Epigenetic and pharmacological targeting of neuroinflammation as novel therapeutic interventions for epilepsy
Iori, V.

Citation for published version (APA):
Iori, V. (2018). Epigenetic and pharmacological targeting of neuroinflammation as novel therapeutic interventions for epilepsy

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 1

General introduction and outline of the thesis
Chapter 1 — Introduction

GENERAL INTRODUCTION

EPILEPSY

Epilepsy is one of the most common chronic neurological diseases, with approximately 65 million people affected worldwide. People with epilepsy suffer from discrimination, social stigma and the stress of living with a chronic unpredictable disease that can lead to loss of autonomy for activities of daily living. The prevalence of active epilepsy is 5-8 per 1000 population in high-income countries and 10 per 1000 population in low-income countries (Moshe et al., 2015).

The International League against Epilepsy (ILAE) has defined epilepsy “a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiologic, cognitive, psychological, and social consequences of this condition” (Fisher et al., 2005, 2014). Since the term “disorder” is poorly understood by the public and minimizes the serious nature of epilepsy, the ILAE has recently agreed that epilepsy is best considered to be a disease (Fisher et al., 2014). Seizures are the hallmarks of epilepsy and have been defined “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” (Fisher et al., 2005). Seizures result from hyperexcitability of neurons that exceeds a physiological threshold and by hypersynchrony of neuronal networks, i.e., a population of neurons firing at the same time at a similar rate (Stafstrom, 2006). The clinical manifestations of seizures depend on which brain area(s) is involved. In 2017 ILAE revised the terminology and classification of seizures types, dividing them into two major classes: focal onset seizures, originating within networks limited to one hemisphere, and generalized onset seizures, arising within, and rapidly engaging, bilaterally distributed networks. Focal seizures can be subdivided into those with retained or impaired awareness and, in addition, as motor, non-motor and focal to bilateral tonic-clonic seizures. Generalized seizures are categorized as motor and non-motor (absence) seizures (Fisher, 2017).

Epilepsies are often classified according to their causes as: (1) genetic when genetic factors have a major role in the causation of the disease, and the causative or susceptibility genes are inherited or results from de novo mutations; (2) structural or metabolic, where acquired epilepsy is the result of changes in neuronal network excitability that follow an initial precipitating insult to the brain (i.e., stroke, trauma, brain tumor, CNS infections, cortical malformations, febrile seizures) or
originates from metabolic abnormalities (i.e. mitochondrial disorders, peroxisomal disorders, pyridoxine dependent epilepsy); and (3) unknown or idiopathic, where no cause has been identified as yet (Moshe et al., 2015; Scheffer et al., 2017).

Anti-epileptic drugs (AEDs), now best defined as anti-seizure drugs (ASDs), mainly target neuronal ion channels or classical neurotransmitter systems; they are the mainstay treatment for epileptic seizures but about one-third of people with epilepsy show intractable seizures (Kwan and Brodie, 2010; Schmidt and Löscher, 2009). More than 50% of patients respond to the first ASD and of the remaining, only 20% respond to alternative or additional anti-convulsants. In addition, some patients develop resistance during the course of the disease (Matin et al., 2015). In selected patients surgical excision of the focus epilepticus may be curative (Cascino, 2004). Whereas ASDs may provide symptomatic relief, they do not modulate the underlying disease mechanisms. Next generation therapies need to target the mechanisms involved in making the brain susceptible to generate spontaneous seizures, namely “epileptogenesis”. The processes underlying epileptogenesis includes molecular and cellular changes that may alter brain excitability, and this process does not stop when the first seizures occur but also contributes to the progression of the disease (Pitkanen and Engel, 2014).

**Temporal lobe epilepsy**

Temporal lobe epilepsy (TLE) represents a common form of focal epilepsy and it is characterized by recurrent seizures primarily involving temporal pole structures and limbic system (Figure 1).

Two major types of TLE are recognized: mesial TLE (MTLE), where the onset of seizures is from the hippocampus, amygdala or other medial structures in the temporal lobe, and lateral TLE, where seizures arises from the temporal neocortex. The potential causes of TLE include mesial temporal sclerosis, febrile status epilepticus (SE), traumatic brain injury, brain infections, such as encephalitis and meningitis, hypoxic brain injury, stroke, cerebral tumors, and familiar syndromes.

**Figure 1. Mesial temporal lobe** is the inner part of temporal lobe. Deep inside is the region of the brain known as limbic system, which includes hippocampus, amygdala, cingulate gyrus, thalamus and hypothalamus, many of which are of particular relevance to the processing of memory (https://themedicalmusingsofdisha.files.wordpress.com)
EXPERIMENTAL MODELS OF SEIZURES

There is a wide range of different experimental models of seizures and epilepsy, which selection is based on the particular questions being asked. Animal models can be divided in acute seizure models and chronic epilepsy models (Pitkänen et al., 2017).

Acute seizure models are characterized by induced self-remitting seizures with no evidence of persisting changes in seizure threshold or onset of spontaneous seizures. Induction protocols are based on single or repetitive exposure to seizure-provoking interventions, including chemoconvulsants (pentylentetrazol (PTZ), low dose of kainic acid (KA) or bicuculline), electrical stimulation (Maximal Electroshock Test), and short hyperthermia. For example, seizure activity following KA or bicuculline- which are molecules increasing glutamate-mediated or decreasing gamma-aminobutyric acid (GABA)-mediated neurotransmission, respectively- is characterized by discrete ictal episodes intermixed with spiking activity, recurring for about 2 hours, after which time animals display a normal EEG pattern similar to pre-ictal baseline. Ictal activity in these models is reproducible and can be quantified by measuring the onset time to the first seizure, the number and duration of ictal events and the interictal activity (Maroso et al., 2010) (Figure 2).

These models are used to investigate the molecular mechanisms underlying ictogenesis and for anti-seizure drug screening but acute seizures do not necessarily respond to therapies that affect epileptogenesis, and might not predict adverse or toxic effects of drugs in human (Simonato et al., 2014).
Chronic epilepsy models are models of epileptogenesis characterized by spontaneous recurrent seizures monitored in long-term video-EEG studies. These seizures can be either induced by brain injuries or result from gene mutations. Table 1 reports rodent models of acquired epilepsies induced by brain injuries during post-natal life and genetic models characterized by seizures arising as a consequence of spontaneous or induced gene modifications.

Table 1. Experimental models of epileptogenesis

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Rodent model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural</strong></td>
<td></td>
</tr>
<tr>
<td>De novo status epilepticus</td>
<td>Chemoconvulsants, electrical stimulation, hyperthermia</td>
</tr>
<tr>
<td>Neurotrauma</td>
<td>Fluid percussion, controlled cortical impact, cortical undercut</td>
</tr>
<tr>
<td>Unilateral hippocampal sclerosis</td>
<td>Intra hippocampal/cortical KA, perforant path stimulation</td>
</tr>
<tr>
<td>Stroke</td>
<td>Cortical phototrombosis, permanent middle cerebral artery occlusion</td>
</tr>
<tr>
<td>Blood-brain barrier damage</td>
<td>Sub-chronic albumin or TGF-β intracerebroventricular infusion</td>
</tr>
<tr>
<td>Developmental epileptic encephalopathies</td>
<td>Hypoxia-ischemia in pups, infantile spasms: multiple hit rat model</td>
</tr>
<tr>
<td>Cortical dysplasia</td>
<td>In utero rat irradiation or alkylant agents</td>
</tr>
<tr>
<td>Tuberous sclerosis complex</td>
<td>Cell specific-conditional Tsc1/2 knock-out mice</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Neocortical transplantation of human glioma cells in scid mice or glioma cell lines in rats</td>
</tr>
<tr>
<td><strong>Genetic</strong></td>
<td></td>
</tr>
<tr>
<td>Spontaneous mutations</td>
<td>Models of absence epilepsy in mice and rats (GAERS, WAG/Rij)</td>
</tr>
<tr>
<td>Induced monogenic mutations</td>
<td>Mouse knock-out or knock-in mutation of voltage-gated ion channels subunits (Na⁺, K⁺, Ca²⁺), neurotransmitter receptor subunits (GABAA, nicotinic) and transporters, accessory synaptic proteins; cystatin B knock-out</td>
</tr>
<tr>
<td>Developmental epileptic encephalopathies</td>
<td>Dravet syndrome: mutation in sodium channels Scn1A, Scn1B Infantile spasms</td>
</tr>
<tr>
<td>Models of brain malformations (cortical dysplasia, megalencephaly)</td>
<td>Pten knock-out mice</td>
</tr>
</tbody>
</table>

