL-6 mRNA levels, 24 hours after exposure to IL-1β in U373 cells (A) and cultured human astrocytes (B) transfected with LNA-antimiR-146a (50 nM) or miR-146a mimic (pre-mir-146a, 50 nM). (C–D) COX-2 mRNA levels, 24 hours after exposure to IL-1β in U373 cells (C) and cultured human astrocytes (D) transfected with LNA-antimiR-146a (50 nM) or miR-146a mimic (pre-mir-146a, 50 nM). Data are expressed relative to the levels in unstimulated cells and are mean ± SEM from two separate experiments performed in triplicate (*p<0.05 compared to control; **p<0.05, LNA or mimic transfected cells stimulated with IL-1β compared to IL-1β alone).
Figure 5. Expression levels of the miR-146a targets (IRAK-1, IRAK-2 and TRAF-6) after transfection with anti-miR-146a LNA or miR-146a mimic.

(A–C) Quantitative real-time PCR of IRAK-1 (A), IRAK-2 (B) and TRAF-6 (C) expression 24 hours after exposure to IL-1β in U373 glioblastoma cell line transfected with LNA-antimiR-146a (25 nM) or miR-146a mimic (pre-mir-146a, 50 nM). (D) Quantitative real-time PCR of IRAK-1, 24 hours after exposure to IL-1β in cultured human astrocytes transfected with LNA anti-miR-146a (50 nM) or miR-146a mimic (pre-mir-146a, 50 nM). Data are expressed relative to the levels in unstimulated cells and are mean ± SEM from two separate experiments performed in triplicate.

(E) IRAK-1 protein expression 24 hours after exposure to IL-1β in glial cells transfected with LNA anti-miR-146a (50 nM); Representative immunoblot (1 control; 2, IL-1β; 3, IL-1β + LNA-antimiR-146a; 4, IL-1β + LNA-antimiR-146a scramble; 5, IL-1β + mimic; 6, IL-1β + mimic scramble) and optical density measurements. Data are expressed relative to the levels in unstimulated cells and are mean ± SEM from two separate experiments (*p<0.05, compared to control; **p<0.05, LNA or mimic transfected cells stimulated with IL-1β compared to IL-1β alone).
Effects of miR-146a inhibition or overexpression on IL-1β mediated induction of IL-6 and COX-2

IL-1β induced expression of two major inflammatory mediators, IL-6 and Cox-2 was modulated by transfection of glial cells with LNA-anti miR-146a or miR-146a mimic. Transfection with miR-146a mimic downregulated IL-1β induced IL-6 and Cox-2 mRNA levels in both U373 glioblastoma cells and human fetal astrocytes in culture (Fig. 6 A–D). In contrast, LNA anti-miR-146a transfection significantly increased the levels of IL-6 and Cox-2 induced by IL-1β (Fig. 6 A–D). Western blot analysis confirmed the different effects of LNA-anti-miR-146a and miR-146a mimic on COX-2 protein expression in IL-1β stimulated cells; transfection with miR-146a mimic reduced whereas LNA-anti-miR-146a increased the COX-2 expression (Fig. 7 A–B).

Figure 7. Effect of anti-miR-146a LNA or miR-146a mimic upon IL-1β-induced COX-2 protein and release of HMGB1.

COX-2 protein expression 24 hours after exposure to IL-1β in U373 cells transfected with LNA-antimiR-146a (50 nM) or miR-146a mimic (pre-mir-146a, 50 nM). (A) Representative immunoblot and (B) densitometric analysis: values (optical density units relative to the optical density of β-actin) are mean ± SEM of two separate experiments performed and are expressed relative to the levels in unstimulated cells. (C) HMGB1 immunoblot (1 control; 2, IL-1β; 3, IL-1β + mimic; 4, IL-1β + mimic scramble; 5 mimic; 6, IL-1β + LNA-antimiR-146a; 7, IL-1β + LNA-antimiR-146a scramble; 8, LNA) and densitometric analysis (D, optical density units of cellular HMGB1 relative to the optical density of β-actin). *p<0.05, compared to control; **p<0.05, LNA or mimic transfected cells stimulated with IL-1β compared to IL-1β alone.

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Effects of miR-146a inhibition or overexpression on IL-1β mediated release of inflammatory mediators

We evaluated the release of high mobility group box (HMGB1; by western blot analysis) and the release of MCP1 (by ELISA) in response to IL-1β stimulation in both U373 and fetal astrocytes. Transfection of U373 with miR-146a mimic decreased both the cellular and extracellular levels of HMGB1 (Fig. 7 C-D) compared to IL-1β stimulated cells; the levels of extracellular HMGB1 were increased after transfection with LNA-anti miR-146a (Fig. 7 D). The release of MCP-1 induced by IL-1β was negatively regulated by transfection with miR-146a mimic (MCP-1 pg/ml: control: 1582 ± 193; IL-1β: 27151 ± 1368; IL-1β + miR-146a mimic: 11054 ± 2027; p< 0.05 compared to IL-1β stimulated cells), whereas transfection with LNA-anti miR-146a did not significantly affect the release induced by IL-1β.

The release of 16 cytokines/chemokines was evaluated using the human magnetic 10-plex panel in U373 glioblastoma cell line. IL-5, IL-17 and IFN-a2 were under detection limits in our culture conditions. The amount of IL-10, IL-13, IL-18 and VEGF did not significantly change after the different treatments. IL-1b significantly increased the release IL-6, IL-8, IFN-g, IP-10, MIP-1b, G-CSF, Rantes and TNF-a (Fig. 8). Transfection with LNA-anti miR-146a before treatment with IL-1b, significantly increased the levels of IL-6 and IP-10; whereas transfection with miR-146a mimic before treatment with IL-1b significantly decreased the levels of IL-6, IL-8, G-CSF, IFN-g, IP-10, MIP-1b, and TNF-a (Fig. 8).

Figure 8. Effect of anti-miR-146a LNA or miR-146a mimic upon IL-1β-induced release of inflammatory molecules.

(A) Cytokine release 24 hours after exposure to IL-1β in U373 cells transfected with LNA-anti-miR-146a (50 nM) or miR-146a mimic (miR-146a mimic, 50 nM). Data are expressed relative to unstimulated control cells (mean ± SEM from three separate experiments). In comparison with IL-1β alone, cultures stimulated with IL-1β and transfected with LNA-anti miR-146a exhibited significant increase of IL-6 and IP-10 release, whereas transfection of glial cells with miR-146a mimic significantly decreased the levels of IL-6, IL-8, G-CSF, IFN-γ, IP-10, MIP-1β, and TNF-α (* p<0.05). LNA-anti miR-146a and miR-146a mimic alone did not significantly affect the levels of cytokines in the culture medium, compared to non treated cells.
DISCUSSION

The miR-146a has recently been shown to be upregulated in experimental models of epilepsy, as well as in human TLE. (Aronica et al. 2010; Song et al. 2011). In a rat model of TLE strong upregulation was detected in astrocytes 1 week after status epilepticus (during epileptogenesis) which corresponds to the latent period and the time of maximal astrogial activation and upregulation of various genes involved in the immune response (Aronica and Gorter 2007; Gorter et al. 2006). These observations are in line with other studies supporting the association between this specific miRNA and human inflammatory diseases (Quinn and O’Neill 2011; Rusca and Monticelli 2011). In particular, upregulation of miR146a has been detected in active multiple sclerosis lesions (Junker et al. 2009) and in human Alzheimer disease (AD) brain (Lukiw et al. 2008), suggesting a key role of this miRNA in governing astrocyte activation and function in both pathologies. However, there is still little information regarding miR146a function in astrocytes, in particular regarding the effect on astroglia mediated inflammatory pathways in relation to epilepsy.

In the present study, we demonstrate the overexpression of miR-146a in glioneuronal lesions from patients with medically intractable epilepsy and provide evidence for a role of miR-146a in the regulation of the astroglia mediated inflammatory response.

MiR-146a expression in GG and FCD IIB

GG and FCD type IIb represent a major cause of drug-resistant epilepsy (Blumcke and Spreafico 2011; Thom et al. 2012) and are characterized by a prominent activation of various inflammatory pathways, including the TIR signaling pathway (Aronica et al. 2007; Boer et al. 2006; Iyer et al. 2010b; Ravizza et al. 2006; Zurolo et al. 2011). In the present study, we provide evidence of upregulation of miR-146a in these epilepsy-associated glioneuronal lesions. Increased levels of miR-146a were detected GG and FCD type IIb compared to both control autopsy material and histologically normal surgical cortex (without alteration in cortical lamination or astrogliosis) from patients with epilepsy. The latter excludes the direct effect of recurrent seizures, antiepileptic drugs or post mortem interval on the detected expression level of miR-146a in FCD specimens. Thus seizures alone may not account for changes in miR-146a expression. In situ hybridization confirmed the increased expression of miR-146a in reactive astrocytes which are abundantly present within the dysplastic cortex in FCD IIb. This observation suggests an important role of miR-146a in astrocytes in epilepsy associated lesions. Expression of miR146a was also detected in balloon cells. Since balloon cells have been shown to represent a major source of proinflammatory molecules and possibly contribute to the TIR signaling (Boer et al. 2008; Ravizza et al. 2006; Zurolo et al. 2011), further evaluation of the miR-146a function in these cell types could be per-
formed in recently described culture system (Yasin et al. 2010). Evaluation of the function of this miRNA in long-term epilepsy associated glioneuronal tumors and in malformations of cortical development (containing balloon cells with a stem cell phenotype), is particularly interesting in view of the recently described function of miR-146a in modulating neural stem cell proliferation and differentiation through regulation of the key neural stem cell factor Notch1 (Mei et al. 2011).

**miR-146a is regulated in human glial cells in culture by IL-1β**

Since miR-146a was up-regulated in astrocytes in the epileptogenic lesions (FCD, present results; TLE (Aronica et al. 2010; Song et al. 2011)) the expression of miR-146a was further analyzed in culture, using U373 glioblastoma cell line and human fetal astrocytes. We evaluated the expression levels of miR146a upon to exposure to inflammatory molecules (IL-1b, IL-6, TNFa or HMGB1), that are known to be up-regulated in epileptogenic tissue from FCD and TLE patients; (for review see (Aronica and Crino 2011)) and to the TLR4 ligand LPS. Both cell line and primary astrocytes were found to exhibit a prominent up-regulation of miR-146a expression in response to IL-1b stimulation. In contrast, LPS induced modest up-regulation whereas IL-6, TNFa and HMGB1 were not effective in inducing significant changes in miR-146a expression. The effect of IL-1b in human astrocytes is in line with recent studies in culture (Junker et al. 2009; Lukiw et al. 2008), supporting an important role of this cytokine in the regulation of astroglial miR-146a expression. The effect of IL-1b was concentration and time dependent and was observed from 16 hours after exposure to the cytokine. The observation that IL-1Ra inhibited the effect of IL-1b on miR-146a is consistent with the fact that IL-1b signals through the type I IL-1b receptor which is upregulated in epileptogenic lesions (Ravizza et al. 2005); for review see [Aronica, 2012].

**Regulation of IL-1b pathway by miR-146a**

To further investigate the function of miR-146a in human glial cells, we studied the effects of overexpression (with miR-146a mimic) or blockade of miR-146a (with antisense miR-146a; LNA- anti-miR-146a) on the expression levels of downstream signaling molecules (IRAK-1, IRAK-2 and TRAF-6) of the TIR signaling pathway that have previously been shown to be miR-146 targets (Cui et al. 2010; Quinn and O'Neill 2011; Sheedy and O'Neill 2008; Taganov et al. 2006). In both the glioblastoma cell line and human astrocytes transfection with miR-146a mimic significantly reduced IRAK-1, IRAK-2 and TRAF-6 mRNA and IRAK-1 protein after stimulation with IL-1b. In a previous study in primary cultures of human astroglial cells exposed to IL-1b and Aβ42 peptide, it has been shown that an antisense miRNA-146a inhibits miRNA-146a and controls IRAK-1, but not IRAK-2 expression, suggesting an independent regulation
of the two targets under these experimental conditions (Lukiw et al. 2010). However, in our study, both IRAK-1 and IRAK-2 mRNA were positively regulated by LNA-anti-miR-146a (and negatively by miR-146a mimic) in IL-1b stimulated human astrocytes. This observation argues against a proinflammatory role of this miRNA-146a, as result of a compensatory upregulation of IRAK2 (at least in the absence of Aβ42 peptide). However, the results at the protein level may suggest a preferential regulation of IRAK-1, which is prominently downregulated by miR-146a mimic. The effect of miR-146a on multiple targets may also vary in different cell types and upon different stimuli. For example, it has been shown that miR-146a is upregulated during viral infection in macrophages and acts as a negative regulator of the retinoic acid-inducible gene I (RIG-I)-like helicases by targeting not only IRAK1 but also TRAF6 and IRAK2 (Hou et al. 2009).

**miR-146a regulates the expression IL-6 and COX-2**

To further evaluate the function of miR-146a in response to inflammatory stimuli, we investigated the effect of miR-146a overexpression or reduction on IL-6 and COX-2, two major inflammatory molecules induced by IL-1β [for reviews see (Aronica et al. in press; Bartfai et al. 2007)]. Astrocytes are a major source of IL-6 and prominent induction of this cytokine on IL-1β stimulation has been previously reported in human fetal astrocytes in culture (Aloisi et al. 1992; Aronica et al. 2005b). Both IL-6 and COX-2 have been reported to be associated with astrogliosis and activation of the innate immune response in different pathological conditions (for reviews see (Aronica and Crino 2011; Aronica et al. in press; Bartfai et al. 2007)). Furthermore, miR-146a has a complementarity with the COX-2 3’ UTR and miR-146a mimic has been recently shown to reduce COX-2 mRNA levels in fibroblasts (Sato et al. 2010) and glial cells (Li et al. 2011).

The *in vitro* over-expression and knock-down studies showed that transfection of with miR-146a mimic reduces the IL-6 and COX-2 mRNA levels in cultures stimulated with IL-1b, whereas LNA-anti-miR-146a had the opposite effect. Western blot analysis further showed that ectopic miR-146a caused a reduction of both basal and IL-1b stimulated expression of COX-2 protein. Conversely, an upregulation of the COX-2 protein was observed after knockdown of miR-146a with LNA-anti-mir146a in IL-1b -treated cells. The anti-inflammatory role of miR-146a is also supported by its ability to regulate the IL-1b induced release of several other proinflammatory factors, such as IL-6, IL-8, G-CSF, IFN-g, IP-10, MIP-1b, and TNF-a, HMGB1 and MCP1.

Our observations, together with other emerging data suggest a role for miR-146a-mediated regulation of inflammation in glial cells and provides an opportunity to develop novel therapeutic strategies in neurological disorders associated with chronic inflammation and immune deregulation.
ACKNOWLEDGEMENTS

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REFERENCES


Astrocytes mediated signaling and epilepsy
4.1 CB1 AND CB2 CANNABINOID RECEPTOR EXPRESSION DURING DEVELOPMENT AND IN EPILEPTOGENIC DEVELOPMENTAL PATHOLOGIES

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ABSTRACT
Recent data support the involvement of the endocannabinoid signaling in early brain development, as well as a key role of cannabinoid receptors (CBR) in pathological conditions associated with unbalanced neuronal excitability and inflammation. Using immunocytochemistry, we explored the expression and cellular pattern of CBR 1 and 2 (CB1 and CB2) during prenatal human cortical development, as well as in focal malformations of cortical development associated with intractable epilepsy (focal cortical dysplasia; cortical tubers in patients with the tuberous sclerosis complex and glioneuronal tumors). Strong CB1 immunoreactivity was detected in the cortical plate in developing human brain from the earliest stages tested (gestational week 9) and it persisted throughout prenatal development. Both cannabinoid receptors were not detected in neural progenitor cells located in the ventricular zone. Only CB1 was expressed in the subventricular zone and in Cajal–Retzius cells in the molecular zone of the developing neocortex. CB2 was detected in cells of the microglia/macrophage lineage during development. In malformations of cortical development, prominent CB1 expression was demonstrated in dysplastic neurons. Both CBR were detected in balloon/giant cells, but CB2 appeared to be more frequently expressed than CB1 in these cell types. Reactive astrocytes were mainly stained with CB1, whereas cells of the microglia/macrophage lineage were stained with CB2. These findings confirm the early expression pattern of cannabinoid receptors in the developing human brain, suggesting a function for CB1 in the early stages of corticogenesis. The expression patterns in malformations of cortical
development highlight the role of cannabinoid receptors as mediators of the endocannabinoid signaling and as potential pharmacological targets to modulate neuronal and glial cell function in epileptogenic developmental pathologies.

Introduction

The endogenous cannabinoid signaling system, including the endogenous ligands (endocannabinoids, eCB) and their receptors has been suggested to play a critical role during brain development (for review see [Fernandez-Ruiz et al., 2000] and [Fernandez-Ruiz et al., 2004]). Increasing evidence indicates that developmental exposure to cannabinoids may induce subtle and long-lasting neurofunctional alterations (for review see; Trezza et al., 2008). Moreover, several studies show expression of cannabinoid receptor 1 (CB1) and their endogenous ligands early during brain development in rodents ([Berrendero et al., 1998], [Buckley et al., 1998], [Fernandez-Ruiz et al., 1999] and [Vitalis et al., 2008]). In particular, high levels of CB1 mRNA expression were observed in the cerebral cortex and in the subventricular zone (SVZ), ([Berrendero et al., 1998], [Fernandez-Ruiz et al., 2000], [Mulder et al., 2008] and [Vitalis et al., 2008]). Abundant levels of CB1 mRNA and CB1 binding have been also detected in early prenatal stages in human brain ([Glass et al., 1997] and [Mato et al., 2003]). Experimental evidence, in vivo and in vitro, further supports the role of this system in the process of neural development, regulating neural progenitor proliferation and migration, axonal elongation, synaptogenesis and myelinogenesis ([Fernandez-Ruiz et al., 1999], [Fernandez-Ruiz et al., 2000], [Fernandez-Ruiz et al., 2004], [Fride, 2004], [Gomez et al., 2008a], [Mulder et al., 2008] and [Vitalis et al., 2008]).

The ubiquitous abundance of eCB and their receptors in the CNS, together with the complexity of the eCB signal transduction pathways, may also suggest a critical role for the cannabinoid receptors (CBR) in various physiological and pathological conditions in the postnatal and adult brain. Thus, depending on the cellular localization and the signal transduction pathways, CBR have been shown to exert neuroprotective actions and regulate both glutamatergic and GABAergic synaptic transmission (for reviews see [Pacher et al., 2006] and [Onaivi, 2009]). In addition, evidence exists to support the possible involvement of the cannabinoid system in a number of neurological conditions, including epilepsy ([Lutz, 2004] and [Armstrong et al., 2009]). Dysregulation of the eCB system, with alterations in the expression of CB1, has been reported in both human and experimental temporal lobe epilepsy (TLE; [Falenski et al., 2007], [Falenski et al., 2009] and [Ludanyi et al., 2008]). Recent studies also demonstrate the existence of eCB-mediated neuron-astrocyte communication, supporting the potential role of cannabinoid receptors expressed by glial cells in both physiological and pathological processes (Navarrete and Araque, 2008). Moreover the cannabinoid system exerts immunomodulatory effects and has been suggested as potential pharmacological target in pathological conditions associated with brain inflammation ([Sheng et
The expression pattern and cellular localization of CBR (CB1 and CB2) during corticogenesis in human brain remains uncharacterized. Furthermore, there is no information about CB1 and cannabinoid receptor 2 (CB2) distribution in human developmental pathologies associated with epilepsy. To gain further insight into the role of CBR in both normal and abnormal corticogenesis, we studied the expression of CB1 and CB2 in the developing human cerebral cortex and in focal malformations of cortical development (MCD) associated with intractable epilepsy.

**Materials and methods**

**Human material**

The subjects included in this study were obtained from the databases of the Department of Neuropathology of the Academic Medical Center (University of Amsterdam; UvA) in Amsterdam, the Netherlands, the Service Histologie-Embryologie-Cytogénétique Hôpital Necker-Enfants malades, Paris, France (Dr. F. Encha-Razavi and Dr. M. Sinico) and the University Medical Center in Utrecht (UMCU). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki.

The developmental expression of CB1 and CB2 was evaluated at the following ages: 9, 10, 13, 16, 17, 20, 22, 23, 25, 29, 31, 36 and 40 gestational weeks (GW) obtained from spontaneous or medically induced abortions with appropriate maternal written consent for brain autopsy. Normal-appearing control cortex/white matter and hippocampus was obtained at autopsy from pediatric patients (3 weeks, 7 months, 8 years) and from 6 young adult patients (male/female: 3/3; mean age 31; range 14-35), without a history of seizures or other neurological diseases. For comparison with the autopsy specimens, we also included 3 surgical cases of glioneuronal tumors (gangliogliomas; GG) that contained sufficient amount of perilesional tissue (mean age 35). Three autopsy specimens from patients with multiple sclerosis (MS) were also included in the study. All autopsies were performed within 12 hours after death.

Expression of CB1 and CB2 in focal epileptogenic lesions was examined in 28 surgical specimens (6 focal cortical dysplasia, FCD; 6 cortical tubers; 6 ganglioglioma, GG; 6 dysembryoplastic neuroepithelial tumor, DNT; and 4 subependymal giant-cell astrocytomas, SEGA; Table1). All FCD cases included in this study fulfilled the histopathological criteria for FCD type IIB, containing dysmorphic neurons (DNs) and balloon cells (BCs) (Palmini et al., 2004). The histopathological features of the cortical tuber specimens included abnormal cortical laminar architecture, DNs, giant cells (GCs) and astrogliosis (Mizuguchi and Takashima, 2001, DiMa-
rio, 2004). For the GG and DNT and SEGA we used the revised WHO classification of tumors of the central nervous system (Louis et al., 2007). The clinical characteristics derived from the patient’s medical records are summarized in Table 1.

<table>
<thead>
<tr>
<th>Pathology type (PM or S)</th>
<th>Number of cases</th>
<th>Mean age at surgery (years/range)</th>
<th>Localization</th>
<th>Mean duration of epilepsy (years/range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCD IIB</td>
<td>6</td>
<td>25.8 (14-43)</td>
<td>Temporal(5)</td>
<td>17.3 (5-22)</td>
</tr>
<tr>
<td>Cortical Tubers (TSC)</td>
<td>6</td>
<td>17.8 (5 – 35)</td>
<td>Frontal (3)</td>
<td>13.5 (2.8 – 34)</td>
</tr>
<tr>
<td>GG</td>
<td>6</td>
<td>32 (16-49)</td>
<td>Temporal</td>
<td>16.1 (12-26)</td>
</tr>
<tr>
<td>DNT</td>
<td>6</td>
<td>31 (18-38)</td>
<td>Temporal</td>
<td>15.6 (2-22)</td>
</tr>
<tr>
<td>SEGA</td>
<td>4</td>
<td>15.1 (8-23)</td>
<td>LV</td>
<td>7.3 (2-20)</td>
</tr>
</tbody>
</table>


**Tissue preparation**

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 µm, mounted on pre-coated glass slides (StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany) and used for immunocytochemical staining as described below.

**Antibody characterization**

Antibodies specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9; DAKO; 1:1000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:2000), neurofilament (NF, SMI311; Sternberger Monoclonals, Lutherville, MD; 1:1000), human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark; 1:400) and microtubule-associated protein 2 (MAP2; mouse clone HM2; Sigma, St Louis, MO; 1:100) were used in the routine immunocytochemical analysis. For the detection of CB1, we used the anti-human CB1 polyclonal rabbit antibody from Affinity BioReagents (Rockford, IL, USA; PA1-743; raised against a fusion protein containing the first 99 amino acid residues from human CB1; 1:200) and the anti-human CB1 polyclonal rabbit from Abcam (Cambridge, MA, USA) raised against a synthetic peptide, corresponding to C terminal amino acids 461-472 of Human
CB1; 1:100). For the detection of CB2 we used a polyclonal rabbit antibody (Cayman, Ann Arbor, MI, USA; raised against the human CB2 receptor sequence amino acids 20-33; 1:50). Specificity of the staining in human tissue was further confirmed by omission of the primary antibody or by its replacement with equivalent amounts of isotype-matched non-immune IgG or with the sera preadsorbed with the immunizing peptide for the anti-CB1 (polyclonal rabbit from Abcam, Cambridge, MA, USA) and CB2 (as previously reported Ellert-Miklaszewska et al., 2007). Western blots of the total homogenates of human control brain (performed as previously described Aronica et al., 2007a) detected major CB1 receptor species of approximately 53-60 kDa for both CB1 antibodies (McIntosh et al., 1998, De Jesus et al., 2006, Grimsey et al., 2008) and a major band at approximately 45 kDa for CB2 (Nithipatikom et al., 2004, Zhang et al., 2007) CB1 and CB2 specific bands that were identified using these sera and the CB immunoreactivity (IR) were absent after preadsorption with the corresponding peptide (not shown).

Other anti-CB1 antibodies tested on human material were: the L15 anti-CB1 (raised in rabbits against the last 15 C-terminal amino acid residues of the rat CB1 receptor; generous gift from Ken Mackie, University of Washington) and the polyclonal rabbit PAI-745 (Affinity BioReagents, Rockford, IL, USA; raised against a fusion protein containing the first 77 amino acid residues from rat CB1). However these two antibodies did not work, under our experimental conditions, on human paraffin embedded material.

Immunocytochemical analysis

For single-labeling, paraffin-embedded sections were deparaffinized, re-hydrated, and incubated for 20 min in 0.3% H₂O₂ diluted in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed by incubation for 10 min at 121 °C in citrate buffer (0.01 M, pH 6.0). Sections were washed with phosphate-buffered saline (PBS), and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK). After incubation with the primary antibodies overnight at 4 °C, the sections were washed in PBS and the antibodies were visualized using the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) as chromogen. Sections were counterstained with hematoxylin, dehydrated and mounted. Sections incubated without the primary antibody and after preadsorption with the corresponding peptide, were essentially blank.

All labeled tissue sections were evaluated with respect to the presence or absence of various histopathological parameters and specific IR for the different markers. The intensity of CB1 and CB2 staining was evaluated using a scale of 0-3 (0: no; 1: weak; 2: moderate; 3: strong staining). All areas of the specimen were examined and the score represents the pre-
dominant cell staining intensity found in each case for the different cell types (neurons, astrocytes, microglial cells and balloon cells). The frequency of CB1 and CB2 positive cells [(1) rare; (2) sparse; (3), high] was also evaluated to give information about the relative number of positive cells within the specimen. As proposed before (Vandeputte et al., 2002, Ravizza et al., 2006), the product of these two values (intensity and frequency scores) was taken to give the overall score (total score) shown in Fig. 5. In the FCD and TSC cases, comparative analysis was carried out for the numbers of CB1 and CB2 immunoreactive balloon/giant cells. An identical region of interest in the white matter, beneath the region of dysplasia or tuber, was outlined at low magnification (x 2.5 objective) on adjacent sections stained for CB1 and CB2. All balloon cells within this region were counted systematically at high magnification (x 40 objective) as positive or negative. The percentage of morphologically identified BCs and GCs stained with CB1 and CB2 was compared (Table 2).

For double-labeling studies, sections were incubated for 2 hours at room temperature after incubation overnight at 4°C with the primary antibodies, with Alexa Fluor® 568-conjugated anti-rabbit IgG and Alexa Fluor® 488 anti-mouse IgG (1:100, Molecular Probes, Breda, The Netherlands). Sections were mounted with Vectashield containing DAPI (targeting DNA in the cell nucleus; blue emission) and analyzed by means of a laser scanning confocal microscope (Leica TCS SP2, Wetzlar, Germany).

### Table 2  CB1 and CB2 expression in balloon/giant cells of FCD and TSC specimens.

<table>
<thead>
<tr>
<th>Ab</th>
<th>BCs</th>
<th>GCs</th>
</tr>
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<tbody>
<tr>
<td>CB1</td>
<td>62.2 ± 2.4</td>
<td>68.4 ± 3.2</td>
</tr>
<tr>
<td>CB2</td>
<td>98.3 ± 4.7</td>
<td>97.2 ± 2.8</td>
</tr>
</tbody>
</table>

Percentage of morphologically identified balloon cells (BCs) and giant cells (GCs) immunoreactive for CB1 and CB2 across all 6 FCD and 6 TSC specimens.

## RESULTS

### Temporal CB1 and CB2 expression during human cortical development

The expression pattern of CB1 and CB2 was studied immunocytochemically at different prenatal ages, 9, 10, 13, 16, 17, 20, 22, 23, 25, 29, 31, 36 and 40 GW, as well as at postnatal ages of 3 weeks, 7 months and 8 years.

### CB1 and CB2 expression during corticogenesis

At all the prenatal ages examined (GW 9-36), the neuroepithelium of the ventricular zone (VZ) was CB1 negative; in contrast, expression was evident in the cortical plate (CP) at early stages of development (9-10 GW; Fig. 1 A-F). Strong expression in the CP was also detected at 13 and 17 GW and was characterized by radially oriented processes (Fig. 1 I-J). Around
Figure 1. CBR immunoreactivity (IR) during cortical development
(Panels A–C) 9 GW: CB1 IR is observed in the CP; high magnification photographs (B, C) show the absence of detectable IR in VZ (B) but expression of CB1 in CP (C). (Panels D–F) 10 GW. (E) no detectable CB1 expression in the VZ. (F) strong CB1 IR is detected in CP. (Panels G–I) GW 13. (H) CB1 expression is observed in the SVZ. (I) the CB1 IR in the CP increases with clear positive process. (Panels J, K): GW 17. (Panel J) shows clear CB1 IR in CP. (K) Cajal-Retzius cells expressing CB1 in the marginal zone. Panel (L) 23 GW with CB1 IR in CP. (Panels M–P) expression of CB1 in pyramidal cells at 31 (M) and 38 (N) GW and at 3 (O) and 7 (P) months postnatally. (Q, 7 mon) residual Cajal–Retzius cells in the molecular layer expressing CB1. Insert in (Q) shows Cajal–Retzius cells (reelin positive; green) expressing CB1 (red). (Panels R, S) CB2 IR, 17 GW; (R) CP without CB2 IR in neural cells, but expression in blood vessels (insert). (S) VZ without detectable CB2 IR; CB2 IR is detected in blood vessels and in a few perivascular cells (arrow). Inserts in (S) a, CD68 (green); b, CB2 (red); c, merged image. VZ, ventricular zone; SVZ, subventricular zone; SP/IZ, subplate/intermediate zone; CP, cortical plate. For single-labeling the chromogen used (DAB) gives a brown color staining (sections are counterstained with Hematoxylin). Scale bar in Q: (A, D, G):100 μm; (B, C, E, F, H, J, K, Q): 35 μm. (I, L, M, P): 25 μm; (N, O): 20 μm; (E): 70 μm; (S): 35 μm.
Figure 2. Distribution of CB1 immunoreactivity (IR) in malformations of cortical development. (Panels A, B) CB1 IR in control cortex (A) and white matter (B) showing diffuse neuropil staining and expression in neuronal cells (insert in A) but no detectable glial labeling. (Panels C–G) CB1 IR in focal cortical dysplasia (FCD) showing IR within the dysplastic cortex. Insert in (C) shows a cytomegalic neuron surrounded by a dense plexus of immunoreactive fibers. High magnification Photograph D shows strong expression in somata of dysplastic neurons (arrows). Insert in (D) co-localization (yellow) of CB1 (red) with the neuronal marker NeuN (green). (Panel E) CB1 IR is observed in balloon cells (arrows) within the white matter. High magnification Photograph F shows a balloon cell (arrows) and a neuron (arrow-head) strongly stained with CB1. Insert (a) in (E) co-localization (yellow) of CB1 (red) with the stem cell marker nestin (green); insert (b) in (E) co-localization (yellow) of CB1 (red) with vimentin (green) in a group of balloon cells. (G) Dysplastic white matter area showing CB1 IR in heterotopic neurons (arrow-head), balloon cells (asterisk) and dysplastic binucleated glial cells (arrows). Insert (a) in (G) shows co-localization (yellow) of CB1 (red) with GFAP (green) in a balloon cell (arrow; asterisk shows a CB2 positive cell, without GFAP IR). Insert (b) in (G) shows moderate IR in astroglial cells. Insert (c) in (G) co-localization (yellow) of CB1 (red) with the astroglial marker GFAP (green). (Panel H, I) CB1 IR in cortical tuber of TSC showing expression within the dysplastic cortex. High magnification photographs (I, J) demonstrate expression in neuronal cells of different shape and morphology (arrows in I); strong membrane staining is observed in large dysplastic neurons (J). Insert in (J) co-localization (yellow) of CB1 (red) with the neuronal marker NeuN (green). (Panel K) Dysplastic white matter area showing CB1 IR in balloon cells. Insert (a) in (K) CB1 (red) is not observed in HLA-DR positive cells (green); inserts (b and c) in (K) co-localization (yellow) of CB1 (red) is observed with nestin in balloon cells (b) and with GFAP (c) in a subpopulation of binucleate cells. (Panel L) shows moderate CB1 IR in astroglial cells; insert (a) in (L) high magnification of a CB1 positive astrocyte; insert (b) in (L) co-localization (yellow) of CB1 (red) with the astroglial marker GFAP (green). Sections (single staining) are counterstained with Hematoxylin. Scale bar in L: (A): 500 μm (C, H): 300 μm. (B, G, I, M, N): 70 μm. (E): 150 μm; (D, F): 25 μm; (J): 20 μm.
mid gestation, CB1 was observed in the neuropil and in cell somas displaying pyramidal shapes and this pattern of IR persisted at later prenatal and postnatal ages (Fig. 1 L-P). At early stages of cortical development (9-17 GW), CB1 expression was observed in the subventricular zone (SVZ; Fig. 1 H, 13 GW).

In the early cortical plate stages (9-17 GW), we also identify CB1 expression in the marginal zone in Cajal-Retzius cells, a population of cells that are strongly reelin immunoreactive (Fig. 1 K, 17 GW). The expression of CB1 was still observed in Cajal-Retzius cells in the perinatal and early post-natal period (< 1 year; Fig. 1 Q).