10
Among the various epileptogenic injuries, SE is the most widely used to induce epilepsy in rodents, and it is responsible for about 10% of all acquired human epilepsies (Loscher, 2002). SE consists of a prolonged seizure or seizures occurring so frequently that they do not allow for full recovery of normal brain function (Trinka et al., 2015). SE can be induced in rodents by sustained electrical stimulation of the hippocampus or hippocampal afferent pathways (such as the perforant pathway) or the amygdala or by intracerebral or systemic administration of chemoconvulsants (usually pilocarpine, a muscarinic agonist, or single or repetitive doses of KA). SE is followed by a latent period that lasts few days to weeks -depending on the severity of the initial insult, the brain area of seizure initiation, age, sex and animal's strain- after which spontaneous recurrent seizures develop and may progress over time. A pattern of hippocampal sclerosis similar to that found in MTLE develops in some of these models, which is characterized by hallmarks such as neuronal cell loss, reactive gliosis and synaptic reorganization. These models better represent the human disorder than the acute seizure models and are therefore more suitable for studying potential targets for new therapies.

MECHANISMS OF EPILEPTOGENESIS

The process of epileptogenesis is characterized by a network of widespread aberrant and compensatory changes rather than on a specific cellular or molecular abnormality. These changes have been observed in seizure-prone brain areas in animal models as well as in brain tissue surgically resected from drug-resistant epilepsy patients and they impact on relevant functional outcomes such as the onset of spontaneous seizure, seizure severity and progression, histopathological changes and neurological comorbidities. Epileptogenic mechanisms induced by diverse precipitating events are complex and heterogeneous. Each mechanism has a specific post-injury onset and offset time before the occurrence of spontaneous seizures and may persist also during disease progression.

The epileptogenic process is characterized by cellular and structural changes, regulation of gene expression through transcriptomic and epigenetic modifications and activation of specific molecular signaling pathways (Figure 3).
Figure 3. Cellular and molecular alterations during the epileptogenic process. Cellular alterations induced by various types of brain insults include neurodegeneration, neurogenesis, gliosis, invasion of inflammatory cells, axonal sprouting, dendritic plasticity, BBB disruption, changes in extracellular matrix, and alteration in voltage- and ligand-gated ion channels in individual neurons. These alterations are accompanied by a variety of molecular changes, regulation of gene expression and epigenetics modifications. As a consequence, several functional impairments in addition to epilepsy can develop, including developmental delay, cognitive and sensory-motor deficits and drug refractoriness (from Pitkanen and Lukasiuk, 2009).

**Neurodegeneration** is observed in hippocampus, amygdala and parahippocampal cortices, as well as in many extra-temporal areas, including thalamus and cerebellum (Jutila et al., 2002). Neuronal cell loss occurs in mTLE patients and is reflected in atrophy of limbic brain areas (Thom et al., 2009). Cell loss does not appear to be associated with the development of spontaneous seizures although it may play a role in cognitive dysfunctions. Although neuroprotective strategies that start before or shortly after the initial insult may ameliorate spontaneous seizures (Loscher and Brandt, 2010; Pitkänen, 2002), this is clear-cut evidence in animal models that the rescue of cell loss alone is not sufficient to prevent epilepsy (for review see Arzimanoglou et al., 2002).

**Sprouting of glutamatergic granule cell axons** (mossy fibers) into the molecular layer of the dentate gyrus potentially contributes to hippocampal hyperexcitability in TLE (Buckmaster, 2012). Mossy fibers sprouting is thought to establish a positive excitatory feed-back loop with somata and dendrites of granule cells (Buckmaster, 2012). An opposing view proposes that aberrant fibers
predominantly innervate inhibitory neurons, thereby reducing neuronal circuit excitability (Buckmaster, 2012, 2014; Sloviter et al., 2006). Recent evidence links mossy fibers sprouting to granule cell neurogenesis. In epileptic rodent brain, seizure activity can disturb the migration of newly born neurons, resulting in their ectopic location in the hilus, thus resulting in aberrant connectivity and enhanced excitability (Scharfman et al., 2000). Altered neurogenesis has been linked to learning and memory impairment and depression (Scharfman et al., 2000). Development of epilepsy and cognitive deficits are both attenuated during treatments that reduce aberrant neurogenesis (Bielefeld et al., 2014; Hester and Danzer, 2013).

Reactive gliosis has been reported in human and experimental epileptogenic tissue and includes the increase in astrocytes size and number and the activation of microglial cell (Aronica et al., 2012; Devinsky et al., 2013). Activated astrocytes display an altered phenotype including the downregulation of gap-junction connexins, glutamate transporter and metabolism, Kir4.1 potassium and aquaporins channels (Devinsky et al., 2013) leading to altered ionic and extracellular glutamate homeostasis (Robel et al., 2015). Changes in adenosine metabolism (Boison, 2016; Clasadonte and Haydon, 2012), enhanced gliotransmitters and cytokines release (Devinsky et al., 2013) can also contribute to network hyperexcitability. These phenotypic alterations play a role in epileptogenesis and promote neuronal network synchronization (Clasadonte and Haydon, 2012; Devinsky et al., 2013).

Microglial cells, promptly activated after epileptogenic insults, release cytokines with ictogenic properties (Rodgers et al., 2009; Vezzani et al., 2011) and undergo ATP-induced P2X7 receptor activation which contribute to both SE and spontaneous seizures (Engel et al., 2016). There is evidence of neuro-microglial interactions, which contribute to neurogenesis, neurodegeneration and synaptic plasticity (Eyo et al., 2017).

Disruption of blood-brain barrier (BBB) causes the leakage of serum protein, such as albumin, and may be associated with leucocyte extravasation into the brain. Albumin activating the TGF-β receptor signaling in astrocytes can decrease seizure threshold, by altering Kir4.1 channels, glutamate re-uptake and promoting cytokine expression and release (Friedman et al., 2009). BBB opening per se may lead to epileptogenesis (Friedman et al., 2009) and promote the generation of seizures (Marchi et al., 2007) similarly to astrocyte activation in the absence of other pathologic
phenomena (Ortinski et al., 2010; Robel et al., 2015). The extent of BBB leakage positively correlates with the frequency of spontaneous seizures in animal models (van Vliet et al., 2007). After epileptogenic insults there is evidence of changes in both ligand- and receptor- gated ion channels. This phenomenon is called “acquired channelopathy” and it contributes to the establishment of lowered seizure threshold (Bernard et al., 2004; Raol et al., 2009).

Genome wide microarray analysis of epileptogenic tissue in animal models allowed to identify common molecular pathways linked to known cellular changes (Dingledine et al., 2017; Pitkanen et al., 2015). The most studied and better validated molecules include those related to:

a) **neuroinflammation and oxidative stress pathways**, which involve the production and release of various cytokines and danger signals (Vezzani et al., 2011, 2015a), activation of the complement system (Aronica et al., 2007) and mitochondrial dysfunction which, together with activation of NADPH oxidase, leads to the generation of reactive oxygen species (Aronica et al., 2017; Pauletti et al., 2017; Rowley and Patel, 2013);

b) **transforming growth factor beta (TGF-β)** signaling activated in astrocytes by brain extravasation of serum albumin following alterations in the BBB permeability (Friedman et al., 2009);

c) **adenosine kinase**, which regulates the expression of adenosine, an endogenous anti-convulsant molecule (Boison, 2016);

d) **mammalian target of rapamycin (mTOR)**, a protein kinase involved in cell metabolism and growth, synaptic plasticity, neurogenesis, dendritic morphology and axonal sprouting (Galanopoulou et al., 2012; Ostendorf and Wong, 2015);

e) **brain derived neurotrophic factor (BDNF)**, which binds to its cognate receptor tyrosine kinase (TrkB), potentiating glutamatergic neurotransmission and impairing inhibitory synapses function (McNamara and Scharfman, 2012).

Recent studies have revealed that epigenetic modifications of DNA or chromatin may also be relevant to the development of epilepsy and/or seizures control (Hauser et al., 2017). The aspect of epigenetic modifications as possible mechanisms involved in epileptogenesis will be discussed later.
NEUROINFLAMMATION IN EPILEPSY

Experimental studies in animal models of epilepsy and clinical findings obtained in human brain specimens from various pharmaco-resistant forms of epilepsy provided evidence for the activation of the innate and adaptive immunity mechanisms and the induction of the associated inflammatory processes in the epileptogenic foci (Aronica and Crino, 2011; Vezzani et al., 2011).

The definition of inflammation includes a complex biological response of tissues against infections or sterile (non-infectious) injuries, associated with activation of innate and/or adaptive immunity cells. It represents a key homeostatic mechanism of body defense, which is crucial for activating tissue repair, via the production of a large array of inflammatory cytokines and related effector molecules.

A significant component of this inflammatory response is confined to brain resident cells (i.e., microglia, astrocytes, neurons and endothelial cells of the BBB) and is defined as “neuroinflammation” (Aronica et al., 2012; Vezzani et al., 2015). Neuroinflammation is not triggered only in response to “classical” factors (i.e. infections, autoimmunity, toxins), but is also ignited by increased neuronal activity, including epileptic seizures. Recently, the term “neurogenic neuroinflammation” has been proposed to describe neuroinflammatory mechanisms evoked by neuronal activity (Xanthos and Sandkuhler, 2014). This process may be homeostatic in nature thus enabling the brain to cope with the enhanced metabolic demands. However, it can become maladaptive when it is not properly resolved or when it spreads to remote sites, thus contributing to tissue pathogenesis (Xanthos and Sandkuhler, 2014).

A notable finding is that the inflammatory mediators released by brain resident cells during epileptic activity (i.e. cytokines, chemokines, alarmins/danger signals, prostaglandins, complement factors) are not only effectors molecules of the immune system promoting local inflammation but they function as neuromodulators therefore directly affecting neuronal function and excitability (Vezzani and Viviani, 2015). Indeed, specific inflammatory molecules were reported to significantly contribute to the mechanisms of seizure generation, to epileptogenesis and to pharmaco-resistance in experimental models (Vezzani et al., 2011).
Interleukin-1 Receptor/Toll-like Receptor (IL-1R/TLR) signaling in the brain

The IL-1R/TLR pathway is a key upstream signal promoting the activation of the innate immunity and associated neuroinflammation. Beside its classical role in the mechanisms of pathogen recognition and their killing and removal from tissue (Ulevitch and Tobias, 1995), the signaling can be activated also by pro-inflammatory mediators or endogenous molecules (i.e., damage-associated molecular patterns [DAMPs]) released by injured or activated brain cells, giving rise to the so-called “sterile inflammation” (Bianchi, 2007).

The IL-1R/TLR is part of a superfamily of single transmembrane domain receptor that share a common cytosolic domain, called Toll/IL-1 receptor (TIR) domain, which defines the downstream signaling (O’Neill and Bowie, 2007). Agonist binding to the extracellular domain of this family of receptors, and the subsequent recruitment of myeloid differentiation factor 88 (MyD88) and other cytosolic adaptor proteins (Akira et al., 2006), activates signaling via IL-1R-associated kinases (IRAKs) 1/4 and TNF receptor-associated factor (TRAF) 6. These events induce the expression of genes involved in immunity and inflammation, under the control of transcription factors such as nuclear factor kB (NF-kB), activator protein-1 (AP-1) and Interferon Regulatory Transcription Factors (IRFs) (O’Neill and Bowie, 2007).

The work included in this thesis focused on specific members of IL-1R/TLR signaling: IL-1R type 1 and TLR4 and their prototypical endogenous ligands, the pro-inflammatory cytokine interleukin(IL)-1β and the danger signal High Mobility Group Box 1 (HMGB1), respectively. These molecules are induced in brain resident cells during seizures and following various experimental epileptogenic insults such as febrile or non febrile SE, stroke, neurotrauma, CNS infection (Vezzani and Friedman, 2011).
IL-1β and IL-1R1

The IL-1R is the receptor which binds with high affinity IL-1β and exists in two forms: the type 1 receptor (IL-1R1) is primarily responsible for transmitting the biological effects of IL-1, while the type 2 receptor may act as a decoy receptor which prevents IL-1 activity by competing with IL-1R1 binding (Dinarello, 1996). The IL-1R1 and IL-1 accessory protein (IL-1R-AcP) form heterodimers that mediate the biological actions of the two agonists, namely the secreted pro-inflammatory cytokine IL-1β, and the intracellular IL-1α, as well as the natural occurring IL-1 competitive receptor antagonist (IL-1ra). IL-1β, synthesized as a precursor protein (pro-IL-1β) localized in the cytoplasm, needs to be processed by IL-1β converting enzyme (ICE/Caspase-1), a serine protease that produces the mature and bio-active protein, which is then secreted in the extracellular space (Dinarello, 2005). The synthesis of the IL-1β precursor is induced by various pro-inflammatory stimuli including brain injuries and infections (Allan et al., 2005).

Using rodent models of SE in which epileptogenesis is triggered by electrical stimulation or chemoconvulsants, it has been shown that IL-1β mRNA level in the hippocampus, and the protein which is barely detectable in healthy brain, are rapidly and persistently increased after SE induction (De Simoni et al., 2000; Dhote et al., 2007; Gorter et al., 2006; Ravizza et al., 2008; Vezzani et al., 1999). The IL-1ra, the endogenous anti-inflammatory counterpart of IL-1β, is also induced in the brain after SE but several hours after IL-1β and never in excess to IL-1β (De Simoni et al., 2000), thus indicating that the biological effects of IL-1β upon its brain production are not rapidly and effectively controlled as expected for an homeostatic response which should undergo a rapid resolution. Long-lasting induction of IL-1β has been also described in the hippocampus of immature rats after exposure to prolonged febrile seizures in animals that develop epilepsy (Dube et al., 2010).

Immunohistochemical studies showed that IL-1β is strongly induced in activated microglia and astrocytes both during the acute and post-injury phases of SE and in the chronic phase of spontaneous seizures in brain areas involved in seizure generation and propagation. IL-1R1 is up-regulated in neurons and astrocytes (Ravizza et al., 2008) suggesting that the released cytokine exerts both autocrine and paracrine actions. IL-1β and IL-1R1 are also expressed during epileptogenesis in both perivascular astrocytes and endothelial cells of the BBB, often in
association with BBB leakage and neuronal damage (Ravizza and Vezzani, 2006; Ravizza et al., 2008), suggesting that IL-1β/IL-1R1 axis contributes to these pathologic changes.

**HMGB1, TLR4 and RAGE**

Toll-like receptors (TLRs) are transmembrane pattern-recognition receptors (PRRs). The toll gene was the first-identified as a TLR family member with an essential role in the establishment of dorsal-ventral orientation during embryonic development and in the innate immune response against fungal infections in *Drosophila melanogaster* (Lemaitre et al., 1996). Subsequently, many homologues for Toll were identified across diverse species (Medzhitov, 2001). To date, 11 (TLR1-11) and 12 (TLR1-9; TLR11-13) functional TLRs have been identified in humans and mice, respectively (Kawai and Akira, 2010; Matin et al., 2015).

TLRs initiate innate immune responses via recognition of pathogen-associated molecular patterns (PAMPs) or endogenous molecules released after cellular stress or tissue injury (DAMPs), including heat shock proteins, S100 proteins, hyaluronan, nucleic acids, heparan sulfate, surfactant protein-A, fibrinogen and HMGB1 (Bianchi, 2007).