At all the prenatal ages examined (GW 9-36), VZ/SVZ and CP were largely devoid of CB2 IR. CB2 IR was however detectable in blood vessels and cells of the macrophage/microglia lineage (Fig. 1 R and S). Expression of CB2 was occasionally observed in cells with glial morphol-

![Image](https://example.com/image.png)

**Figure 3.** CB1 immunoreactivity (IR) in glioneuronal tumors (GG and DNT) and SEGA. (A, B) CB1 IR is observed in the neuronal component of both GG and DNT (arrows; insert in B). No detectable CB1 expression is observed in the oligodendroglia-like cells within DNT (B, insert in B). Insert (a) in A shows co-localization (yellow) of CB1 (red) with the neuronal marker NeuN (green) in GG. Tumor astrocytes in GG display variable, often light staining (insert b). Insert (c) in A shows co-localization (yellow) of CB1 (red) with the astroglial marker GFAP (green). (Panels C, D) strong CB1 IR in SEGA with both membrane and cytoplasmic staining. Inserts in (D) show co-localization (yellow) of CB1 (red) with GFAP (green; insert a) but not with HLA-DR (green; insert b). Sections (single staining) are counterstained with Hematoxylin. GG, ganglioglioma; DNT, dysembryoplastic neuroepithelial tumor; SEGA, subependymal giant-cell astrocytoma. Scale bar in D: (A, B): 70 μm. (C): 150 μm; (D): 25 μm.
ogy within the SVZ at early postnatal ages (data not shown).

**CB1 in focal developmental lesions**

In agreement with previous observations (Tsou et al., 1998, Eggan and Lewis, 2007, Koethe et al., 2007), CB1 IR as present throughout all cortical layers as diffuse neuropil staining in human control autopsy specimens (Fig. 2 A), as well as in the normal-appearing cortex adjacent to the tuber or to GG (not shown). CB1 IR was found in axons, dendrites and it appeared as punctate labeling in neuronal somata. Intensely immunoreactive cell bodies were more often restricted in neocortical layers 2 and 3. Control specimens (autopsy and perilesional cortex) did not display detectable glial IR in both grey and white matter (Fig. 2 B). In FCD and cortical tuber specimens, similar to control specimens, IR for CB1 was characterized by intense neuropil staining (Fig. 2 C-L). Strong CB1 IR was often observed in DNs (Fig. 2 D, I, J; Fig. 5). CB1 IR was also observed in heterotopic neurons within the white matter and balloon cells in FCD or giant cells in cortical tubers (Fig. 2 E-G; Fig. 2 L; Fig. 5). Reactive astrocytes, present in the dysplastic cortex, displayed variable CB1 IR (Fig. 2 G and M; Fig. 5). Double-labeling experiments confirmed the co-localization of CB1 IR with neuronal and glial markers, as well as with stem cell markers, such as nestin or vimentin (Fig. 2).

CB1 expression was encountered in the neuronal component of GG and DNT, as well as in a subpopulation of astroglial tumor cells in GG (Fig. 4 A-B; Fig. 5). No detectable CB1 IR was observed in oligodendroglial tumor cells (Fig. 4 A-B). Strong CB1 IR was observed in all the SEGA specimens examined; double-labeling experiments showed co-localization of CB1 IR with GFAP, whereas CB1 IR was not observed in cells of the microglia/macrophage lineage (Fig. 4 C-D).

**CB2 in focal developmental lesions**

In agreement with previous observations (Benito et al., 2003), CB2 IR was very low or not detectable in human control autopsy specimens (Fig. 3 A), as well as in the normal-appearing cortex adjacent to the tuber or to GG (not shown). Control specimens (autopsy and perilesional cortex) did not display detectable glial IR in both grey and white matter (Fig. 3 B). However, as previously reported (Benito et al., 2007), strong expression of CB2 was observed in white matter of MS tissue samples (within MS lesions with positive astrocytes and reactive microglial cells; not shown). In FCD and cortical tuber specimens, CB2 IR was observed within the gray and white matter dysplastic areas (Fig. 3 C-J; Fig. 5). The staining was localized in some DNs (Fig. 3 E, H; Fig. 5) and in the large majority of balloon and giant cells (Fig. 3 F-G; Fig. 5), expressing GFAP, as well as stem cell markers (nestin, vimentin, not shown). Only light IR was observed in few reactive astrocytes, whereas strong expression was detected in cells with the morphology
of microglia/macrophages (Fig. 3C-J; Fig. 5). Double-labeling experiments confirmed the expression of CB2 in cells of the microglia/macrophage lineage (Fig. 3E, J).

CB2 expression was occasionally encountered in the astroglial component of GG (Fig. 4 E; Fig. 5). No detectable CB2 IR was observed in oligodendroglial tumor cells (Fig. 4 F). Expression was detected in cells of the microglia/macrophage lineage in both GG and DNT (not shown; Fig. 5). Strong CB2 IR was observed in all the SEGA specimens examined; double-labeling experiments showed co-expression of CB2 in GFAP positive tumor cells, as well as in cells of the microglia/macrophage lineage (Fig. 4G-H).

Figure 4. Evaluation of CB1 immunoreactivity (IR) in FCD, TSC and GG. Distribution of CB1 IR scores (total IR score; for details see Experimental Procedure’s section) in different cell types of normal control cortex (Ctx), FCD, cortical tubers in patients with TSC and in gangliogliomas (GG). (A) astrocytes and microglia/macrophage. (B) neurons (DNs, dysmorphic neurons in FCD/TSC) and balloon/giant cells (BCs/GCs). Astrocyte IR score was significantly elevated in FCD, TSC and GG relative to controls for CB1; neuronal IR score was significantly elevated compared to controls only in GG for CB1 (* P<0.05). No differences in both neuronal and glia IR scores were observed between patient groups.

Figure 5. Distribution of CB2 immunoreactivity (IR) in malformations of cortical development. (Panels A, B) CB2 IR in control cortex (A) and white matter (B) showing very low neuronal expression and no detectable glial labeling. (Panels C–G) CB2 IR in FCD showing IR within the dysplastic cortex. High magnification photographs (D, E; FCD) show variable CB2 expression in dysplastic neurons; the large majority of neurons display almost no detectable levels of CB2 IR (asterisk in D) and low expression is observed in the surrounding glial cells (arrows in D, E); few large dysplastic neurons are CB2 positive (asterisk in E); CB2 IR is detected in a population of cells with round (microglia/macrophage-like) morphology (arrow-heads in E). Insert (a) in E (FCD): absence of CB2 expression (red) in GFAP positive cells (green). Insert (b) in E (FCD): co-localization (yellow) of CB2 (red) with the HLA-DR (green) in cells of the microglia/macrophage lineage. (Panels F, G; FCD) strong CB2 IR is observed in balloon cells. Insert in G shows co-localization (yellow) of CB2 (red) with GFAP (green) in a balloon cell. (Panels H–J) CB2 IR in cortical tuber of TSC showing expression particularly within the white matter (Wm) area of the tuber. Insert in (H) few large dysplastic neurons are CB2 positive (arrow); astroglial cells do not express detectable levels of CB2 IR (arrow-head). High magnification photographs (Panels I, J; TSC) show strong CB2 IR in giant cells (arrows in J; higher magnification in J). Inserts (a) in I (TSC) shows co-localization (yellow) of CB2 (red) with GFAP (green) in a giant cell; insert (b) in I (TSC) shows neuronal expression (red) but absence of co-localization in astrocytes (GFAP positive, green). Insert (a in J; TSC): CB2 IR is detected in a population of cells with round (microglia/macrophage-like) morphology (arrow-head); astroglial cells do not express detectable levels of CB2 IR (arrow). Insert (b in J; TSC): co-localization (yellow) of CB2 (red) with the HLA-DR (green) in cells of the microglia/macrophage lineage in TSC. Sections (single staining) are counterstained with Hematoxylin. Scale bar in J: (A): 150 μm; (B): 70 μm; (I): 50 μm; (D, E, G): 25 μm; (J): 20 μm.
Despite the emerging role of the eCB signaling in brain development (Fernandez-Ruiz et al., 2000, Gomez et al., 2008a), data regarding the expression and cellular distribution of CBR in the developing human brain, particularly during the early stages of corticogenesis, are still limited. In addition, no information is available concerning the expression patterns in human developmental disorders, such as focal MCD in which a primary or acquired alteration of the early stage of corticogenesis has been proposed as pathogenetic mechanism (Barkovich et al., 2005, Wong, 2008). Knowledge concerning the expression of CBR subtypes in these highly epileptogenic developmental glioneuronal lesions is also interesting considering the key function of eCB signaling in regulating glial function and neuronal excitability.

**DISCUSSION**

Fig. 6. CB2 immunoreactivity (IR) in glioneuronal tumors (GG and DNT) and SEGA. (A, B) CB2 in the neuronal component of both GG and DNT does not display detectable IR (arrow in A; insert in B). No detectable CB2 expression is observed in the oligodendroglia-like cells within DNT (B, insert in B). Occasionally few glial cells are CB2 positive in GG (insert a in A). Insert (b in A) shows co-localization (yellow) of CB2 (red) with GFAP (green) in few cells. (Panels C, D) strong CB2 IR in SEGA with prominent cytoplasmic staining. Inserts in (D) show co-localization (yellow) of CB2 (red) with GFAP (green; insert a) and with HLA-DR (green; insert b). Sections (single staining) are counterstained with Hematoxylin. GG, ganglioglioma; DNT, dysembryoplastic neuroepithelial tumor; SEGA, subependymal giant-cell astrocytoma. Scale bar in D: (B, C): 70 μm; (A, D): 25 μm.
In the present study we analyzed the expression patterns of CBR in human brain during corticogenesis and we provide evidence of CB1 and CB2 expression in focal MCD. The cell-specific distribution in relation with the histopathological features of the different entities is discussed below.

**CB1 and CB2 expression during human cortical development**

We provide evidence of expression of CB1 in human neocortex early during development. The period of prenatal development studied (9-40 GW) includes all the critical stages of human cortical development (proliferation, migration and maturation/organization) which are involved in the formation and differentiation of the CP (Kostovic and Rakic, 1990, Rakic and Lombroso, 1998). We observed expression of CB1 in the CP as early as 9 weeks of gestation. This early expression of CB1 is in agreement with previous autoradiographic studies in fetal human brain, including early developmental stages (Glass et al., 1997, Biegon and Kerman, 2001, Mato et al., 2003). Interestingly, very high levels of CB1 were observed at the neocortical SVZ in fetal cases (Mato et al., 2003), however, the resolution of these previous studies was not sufficient to detect the specific cellular distribution within the developing cortex. In our study we observed expression in postmitotic neural cells in the CP, starting from the earliest stage investigated (9 GW), whereas the VZ was largely devoid of CB1 IR. This finding is in good agreement with the previously reported CB1 mRNA and protein expression in rodent embryonic brain (Mulder et al., 2008, Vitalis et al., 2008).

![Figure 7. Evaluation of CB2 immunoreactivity (IR) in FCD, TSC and GG.](image)

Distribution of CB2 IR scores (total IR score; see for details Method's section) in different cell types of normal control Ctx, FCD, cortical tubers in patients with TSC and in gangliogliomas (GG). (A) astrocytes and microglia/macrophage. (B) neurons (DNs, dysmorphic neurons in FCD/TSC) and balloon/giant cells (BCs/GCs). Microglia/macrophage IR score was significantly elevated in FCD, TSC and GG relative to controls for CB2 (* P<0.05). No differences in both neuronal and glia IR scores were observed between patient groups.
CB1 and CB2 expression in focal developmental lesions

CB1 expression in dysmorphic neurons (DNs)

We demonstrate that CB1 are expressed within focal malformations (such as FCD, TSC) in DNs. Although IR for both receptor subtypes (CB1 and CB2) could be detected in these cell types, CB1 was observed in a higher proportion of neuronal cells compared to CB2. The prominent neuronal expression of CB1 is in line with previous observations, showing broad expression of functional CB1 in neocortical neurons (Hill et al., 2007). We do not have a definitive explanation for the presence of the intense CB1 IR in the neuronal population of FCD and TSC specimens. It is however possible that the increased excitatory synaptic network, with DNs displaying a glutamatergic cell phenotype (Lamparello et al., 2007), requires high
turnover and synthesis of CBR proteins. Previous studies suggest that DNs more efficiently synthesize or post-translationally modify receptor proteins in both cell bodies as well as in dendritic processes (Ying et al., 1998, Hilbig et al., 1999). Activation of neuronal CB1 may than represent a key mechanism to control neuronal excitability within these highly epileptogenic lesions. Accordingly, increasing evidence indicates that seizure activity regulates the expression of CB1 and that pharmacological modulation of the CBR signaling, using selective agonists/antagonists of CB1, critically affects neuronal injury and epileptogenesis (Wallace et al., 2003, Shafaroodi et al., 2004, Bernard et al., 2005, Monory et al., 2006, Chen et al., 2007). Thus, these neuroprotective and anticonvulsant effects together with the prominent expression of CB1 observed in the neuronal epileptogenic component of FCD and TSC specimens, could potentially impact future therapeutic approaches (i.e. through selective enhancement of eCB levels) also in patients with focal MCD. However, CBR, and particularly CB1 signaling, have also been shown to critically regulate neuronal connectivity during early development, modulating the outgrowth of dendrites and axons (Bernard et al., 2005, Mulder et al., 2008, Vitalis et al., 2008). Thus, the possibility that the strong expression of CB1 may be critical with respect to morphology and dysfunction of neuronal cells has also to be taken in consideration in any attempt to pharmacologically modulate the CB1 signaling during brain development.

CBR expression in balloon and giant cells (BCs/GCs)
Recently, attention has been focused on these morphologically aberrant cells concerning their nature, origin and role in epileptogenicity of FCD and TSC (for reviews see Najm et al., 2007; Wong, 2008). A recent study suggests that BCs in FCD are derived from radial glial progenitor cells phenotype (Lamparello et al., 2007). In the present study we report expression of CBR in both BCs and GCs of FCD and TSC specimens containing this cell type. In particular, the large majority of balloon and giant cells are mainly immunoreactive for CB2. The functions of CBR in this population of cells, which do not establish synaptic contacts and display minimal signs of hyperexcitability is still unclear (Cepeda et al., 2003, 2005, Alonso-Nanclares and De Felipe, 2005,. However, since CBR are known to suppress the production of inflammatory proteins (for review see Arevalo-Martin et al., 2008, Cabral and Griffin-Thomas, 2008) and BCs/GCs are involved in the inflammatory processes observed in FCD and TSC (Ravizza et al., 2006, Boer et al., 2008), their expression in BCs/GCs could represent an attempt to modulate the inflammatory response within the dysplastic cortex.

CBR expression in glial cells
Besides neuronal cells, glial cells are also an important component of focal MCD.
A variable degree of reactive astrocytosis is present within the dysplastic cortex and the subcortical white matter in both FCD and TSC specimens (Mizuguchi and Takashima, 2001, Sosunov et al., 2008, Blümcke et al., 2009, Martinian et al., 2009). Recently, it has been shown that hippocampal astrocytes express CB1 receptors and respond to eCB, revealing the existence of eCB-mediated neuron-astrocyte communication (Navarrete and Araque, 2008). Therefore, astroglial CBR within the FCD may represent an additional target for eCB to regulate synaptic transmission. In addition, activation of glial CBR has been shown to have immunosuppressive activity and to modulate the function of inflammatory cells (Sheng et al., 2005, Arevalo-Martín et al., 2008). In particular, increasing evidence (both in vitro and in vivo) indicates that CB2 are up-regulated in activated microglia in different pathological conditions and supports their key role in microglia/macrophage functions (Benito et al., 2008, Stella, 2009). Interestingly, activation of cells of the microglia/macrophage lineage and induction of different inflammatory pathways have been described in both animal models and human focal chronic epilepsy, including MCD (Aronica et al., 2005, Boer et al., 2006, Ravizza et al., 2006, Aronica et al., 2007b, Boer et al., 2008). Moreover, recent evidence strongly suggests the involvement of inflammatory processes in the etiopathogenesis of seizures (Vezzani and Granata, 2005, Vezzani et al., 2008). In our study we confirm the inducible nature of expression of CB2 in microglia/macrophages in human epileptogenic lesions. Thus, the CB2 could represent attractive target to modulate the inflammatory response associated with epilepsy, without inducing the psychotropic effects associated with activation of CB1.

**CBR expression in glioneuronal tumors and SEGA**

Recently, the expression of both CB1 and CB2 has been reported in human brain tumors. In particular expression of CBR was observed in both adult and pediatric astroglial tumors (Held-Feindt et al., 2006, Calatozzolo et al., 2007, Ellert-Miklaszewska et al., 2007, Schley et al., 2009). Our results demonstrated expression of only CB1 in the neuronal component of both GG and DNT, whereas variable expression of both CBR was detected in the astroglial component of GG. Interestingly, we observed a strong expression of CBR in SEGA. Prominent expression of CB2 has been previously reported in SEGA, (Ellert-Miklaszewska et al., 2007). The expression of both CBR in a low grade tumors, such as SEGA argues against the notion that CBR expression is associated with tumor malignancy (Ellert-Miklaszewska et al., 2007). However, the high levels of CBR observed in tumor cells in SEGA could suggest a potential role of cannabinoids, particularly of CB2 agonists devoid of psychotropic side effects, in this type of tumor. Accordingly, cannabinoids have been proposed as potential antitumoral agents displaying the ability to reduce glioma tumor growth both in vitro and in animal...
models (for review see Parolaro and Massi, 2008).