According to their subcellular localization and respective ligands, TLRs can be divided into two groups: (1) cell surface TLRs (TLR1, 2, 4, 5, 6, and 11) that recognize microbial membrane components, such as lipids, lipoproteins and protein and (2) intracellular TLRs (TLR3, 7, 8 and 9) that recognize viral or endogenous nucleic acids. However, some TLRs such as TLR3 and TLR7 could be localized both on the cell surface and in intracellular compartments (Akira et al., 2006) (Figure 4).

Upon TLR stimulation, the receptor dimerizes and specific adaptor proteins are recruited. All TLRs signal through MyD88, with the exception of TLR3 which uses TIR domain-containing adaptor inducing IFN-β (TRIF)-dependent pathway. This pathway is also shared by TLR4. In MyD88-dependent pathway, MyD88 recruits IRAKs, which interact with TRAF6, leading to the phosphorylation of the inhibitor of NF-κB (IκB) and its degradation and subsequent release and nucleus translocation of NF-κB for gene transcription. The MyD88-dependent pathway activates the mitogen-activated protein kinase (MAPK) signaling pathways, leading to the activation of AP-1 and IRFs (Akira et al., 2006; Liu and Ji, 2014) (Figure 4).
Figure 4. TLRs signaling pathway. TLR1/6, TLR2, TLR4 and TLR5 are expressed on the cell surface whereas TLR3, TLR7/8 and TLR9 are mainly localized in the endosomes. All TLRs, except TLR3, recruit the adaptor MyD88. Subsequently IRAKs and TRAF6 form a complex, IκB is phosphorylated and degraded, resulting in NF-κB translocation to the nucleus, where it initiates gene expression. TLR3 and TLR4 signal through the TRIF-dependent pathway and activates NF-κB and IRF3, leading to the induction of pro-inflammatory cytokines and type I IFN. Stimulation of TLR7 and TLR9 can also recruit MyD88 and activate the NF-κB and IRF7 pathway for the induction of pro-inflammatory cytokines and type I IFN (from Liu and Ji, 2014).

TLR4 was the first mammalian homolog of Toll identified as a pattern recognition receptor required for innate and adaptive immune responses to infections (Medzhitov et al., 1997). TLR4 in particular detects lipopolysaccharide (LPS) (Figure 5), a major outer membrane component of Gram-negative bacteria (Poltorak et al., 1998). TLR4 activation by LPS decreases seizure threshold in adult and immature rodents (Sayyah et al., 2003) and is associated with a chronic increase in hippocampal neuronal network excitability (Galic et al., 2008; Rodgers et al., 2009; Sayyah et al., 2003). TLR4 uses both MyD88-dependent and TRIF-dependent pathways. In the MyD88-independent pathway, TRIF interacts with TRAF3 to activate IRF3 and IRF7 and initiates the production of Type I interferons (e.g., IFN-α/β), which are hallmarks of the host innate immune response (Yamamoto et al., 2003). TRIF also binds to receptor-interacting protein 1 and TRAF6, leading to the activation of the NF-κB and/or MAPK pathways for the late-phase induction of pro-inflammatory genes (Liu and Ji, 2014). TLR3, which recognizes double-stranded RNA, uses only TRIF-dependent pathway for its signaling (Akira et al., 2006).
High mobility group box 1 (HMGB1) is a prototypical member of the DAMP family and it was identified by our laboratory (Maroso et al., 2010) as a mediator of sterile neuroinflammation evoked by epileptogenic injuries (see also Iori et al., 2013; Pauletti et al., 2017; Zurolo et al., 2011). HMGB1 is a highly conserved non-histone nuclear protein expressed by most eukaryotic cells, where it binds to chromatin and regulates gene transcription (Bianchi and Manfredi, 2007). HMGB1 contains two DNA-binding domains, called A and B boxes, and an acidic tail comprising glutamic and aspartic acids (Venereau et al., 2016) (Figure 6).

Importantly, HMGB1 can act not only in the nucleus, but also outside the cells. HMGB1 is passively released by dying necrotic cells, while it is retained by nuclei of apoptotic cells, or it is actively released upon its re-location from the nucleus to the cytoplasm during pyroptosis and inflammasome activation (Lu et al., 2014). Post-translational modifications of HMGB1 play a key role in its pleiotropic functions. In particular, the functional activity of HMGB1 is determined by the redox state of three key cysteine residues: C23, C45 and C106. Intracellular HMGB1 is found in fully reduced (all-thiol) state and when released extracellularly has chemoattractive properties by interacting with the chemokine CXCL12 and its receptors (Venereau et al., 2012). Extra-nuclear HMGB1 can be partially oxidized by formation of the disulfide bond between C23 and C45, thus generating the disulfide HMGB1 isoform which is not chemotactic but activates TLR4, thus mediating pro-inflammatory activities (Balosso et al., 2014) (Figure 6). Since the generation of reactive oxygen species (ROS) during oxidative stress promotes the stabilization of HMGB1 in its disulfide form, there is a vicious cycle that potentially links ROS to disulfide HMGB1 generation and inflammation.

![Figure 6. HMGB1 structure, isoforms and functions](image)
We recently showed that HMGB1 progressively translocates to the cytoplasm of activated astrocytes as well as in microglia, neurons and endothelial cells of the BBB in adult rats exposed to SE during disease development (Walker et al., 2017). HMGB1 translocation in astrocytes was observed also in immature rats exposed to febrile SE (Patterson et al., 2015) and after epileptogenic injuries such as TBI (Parker et al., 2017) and stroke (Tsukagawa et al., 2017).

Upon brain insults, high levels of ATP at sites of tissue damage induce P2X7 receptor-mediated activation of inflammasome, which results in the extracellular release of both IL-1β and HMGB1 (Lu et al., 2012, 2014). Once released HMGB1 interacts with TLR4 and Receptor for Advanced Glycation End products (RAGE) (Figure 5).

RAGE is a member of the immunoglobulin superfamily of cell surface receptors and it binds to structurally diverse molecules that include not only AGEs, but also fibrillar proteins such as amyloid-β peptides, S100 proteins and HMGB1 (Bierhaus et al., 2005). Consequently, RAGE can be considered a pattern-recognition receptor that binds predominantly endogenous molecules that are either generated or released during cellular or physiological stress.

Both TLR4 and RAGE are rapidly and lastingly induced in neurons and activated astrocytes after epileptogenic insults in rodent epileptogenic brain areas (Maroso et al., 2010; Vezzani et al., 2011). Binding of HMGB1 with these receptors expressed by immune cells leads to the production of cytokines mediated by NF-kB activation (Park et al., 2004) (Figure 5), while activation of neuronal receptors induces rapid changes in neuronal excitability as discussed in the next paragraph.
Figure 5. Pro-inflammatory and chemoattractive effects of HMGB1 are mediated by TLR4 and RAGE. Following pathophysiological cell activation or injury, HMGB1 rapidly undergoes nucleus-to-cytoplasm translocation and subsequent cellular release. Once released HMGB1 can interact with different receptors such as TLR4 and RAGE expressed by immune cells, leading to NF-kB-mediated production of pro-inflammatory cytokines (http://www.invivogen.com/review-damp).
Experimental evidence for a role of IL-1R/TLRs in seizure mechanisms

**IL-1R1/TLR4 signaling.** A major advance in understanding the actions of immune mechanisms in the brain, stems from the evidence showing that **IL-1R1/TLR4 signaling activation in neurons** has non-canonical effects which mediate rapid post-translational changes in voltage- and receptor-gated ion channels thus resulting in altered neuronal excitability (Vezzani and Viviani, 2015; Vezzani et al., 2011). These effects are pivotal for hyperexcitability phenomena underlying seizure generation as shown in animal models. The first evidence that IL-1R1/TLR4 signaling contributes to seizures was provided by the exacerbation of seizures induced by IL-1β or HMGB1 after their injection into the hippocampus or intracerebroventricularly shortly before the animal exposure to a local convulsive challenge. The frequency of both acute seizures and established chronic seizures was drastically reduced by 50-70% by pharmacological interventions preventing or reversing the signaling activation in the brain (Vezzani et al., 2011).

In particular, IL-1β injection in rodent hippocampus 5-10 min before KA or bicuculline application resulted in pro-convulsive effects mediated by IL-1R1 (Vezzani et al., 1999, 2000, 2002), while the intracerebral injection of IL-1ra mediated powerful anti-convulsive effects (Vezzani et al., 1999, 2000). In accordance, transgenic mice overexpressing IL-1ra in astrocytes showed a reduced susceptibility to evoked seizures (Vezzani et al., 2000). IL-1β acting on IL-1R1 also lowered core temperature threshold required to induce seizures in a post-natal day 14 mouse model of febrile convulsions (Dube et al., 2005).

TLR4 activation by systemic administration of LPS in 14-day old rats induced a rapid and transient increase in blood and hippocampal cytokines associated with a chronic decrease in seizure threshold. In fact, the animals displayed an increased seizure susceptibility to pilocarpine, KA and PTZ in their adulthood together with cognitive deficits, anxiety-like behaviors and increased neuronal degeneration in the hippocampus (Galic et al., 2008).

The release of HMGB1 from neurons and glia was subsequently identified as a molecular event that, together with IL-1β, lowers seizure threshold (Balosso et al., 2014; Maroso et al., 2010).

Pharmacologic or genetic interference with the IL-1R1/TLR4 signaling provided additional demonstration of its involvement in seizure mechanisms. Thus, the blockade of IL-1β biosynthesis with specific interleukin converting enzyme(ICE)/Caspase-1 inhibitors (Pralnacasan, VX-765) (Maroso et al., 2011a; Ravizza et al., 2006a; Vezzani et al., 2010) or the inactivation of HMGB1’s
action by its broad spectrum receptor antagonist BoxA or by selective TLR4 antagonists, significant delayed seizure onset and reduced acute seizure frequency induced by KA or bicuculline in rodents (Maroso et al., 2010), as well as chronic seizures in epileptic mice (unpublished data). Moreover, the systemic administration of VX-765 in a GAERS, a rat model of absence seizures, significantly reduced the number and duration of spike-and-waves discharges (Akin et al., 2011). The administration of an inactivating anti-HMGB1 monoclonal antibody attenuated seizures induced either by maximal electroshock or systemic injections of PTZ (Zhao et al., 2017). Decreased intrinsic seizure susceptibility was observed in mice lacking Caspase-1 or IL-1r1 or Tlr4 genes, thus reinforcing the pharmacological data (Maroso et al., 2010; Ravizza et al., 2006a; Vezzani et al., 2000).

The rapid onset effects on seizures (<10 min) observed by interfering with IL-1R1/TLR4 signaling are compatible with the activation of non-transcriptional pathways. In this respect, IL-1β- and HMGB1-dependent ictogenic activities involve a rapid activation of the Src tyrosine kinase family and the consequent phosphorylation of N-methyl-D-aspartate (NMDA)-NR2B receptors, associated with increased neuronal Ca\(^{2+}\) influx (Balosso et al., 2008, 2013; Maroso et al., 2010).

Additional molecular mechanisms that possibly contribute to IL-1β and HMGB1 hyperexcitability underlying seizures include reduction of GABA\(_A\) receptor-mediated currents (Roseti et al., 2015) and extracellular glutamate increase due to inhibition of astrocytic glutamate re-uptake or enhanced astrocytic release (Coulter and Steinhäuser, 2015; Devinsky et al., 2013; Pedrazzi et al., 2006).

**TLR3 signaling.** The involvement of TLR3-mediated signaling in seizures is suggested by the clinical evidence that febrile seizures often occur as a result of viral infections, and virus express molecular motifs recognized by TLR3. Previous studies suggest an intrinsic enhancement of the TLR3-signaling in patients who experienced febrile seizures (Matsuo et al., 2006; reviewed in Vezzani et al., 2011).

Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic double-stranded RNA molecule, which stimulates TLR3 and evokes an immune response much like viruses (Vercammen et al., 2008). Intracerebroventricular administration of Poly I:C in 14-day old rats causes fever and increased IL-1β in rat forebrain (Galic et al., 2009). In their adulthood, rats were more susceptible to pilocarpine and PTZ-induced seizures, showed memory deficits and increased NMDA and α-amino-
3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit mRNA expression in forebrain (Galic et al., 2009). Mice pretreated with Poly I:C showed increased KA-induced seizures and forebrain upregulation of several cytokine mRNAs, such as IL-6, TNF-alpha, IL-1β and chemokines (Kirschman et al., 2011; Michalovicz and Konat, 2014).

Increased excitability and susceptibility to seizure induction was reported also in hippocampal slices obtained from mice injected intraperitoneally with Poly I:C. These effects were concomitant with alterations in the expression of phosphorylated NR2B subunit of NMDA receptor and of an astrocyte-specific glutamate transporter, which regulates extracellular glutamate concentrations (Costello and Lynch, 2013).

Activation of TLR3 has also been shown to affect memory consolidation, anxiety and depressive-like behaviours (Gibney et al., 2013; Kent et al., 2007). Poly I:C-induced activation of TLR3 in mice induced impairment of working memory and inhibition of neurogenesis (Okun et al., 2010).

TLR3 also contributes to epilepsy progression since its deficiency reduced the number of chronic spontaneous seizures in mice exposed to pilocarpine-induced SE, along with a reduced expression of pro-inflammatory cytokines in the hippocampus (Gross et al., 2017).

Clinical evidence

Experimental data have been corroborated by evidence of IL-1R/TLR signaling activation in brain specimens resected at therapeutic surgery from patients with differing forms of drug-resistant epilepsy (Aronica and Crino, 2011, 2014). IL-1β/ILR1 and HMGB1/TLR4 axes, and their key downstream signaling molecules, have been studied by immunohistochemical and biochemical analysis in TLE (Maroso et al., 2010; Pernhorst et al., 2013; Ravizza et al., 2008; Roseti et al., 2015) (Figure 7 and 8), epilepsy associated with glioneuronal tumors (ganglioglioma) (Prabowo et al., 2013, 2014), malformations of cortical development (focal cortical dysplasia and cortical tubers in tuberous sclerosis, TSC) (Ravizza et al., 2006b; van Scheppingen et al., 2016; Zurolo et al., 2011) and Rasmussen’s encephalitis (Luan et al., 2016). In mesial TLE specimens, a prominent overexpression of IL-1β and IL-1R1 (Ravizza et al., 2008) (Figure 7), increased expression of NLRP1 inflammasome and caspase-1 (Tan et al., 2015), as well as of TLR4 and HMGB1 (Maroso et al., 2010) (Figure 8), have been detected in glial cells, neurons and endothelial cells of the BBB.
Notably, HMGB1 cytoplasmatic staining, indicative of its translocation from nuclei, was increased in neurons and astrocytes (Maroso et al., 2010) (Figure 8), thus confirming the findings in experimental models. The activation of this signaling in human epilepsy was supported by gene expression analysis. In particular, hippocampal levels of \textit{Tlr4} gene in patients with TLE positively correlated with the frequency of spontaneous seizures before surgery (Pernhorst et al., 2013). The activation of the TLR4 and IL-1R1 signaling was further supported by an increased expression of IRAK1 signaling molecule in TLE specimens (Roseti et al., 2015).

Neuropathological examination of surgical specimens from patients with focal malformations of cortical development provided evidence of activation of immune responses and concomitant induction of various inflammatory pathways and molecules, including IL-1\(\beta\), TLR4, RAGE, HMGB1 and chemokines (Boer et al., 2006; Ravizza et al., 2006b; Zurolo et al., 2011). Interestingly, in a cohort of focal cortical dysplasia patients, the density of activated microglial cells significantly correlates with the duration of epilepsy, as well as with the frequency of seizures before surgical resection (Boer et al., 2006). Similarly, the number of IL-1\(\beta\)- and IL-1R1- positive neurons in glioneuronal tumors was positively correlated with the frequency of seizures (Prabowo et al., 2015), whereas the number of IL-1ra-positive neurons and astrocytes was negatively correlated with the duration of epilepsy prior to surgery (Ravizza et al., 2006b).
Figure 7. Distribution of IL-1β and IL-1R1 immunoreactivity in the hippocampus of TLE patients with HS. 