Conclusions

Despite the emerging role of the eCB signaling in brain development ([Fernandez-Ruiz et al., 2000] and [Gomez et al., 2008a]), data regarding the expression and cellular distribution of CBR in the developing human brain, particularly during the early stages of corticogenesis, are still limited. In addition, no information is available concerning the expression patterns in human developmental disorders, such as focal MCD in which a primary or acquired alteration of the early stage of corticogenesis has been proposed as pathogenetic mechanism ([Barkovich et al., 2005] and [Wong, 2008]). Knowledge concerning the expression of CBR subtypes in these highly epileptogenic developmental glioneuronal lesions is also interesting considering the key function of eCB signaling in regulating glial function and neuronal excitability ([Fride, 2005] and [Navarrete and Araque, 2008]). In the present study we analyzed the expression patterns of CBR in human brain during corticogenesis and we provided evidence of CB1 and CB2 expression in focal MCD. The cell-specific distribution in relation with the histopathological features of the different entities is discussed below.

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4.2 UPREGULATION OF ADENOSINE KINASE IN ASTROCYTES IN EXPERIMENTAL AND HUMAN TEMPORAL LOBE EPILEPSY

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ABSTRACT

Purpose: Adenosine kinase (ADK) represents the key metabolic enzyme for the regulation of extracellular adenosine levels in the brain. In adult brain ADK is primarily present in astrocytes. Several lines of experimental evidence support a critical role of ADK in different types of brain injury associated with astrogliosis, which is also a prominent morphological feature of temporal lobe epilepsy (TLE). We hypothesized that dysregulation of ADK is a ubiquitous pathological hallmark of TLE.

Methods: Using immunocytochemistry and Western blot analysis, we investigated ADK protein expression in a rat model of TLE during epileptogenesis and the chronic epileptic phase and compared those findings with tissue resected from TLE patients with mesial temporal sclerosis (MTS).

Key findings: In rat control hippocampus and cortex, a low baseline expression of ADK was found with mainly nuclear localization. One week after the electrical induction of status epilepticus (SE), prominent up-regulation of ADK became evident in astrocytes with a characteristic cytoplasmic localization. This increase in ADK persisted at least for 3–4 months after SE in rats developing a progressive form of epilepsy. In line with the findings from the rat model, expression of astrocytic ADK was also found to be increased in the hippocampus and temporal cortex of patients with TLE. In addition, in vitro experiments in human astrocyte
cultures showed that ADK expression was increased by several proinflammatory molecules (interleukin-1β and lipopolysaccharide).

**Significance:** These results suggest that dysregulation of ADK in astrocytes is a common pathological hallmark of TLE. Moreover, the in vitro data suggest the existence of an additional layer of modulatory crosstalk between the astrocyte-based adenosine cycle and inflammation. Whether this interaction also can play a role in vivo needs to be further investigated.

**INTRODUCTION**

Temporal lobe epilepsy (TLE) is a common neurological disorder and mesial temporal sclerosis (MTS) represents a frequent pathophysiological substrate of TLE (Engel 2001, Wieser 2004). However, MTS represents the pathological substrate of a process that occurs over a long period of time, following an initial injury (i.e. febrile seizures, status epilepticus (SE) of several causes, cerebral trauma) (Engel, et al. 2003, Pitkanen 2010).

Large-scale analysis of gene expression studies performed in different experimental models of TLE revealed that the biological process that emerges as the most prominent during epileptogenesis is related to glial activation (Aronica & Gorter 2007, Lukasiuk, et al. 2006). Astrogliosis is indeed a major pathological feature of MTS (Blümcke, et al. 2009, Wieser 2004) and recent data suggest a role for astroglial cells in epilepsy, indicating that astroglia can display different functional phenotypes within the epileptic focus, contributing to seizure development (Binder & Steinhauser 2006, Seifert, et al. 2010, Wetherington, et al. 2008).

Recently attention has focused on the role of astrocytic adenosine kinase (ADK). This enzyme is responsible for the phosphorylation of adenosine into AMP and has been shown to critically regulate the extracellular adenosine levels in brain (Boison 2006, Etherington, et al. 2009). In particular, increased levels of astrocytic ADK have been reported in the kainic acid-induced mouse model of TLE and inhibition of ADK has been shown to be an effective anticonvulsant strategy in this model (Fedele, et al. 2005a, Gouder, et al. 2004); reviewed in (Boison 2008, Boison 2010)). A key challenge in translating the link between ADK expression and epilepsy into the clinic is represented by the generalization of these findings to different animal models and validation of those findings in appropriate patient populations.

In order to understand the dynamics of ADK expression during development and progression of epilepsy we evaluated the expression and cellular distribution of ADK in a rat model of TLE (post-SE model induced by electrical stimulation), as well as in hippocampal and cortical specimens of MTS patients. Moreover, we investigated whether ADK could be regulated by IL-1β, a proinflammatory cytokine that has been shown to be activated in epileptic tissue (Vezzani & Baram 2007).
MATERIAL AND METHODS

Experimental animals

Adult male Sprague Dawley rats (Harlan CPB laboratories, Zeist, The Netherlands) weighing 300-500 grams were used in this study which was approved by the Animal Welfare committee of the University of Amsterdam, and which is in accordance with the NIH guidelines for the care and use of experimental animals. All experimental protocols followed the European Communities Council directive 86/609/EEC and the Dutch Experiments on Animal Act (1997), and were approved by the Dutch animal welfare committee (DEC).

The rats were housed individually in a controlled environment (21±1°C; humidity 50-60%; lights on 08:00 AM - 8:00 PM; food and water available ad libitum).

Electrode implantation and seizure induction

Adequate measures were taken to minimize pain or discomfort. Rats were anaesthetized with an intramuscular injection of ketamine (57 mg/kg; Alfasan, Cuyk, The Netherlands) and xylazine (9 mg/kg; Bayer AG, Germany) and placed in a stereotactic apparatus. In order to record hippocampal EEG, a pair of insulated stainless steel electrodes (70 µm wire diameter, tips were 80 µm apart) were implanted into the left dentate gyrus (DG) under electrophysiological control as previously described (Gorter, et al. 2001). A pair of stimulation electrodes was implanted in the angular bundle. Two weeks after implantation rats underwent tetanic stimulations (50 Hz) of the hippocampus in the form of a succession of trains of pulses every 13 seconds. Each train had a duration of 10 seconds and consisted of biphasic pulses (pulse duration 0.5 ms, maximal intensity 500 µA). Stimulation was stopped when the rats displayed sustained forelimb clonus and salivation for minutes, which usually occurred within 1 hour. However, stimulation never lasted longer than 90 minutes. Differential EEG signals were amplified (10x) via a FET transistor that connected the headset to a differential amplifier (20x; CyberAmp, Axon Instruments, Burlingame, CA, USA), filtered (1-60 Hz), and digitized by a computer. A seizure detection program (Harmonie, Stellate Systems, Montreal, Canada) sampled the incoming signal at a frequency of 200 Hz per channel. EEG recordings were monitored also visually and screened for seizure activity. Behavior was observed during electrical stimulation and several hours thereafter. Immediately after termination of the stimulation, periodic epileptiform discharges (PEDs) occurred at a frequency of 1-2 Hz and they were accompanied by behavioral and EEG seizures (status epilepticus; SE). Most rats were monitored continuously from the cessation of SE to the time of sacrifice (24 h to 1 week). The chronic epileptic group (3-4 months after SE) was monitored during and shortly after SE and during 3-5 days before sacrifice in order to determine the frequency of spontaneous seizures. Sham-operated control rats were handled and recorded identically, but...
did not receive electrical stimulation. None of these rats needed to be reimplanted. Chronic epileptic rats had frequent daily seizures (range, 5–12). The time between the last spontaneous seizure and sacrifice was < 5 h.

**Rat tissue preparation**

Rats were disconnected from the EEG recording set-up and deeply anesthetized with pentobarbital (Nembutal, intraperitoneally, 60 mg/kg). For immunocytochemistry, the animals were perfused through the ascending aorta with 300 ml of 0.37 % Na₂S solution, followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Thereafter, the brains were removed, incubated for 72 hours in 0.3 M EDTA, pH 6.7 (Merck, Amsterdam, The Netherlands) and paraffin embedded. Paraffin-embedded tissue was sectioned at 6 µm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel GmbH, Braunschweig, Germany) and were used for immunocytochemistry. Horizontal sections were analyzed at a midlevel of the brain (5300–6100 µm below cortex surface). Immunocytochemistry was performed on two adjacent serial sections from each group [control, n=6; 24 h, n= 5; 1 week, n=6; 3–4 months, long term (LT) epilepsy, progressive pLT, n= 5 and non-progressive, npLT, n=5 and non-SE rats, n=3). Progressive epileptic rats (pLT) were characterized by frequent daily seizures that had progressed over time until epilepsy had developed with a rather stable frequency (~10 seizures/day). Non-progressive epileptic rats (npLT), were characterized by exhibiting an occasional seizure per week, the frequency of which did not increase over time (~1 seizure/week). Non-SE rats did not develop a SE during stimulation and were included as controls and were sacrificed 3-4 months after stimulation. Two additional serial slices were used for the double staining, as described below.

For Western blot analysis, animals were decapitated in the acute phase (one day after SE, n=4), in the latent period (1 week after SE, n=6; the rats in this group did not yet exhibit spontaneous seizures) and in the chronic epileptic phase, LT (progressive pLT n=5 and non-progressive, npLT n=4). Control rats (n=6) were implanted but not stimulated. The temporal cortex was dissected and immediately frozen on dry ice and stored at -80 °C until use (Western blot analysis).

**Human material**

The human cases included in this study were obtained from the files of the Departments of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and the VU University Medical Center (VUMC). Eleven patients underwent resection of the hippocampus for medically intractable TLE. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All samples were obtained...
and used in a manner compliant with the Declaration of Helsinki. Two neuropathologists reviewed all cases independently. In 6 cases a pathological diagnosis of HS (without extrahippocampal pathology) was made. The HS specimens include 4 cases of classical HS (grade 3, mesial temporal sclerosis type 1a) and 2 cases of severe HS (grade IV; mesial temporal sclerosis type 1b; Blumcke, et al. 2007, Wyler, et al. 1992). Five non-HS cases, in which a focal lesion [gangliogioma not involving the hippocampus proper] was identified, were also included to provide a comparison group to HS cases. Control hippocampal tissue was obtained at autopsy from 6 patients without history of seizures or other neurological diseases. All autopsies were performed within 12 hours after death. Table 1 (supplementary) summarizes the clinical features of TLE and control cases.

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 µm, mounted on organosilane-coated slides (Star Frost, Waldemar Knittel GmbH, Brunschweig, Germany) and used for in situ hybridization and immunocytochemistry as described below. Temporal cortex from control patients (n=5) and surgical cortex from MTS patients (n=5) was snap frozen in liquid nitrogen and stored at −80°C until further use (western blot analysis).

**Immunocytochemistry**

Glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9, DAKO; 1:1000), neuronal nuclear protein (NeuN; mouse clone MAB377, IgG; Chemicon, Temecula, CA, USA; 1:2000) and (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400) were used in the routine immunocytochemical analysis of TLE human specimens.

For the detection of ADK, we used a polyclonal rabbit antibody (1:500; Ren, et al. 2007, Studer, et al. 2006). Single-label immunocytochemistry was performed with Powervision (Immunologic, Duiven, The Netherlands). 3,3-Diaminobenzidine was used as chromogen. Sections were counterstained with Haematoxylin. For double-labeling studies, sections, after incubation with primary antibodies, were incubated for 2 h at RT with Alexa Fluor® 568 and Alexa Fluor® 488 (anti-rabbit IgG or anti-mouse IgG; 1:200; Molecular probes, Eugene, USA).

For double labeling we also used the anti-doublecortin (DCX; Abcam 1:4000; Cambridge, UK). Sections were analyzed by means of a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA; MRC1024) equipped with an argon-ion laser.

Quantitative analysis was also performed in rat hippocampus and the number of positive cells was quantified as previously described (Maroso, et al. 2010). Briefly, two representative adjacent non-overlapping fields of the hippocampus (CA1, CA3 and hilar region of
the dentate gyrus, DG) were captured (magnification 40x; total area of each field: 171,600 μm²) and digitized using a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA; MRC1024). We counted the total number of GFAP-positive cells, and those showing nuclear or extra-nuclear ADK staining.

Cell cultures
For cell culture experiments (astrocyte-enriched human cultures), fetal brain tissue (22–23 weeks of gestation) was obtained from spontaneous or medically induced abortions with appropriate maternal written consent for brain autopsy. Resected tissue samples were collected in Dulbecco's modified Eagle's medium, DMEM/HAM F10 (1:1) medium (Gibco, Grand Island, NY). Cell isolation was performed as previously described (Aronica et al., 2003; Aronica et al., 2005c). Briefly, after removal of meninges and blood vessels, tissue was dissociated by incubation at 37°C for 20 min in a Hank's balanced salt solution containing 2.5 mg/ml trypsin (Sigma, St. Louis, MO, USA) and 0.1 mg/ml bovine pancreatic Dnase I (Boehringer Mannheim, Germany). Tissue was triturated and washed with DMEM/HAM F10 medium, supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin and 10% fetal calf serum (FCS). Cell suspensions (containing ~0.5 g wet weight tissue/10 ml culture medium) were passed through a 70 µm cell sieve (Becton Dickinson, USA) and plated into poly-L-lysine (PLL; 15 µg/ml, Sigma), pre-coated 25 cm² flasks (Falcon, Lincoln Park, NJ) and maintained in a 5% CO2 incubator at 37°C. After 48 h the culture medium was replaced by fresh medium and cultures were subsequently fed twice a week. Cultures reached confluence after 2-3 weeks. Secondary astrocyte cultures were established by trypsinizing confluent cultures and sub-plating onto PLL-precoated 6 wellplates or 75 cm² flasks (0.5-1 X 106 cell/ml; for Western blot analysis or for the generation of serial passages respectively) More than 98% of the cells in primary culture, as well as in the successive 12 passages were strongly immunoreactive for the astrocytic marker GFAP. In the present study astrocytes were used for Western blot analysis at passage 3-4. IL-1 β (10 ng/ml; IL-1b, Peprotech, NJ, USA) or LPS (lipo polysaccharide; 100 ng/ml; Sigma, St. Louis, MO, USA) were applied and maintained in serum free medium for 24 h before harvesting them for Western blot analysis. As previously shown (Aronica et al., 2005c), the viability of human astrocytes in culture was not influenced by the treatments.

Western blot analysis
For immunoblot analysis, samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, Na-orthevanadate (10.4 mg/ml), 5 mM EDTA (pH 8.0), 5 mM NaF and protease inhibitor cocktail (Boehringer Mannheim, Germany).
Protein content was determined using the bicinchoninic acid method. For electrophoresis, equal amounts of proteins (30 µg/lane) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis. Separated proteins were transferred to nitrocellulose paper for 1 h and 30 min, using a semi-dry electroblotting system (BioRad, Transblot SD, Hercules, CA, USA). Blots were incubated over night in TTBS (20 mM Tris, 150 mM NaCl, 0.1% Tween, pH 7.5)/5% non fat dry milk, containing the primary antibody (1:5000). After several washes in TTBS, the membranes were incubated in TTBS/5% non fat dry milk/1% BSA, containing the goat anti-rabbit or goat anti-mouse antibodies coupled to horse radish peroxidase (1:2500; Dako, Denmark) for 1 h. After washes in TTBS, immunoreactivity was visualized using Lumi–light PLUS western blotting substrate (Roche Diagnostics, Mannheim, Germany) and digitized using a Luminescent Image Analyzer (LAS-3000, Fuji Film, Japan). Expression of β-actin (monoclonal mouse, Sigma, St. Louis, MO, USA 1:50.000) was used as reference. Statistical analyses were performed with SPSS for Windows (SPSS 11.5, SPSS Inc., Chicago, IL, USA) using two-tailed Student’s t-test; to assess differences between more than two groups, ANOVA and a non-parametric Kruskal–Wallis test followed by Mann–Whitney U-test were performed. P < 0.05 was considered significant.