(A, B) Control hippocampus from autopsy specimens and histologically non-sclerotic hippocampus without detectable IL-1β immunoreactivity. (C) IL-1β staining in TLE-HS showing strong expression in neurons (arrow and inset), astrocytes (arrowheads) and in blood vessels (double-arrowheads). IL-1β staining in TLE-HS showing immunoreactivity (D) in a blood vessel in perivascular astrocytic endfeet, (E) in reactive astrocytes (inset shows co-localization of IL-1β with vimentin), (F) in cells of the microglia/macrophage lineage (inset shows co-localization of IL-1β with HLA-DR). (G,H) Control hippocampus from autopsy specimens and histologically non-sclerotic hippocampus without detectable IL-1R1 immunoreactivity. (I) IL-1R1 staining in TLE-HS showing strong expression in neurons (arrow and inset) and astrocytes (arrowheads). IL-1R1 staining in TLE-HS showing immunoreactivity (J) in astrocytic endfeet (inset shows prominent albumin staining around a blood vessel within the hilar region), (K) in reactive astrocytes (inset shows co-localization of IL-1R1 with vimentin), (L) in cells of the microglia/macrophage lineage (inset shows co-localization of IL-1R1 with HLA-DR). Scale bar: A, G 90 μm; B, C and H, I, 40 μm; D–F and J–L, 100 μm (from Ravizza et al., 2008).
Figure 8. HMGB1 and TLR4 immunoreactivity in the hippocampus of TLE patients with HS. (a) Immunohistochemical staining for HMGB1 in the CA1 region of control individuals and individuals with TLE. Arrows indicate pyramidal neurons. Double arrows point to neurons with prominent cytoplasmic immunoreactivity; arrowheads point to cells with glial morphology with nuclear staining and double arrowheads point to cells with glial morphology with cytoplasmic staining. (b) Immunofluorescence of HMGB1, GFAP and HLA-DR in hippocampi from control individuals and individuals with TLE-HS. (c) TLR4 immunostaining in the CA1 region; arrows point to neurons and arrowheads to reactive glial cells. (d) Confocal images showing colocalization of TLR4 with NeuN in neuronal cells, or with GFAP in reactive astrocytes, and lack of colocalization of TLR4 with HLA-DR in cells of the microglia/macrophage lineage. Scale bars: a,c, 50 μm; b,d, 20 μm (from Maroso et al., 2010).
NEUROINFLAMMATION IN PRION DISEASES

Prion diseases are fatal neurodegenerative and infectious conditions caused by misfolding of the prion protein (PrP\(_c\)), a cell surface glycoprotein (Chiesa, 2015). Creutzfeldt-Jakob disease (CJD) is the most common form in human. CJD occurs as a sporadic, familiar or infectious/acquired disease. Sporadic CJD is the most common subtype, occurring worldwide in 84% of cases with an annual mortality rate of 1.39 per million (Ladogana et al., 2005). It is believed that the underlying mechanism leading to prion pathogenesis is the conversion of the PrP\(_c\) into abnormal disease-related form (PrP\(_\Sc\)), which accumulates in the brain (Colby and Prusiner, 2011).

In about two-thirds of patients, electroencephalography (EEG) detects typical periodic sharp wave complexes (PSWCs), either lateralized or generalized (Wieser et al., 2006). Epileptiform discharges and focal motor or generalized seizures may also be observed, typically in the late stage of the disease (Wieser et al., 2006).

Studies in animal models have suggested several toxic mechanisms activated by abnormally folded PrP that may lead to neuronal dysfunction and death, including NMDAR dysfunction (Chiesa, 2015). Increased NMDAR-dependent excitation has been reported in mice inoculated with CJD prions (Ratté et al., 2008), and when PrP\(_\Sc\) was exogenously presented to cultured neurons, the resulting neurotoxicity could be blocked by NMDAR antagonists (Resenberger et al., 2011; Thellung et al., 2013). Loss of a physiological PrP\(_c\) function in regulating NMDAR activity may also contribute to the pathogenic process. Genetic PrP\(_c\) depletion results in increased hippocampal NMDAR-mediated excitation and glutamate excitotoxicity (Khosravani et al., 2008).

Deposition of misfolded/aggregated PrP and astro- and micro-gliosis are typical neuropathological hallmarks of CJD (Wojtera et al., 2012). In addition, high levels of several inflammatory cytokines, including IL-1\(\beta\), IL-6 and TNF-\(\alpha\), have been measured in CJD brains (Llorens et al., 2014; Shi et al., 2013).
EPIGENETIC CONTROL OF NEUROINFLAMMATION

The term "epigenetic" summarizes processes that influence gene transcription by altering the chromatin state and that persist long after the initial stimulus has ceased (Henshall and Kobow, 2015; Kobow and Blümcke, 2017). Key molecular epigenetic mediators include methylation of DNA, histone modifications and noncoding RNAs (Henshall and Kobow, 2015). Emerging findings in animal models and human brain tissue have revealed that epilepsy and epileptogenesis are associated with significant changes in each of these molecular contributors to the epigenome (Dębski et al., 2016; Sweatt, 2013).

DNA methylation is accomplished by a group of enzymes called DNA methyltransferases and involves covalent addition of a methyl group from S-adenosylmethionine to a cytosine base to form 5-methylcytosine (Robertson, 2005). This epigenetic modification is typically associated with a silencing effect on transcriptional activity. DNA methylation appears to play a critical role in the onset and severity of seizure activity, and it may contribute to the development of epilepsy (Kobow and Blumcke, 2011). A significant modulation of DNA methylation has been reported during disease development in pilocarpine- and KA-induced rat model of chronic epilepsy (Kobow et al., 2009; Williams-Karnesky et al., 2013).

Histones. Post-translational modifications of histones affect the compaction of chromatin and change gene accessibility to the transcriptional machinery. These modifications include acetylation, methylation, phosphorylation and ubiquitination. A rapid increase in phosphorylation and acetylation of specific histone subunits has been reported after pilocarpine- and KA-induced seizures in mice (Crosio et al., 2003; Sng et al., 2006).

Noncoding RNAs refer to any transcribed RNAs which do not code or cannot be translated into protein; these are usually divided according to their size into short (<200 nucleotides) and long (>200 nucleotides) forms. There is still scarce information about long noncoding RNA expression in epilepsy, while several studies have profiled microRNAs (miRNAs), a subgroup of short noncoding RNAs, in experimental models and human epileptic tissue (Henshall, 2014).
microRNAs

miRNAs are a class of small (~22 nucleotides) single-stranded non-protein-coding RNA molecules that regulate gene expression either by promoting mRNA degradation or by attenuating protein translation at the post-transcriptional level.

Figure 9 depicts the processes involved in miRNAs biogenesis. miRNAs are transcribed as primary transcripts (pri-miRNA), mainly by RNA Polymerase II. In the nucleus, the pri-miRNA is processed to produce an approximately 60–70-nucleotide hairpin structure, the pre-miRNA, by a microprocessor complex containing the RNase Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8). The pre-miRNA is then exported to the cytoplasm by exportin 5 and processed further by Dicer, another RNase, to produce the mature, approximately 22-nucleotide, duplex miRNA, a process enhanced by transactivation-responsive RNA binding protein (TRBP). The functional strand of the mature miRNA is uploaded into the RNA-induced silencing complex (RISC) which contains the protein Argonaute 2. Once loaded, the RISC traffics along target mRNAs until sufficient complementary binding is present. The target selection is specified by a 7–8 nucleotide complementarity sequence match, the so-called seed interaction between the miRNA and the mRNA. This binding produces stable miRNA–mRNA complexes that facilitate mRNA decay or translational repression. It is estimated that a third or more mRNAs are regulated by miRNA. A given miRNA can have several binding sites to the same mRNA and a single mRNA may be targeted by multiple miRNAs, thereby producing stronger epigenetic effects. In line with this view, a single miRNA is able to regulate the expression of possibly hundreds of genes, thereby exerting important effects on cellular functions (Henshall et al., 2016; Reschke and Henshall, 2015).

Studies in genetically modified mice lacking key biogenesis genes have revealed that miRNAs are essential for normal organism development. More recent work in conditional models has determined that loss of miRNAs biogenesis components during brain maturation results in progressive tissue dysmorphogenesis, neurodegeneration and seizures (Davis et al., 2008; Fiorenza et al., 2016).
Figure 9. miRNA biogenesis. miRNA biogenesis begins in the nucleus with the transcription of a pri-miRNA, cleaved to produce the pre-miRNA, which is exported to the cytoplasm by exportin 5 and processed to produce the mature duplex miRNA. The guide strand of the miRNA is bound by an argonaute protein, forming the miRNA-induced silencing complex (RISC). Once loaded, the RISC traffics along target mRNAs until sufficient complementary binding is present. This binding produces stable miRNA–mRNA complexes that facilitate mRNA decay or translational repression (from Henshall et al., 2016).
Epilepsy-related miRNAs

Profiling studies of miRNA brain expression in animal models of epilepsy and in brain tissue from pharmaco-resistant epilepsy patients have identified deregulation of >100 different miRNAs. The reproducibility of such studies among different laboratories is demanding for different reasons, including the diversity in study design, time points, animal models, type and origin of tissue samples and technique used to profiling miRNA expression. However, a subset of miRNAs has been repeatedly found to be altered in rodent hippocampus after SE and in resected human brain tissue from TLE patients, and importantly, to be involved in epileptogenesis-related processes, such as neuroinflammation, cell death and synaptic remodeling (Table 2).

miRNAs and neuroinflammation. Some miRNAs have emerged as specific regulators of neuroinflammation since they target molecular constituents of both the innate and adaptive immune responses. Among these miRNAs, prominent changes in epilepsy have been reported for miR-146a, miR-155 and miR-21. Their levels were increased in experimental and human TLE and in epilepsy-associated glioneuronal lesions, like focal cortical dysplasia and ganglioglioma (Aronica et al., 2010; van Scheppingen et al., 2016). In particular, miR-146a and miR-155 act as key players in the regulation of the astrocyte-mediated inflammatory response by modulating the release of inflammatory mediators (van Scheppingen et al., 2016). Levels of miR-187 were shown to be downregulated in rodents with spontaneous seizures and in the hippocampus of TLE patients, and inversely correlated with IL-10 levels, an anti-inflammatory cytokine (Alsharafi et al., 2015). miR-23a was found to be up-regulated by several profiling studies and is predicted to target genes putatively involved in the regulation of inflammation (Roncon et al., 2015).

Several miRNAs have been reported to be involved in seizure-induced neuronal death. Levels of miR-34a, miR-132, miR-134 and miR-184 were found to be increased in experimental and human TLE. Experimental manipulation that decrease their expression in experimental models resulted in reduction (miR-34a, miR-132 and miR-134) or increase (miR-184) in neuronal death caused by SE (Hu et al., 2012; Jimenez-Mateos et al., 2011, 2012; McKiernan et al., 2012; Peng et al., 2013; Sano et al., 2012; Wang et al., 2014).

Some miRNAs have been shown to be important in maintaining synaptic plasticity within the physiologic range of neuronal excitability. miR-132 and miR-134, in addition to the previously
mentioned effect on seizure-induced neuronal death, increase spine density of pyramidal neurons, thus contributing to neuronal hyperexcitability (Hansen et al., 2010; Jimenez-Mateos et al., 2012).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Direction of regulation</th>
<th>Origin of tissue sample</th>
<th>Associated cellular processes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>Up</td>
<td>TLE animal model TLE human Glioneuronal tumors Malformations of cortical development</td>
<td>Inflammation</td>
<td>(Gorter et al., 2014) (Aronica et al., 2010) (van Scheppingen et al., 2016) (Prabowo et al., 2015) (Iyer et al., 2012)</td>
</tr>
<tr>
<td>miR-146a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-155</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-23a</td>
<td>Up</td>
<td>TLE animal model TLE human</td>
<td>Inflammation</td>
<td>(Song et al., 2011) (Gorter et al., 2014) (Kretschmann et al., 2014) (Roncon et al., 2015)</td>
</tr>
<tr>
<td>miR-187</td>
<td>Down</td>
<td>TLE animal model TLE human</td>
<td>Inflammation</td>
<td>(McKiernan et al., 2012) (Gorter et al., 2014) (Alsharafi et al., 2015) (Kretschmann et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Up</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-221</td>
<td>Down</td>
<td>TLE human</td>
<td>Inflammation</td>
<td>(Kan et al., 2012)</td>
</tr>
<tr>
<td>miR-222</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-34a</td>
<td>Up</td>
<td>SE and TLE animal model</td>
<td>Cell death/ Apoptosis</td>
<td>(Hu et al., 2012) (Sano et al., 2012) (Gorter et al., 2014) (Risbud and Porter, 2013) (Kretschmann et al., 2014)</td>
</tr>
<tr>
<td>miR-132</td>
<td>Up</td>
<td>SE and TLE animal model TLE human</td>
<td>Cell death/ Apoptosis</td>
<td>(Jimenez-Mateos et al., 2011) (Gorter et al., 2014) (Kretschmann et al., 2014)</td>
</tr>
<tr>
<td>miR</td>
<td>Change</td>
<td>Animal Model</td>
<td>Human Model</td>
<td>Effect</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
<td>------------------------------------------------</td>
<td>-------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>miR-134</td>
<td>Up</td>
<td>SE and TLE animal model</td>
<td>TLE human</td>
<td>Cell death/Apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-181a</td>
<td>Up</td>
<td>SE animal model</td>
<td>TLE human</td>
<td>Cell death/Apoptosis</td>
</tr>
<tr>
<td>miR-184</td>
<td>Up</td>
<td>SE animal model</td>
<td>TLE human</td>
<td>Cell death/Apoptosis</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-199a</td>
<td>Up</td>
<td>SE and TLE animal model</td>
<td>TLE human</td>
<td>Cell death/Apoptosis</td>
</tr>
<tr>
<td>miR-124</td>
<td>Down</td>
<td>SE and TLE animal model</td>
<td>TLE human</td>
<td>Synaptic remodelling</td>
</tr>
<tr>
<td></td>
<td>Up</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-203</td>
<td>Up</td>
<td>TLE animal model</td>
<td>TLE human</td>
<td>Synaptic remodelling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-204/miR-218</td>
<td>Down</td>
<td>TLE animal model</td>
<td>TLE human</td>
<td>Synaptic remodelling</td>
</tr>
<tr>
<td>miR-219</td>
<td>Down</td>
<td>SE animal model</td>
<td>TLE human</td>
<td>Neural activity</td>
</tr>
</tbody>
</table>
miR-146a

Among neuroinflammation-associated miRNAs, we focused our attention on miR-146a since it has been shown to critically modulate innate immunity through regulation of TLR signaling and cytokine responses (Pedersen and David, 2008; Sheedy and O’Neill, 2008; Taganov et al., 2006). The human miR-146 family of miRNAs consists of two member genes, miR-146a and miR-146b, located on chromosomes 5 and 10, respectively, and their mature products differ only by 2 nucleotides. miR-146a was the first miRNA associated to neuroinflammation to be studied in epilepsy. Genome-wide miRNA expression profiling studies revealed brain region and temporal specific changes in miR-146a expression in the epileptic rodents (Gorter et al., 2014; Roncon et al., 2015). These studies were further supported by PCR and in situ hybridization analysis carried out for studying miR-146a quantification and localization. Following SE, hippocampal levels of miR-146a are increased both in immature and adult rat epilepsy models (Aronica et al., 2010; Omran et al., 2012). Notably, miR-146a levels are also increased in the resected hippocampus from adults and children with TLE (Aronica et al., 2010; Omran et al., 2012). In the control hippocampus, miR-146a is expressed by neuronal cells, including pyramidal cells of CA1 and CA3 regions, as well as granule cells and hilar neurons of the dentate gyrus (Aronica et al., 2010). During epileptogenesis and in the chronic epilepsy phase, a prominent upregulation of miR-146a expression was detected in CA1 and CA3 pyramidal neurons and granule cells of the dentate gyrus and also in glial cells in the inner molecular layer of the dentate gyrus and in the hilar region. Co-localization studies indicated that miR-146a expression was induced in astrocytes and not in microglia (Aronica et al., 2010). A similar pattern of expression and cellular distribution was observed in tissue specimens from TLE patients with HS. Other evidence of miR-146a overexpression has been reported in glioneuronal lesions from patients with medically intractable epilepsy (Iyer et al., 2012; Prabowo et al., 2015; van Scheppingen et al., 2016). Notably miR-146a was found to be increased also in serum from epileptic patients therefore being a potential biomarker candidate (An et al., 2016).