Results
Overexpression of ADK in hippocampus of rats with progressive course of TLE
To determine the temporal-spatial expression and cellular distribution of ADK we performed immunocytochemistry in tissue samples of control rats and rats that were sacrificed at different time points after SE (1 day, 1 week and 3 months post-SE). Control hippocampus displayed weak ADK immunoreactivity (IR) in the different hippocampal subfields (Fig. 1 A-B). No ADK IR was detected in neurons, however nuclear ADK IR was observed in resting glial cells. Only sparse nuclear ADK IR was observed at one day post-SE (not shown). At 1 week post-SE (Fig. 1C-H), ADK IR (with cytoplasmic staining) was detected within the different hippocampal regions in glial cells with the morphology of reactive astrocytes (Fig. 2D-H). Double labelling confirmed ADK expression in GFAP-positive reactive astrocytes (Figure 1H). The large majority of pyramidal neurons in CA1 and CA3 regions did not display ADK expression (Fig. 1D-E, insets), however occasionally neuronal IR was observed (Fig. 1D, inset). Strongly stained ADK positive cells in the subgranular zone of the DG did not appear to co-localize with DCX (Fig. 1G, inset).
Chronic epileptic rats can be divided in rats with frequent daily seizures and a progressive form of epilepsy (pLT) and rats that had rare seizures that did not progress over time (npLT). In chronic epileptic rats that had a progressive form of epilepsy (3-4 months post-SE; pLT), ADK was still increased in astrocytes and a weak ADK IR was detected in some neurons (Fig.
Figure 1. Immunostaining of ADK in hippocampal tissue of control rat and after induction of status epilepticus (SE). Immunostaining of ADK in hippocampal tissue of control rat and after induction of SE. (A, B) Control hippocampus showing low ADK IR in the different hippocampal subfields. (B) CA3 region showing no detectable IR in neurons and sparse nuclear IR in glial cells (inset). (C–H) Hippocampus 1 week post-SE showing increased ADK expression in glial cells within the different hippocampal subfields, including CA3 (D), CA1 (E–F; arrows), DG (G–H, arrows; arrowheads in (G) indicate ADK positive cells in the subgranular zone) regions; inset a in (D) ADK positive astrocytes in CA3; inset b in (D) merged confocal image, showing ADK positive glial cells (red) surrounding the NeuN (green) positive neuronal cells in CA3. Inset c in (D) ADK (red) NeuN (green) colocalization. (E) Merged confocal image, showing ADK positive glial cells (red) surrounding the NeuN (green) in CA1; inset in (G) absence of colocalization of ADK (red) and DCX (green) in the subgranular zone; inset in (H) expression of ADK (red) in astrocytes (GFAP positive, green). Scale bars: A, C: 560 μm; B, D: 140 μm; E: 20 μm; G: 70 μm; F, H: 40 μm.
Figure 2. ADK IR in rat hippocampus 3–4 month after SE. (A, C, E, G) Hippocampus of rats with long-term epilepsy, progressive (pLT; approximately 10 seizures/day; last seizure <2 h before sacrifice) showing both nuclear and cytoplasmic ADK IR in different hippocampal subfields, including CA3 (C; inset), CA1 (E; inset a), and dentate gyrus (DG, G; inset); Inset in (C) ADK-positive glial cells surrounding a neuron with weak ADK. Inset b in (E) expression of ADK (red) in astrocytes (GFAP positive, green). (B, D, F, H) Hippocampus of rats with long-term epilepsy, nonprogressive (npLT; 1 seizure every other day; last seizure at least 1 day before sacrifice), showing low ADK IR in different hippocampal subfields; mainly nuclear IR is observed in sparse glial cells in CA3 (D, inset), CA1 (F) and DG (H; inset). Scale bars: A, C: 280 μm; C, D: 140 μm; E–H: 70 μm.
Co-localization studies indicated that ADK was expressed in GFAP positive cells (astrocytes; Fig. 2 E, inset b). In contrast, in chronic epileptic rats that had a non-progressive form of epilepsy (3-4 months post-SE; npLT) the expression pattern was similar to control hippocampus, with no neuronal expression and a few positive glial cells with nuclear IR (Fig. 2 B, D, F, H). Similar IR pattern, without neuronal expression, was observed in non-SE rat hippocampus (not shown). Quantitative analysis confirmed the increased expression of ADK (nuclear and extra-nuclear staining) at both 1 week and 3-4 months (chronic epileptic rats with progressive form of epilepsy) post SE in astrocytes within different hippocampal regions (Fig. 3 A-C).

**Figure 3. Evaluation of ADK astrogial immunoreactivity in hippocampal regions of control rat and after induction of status epilepticus (SE).** Bar diagrams of ADK-positive cells in CA1 (A), CA3 (B), and dentate gyrus (hilar region; C) of control hippocampus and hippocampus at 24 h, 1 week and 3–4 months post-SE (rats with long-term epilepsy, progressive, pLT (approximately 10 seizures/day; last seizure <2 h before sacrifice) and rats with long-term epilepsy, nonprogressive, npLT (1 seizure every other day; last seizure at least 1 day before sacrifice)). Nuclear and extranuclear staining: *p < 0.05 versus control, one-way ANOVA followed by Tukey’s test.
ADK expression in rat temporal cortex

To determine the temporal-spatial expression and cellular distribution of ADK in temporal cortex, we performed immunocytochemistry in tissue samples of control rats and rats that were sacrificed at different time points after SE (1 day, 1 week, and 3–4 months post-SE). Control cortex displayed weak ADK IR (Fig. S1A). No ADK IR was detected in neurons; however, sparse, mainly nuclear ADK IR was observed in resting glial cells (inset in A). Only sparse nuclear ADK IR was observed at 1 day post-SE (not shown). At 1 week post-SE (Fig. S1B,C), increased ADK IR was observed (with cytoplasmic staining) in glial cells. ADK IR was also observed in a few neuronal cells (Fig. S1C, insets a,c). In chronic epileptic rats that had a progressive form of epilepsy (3–4 months post-SE; pLT; Fig. S1D,E), increased ADK IR, with cytoplasmic localization, was still detected in glial cells. Colocalization studies confirmed ADK-expression in GFAP-positive cells (astrocytes; Fig. S1E, inset). In contrast, in chronic epileptic rats that had a nonprogressive form of epilepsy (3–4 months post-SE; npLT) only sparse nuclear IR was detected in glial cells (Fig. S1F).

Figure 4. Western blot analysis of ADK in temporal cortex of control rat and after induction of SE. (A) Representative immunoblots of total homogenates from control rat cortex and after induction of SE (24 h, 1 week, 3–4 months long term progressive epilepsy, pLT, and nonprogressive, n-pLT). (B) Den-
sitometric analysis: values (optical density units, O.D.) are mean ± standard error of the mean (SEM), (control, n = 6; 24 h, n = 4; 1 week, n = 6; pLT, n = 5; npLT, n = 4), relative to the optical density of β-actin; *p < 0.05 compared to controls.
Figure 5. Distribution of ADK immunoreactivity in the hippocampus and temporal cortex (Ctx) of control and TLE patients with MTS. (A, C, E) Control hippocampus (A, CA3; C, dentate gyrus, hilar region) and Ctx (E) showing weak ADK IR in both glial and neuronal cells (insets a, b in E). Sections are counterstained with hematoxylin. Histologically normal surgical hippocampus and cortex displayed a pattern of IR similar to that observed in control autopsy hippocampus (not shown). (B, F) MTS, hippocampal sclerosis (B, CA3; F, dentate gyrus, hilar region), showing increased ADK expression. Expression was observed in residual neurons (arrowhead in D) and in reactive astrocytes (arrows in D). (F) MTS, temporal cortex showing increased ADK expression with both neuronal and glial IR (insets). Scale bars: A, B: 160 μm; C, D: 40 μm; E, F: 40 μm. Western blot analysis of ADK in temporal cortex: representative immunoblots (G) and densitometric analysis (H) of total homogenates from autopsy control temporal cortex and surgical cortex from MTS and non-MTS specimens. (H) Densitometric analysis: values (optical density units, O.D.) are mean ± SEM, five controls, five MTS, and four non-MTS, relative to the optical density of β-actin; * p < 0.05.
Western blot analysis of total homogenates of rat temporal cortex revealed a band at molecular weight of approximately 40 kDa (Fig. 4A). Densitometric analysis (Fig. 4B) of control cortex and cortex from rats at different time points after SE (1 day, 1 week and 3–4 months post-SE) revealed a significant increase in ADK expression at 1 week and in chronic epileptic rats that had a progressive form of epilepsy (pLT).

**ADK expression in hippocampal sclerosis**

The expression and cellular distribution of ADK was studied by immunocytochemistry in hippocampal specimens of control and TLE patients with MTS. In control (autopsy) hippocampus, ADK displayed weak staining in the different hippocampal subfields, including CA3–CA4 regions (Fig. 5A,C) and cortex, (Fig. 5E). A similar immunoreactivity pattern was observed in non-MTS specimens (not shown). In HS specimens, ADK IR was observed in cells with typical astroglial morphology (Fig. 5B,F). Double labeling confirmed ADK expression in GFAP-positive reactive astrocytes (Fig. 5D, inset). Weak to moderate ADK IR was also observed in a few neuronal cells (Fig. 5F). No detectable ADK IR was observed in cells of the microglial/macrophage lineage.

Western blot analysis was also performed to quantify the total amount of ADK in total homogenates of control autopsy cortex and surgical cortex from MTS and non-MTS patients (Fig. 5G). Densitometric analysis revealed a significant increase of ADK expression in MTS compared to control and non-MTS specimens (Fig. 5H). No significant correlation was observed between Western blot data (optical density values) and different clinical variables.

**Figure 6. ADK in human astrocytes induced by IL-1β and LPS.**

ADK in human astrocytes induced by IL-1β and LPS. (A) Representative immunoblot of total homogenates from human fetal astrocytes untreated and treated for 24 h with 10 ng/ml IL-1β or 100 ng/ml LPS. (B) Densitometric analysis: values (optical density units, O.D.) are mean ± SEM, (control, n = 3; IL-1β, n = 4 and LPS, n = 4 treated astrocytes), relative to the optical density of β-actin; *p < 0.05, compared to controls.
ADK in cultured astrocytes

Because IL-1β is known to be activated in both experimental and human TLE (Ravizza et al., 2008; Vezzani et al., 2008), we also investigated whether this inflammatory cytokine could play a role in the regulation of the expression of ADK. Astrocyte-enriched human cell cultures were exposed to LPS and to IL-1β. Western blot analysis performed in total homogenates demonstrated that both IL-1β and LPS increased the expression of ADK in cultured human astrocytes (Fig. 6A,B).

Discussion

Astrocytes play a major role in the regulation brain levels of adenosine, which is an endogenous neuroprotectant and anticonvulsant (Boison 2006, Boison, et al. 2010, Etherington, et al. 2009). Mouse models of epilepsy support a role for astrocytic ADK regulation in epileptogenesis (Boison 2008, Boison 2010). Until now however it has not yet been established whether dysregulation of ADK is a common mechanism being operative in several forms of epilepsy. We therefore assessed the cellular distribution and expression of ADK in both rat and human chronic epileptic tissue.

ADK is known to be rapidly downregulated under different conditions of acute brain injury (Pignataro, et al. 2008). Accordingly, in a previous micro-array study which was performed in the electrical post-SE rat model we observed that the ADK gene is down-regulated 24 hrs after induction of SE in the CA3 region of the hippocampus (Gorter, et al. 2006). This regulation likely represents an attempt to increase the protective levels of adenosine (Pignataro, et al. 2008), but my also contribute, in concert with the regulation of glial adenosine receptor (AR) expression to the development of astrogliosis (Boison 2010, Hask, et al. 2005). In the post-SE rat model (similarly to the mouse models of epileptogenesis (Boison 2010)) we detected an upregulation of ADK protein during the latent phase (1 week post SE) both in hippocampus and temporal cortex, which precedes the development of spontaneous electrographic seizures and is characterized by prominent astrogliosis. Immunocytochemical analysis showed ADK expression in reactive astrocytes with both nucleus and cytoplasmic labeling. Two isoforms of ADK have been identified in mammalian organisms and they have a different subcellular localizations (ADK-long and –short isoforms with respectively nuclear and cytoplasmic localization) and different functions (Cui, et al. 2009). Nuclear ADK is likely involved in epigenetic mechanisms, such as methylation reactions, whereas the cytoplasmic isoform is thought to regulate the extracellular levels of adenosine. Accordingly, mice constitutively overexpressing a transgene for the cytoplasmic isoform of ADK are characterized by a reduced adenosine tone, and display spontaneous seizures, as well as increased susceptibility to seizure-induced neuronal cell death (Fedele, et al. 2005b, Li, et al. 2008a.
Li, et al. 2008b, Pignataro, et al. 2007). This dual functionality is also suggested by previous developmental studies (Studer, et al. 2006). In addition, a more recent study confirms the differential role of ADK isoforms, showing that the cytoplasmic, but not the nuclear isoform of ADK is implicated in sleep regulation (Palchykova, et al. 2010).

The upregulation of ADK in activated astrocytes was also observed in the temporal cortex and persisted in both hippocampus and cortex into the chronic epileptic phase in rats with a progressive form of epilepsy. ADK expression in rats with a non-progressive form of epilepsy was very similar to control expression. These observations support the implication of glial ADK expression not only in the development, but also in the progression of epilepsy. In addition, ADK expression in reactive astrocytes has been confirmed in human MTS specimens of patients undergoing surgery for pharmacologically refractory TLE. Increased ADK in human astrocytes may explain the relatively lower adenosine baseline levels detected in microdialysis samples of epileptic patients compared to control human hippocampus (During & Spencer 1992).

ADK was predominantly expressed in astrocytes, however neuronal expression was occasionally observed in both rat and human epileptic tissue. Interestingly, ADK is known to be developmentally regulated and ADK expression in neurons has been observed at early postnatal stages in both cerebral cortex and hippocampus (Studer, et al. 2006). Whether this expression may reflect a return to an earlier developmental state, as part of the process of epileptogenesis, and may contribute to a reduction of neuronal adenosine release, supporting a prolonged excitatory activity, requires further investigation. However, DCX positive neuronal progenitor cells, within the dentate gyrus of adult epileptic hippocampus did not express ADK. This observation is in agreement with the previously reported absence of ADK in neuronal progenitor cells in adult mouse hippocampus (Studer, et al. 2006).

We also show that inflammatory molecules, such as LPS and IL-1b may induce increased expression of ADK in human cultured astroglial cells. Inflammatory responses are known to be activated during epileptogenesis and IL-1b is up-regulated in epileptogenic tissue from TLE patients (Ravizza, et al. 2008b, Vezzani, et al. 2008). Thus, this cytokine could play a role in the regulation of ADK levels in astrocytes providing an additional layer of modulatory crosstalk between the astrocyte-based adenosine cycle and inflammation, that deserves further investigation.

In conclusion, the critical role of astroglial ADK in epileptic tissue is supported by these findings, providing additional evidence of ADK regulation in astrocytes during epileptogenesis, as well as during chronic epilepsy in both rat and human TLE. Our findings suggest that overexpression of ADK is a common pathological hallmark of medically intractable chronic epilepsy.
ACKNOWLEDGEMENTS

We are grateful to J.T. van Heteren for her technical help. This work has been supported by National Epilepsy Funds, NEF 09-05 (EA), NEF07-19 (JAG); EU FP7 project NeuroGlia, Grant Agreement N° 202167, and the National Institutes of Health (NIH, USA) through grant R01-NS061844.

REFERENCES

Astrogliosis in epilepsy leads to overexpression of adenosine kinase, resulting in seizure aggravation. *Brain* 128:2383-2395.


**SUPPLEMENTARY INFORMATION**

**Supplementary Table I.** Summary of clinical findings of epilepsy patients and controls.

<table>
<thead>
<tr>
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<th>HS (n = 6)</th>
<th>Non-HS (n = 5)</th>
<th>Control (n = 6)</th>
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<td>Duration of epilepsy yrs</td>
<td>11.8 (9 – 20)</td>
<td>13.6 (9 – 21)</td>
<td>NA</td>
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HS, hippocampal sclerosis; CPS, complex partial seizures; SGS, secondary generalized seizures; HS, hippocampal sclerosis; non-HS: non-sclerotic hippocampus; NA: not applicable.

**Supplementary Figure 1.** Evaluation of ADK astroglial immunoreactivity in hippocampal regions of control rat and after induction of status epilepticus (SE). Quantification bar graphs of ADK-positive cells in CA1 (A), CA3 (B) and dentate gyrus (hilar region; C) if control hippocampus and hippocampus at 24 h, 1 week and 3 months post-SE (rats with long term epilepsy, progressive, pLT). Nuclear and extranuclear staining: *p< 0.05 vs control, one-way ANOVA followed by Tukey’s test.
4.3 Overexpression of ADK in Human Astrocytic Tumors and Peritumoral Tissue Is Related to Tumor-Associated Epilepsy

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ABSTRACT

Purpose: Adenosine kinase (ADK), a largely astrocyte-based metabolic enzyme, regulates adenosine homeostasis in the brain. Overexpression of ADK decreases extracellular adenosine and consequently leads to seizures. We hypothesized that dysfunction in the metabolism of tumor astrocytes is related to changes in ADK expression and that those changes might be associated with the development of epilepsy in patients with tumors.

Methods: We compared ADK expression and cellular distribution in surgically removed tumor tissue (n = 45) and peritumoral cortex (n = 20) of patients with glial and glioneuronal tumors to normal control tissue obtained at autopsy (n = 11). In addition, we compared ADK expression in tumor patients with and without epilepsy. To investigate ADK expression, we used immunohistochemistry and Western blot analysis. ADK activity measurement was performed in surgical specimens of astrocytomas World Health Organization (WHO) grade III (n = 3), peritumoral cortex (n= 3), and nonepileptic cortex (n = 3).

Key findings: Immunohistochemistry predominantly showed cytoplasmic labeling in tumors and peritumoral tissue containing infiltrating tumor cells. ADK immunoreactivity was significantly stronger in tumor and peritumoral tissue compared to normal white matter and normal cortex, especially in astrocytoma WHO grade III, as confirmed by Western blot analysis and ADK activity measurements. Importantly, we found a significantly higher expression of ADK in the peritumoral infiltrated tissue of patients with epilepsy than in patients without epilepsy.

Significance: These results suggest a dysregulation of ADK in astrocytic brain tumors. Moreover, the upregulation of ADK observed in peritumoral infiltrated tissue of glioma patients with epilepsy supports the role of this enzyme in tumor-associated epilepsy.
**INTRODUCTION**

Patients with primary brain tumors commonly have epileptic seizures. The incidence varies between 30-100% depending on tumor pathology (van Breemen, et al. 2007). Although any brain tumor (including also metastases) can cause seizures, patients with glial tumors (particularly slow-growing, low-grade tumors) are more likely to develop epilepsy (van Breemen, Wilms & Vecht 2007). It remains unclear how various types of brain tumors induce epileptogenesis and several hypothesis have been proposed (Beaumont & Whittle 2000, Rajneesh & Binder 2009, Shamji, et al. 2009b). Besides that the mechanism of epileptogenesis is unknown, the majority of patients are refractory to anti-epileptic drugs (AEDs), on which the therapy of epilepsy largely relies. Identifying factors in the pathway that leads to epilepsy may help find preventive therapies.

A dysfunction of adenosine-mediated neuromodulation has recently been suggested to play a role in the development of epilepsy (i.e. epileptogenesis) (Boison & Stewart 2009). Under physiological conditions, adenosine exerts control over a large range of brain functions, acting as endogenous neuromodulator with mainly inhibitory effects on neuronal activity (Boison 2005, Boison 2007, Boison 2008a). Astrocyte-expressed adenosine kinase (ADK), represents the key metabolic enzyme for the regulation of extracellular adenosine levels in the brain by phosphorylating adenosine to 5’-AMP intracellularly, using ATP as a phosphate donor (Boison 2006, Etherington, et al. 2009). A dynamic regulation of ADK expression has been reported under different pathological conditions. Acute injuries (e.g. status epilepticus, ischemia) can rapidly down-regulate ADK, providing a neuroprotective environment, (Boison 2006, Boison 2008c, Pignataro, et al. 2008). In contrast, upregulation of astrocytic ADK has been observed in different experimental models of chronic epilepsy, as well as in human temporal lobe epilepsy (TLE; (Boison 2008c, de Groot, et al. in press, Fedele, et al. 2005b, Gouder, et al. 2004a, Masino, et al. in press). Overexpression of ADK leads to a decrease of extracellular adenosine levels and consequently to seizures and inhibition of ADK has been proposed as therapeutic strategy in epilepsy (Fedele, Gouder, x00Fcttinger, Gabernet, Scheurer, x00Fct, licke, Crestani & Boison 2005b, Theofilas, et al. 2011).

Tumor cells in astrocytoma share some common features with reactive astrocytes, suggesting the existence of common astrocytic programs after brain injury or disease and during brain tumorigenesis (Silver & Steindler 2009). However, little is known about the role of adenosine and ADK in brain tumors (Boison 2008b). Any dysfunction in the metabolism of astrocytes will affect the metabolism of adenosine (Boison 2010b). In brain tumors cytogenetic abnormalities tend to involve chromosomes carrying genes encoding enzymes of adenine metabolism such as ADK (Bardot, et al. 1994). A possible modification of the adenine metabolism by dysfunctional tumor astrocytes could alter the levels of adenosine in tumor...
and/or in the peritumoral tissue. Accordingly, the concentrations of adenosine have been shown to differ between the tumor and peritumoral region (Melani, et al. 2003).

In the present study, we report the expression and cellular distribution of ADK in human astrocytic tumors. ADK protein expression has not been previously studied in primary human glial tumors. In order to relate changes in ADK expression to type and progression of the tumor, both high-, as well as low-grade glial tumors have been included in our study. To explore whether ADK plays a role in tumor-associated-epilepsy, we compared the expression of ADK in tumor tissue and peritumoral cortex of patients with glial and glioneuronal tumors with the expression in control cortex and we evaluated the difference between the expression of ADK in tumor patients with epilepsy and without epilepsy.

MATERIALS AND METHODS

Subjects

We examined immunocytochemically 45 surgical specimens of brain tumor from patients with astrocytic tumors (10 WHO grade I pilocytic astrocytoma, 5 WHO grade II astrocytoma; 8 WHO grade III astrocytoma; 12 glioblastoma multiforme, GBM) and 10 patients with glioneuronal tumors (ganglioglioma, GG); Table 1. In 20 patients, a significant amount of peritumoral tissue/cortex (macroscopically normal-appearing cortex/white matter adjacent to the mass lesion, but microscopically containing infiltrating tumor cells in astrocytoma and GBM cases) was resected as well (Table 1). Normal-appearing control cortex/white matter was obtained at autopsy from 11 adult control patients without a history of seizures or other neurologic diseases. All autopsies were performed within 12 h after death. Cortical samples (cortex/white matter adjacent to the lesion with reactive changes, such as astrogliosis, but not tumor cells) of five patients with nonglial brain tumors (three meningiomas, one metastasis of carcinoma, and one lymphoma) and without refractory epilepsy were also analyzed (control cortex/surgical, Table 1). A chart review was conducted of all patients. Epilepsy was defined as the experience of one or more seizures, and data regarding seizure frequency and seizure type were obtained from patient histories. We collected additional data including age, gender, tumor location, and epilepsy duration. Patients with GG subgroup fulfilled criteria of “long-term epilepsy” [long-term epilepsy-associated tumors; LEATs; according to the definition provided by Luyken et al. (2003)] including patients with epilepsy >2 years (mean duration 11.3 years). Patient data and specimens were obtained from the databases of the departments of Neuropathology of the Academic Medical Center (University of Amsterdam; UVA) in Amsterdam and the University Medical Center in Utrecht (UMCU). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with
the Declaration of Helsinki. Two neuropathologists reviewed all cases independently, and the diagnosis was confirmed according to the revised WHO classification of tumors of the central nervous system (Louis et al., 2007).

Table 1. Clinical and histopathological features

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</table>

All data in number of patients (percentages) or as indicated; A II: Astrocytoma WHO grade II, A III: Astrocytoma WHO grade III, GBM: Glioblastoma multiforme, GG: Ganglioglioma.

Tissue preparation for immunocytochemistry

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 5 μm, mounted on organosilane-coated slides (SIGMA, St. Louis, MO) and used for immunohistochemical staining as described below.

Antibodies

Antibodies specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, 1:4,000; DAKO, Glostrup, Denmark; monoclonal mouse, 1:50; DAKO), vimentin (mouse clone V9, 1:1,000; DAKO), neuronal nuclear protein (NeuN; mouse clone MAB377, 1:2,000; Chemicon, Temecula, CA, U.S.A.), synaptophysin (mouse clone Sy38, 1:200; DAKO; rabbit anti-synaptophysin, 1:200; DAKO), CD34 (mouse clone QBEnd10, 1:600; Immunotech, Marseille, Cedex, France), Ki67 (mouse clone MIB-1, 1:200; DAKO), (HLA)-DP, DQ, DR (mouse clone CR3/43, 1:400; DAKO), MAP2 (mouse clone HM2, 1:100; Sigma), and p53 (Clone DO-7 + BP53-12, 1:2,000; Neomarkers, Fremont, CA, U.S.A.) were used in the routine immunohistochemical analysis of glial and glioneuronal tumors. For the detection of ADK, we used a polyclonal rabbit antibody [1:500; (Gouder et al., 2004; Studer et al., 2006; Ren et al., 2007; Aronica et al., 2011)].
Immunohistochemistry
For single-label immunohistochemistry, paraffin-embedded sections were deparaffinized, rehydrated, and incubated for 20 min in 0.3% H2O2 diluted in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed by incubation for 10 min at 121°C in citrate buffer (0.01 m, pH 6.0); sections were washed with phosphate-buffered saline (PBS) and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, United Kingdom). Sections were incubated with the primary antibodies overnight at 4°C. Hereafter, sections were washed in PBS and we used the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3’-diaminobenzidine (DAB; Sigma) as chromogen. Sections were counterstained with hematoxylin, dehydrated, and coverslipped. Sections incubated without the primary antibody were essentially blank.

For double-labeling studies, after incubation of ADK combined with GFAP (or NeuN, Ki67, p53, MAP2) overnight at 4°C, sections were incubated for 2 h at RT with Alexa Fluor® 568-conjugated anti-rabbit IgG and Alexa Fluor 488 anti-mouse IgG (1:100; Molecular Probes, Leiden, The Netherlands). Sections were then analyzed by means of a laser scanning confocal microscope (Leica TCS Sp2; Wetzlar, Germany) equipped with an argon-ion laser.

Evaluation of immunostaining
All labeled tissue sections were evaluated by two independent observers, with respect to the presence or absence of various histopathologic parameters and specific immunoreactivity (IR) for the different markers. Semiquantitative evaluation of IR was performed as reported previously (Vandeputte et al., 2002; Ravizza et al., 2006); for more details see Data S1. Because distinction of tumor astrocytes from reactive astrocytes according to only morphology is difficult, immunocytochemistry for NeuN, Ki67, p53, and CD34 (in GG) was performed for the evaluation and characterization of the peritumoral tissue. The peritumoral cortex of GG did not contain Ki67, P53, or CD34 positive cells. The peritumoral cortex of astrocytoma and GBM cases used in the study contained Ki67 and P53 positive cells, indicating tumor infiltration.

Western blot
For immunoblot analysis, freshly frozen human histologically normal cortex (n = 6, autopsy material; n = 1, surgical temporal cortex), astrocytomas grade II (n = 5), astrocytomas grade III (n = 5), and GBM (n = 12) samples were homogenized in lysis buffer containing 10 mm Tris (pH 8.0), 150 mm NaCl, 10% glycerol, 1% NP-40, 0.4 mg/ml Na-orthovanadate, 5 mm EDTA (pH 8.0), 5 mm NaF, and protease inhibitor cocktail (Boehringer, Mannheim, Germany). Pro-
tein content was determined using the bicinchoninic acid method (Smith et al., 1985); for more details see Data S1.

**ADK enzyme activity**

The ADK enzyme activity was evaluated in three tumor and peritumoral specimens removed from epileptic patients with grade III astrocytoma (gender: male; age: 43, 51, 54 years; location: one frontal, two temporal) and three histologic normal specimens from nonepileptic patients with meningioma (gender: male; age: 53, 39, 58 years; location: two frontal, one temporal). Because we cannot, with absolute certainty, differentiate the tumor from peritumoral cortex (normal or infiltrated) macroscopically, histologic analysis was performed in sections adjacent to the analyzed sections; hematoxylin and eosin (H&E) stain and GFAP, NeuN, Ki67, and p53 immunocytochemistry were used for the evaluation and characterization of the tissue. The tumor specimens included in the analysis contain only tumor astrocytes, and the diagnosis of grade III astrocytoma had been confirmed on additional formalin-fixed paraffin-embedded material. The peritumoral specimens of astrocytoma consist of macroscopically normal-appearing cortex/white matter adjacent to the lesion, but microscopically infiltrated, but not entirely replaced by tumor cells. The peritumoral specimens of meningioma consist of microscopically normal-appearing cortex/white matter adjacent to the lesion. The evaluation of the enzymatic activity for ADK was performed as described previously (Gouder et al., 2004); for more details see Data S1.

**Statistical analysis**

Statistical analyses were performed with SPSS for Windows (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL, U.S.A.) using a two-tailed Student’s t-test; to assess differences between more than two groups, analysis of variance (ANOVA) and a nonparametric Kruskal-Wallis test, followed by the Dunn’s post hoc test, were performed. Correlations between immunostaining and different clinical variables were assessed using the Spearman’s rank correlation test. The value of p < 0.05 was defined as statistically significant.

**RESULTS**

**Patients**

The clinical and histopathologic characteristics of the patients included are shown in Table 1. Of the 45 tumor patients, 26 patients (10 GG, 15 gliomas, and one pilocytic astrocytoma) had epilepsy and of the 20 patients (4 gangliogliomas and 16 gliomas) of whom peritumoral tissue was analyzed, 15 had epilepsy. The majority of the patients with epilepsy had secondary generalized seizures. All patients with epilepsy used antiepileptic drugs (valproic acid,
levetiracetam, phenytoin, carbamazepine, or oxcarbazepine). Of the patients with glioma, 8 patients (53%) had seizures despite maximal tolerated antiepileptic drugs. Patients with ganglioglioma all had seizures despite maximal tolerated doses of antiepileptic drugs.

**ADK immunoreactivity**

**Control tissue**

In control white matter, ADK IR was present in sparse glial cells with only a weak staining (Fig. 1A). Similar to white matter, control cortical gray matter displayed a weak astroglial staining (Fig. 1B). The IR score was similar in control cortex from autopsy and surgical samples (Table 2).

**Tumor tissue**

Thirty-five tumor specimens were studied and the mean IR score for each tumor type is summarized in Table 2. GG showed weak to moderate ADK IR within the tumor area; ADK was detected in the astroglial component of the tumor with a predominant cytoplasmic localization (Fig. 1C). Cytoplasmic and, to a lesser extent, nuclear expression was observed in pilocytic astrocytoma (grade I; Fig. 1D), astrocytomas grade II and grade III (Fig. 1E,F; Table 2), and GBM (Fig. 1H; Table 2). Double labeling confirmed ADK expression in GFAP-positive tumor cells (Fig. 1F, inset b) as well as in tumor cells that express the proliferation marker Ki-67 (Fig. 1F, inset c) and show nuclear accumulation of p53 protein (Fig. 1F, inset d) and MAP2 positivity (Fig. 1F, inset e). The percentage of p53 positive glial cells coexpressing ADK was quantified in three patients with astrocytoma grade III, showing a high percentage of p53 tumor cells with ADK positivity (95 ± 4). Cells of the microglial/macrophages lineage (HLA-DR positive cells) did not display ADK IR (not shown). Astrocytoma grade II showed ADK positive cells (Fig. 1E); however, the amount of positive cells was variable among the specimens, as reflected by the IR score (Table 2). More consistent ADK expression was observed in the astrocytoma grade III (Fig. 1F; Table 2). GBM showed a variable ADK IR; although both nuclear

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**Table 2. ADK immunoreactivity in glial and glioneuronal tumors**

<table>
<thead>
<tr>
<th></th>
<th>A II (n=5)</th>
<th>A III (n=8)</th>
<th>GBM (n=12)</th>
<th>GG (n=10)</th>
<th>Control/autopsy (n=11)</th>
<th>Pentumoral cortex (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR Cytoplasm</td>
<td>2.6 ± 0.6 *</td>
<td>4.0 ± 0.5 **</td>
<td>2.2 ± 0.4 *</td>
<td>2.8 ± 0.3 *</td>
<td>1.1 ± 0.1</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>IR Nucleus</td>
<td>0.6 ± 0.2</td>
<td>2.8 ± 0.2 *</td>
<td>0.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
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</table>

A II: Astrocytoma WHO grade II, A III: Astrocytoma WHO III, GBM: Glioblastoma multiforme, GG: Ganglioglioma. Values represent the mean ± SEM of the number of samples indicated in parenthesis. * = P< 0.05 (compared to control cortex); ** = P< 0.05: significant difference when compared to control cortex and GBM.

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Fig. 1. Expression of ADK immunoreactivity (IR) in glial and glioneuronal tumors. Representative photomicrographs of ADK IR in histologically normal white matter (WM, A) and cortical gray matter (CTX, B), ganglioglioma (GG, C), pilocytic astrocytoma (PA, D), astrocytoma grade II (A II, E), astrocytoma grade III (A III, F–J), and glioblastoma multiforme (GBM, H–J). Sections were counterstained with hematoxylin. Control white matter and cortex show only a weak ADK IR in sparse cells. ADK IR is clearly detectable in tumor cells in GG, PA, A II, and A III with both nuclear and cytoplasmic IR (arrows and insets in C, E, F). Inset b in (F): merged confocal image, showing ADK (red) expression in GFAP (green) positive tumor cells; inset c in (F): merged confocal image, showing ADK (red) expression in Ki67 (green) positive tumor cells. Inset d in (F): merged confocal image, showing ADK (red) expression in p53 (green) positive tumor cells. Inset e in (F): merged confocal image, showing ADK (red) expression in MAP2 (green) positive tumor cells. Panel (G) shows strong IR in the peritumoral region with tumor infiltration of A III. Inset in (G): merged confocal image, showing ADK (red) expression in p53 (green) positive tumor cells. Variable ADK IR is observed in GBM tumor cells (H–J). Panel (H) shows both nuclear and cytoplasmic ADK IR in GBM (high magnification in inset); however, low IR is also observed in GBM, particularly in the central area of the tumor (I). Panels (J and K) show ADK-positive cells in the peritumoral region with tumor infiltration of a GBM (arrows; inset in J and inset a in K); inset b in (K) shows ADK expression in tumor astrocytes (red) surrounding a preexisting neuron (green). Scale bar in A: 40 μm; B–H: 80 μm; I–K: 160 μm.
and cytoplasmic staining was observed in GBM, the central area of the tumor showed often low IR (Fig. 1H,I). The tumor cytoplasmic IR scores were higher in pilocytic astrocytoma, and astrocytoma grade II, III, and GG compared to control cortex (Table 2). Only in astrocytoma grade III the nuclear IR score was higher compared to that of control tissue. ADK cytoplasmic IR score was not significantly different between the different tumor subtypes.