It has been shown that miR-146a is a key feedback modulator of NF-kB induction; in fact, it is induced by NF-kB and, in turn, it downregulates the levels of signaling molecules downstream of IL-1R/TLR activation thereby reducing the activity of this inflammatory pathway (Taganov et al., 2006). In particular, miR-146a suppressed IRAK-1 and -2 and TRAF-6 in cultured human astrocytes exposed to IL-1β (Iyer et al., 2012). This evidence suggests that, in response to inflammatory cues,
miR-146a is induced as a negative-feedback regulator of astrocyte-mediated inflammatory response (Iyer et al., 2012; van Scheppingen et al., 2016).

**Targeting miRNAs in models of epilepsy**

To study the involvement of specific miRNAs in seizure generation or in the associated neuropathology two main oligonucleotide-based approaches have been used: 1) injecting an antagomir to block the specific miRNA, or 2) injecting a synthetic miRNA mimic to increase the endogenous miRNA level (Figure 10). These oligonucleotides may be delivered: A) before inducing SE for studying the acute effects on seizures; B) after inducing SE and before the onset of the disease, therefore during epileptogenesis, for studying the effects on disease onset and its clinical course; C) after the onset of the disease to study the progression of the disease. The first type of study (A) aims at assessing the effect of miRNA on intrinsic seizure susceptibility, or seizure threshold, in an acute setting. The second and the third approaches (B and C) aim at identifying potential disease-modifying (anti-epileptogenic) effects of miRNA.

![Figure 10. Techniques used to manipulate miRNAs in in vivo models of epilepsy. Overexpressing miRNA is based on introducing a synthetic oligonucleotide mimic, whereas blockade of miRNA function can be attained by introducing an anti-sense oligonucleotide with complementary base-pairing (antagomir) (from Henshall, 2017).](image)

Hereafter, some examples of miRNAs experimental manipulation are shown.

**AntagomiR-based manipulations.** The first evidence that seizures could be altered by targeting an individual miRNA was reported for miR-134. Intracerebroventricular injection of antagomir-134 resulted in suppression of evoked and spontaneous seizures in a mouse kainate model (Jimenez-Mateos et al., 2012). Additional studies found that antagomirs targeting miR-34a (Hu et al., 2012) and miR-132 (Jimenez-Mateos et al., 2011) reduced seizure-induced neuronal death in SE models.
Inhibiting miR-132 after SE has also been reported to reduce the frequency of spontaneous seizures (Yuan et al., 2016). A recent study showed that delivery of antagomirs targeting miR-155 prior to pilocarpine-induced SE in mice resulted in a trend toward lower mortality and Racine seizure score and suggested brain-derived neurotrophic factor as a possible target (Cai et al., 2016). Levels of miR-199a were found to be increased following pilocarpine-induced SE in rats. Pre-treating rats with an antagomir targeting miR-199a prior to SE reduced acute seizure severity and neuronal damage (Jiang et al., 2016). In a profiling study using a mouse model of pilocarpine-induced SE, miR-203 was upregulated during the epileptic phase and also increased in hippocampi from patients with epilepsy (Lee et al., 2017). Using intranasal delivery, the antagomir targeting miR-203 reduced the frequency of spontaneous seizures in epileptic mice and the glycine receptor B was identified as a potential target (Lee et al., 2017). Finally, antagomir targeting miR-181a were reported to reduce neuronal death after SE in rats (Ren et al., 2016).

miR mimic-based manipulations. Intracerebroventricular injection of a mimic of miR-22, which was found to be upregulated after SE, transiently reduced spontaneous seizures evoked in mice by KA. Conversely, an antagomir targeting miR-22 increased spontaneous seizures and exacerbated astrogliosis within the hippocampus (Jimenez-Mateos et al., 2015). A key target of this miRNA was shown to be the ATP-gated P2X7 receptor, which is known to activate the inflammasome thereby promoting the release of IL-1β and HMGB1. Thus, miR-22 may function to suppress ictogenic neuroinflammatory signaling in epilepsy (Jimenez-Mateos et al., 2015). Levels of miR-23b were reported to be downregulated in a mouse KA model, and delivery of miR-23b mimic into the mouse ventricle reduced post-kainate acute spiking (Zhan et al., 2016). miR-124 is another miRNA downregulated in experimental epilepsy. Pre-treating rats with miR-124 mimic delayed seizure onset and resulted in less severe seizures in the pilocarpine model (Wang et al., 2016). Potential mechanisms include targeting of glutamate-related signaling components and the cyclic-AMP response element binding protein. Lower levels of miR-219 were reported in experimental and human epilepsy and injection of a miR-219 mimic protected against KA seizures in mice. Inhibiting miR-219 with antagonir was, on its own, sufficient to produce epileptiform spiking in mice. The effects of miR-219 were suggested to be mediated by targeting of glutamatergic signaling components (Zheng et al., 2016).
AIM AND OUTLINE OF THE THESIS

A major clinical need for epilepsy is to develop new drugs for controlling seizures in people with pharmaco-resistant epilepsy. Current AEDs provide only symptomatic control of seizures, have multiple adverse effects and are ineffective in up to 40% of patients. Next generation therapies need to possess disease-modifying properties by targeting the mechanisms intimately involved in making the brain susceptible to generate spontaneous seizures. Such drugs are still lacking and they could potentially be used to halt or reverse the progression of epilepsy in patients with an established diagnosis, or delay or prevent the onset of epilepsy in susceptible individuals.

Based on these considerations, the major aim of the work presented in this thesis is to study in-depth the role of the IL-1R1/TLR4 pathway in seizure generation by gathering still missing information on its potential contribution to onset and the progression of epilepsy. We tested novel therapeutic interventions targeting this pathway to study their potential disease-modifying effects in animal models.

In chapter 2 we investigated the relative contribution of TLR4 and RAGE to acute seizures and to the ictogenic effects of HMGB1. Then, we studied the role of these two receptors in the development of spontaneous seizures, cognitive impairment and neuropathology.

In chapter 3 we tested novel therapies inhibiting the IL-1R1/TLR4 signaling in a murine model of acquired epilepsy. We used a novel epigenetic approach by injecting a synthetic mimic of miR-146a in the attempt to impair IL-1R1/TLR4 signal transduction (summarized also in chapter 4). In a parallel study, we blocked signaling activation with selective anti-inflammatory drugs and finally compare the therapeutic effects of these two novel pharmacological interventions with a classically used ASD such as carbamazepine.

In chapter 5 we investigated the involvement of the IL-1β-IL-1R1 signaling in NMDA-dependent neurotransmission and in seizure susceptibility in a mouse model of CJD disease. In this context, we tested the possibility that targeting IL-1β-IL-1R1 axis with clinically available drugs might be beneficial in the symptomatic treatment of the disease.

In chapter 6 we review the neuromodulatory properties of cytokines and danger signals which contribute to the generation of an hyperexcitable neuronal network and to seizure mechanisms, neuropathology and comorbidities in experimental models.
In chapter 7 we report and discuss still unpublished results regarding the role of TLR3 signaling in astrocytes in seizure susceptibility by using its mimetic Poly I:C and genetically modified mice.