On Western blot, homogenates from astrocytomas grade II and astrocytomas grade III cases displayed a denser band than that observed in control cortex (Fig. 2A,B). Densitometric analysis confirmed the higher expression of ADK in astrocytoma grade III compared to control cortex ($p < 0.05$) and astrocytoma grade II showed a trend in the same direction ($p = 0.0508$); ADK expression in homogenates from GBM was not significantly different from that of controls, and ADK expression was not significantly different between individual tumor groups (Fig. 2C).
Peritumoral tissue
The ADK IR score in peritumoral cortex was significantly stronger compared to control cortex (Table 2; p < 0.05). A particularly strong expression was observed in the peritumoral brain tissue, which is infiltrated by tumor cells (Fig. 1G,J,K). Double labeling confirmed ADK expression in tumor cells with nuclear accumulation of p53 protein (inset in Fig. 1G) and surrounding (NeuN positive/ADK negative) preexisting neurons (inset b in Fig. 1K). The percentage of p53-positive glial cells coexpressing ADK was quantified in the peritumoral cortex of three patients with astrocytoma grade III, showing a high percentage of p53 tumor cells with ADK positivity (91 ± 3).

ADK activity
To study the possible relationship between increased ADK IR observed in astrocytic tumors and in peritumoral infiltrated tissue with enhanced adenosine metabolism, we evaluated the enzymatic activity of ADK in homogenates derived from tumor and peritumoral specimens removed from epileptic patients with grade III astrocytoma and three histologically normal specimens from nonepileptic patients. Enzyme activity of ADK was determined by performing an enzyme-coupled bioluminescent assay. Tumor and peritumoral samples from epileptic patients with astrocytoma grade III displayed a significant enhancement of ADK activity compared to the ADK activity detected in nonepileptic cortical control samples from patients with a nonastrocytic tumor (Fig. 2D). Because fresh tumor and peritumoral tissue samples from nonepileptic patients with astrocytoma were not available, we could not establish the relationship between ADK activity and epilepsy with this method.

ADK expression and epilepsy
We compared the expression and distribution of ADK IR in tumor tissue and peritumoral tissue of glial tumors patients (astrocytoma grade III and GBM) with epilepsy and without epilepsy. Because all patients with ganglioglioma had epilepsy, and in our cohort only one patient with supratentorial pilocytic astrocytoma had epilepsy, we could not analyze these groups. No differences were observed within the tumor area between glioma patients with and without epilepsy analyzed (Table 3). In contrast we found a significant higher ADK expression in peritumoral tissue of glioma patients with epilepsy compared to the patients without epilepsy (Table 3; p < 0.05). The number of astrocytomas grade II and III with and without epilepsy was, however, too small to perform meaningful statistical comparisons in subgroups and to assess whether ADK expression is more directly dependent on the presence or absence of seizures or tumor type. Because fresh peritumoral tissue samples from these patients were not available, we could not establish this finding on Western blot or by
determination of ADK activity. Furthermore, no significant correlations were found between ADK IR and other clinical variables such as age at surgery, age at seizure onset, duration of epilepsy, and AED regimens.

DISCUSSION
We assessed the cellular distribution and expression of ADK in epilepsy-associated primary glial brain tumors. We detected changes in ADK protein expression and function in astrocytic tumors and peritumoral cortex compared to control tissue. In addition, ADK expression in the peritumoral cortex of glioma patients with epilepsy was significantly higher than in glioma patients without epilepsy.

To our knowledge this is the first study to describe the cellular distribution and expression of ADK in primary brain tumors. A previous study (Melani et al., 2003) evaluated adenosine concentration in the extracellular fluid of tumor and peritumoral tissue of patients with high-grade gliomas by intraoperative microdialysis. In this study the concentration of adenosine has been shown to be significantly reduced in the tumor tissue when compared to the control tissue, suggesting an altered purine metabolism in the tumor area (Melani et al., 2003). The extracellular adenosine levels may reflect differences in ADK expression; accordingly we observed higher ADK expression in tumors compared to control, noninfiltrated cortex. The variable expression levels observed within glial tumors may reflect differences in intratumoral vascular perfusion and hypoxia gradients, and indeed hypoxia has been shown to downregulate the expression of ADK in astroglial cells (Boison, 2008b; Pignataro et al., 2008). Interestingly, increased ADK expression and activity (compared to control cortex) was detected at the margin of the tumor and in the invasion front.

Immunocytochemical analysis showed ADK expression in tumor astrocytes with both nuclear and cytoplasmic labeling; however, expression was predominant in the cytoplasm. ADK exists in two isoforms: ADK-long and ADK-short isoforms (Cui et al., 2009). It has been demonstrated that ADK-long is mainly localized in the nucleus and has an essential role in methylation reactions, being possibly involved in epigenetic controlling mechanisms. ADK-short, on the other hand, is cytoplasmically localized and regulates the extracellular adenosine concentrations (Boison, 2007; Cui et al., 2009). Therefore, the latter one is believed to be more involved in the regulation of neuronal excitability. Accordingly, several studies demonstrated that overexpression of ADK in mice resulted in a decrease in the adenosinergic tone and subsequently increased seizure activity (Fedele et al., 2005; Pignataro et al., 2007; Li et al., 2008a,b). Theofilas et al. (2011) showed that overexpression of the cytoplasmic ADK-short isoform alone is sufficient to evoke seizures. Furthermore, both experimental and human studies indicate that dysregulation of ADK is a common mechanism, given that it is
operative in several forms of epilepsy (Aronica et al., 2011). The dysregulation of ADK in astrocytic brain tumors together with the upregulation of ADK observed in peritumoral infiltrated tissue of glioma patients with epilepsy supports the role of this enzyme in tumor-associated epilepsy. Importantly, significantly higher expression of ADK was detected in peritumoral tissue of glioma patients with epilepsy than in the peritumoral tissue of patients without epilepsy. Colocalization with tumor markers (such as p53) support the expression in tumor astrocytes; however, since ADK upregulation has been detected in reactive astrocytes (Aronica et al., 2011), we cannot exclude the contribution of a reactive glial cell population to the increase expression/activity observed within the peritumoral cortex.

The peritumoral region has been shown to be relevant for the generation and propagation of seizure activity (van Breemen et al., 2007). The epileptogenicity of the peritumoral zone is supported by both functional and immunocytochemical studies, showing network alterations and revealing cytoarchitectural and neurochemical changes in the cortex resected from patients with intractable epilepsy associated with different types of glial tumors (van Breemen et al., 2007; Shamji et al., 2009). The observed changes in ADK expression may additionally contribute to the epileptogenicity of this region, supporting a surgical approach that should aim to maximize simultaneous resection of both the tumor and (if possible) the peritumoral epileptic focus.

No significant correlation was found between ADK IR and duration of epilepsy in our cohort; however, because our study does not focus on long-term epilepsy-associated tumors (LEATs; Luyken et al., 2003) future investigations on a large cohort of LEATs are necessary to address the relationship between ADK expression and/or activity and duration and/or severity of epilepsy. Additional analysis of large series of tumors that could be stratified on the basis of the presence and absence of chronic epilepsy is also essential to further assess the value of ADK expression/activity as biomarker of epileptogenicity.

A key question is whether the increased ADK protein expression leads to an increase in enzymatic activity. Bardot et al. (1994) evaluated purine metabolic enzyme activities and found no differences in enzyme activity of ADK between low- and high-grade tumors and tissue taken far from the tumor tissue in human patients. However, in this study the low- and high-grade tumors studied included both astrocytomas and oligodendrogliomas; a histologic characterization of the control tissue was not provided. As discussed above, variable levels of ADK expression were observed within the tumor and particularly in GBM. However, higher levels of ADK activity could be detected in astrocytoma grade III and in peritumoral cortex compared to control tissue. Future studies are required to further understand whether the different expression levels observed in GBM reflect only differences in hypoxia gradients.
within the tumor, or may be associated with different glioma cell phenotypes. We acknowledge limitations to the interpretation of these results, since we analyzed the ADK activity in a small cohort of patients and we could not establish the relationship between ADK activity and epilepsy, because fresh tumor and peritumoral tissue samples from nonepileptic patients with astrocytoma were not available. Moreover, the expression patterns and regulation of adenosine receptors (A1, A2A, A2B, and A3) in both tumor and peritumoral areas deserve further investigation. In addition to epileptogenesis, ADK might also play a role in tumor growth and apoptotic cell death in astrocytoma, regulating proliferation of glial and endothelial cells, as well as the antitumor immune response through activation of receptors expressed in both astroglial and microglial cells (Abbracchio et al., 1997; Synowitz et al., 2006; Dehnhardt et al., 2007; Gessi et al., 2010, 2011). Interestingly, increased ADK expression based on quantitative real-time polymerase chain reaction (PCR) data was also found in human cancer samples outside the brain, such as in colorectal cancer (Giglioni et al., 2008). It was further demonstrated that extracellular adenosine reduced the viability of cultured astrocytoma cells (Sai et al., 2006), suggesting that overexpression of ADK might be a strategy of tumor cells to improve survival capabilities.

In conclusion, this study provides information on the cellular distribution and expression of ADK in primary brain tumors, suggesting a dysregulation of ADK in astrocytic brain tumors, as well as a potential involvement in the epileptogenicity of these tumors. Further understanding of the role of adenosine dysfunction and ADK in tumor-associated epilepsies requires the development of suitable animal models displaying both the clinical manifestations and neurochemical changes similar to those observed in human cerebral tumors. Because inhibition of ADK has proven to be an effective therapy for epilepsy in different animal models (Boison, 2010), the use of appropriate experimental models of tumor-associated epileptogenesis is essential to evaluate the possible use of adenosine augmentation therapies in patients with brain tumors and epilepsy.

Consequently, adenosine-augmenting therapeutic strategies might combine antiproliferative effects with the well-known anticonvulsivec effects of adenosine. **Acknowledgements**

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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.
REFERENCES


chim Biophys Acta 1808:1400-1412.


Summary and General Discussion
DISCUSSION

Astrocytes are the most abundant glial cells in the brain and their role in physiology and pathology is still not completely understood. The discovery of gliotransmitters and the fact that astrocytes express receptors for neurotransmitters changed the classical view according to which astrocytes were the passive cells of the brain, only supporting the main players: the neurons. Indeed there is growing evidence of bidirectional communication between neurons and astrocytes and presently the term *tripartite synapse* is commonly used to indicate that communication within the brain not only involves pre- and post-synaptic neurons, but also the surrounding astrocytic processes (Perea, Navarrete et al. 2009).

The active communication between neurons and astrocytes points also to the latter cell type as pathalogical substrate for different CNS disorders. Moreover astrocytes have the potential to secrete various types of cytokines and chemokines which result in a long term communication. Indeed astrocytes are key players in inflammatory processes in the brain and they have been associated with different CNS disorders with an inflammatory component (reviewed in chapter 1.2). Increasing data suggest that inflammatory processes can influence neuronal excitability (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010), seizure duration and intensity. Epilepsy for instance, especially in its pharmacoresistant forms, has been associated with increased expression of inflammatory markers and astrocytosis, which is a reactive process that astrocytes undergo in different pathological conditions. Moreover the fact that almost 30% of epilepsies are drug-resistant emphasizes the need to elucidate the mechanisms underlying the etiopathogenesis of this complex disease. This thesis aimed to investigate the role of astrocytes in epilepsy, focusing on molecular pathways involved in the etiopathogenesis of this disease, thus contributing to the identification of new molecular targets.

Inflammatory pathways in epilepsy

Epilepsy is a common neurological disorder characterized by recurrent seizures which have two main characteristics: hyper-excitability and synchronization of neurons. Astrocytes can influence both parameters releasing molecules that can increase the neuronal response or synchronize populations of neurons (Fellin, Pascual et al. 2006). Moreover astrocytes are an important cell type responsible for the amplification and propagation of the inflammatory stimuli in the brain. Increasing evidences support the concept of activation of innate immune response in experimental and human epilepsy (Vezzani, Balosso et al. 2010; Aronica and Crino 2011; Vezzani, Aronica et al. 2011). In chapter 2.1 of this thesis we showed that activation of plasminogen system occurs in refractory epilepsy. Upregulation of PAs and uPAR might contribute to the mechanisms underlying the epileptogenicity of focal lesions,
through direct modulation of neuronal activity or indirectly through regulation of inflammatory response and tissue remodeling. Indeed brain inflammation can contribute significantly in determining seizure threshold in susceptible brain regions, thus playing a role in seizure precipitation and their recurrence (Dube, Vezzani et al. 2005; Kulkarni and Dhir 2009; Riazi, Galic et al. 2010; Vezzani, Aronica et al. 2011; Vezzani, Maroso et al. 2011). Various in vitro and in vivo findings suggest that specific sets of inflammatory molecules and their cognate receptors, can contribute to the epilepsy associated molecular changes and synaptic plasticity. The IL-1/TLR signaling for example has been shown to influence seizure precipitation in different experimental paradigms (Viviani, Bartsaghi et al. 2003; Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010). In chapter 2.2 we investigated the inflammatory pathways activated in FCD I and II confirming the occurrence of complex inflammatory changes involving both innate and adaptive immune response. In particular in FCD II we found a greater activation of inflammation, as suggested by the presence of microglia, T lymphocytes and dendritic cells. These findings suggest that the activation of inflammation is not simply an effect of seizure activity (FCD I and II had a comparable history of seizure), but an active mechanism that involves different molecules and pathways. In chapter 2.3 we showed that HMGB1-TLR4 pathway is upregulated in human epileptic developmental lesions in particular in astrocytes confirming previous data in experimental models and human TLE (Maroso, Balosso et al. 2010). We also showed that there is translocation of HMGB1 from

![Fig. 1. Ups and downs in epilepsy. Summary of our observations in brains from epileptic patients: increased inflammation-related molecules are shown in green boxes, decreased expression in red, and translocation is depicted in yellow.](image)
the nucleus to the cytoplasm in astrocytes of patients with MCD, and that IL-1β can induce HMGB1 release in cultured astrocytes suggesting that astrocytes may play a key role in the amplification of the inflammatory response. HMGB1 represents a crucial regulator of chromatin conformation and inflammatory gene transcription. Upon its nuclear-to-cytoplasmatic translocation, which is induced by injury or specific biological stressors, and its subsequent release HMGB1 acquires the properties of a proinflammatory cytokine mediated by both TLRs and RAGE. Activation of RAGE by HMGB1 also induces differentiation of immature neurons and has a role in axonal and dendritic elongation (Rauvala and Rouhiainen 2010), an effect also shared by other endogenous ligands of the S100/calgranulin family (Ding and Keller 2005). S100b is a member of a multigenic Ca2+ regulated S100 proteins, and is abundant in CNS where is mainly expressed by astrocytes. It can be released by astrocytes and affects neurons in a paracrine manner. Although both HMGB1- and S100b-mediated activation of TLRs or RAGE subserves physiological and homeostatic functions, excessive release of these molecules and the concomitant overactivation of their receptors can result in neuropathology. These molecules have been implicated in disease progression in several CNS pathological conditions (Schmidt, Yan et al. 2000; Stern, Yan et al. 2002; Huttunen and Rauvala 2004; Rauvala and Rouhiainen 2010). For example, HMGB1 release from mature neurons is involved in the development of ischemic injury and blood-brain barrier (BBB) disruption induced by stroke, and both RAGE and TLRs may mediate these deleterious effects by activation of NF-kb and ERK-signaling, leading to the synthesis of key proinflammatory molecules (Qiu, Nishimura et al. 2008).

Previous studies have demonstrated a long-term increase in brain excitability in mice overexpressing cytokines in astroglia (Campbell, Abraham et al. 1993; Akassoglou, Probert et al. 1997; Stalder, Carson et al. 1998), or in rodent brain after the induction of an inflammatory challenge, particularly if this event occurs during the early post-natal life (Riazi et al., 2010). With this background we could envision that inflammation influences seizure susceptibility in two different ways, with an immediate effect, that influences directly neuronal excitability or a long term mechanisms resulting in long lasting modifications of the brain through the astrocytic network.

In chapter 2.4 we described an acute effect of two proinflammatory molecules in an ex vivo model of focal epilepsy: HMGB1 and IL-1β could increase the focal ictal discharge (fID) in acute brain slices from mice. These two molecules were already shown to be proconvulsant in a mouse epilepsy model (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010) enhancing the total time spent in seizure activity. In our study, HMGB1 and IL-1β were found to be proictogenic, increasing the excitability of the neurons in response to NMDA stimulation. The increased excitability of the network corresponded to an increase in the calcium
signaling in particular in astrocytes, which have been previously shown to have a pivotal role in the generation of fID in enthorinal cortex slices (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). The proictogenic effect of HMGB1 and IL-1β was prevented by application of tetrodotoxin (TTX, that blocks the synaptic transmission by blocking voltage-gated Na⁺ channels) suggesting that there is an active communication between neurons and astrocytes which can be targeted to reduce excitability.

Astrocytes are also important in K⁺ homeostasis, in particular they express Kir 4.1, an inward rectifier potassium channel which is the main responsible for the control of potassium influx and resting potential (Kucheryavykh, Kucheryavykh et al. 2007; Inyushin, Kucheryavykh et al. 2010). To further investigate the role of inflammation in epilepsy, we analyzed the expression of IL-1β in a rat model for TLE (chapter 2.5) and in different tumors associated with epilepsy in relation to Kir4.1. In the present study, we observed that IL-1β levels peaked in the temporal cortex at 1 day after SE, which corresponds to the time point of prominent reduction of Kir4.1 expression. These observations suggest a role for IL-1β in the regulation of Kir4.1 mRNA expression, which was further investigated in vitro, using glial cells in culture. Further experiments on cultured astrocytes showed that application of IL-1β could reduce the expression levels of Kir4.1 mRNA and protein in human astrocytes. Previous studies showed that ablation of Kir4.1 protein results in altered astrocytes function, changed resting potential and impaired K⁺ buffering (Inyushin, Kucheryavykh et al. 2010) and that Kir 4.1 KO leads to a lethal phenotype characterized by seizures recurrence (Djukic, Casper et al. 2007). The reduction of Kir 4.1 after IL-1β could be an additional mechanism by which the inflammation contributes to hyperexcitability of the neurons in an indirect way: lack of potassium buffering could increase the concentration of K⁺ in the extracellular space, setting the resting potential of neurons to more depolarized values, closer to threshold of action potential.

**Regulation of inflammation in astrocytes: the role of miR-146a**

Micro RNAs are small (≈20 nucleotides) regulatory molecules which act as post-translational suppressors of protein expression. Since their discovery in 1993 (Lee, Feinbaum et al. 1993), they have been the focus of attention of the scientific community for their ability to specifically suppress protein synthesis, by preventing the mRNA translation. Several miRNAs have been found in the human brain, and they are found to play a crucial role in a wide range of biological processes, including the regulation of the innate and adaptive immune response (Pedersen and David 2008; Sonkoly, Stahle et al. 2008; Pauley, Cha et al. 2009). In this thesis we showed that miR-146a is upregulated in experimental models of epilepsy, as well as in human TLE (chapter 3.1). In particular, in a rat model of TLE strong upregulation was detected in astrocytes 1 week after status epilepticus (during epileptogenesis) which corre-
responds to the latent period and the time of maximal astroglial activation and upregulation of various genes involved in the immune response (Gorter, Van Vliet et al. 2006; Aronica and Gorter 2007). These observations are in line with other studies supporting the association between this specific miRNA and human inflammatory diseases (Quinn and O’Neill 2011; Rusca and Monticelli 2011). Cell specific upregulation suggests a key role of miRNA-146a in governing astrocyte activation and function. We further demonstrated that the pro-inflammatory cytokine IL-1β can increase the expression levels of mir146a on human cultured astrocytes (chapter 3.2) and we analyzed the two targets of mir146a: IRAK1 and TRAF6, in the pathway that leads to NF-kB activation. These results suggest that inflammation is a key component of epilepsy associated disease, involving astrocytes which in turn activate miR-146a pathway to reduce the inflammatory signaling. In the future this pathway could be targeted to reduce inflammation, for instance by overexpressing miR-146a: our results in vitro demonstrate that increased miR146a leads to downregulation of IRAK1 and TRAF6. The increase in miR-146a that occurs after IL-1β stimulation could be a regulatory mechanism to dampen inflammation to limit tissue damage. Moreover miR-146a serves as a marker for inflammation and could be targeted to reduce the inflammatory response. To further investigate the effect of this microRNA more studies are needed, especially regarding in vivo overexpression of miR-146a in relation to epilepsy and inflammation. Recent data support the possibility of using miRs for in vivo therapy, with the result of silencing protein expression. AntagomiR-134 for example, was shown to suppress seizures in a mouse model of epilepsy when injected after KA administration and subsequent status epilepticus (Jimenez-Mateos, Engel et al. 2012).

However one of the main problems of application of miRNA is the so called off-target effect, which derives from the fact that each microRNA can have more that one target, in some cases hundreds of them. For instance miR-146a can also target complement factor H (CFH), which is an anti-inflammatory component of the complement system, resulting in increase of inflammation. For these reason artificial miRs, small molecules specifically designed to target only one sequence, have a better perspective of use in the therapy.

Astrocytes-mediated signaling and epilepsy
Astrocytes can send information to neighboring cells via release of gliotransmitters. One of the first to be recognized was glutamate, identified in 1994 (Parpura, Basarsky et al. 1994) and found to regulate synaptic transmission in cultured hippocampal cells. Nowadays we know that astrocytes may release different neuroactive molecules such as glutamate, D-serine, ATP, adenosine, GABA, TNFα, prostaglandins (Perea, Navarrete et al. 2009). Adenosine, for instance is an important modulator of synaptic strength and it is recognized as endog-
Endogenous anticonvulsant and neuroprotectant (Ribeiro 2005; Stone, Ceruti et al. 2009; Boison 2010) mainly acting on A₁ receptors causing presynaptic inhibition (Fredholm, Chen et al. 2005). The levels of extracellular adenosine are dependent on the expression levels of the enzyme adenosine kinase (ADK) (for rev. see (Boison 2012)) which, in the adult brain is predominantly expressed in astrocytes (Studer, Fedele et al. 2006) and reduces the adenosine in the extrasynaptic cleft. Experimental up-regulation of ADK within astrocytes was shown to be sufficient to trigger spontaneous recurrent seizures in the absence of any other epileptogenic event, whereas ADK downregulation almost completely abolished spontaneous recurrent seizures (Theofilas, Brar et al. 2011). In the last part of the thesis we showed increased expression of the enzyme ADK in an experimental model as well as in patients with TLE (chapter 4.1) and also in human astrocytic tumors associated with epilepsy (chapter 4.2). These findings suggest that overexpression of ADK is a common pathological hallmark of medically intractable chronic epilepsy and could be an important target for treatment. More studies are needed, in particular to assess the functionality of the enzyme ADK in relation to the excitability of the neurons.

Another signaling system in which astrocytes play an important role is constituted by the endocannabinoids (ECBs) and their receptors. ECBs were classically considered inhibitory as they bind CB1 receptors in neurons inhibiting neurotransmitter release from presynaptic terminals (Chevaleyre, Takahashi et al. 2006; Hashimotodani, Ohno-Shosaku et al. 2007). The discovery that astrocytes express functional CB1R suggested a role for this cell type in ECB signaling. Indeed astrocytes were shown to modulate neuronal excitability upon activation of CB1R (Navarrete and Araque 2008) responding to ECBs with Ca²⁺ elevations and subsequent release of glutamate that activates neurons more distant from the site of ECBs release (Navarrete and Araque 2010). The model that they propose is that ECBs can have either inhibitory effect acting directly on presynaptic neurons or an excitatory effect through activation of astrocytes that in turn activate neurons. In chapter 4.3 of this thesis we analyzed the expression pattern and distribution of CBRs in developing human cerebral cortex and showed an increase of their expression levels in focal malformation of cortical development (MCD) in particular in astrocytes that could contribute to the epileptogenicity of those lesions. In fact dysregulation of the ECB system, with abnormal expression of CB1, has been reported in both human and experimental temporal lobe epilepsy (TLE; (Falenski, Blair et al. 2007; Ludanyi, Eross et al. 2008)). Moreover in vitro studies show that the ECB system is necessary to stimulate astrocytes Ca²⁺ rises in order to maintain the epileptiform activity in hippocampal cultured slices (Coiret, Ster et al. 2012). However, the complexity of the ECB system, the differential expression of their receptors, and the fact that ECB-mediated activation of astrocytes has an excitatory rather that inhibitory effect on neurons, emphasize the
need for further studies to clarify pathways that could be a target for development of new therapeutic strategies (Hofmann and Frazier 2011).

**Clinical implications and future directions**

During the past decade, detailed molecular characterization of astrocytes, in particular reactive astrocytes, demonstrates that these cells are active players in the development and progression of the immune/inflammatory response that takes place in epileptic brain tissue. Both human and experimental data suggest the activation of specific proinflammatory pathways in astrocytes, with consequent release of inflammatory molecules that can alter neuronal network excitability. The identification of “harmful” pro-inflammatory pathways contributing to seizure onset and recurrence highlights the possibility of developing a therapeutic strategy targeting the astrocyte-mediated inflammatory signaling. However, we need to wait for the outcome of clinical studies before we can consider whether this approach is the right strategy. If this is so, it may not only improve control of seizures, but may also act as disease-modifying therapy in patients with epilepsy resistant to conventional antiepileptic drugs.

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**Fig. 2. Summary of the results of this thesis.** The color legend indicates the different processes in which inflammation and astrocytes can play a role in epilepsy.
REFERENCES


Summary

Neurons are not the only cell type in the central nervous system (CNS). In fact they are outnumbered by different non neuronal cells, collectively called glia. For a long time, it was believed that glial cells only functioned as structural and trophic support for neurons, rather than actually contributing to network activity. However, recently they were found to have numerous important functions that allow them to be closely involved in neuronal signaling. Moreover astrocytes represent an important source of immunologically relevant cytokines and chemokines, and reactive astrogliosis (a process of activation of astrocytes) is a pathological hallmark of various types of medically refractory focal epilepsy. The activation of inflammatory pathways and the consequent release of inflammatory molecules by astrocytes can alter neural network excitability via induction of various mechanisms, with either direct or indirect impact on neuronal functions.

In this thesis we investigate the contribution of astrocytes to epilepsy in order to gain more insight into the pathways involved in the etiopathogenesis of this disease. The thesis is divided in the following chapters: Introduction, Inflammatory pathways and epilepsy, Regulation of inflammation in astrocytes: the role of miR 146a, Astrocytes mediated signaling and epilepsy, General Discussion. Chapter 1.1 is a general introduction on astrocytes physiology and their dysfunctions in epilepsy and in chapter 1.2 we review the current evidence regarding the role of astrocytes in the regulation of the innate immune responses in epilepsy. In chapter 2 we investigate some of the inflammatory pathways that could contribute to epilepsy. In particular in chapter 2.1 we show that tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and uPA receptor are upregulated in human hippocampal sclerosis (HS) specimens and in a model of rat temporal lobe epilepsy (TLE). In chapter 2.2 we demonstrate that focal cortical dysplasia (FCD) I and II, even though they have a comparable history of seizures, are characterized by different activation of inflammatory processes, suggesting a specific role of inflammation on seizure generation, rather than it being a consequence of the seizure activity. In chapter 2.3 we show that the inflammatory pathway HMGB1-TLRs/RAGE (already shown to be involved in epilepsy) is upregulated in tissue from patients affected by different types of MCD associated with epilepsy. In chapter 2.4 we observe a pro-epileptic effect of the proinflammatory molecules HMGB1 and IL-1β in a model of focal epilepsy in acute brain slices from rats. Further we showed that chronic exposure to IL-1β can induce a decrease of the expression of Kir 4.1 in cultured astrocytes (chapter 2.5).

In chapter 3 we study a mechanism of regulation of inflammation occurring in astrocytes in epileptic patients and in rat TLE. In chapter 3.1 we show a strong upregulation of mir146a in the astrocytes of both human and rat TLE. We then moved to an in vitro system where we can modulate the expression of mir146a in cultured astrocytes showing that its upregula-
tion can decrease the IL-1β signaling and the inflammatory markers IL-6 and COX2 (chapter 3.2). In chapter 4 we show different pathways that are involved in the astrocytes-mediated signaling in relation to epilepsy. In particular in chapter 4.1 we study the distribution pattern of cannabinoids receptors (CBRs) during the normal brain development and we show a strong upregulation of both CBRs in MCD associated with epilepsy, in different cellular types. We further investigate the adenosine kinase (ADK) levels in epilepsy showing a prominent upregulation in tissue from epileptic patients and from experimental rat model of TLE (chapter 4.2). Moreover we demonstrate that astrocytes from epilepsy- associated tumors have an increased expression of ADK, in comparison to tumors without epilepsy (chapter 4.3). Finally, in chapter 5 we discuss our findings from patient studies and experimental models proposing that the activation of specific proinflammatory pathways in astrocytes could contribute to neuronal activity and synchronization thus promoting epileptical activity.
SAMENVATTING

Lang werd aangenomen dat astrocyten alleen dienden voor structurele ondersteuning en voeding van cerebrale neuronen en niet bijdroegen aan de neurale netwerk activiteit. Dit concept is de laatste decades drastisch veranderd. Astrocyten bleken talrijke belangrijke functies te hebben die nauw met de neuronale overdracht te maken hebben. Daarnaast zijn astrocyten een belangrijke bron van immunologisch relevante cytokinen en chemokinen. Ook reactive gliose, een activatie proces van astrocyten, is een pathologisch kenmerk bij verschillende vormen van refractaire focale epilepsie. De activatie van inflammatoire signaleringsroutes en de daaruit volgende afgifte van ontstekingsmoleculen door astrocyten kan de gevoeligheid van neurale netwerk activiteit beïnvloeden. Dit via de inductie van verschillende mechanismen met een directe of indirecte invloed op de functie van neuronen. In dit proefschrift onderzoeken wij de bijdrage van astrocyten aan epilepsie. Dit om meer begrip te krijgen van de verschillende eiwitsignaleringsroutes betrokken bij de etiopathogenese van deze ziekte. Het eerste hoofdstuk is verdeeld in twee delen. 1.1 betreft een algemene introductie van de fysiologie van astrocyten en de afwijkingen optredend bij epilepsie. In 1.2 geven we een overzicht over de rol van de astrocyten in de zogenaamde aangeboren immuunreactie bij epilepsie. In hoofdstuk 2.0 worden de inflammatoire eiwitsignaalroutes beschreven die een negatieve invloed zouden kunnen hebben op epilepsie. In 2.1 wordt aangetoond dat de weefsel plasminogeenactivator (tPA), de urokinase plasminogeenactivator (uPA) en de uPA receptor geactiveerd zijn in hippocampale sclerose (HS) monsters van patiënten met temporale lob-epilepsie (TLE) en bij een experimenteel model van TLE bij de rat. In 2.2 wordt aangetoond dat Focale Corticale Dysplasie (FCD) I en II, hoewel zij een klinisch vergelijkbare vorm van epilepsie geven, gekarakteriseerd worden door verschillende vormen van activatie van het ontstekingsproces. Dit toont aan dat er een specifieke rol is weggelegd voor het ontstekingsproces bij de opwekking van epilepsie en dat het geen consequentie is van de epileptische activiteit op zich. In 2.3 tonen we dat de eiwitsignaleringstroutes, HMGB1-TLR/RAGE, betrokken bij epilepsie, geactiveerd is in weefsel van patiënten met verschillende vormen van epilepsie geassocieerde malformatie van de corticale ontwikkeling (MCD). In 2.4 beschrijven wij een pro-epileptisch effect van de ontstekingsmoleculen HMGB1 en IL-1β in een epilepsiemodel vervaardigd van verse plakken ratten hersenweefsel. Daarnaast tonen wij aan, 2.5, dat chronische blootstelling aan IL-1β een expressie verminder ing geeft van Kir 4.1 in gekweekte astrocyten. In hoofdstuk 3 bestuderen we een ontstekingsreguleringsmechanisme, optredend in astrocyten bij epilepsiepatiënten en in TLE bij de rat. In 3.1 tonen we aan dat er een sterke activatie plaatsvindt van miR146 in de astrocyten van zowel weefsel van epilepsiepatiënten als in TLE bij de rat. Daarna verlegden wij onze aandacht naar een in vitro systeem van in kweek
gebrachte astrocyten waarin de expressie van miR146 gewijzigd kan worden. Hiermee konden wij aantonen dat opregulatie van miR146 leidt tot een verminderde IL-1β signalering en van een vermindering van de ontstekingsziekten IL6 en COX2 (3.2). In hoofdstuk 4 tonen we aan dat verschillende eiwitsignaleringroutes zijn betrokken in de astrocyten gemedierte signalering in relatie tot epilepsie. Met name in 4.1 wordt het distributiepatroon van cannabinoïde receptoren (CBR) tijdens de normale cerebrale ontwikkeling bestudeerd. Daarnaast tonen we een sterke opregulatie aan van beide CBR receptoren in verschillende cellulaire typen bij MCD geassocieerd met epilepsie.

In 4.2 werd aangetoond dat de adenosine kinase (ADK) niveaus een sterke opregulatie tonen in weefsel van patiënten met epilepsie alsmede in weefsel van het experimenteel TLE rattenmodel. Daarnaast tonen we in 4.3 aan dat astrocyten in met epilepsie geassocieerde cerebrale tumoren een toenemen van expressie tonen van ADK t.o.v. cerebrale tumoren zonder epilepsie.

In het laatste deel, hoofdstuk 5, bediscussiëren wij onze bevindingen in patiëntgebonden studies en in experimentele modellen waarbij de stelling geponeerd wordt dat specifieke eiwitsignaleringroutes in astrocyten, betrokken bij ontsteking, bijdragen aan neuronale activiteit en synchronisatie in het neurale netwerk en zodoende een bijdrage leveren aan de epileptische activiteit.
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CURRICULUM VITAE

Emanuele Zurolo was born in Castellammare di Stabia, Italy on the 8th of March 1984. After finishing his High School in 2002, he started his study in Biological Sciences at the University of Naples "Federico II" where he did his first research internship at the Department of Molecular Biology with Prof. F. Aniello. After obtaining his Bachelor degree in 2006 he started a Master in Neurobiology and obtained his Master degree cum laude in 2008 at the University of Rome where he performed a research traineeship at the Department of Human Physiology and Pharmacology (Prof. Dr. F. Eusebi) where he became interested in the physiology of glial cells. Then he moved to Amsterdam to start his PhD at the Department of (Neuro) Pathology in the Academic Medical Center in Amsterdam (University of Amsterdam) under supervision of Prof. Dr. D. Troost and Prof. Dr. E. Aronica. During this period he was also visiting scientist (in 2010 and 2011) at Institute of Neuroscience of the CNR of Padova, where he worked under the supervision of Dr. G. Carmignoto. The results of his research project on the contribution of astroglia to epilepsy are described in this thesis.