"FEDERICO II"

UNIVERSITY OF NAPLES

PhD Program in

Clinical and Experimental Medicine

Curriculum in Human Development and Growth
XXIX Cycle

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PhD Thesis

"New drugs and therapeutic strategies in pharmacoresistant epilepsy"

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Academic Year 2016-2017

INDEX

BACKGROUND AND AIMS

Epilepsy is a brain disorder characterized by an enduring predisposition to generate recurrent epileptic seizures and by the neurobiologic, cognitive, psychological and social consequences of this condition [1].

This disorder affects over 50 million people worldwide and is characterized by significant co-morbidities, unique stigmatization of affected individuals, and high societal cost.

Current antiepileptic drugs provide symptomatic relief from seizures, have multiple adverse effects, and fail to control seizures in up to 30% of people [2]. This represents a major unmet clinical need. New anti-seizure treatments for epilepsy are unlikely to bridge this treatment gap. The next generation of drugs should potentially be able to delay or prevent the onset of epilepsy in susceptible individuals (anti-epileptogenesis) or to halt or reverse its progression and/or improve the neuropathology and the associated comorbidities (disease-modifying) [3]. In order to develop such drugs, there is the need to understand the pathological processes occurring in the brain of people exposed to epileptogenic injuries, or with an established diagnosis of epilepsy.

Role of inflammation in pathophysiology of epilepsy: *from bench to the bedside and viceversa*

Clinical and experimental evidence provided strong support to the hypothesis that inflammatory processes are involved in the pathophysiology of epilepsy [4–7]. In the clinical setting, the first evidence of the potential role of inflammation in human epilepsy were derived from the use of steroids and other anti-inflammatory treatments as anticonvulsant agents in some drug-resistant epilepsies [8,9]. Moreover, chronic brain inflammation, involving activated microglia, astrocytes, endothelial cells of the blood–

brain barrier, peripheral immune cells, and the concomitant production of inflammatory mediators—was observed in patients with Rasmussen encephalitis [10].

Other evidence of the involvement of immune and inflammatory mechanisms in epilepsy came from the high incidence of seizures in autoimmune diseases, and the discovery of limbic encephalitis as a cause of epilepsy [11,12].

Inflammatory mediators have also been implicated as contributors to the onset of febrile seizures (FS) and to the progression from FS, in particular prolonged, to epilepsy, influencing the process of epileptogenesis [13].

Several inflammatory mediators have also been detected in surgically resected brain tissue from patients with temporal lobe epilepsy (TLE) and cortical dysplasia-related epilepsy [4,7]. The finding that brain inflammation occurred in epilepsies not classically linked to immunological dysfunction highlighted the possibility that chronic inflammation might be intrinsic to some epilepsies, irrespective of the initial insult or cause, rather than being only a consequence of a specific underlying inflammatory or autoimmune etiology.

The mounting evidence for a role for inflammatory processes in human epilepsy has led to the use of experimental rodent models to identify putative triggers of brain inflammation in epilepsy, and to provide mechanistic insights into the causal links between inflammation and seizures [4,6,14].

Experimental studies have shown that epileptogenic brain injuries (i.e. status epilepticus, prolonged febrile seizures) can trigger the release of several inflammatory mediators such as cytokines (i.e. interleukin-1β or TNF-α) and "danger signal molecule" (i.e. high-mobility-group box 1) in brain regions, involved in the generation and propagation of epileptic activity [15-17]. These molecules have shown to provide proconvulsant activity and to have a crucial role in the epileptogenesis in various seizure models [15-17].

Notably, the finding that inflammatory events persist during epileptogenesis in experimental models, thus outlasting the initial precipitating event, suggests that inflammatory processes may precede the onset of epilepsy in humans, possibly playing an etiopathogenetic role in the occurrence of spontaneous seizures.

Therefore, an essential and crucial question is whether targeting inflammatory molecules and pathways may result in anti-ictogenesis, anti-epileptogenesis and/or disease-modification effects. Therefore, preclinical testing in models mimicking relevant aspects of epileptogenesis are needed to guide integrated experimental and clinical trial design.

Moreover, experimental models are also needed to study the mechanisms ensuing in the brain during prolonged seizures and status epilepticus (SE). Experimental evidence highlighted that inflammation may play a key role in brain during SE and its pathological long-term consequences [18-21]. SE is a life-threatening condition, operationally defined as ongoing seizures, or repetitive seizures without recovery of baseline clinical conditions in between, lasting for at least 5 min [22].

Also in the therapeutic management of SE, there is an urgent need of novel therapies considering that about one-third of patients with SE still continue seizing despite the first two treatment lines, evolving to refractory SE, and half of these subsequently develop super-refractory SE.

Therefore, the focus of my research was on preclinical characterization and development of novel antiepileptic and disease-modifying treatments in animal models of epilepsy or SE, with a high translational potential for clinical applications.

In particular, my PhD program has been focused on the study of the following lines of research:

-study of the epileptogenic mechanisms in experimental models of epilepsy;

-study of new anti-epileptogenic and disease-modifying drugs targeting oxidative stress in a rat model of acquired epilepsy induced by electrical status epilepticus (SE);

-study of new therapeutic strategies in an experimental model of benzodiazepine-refractory status epilepticus.

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CHAPTER I

"MECHANISMS OF EPILEPTOGENESIS"

1.1 New definition of epileptogenesis

Epileptogenesis is characterized by the development and extension of brain tissue capable of generating spontaneous seizures, thus resulting in the development of an epileptic condition and/or progression of the epilepsy after it is established [1]. Epileptogenesis can be triggered by congenital, genetic or acquired insults, e.g. neurotrauma, stroke, infections, status epilepticus. The current definition takes into consideration the new knowledge related to the cellular, molecular and functional changes occurring in seizure-prone brain areas both before and during the development of epilepsy, as emerging from clinical [2] and basic science investigations in animal models [3-5]. Consequently, the concepts related to epileptogenesis have evolved significantly over the past few years. The complexity of this process has been acknowledged as well as the evidence that epileptogenesis is not a stepwise process rather a continuum of modifications [6]. Moreover, epileptogenesis is not merely limited to the prodromal phase preceding the onset of spontaneous seizures, but also underlies the development of the disease after its diagnosis [1]. This novel view is compatible with clinical evidence reporting that human epilepsy has a progressive course in a significant percent of cases [2]. This concept is not only mechanistically but also therapeutically relevant since it means that epileptogenesis can potentially be targeted also after spontaneous seizures arise. In accord, anti-epileptogenic interventions could be designed not only for preventing the onset of the disease but also for providing seizure modification (i.e. less frequent or shorter seizures, milder seizure type, change from drug-resistant to drug-responsive) and improving the related pathological outcomes (***disease-modifying treatments***). Another relevant aspect

emerging from basic science investigations relates to comorbidities such as anxiety, cognitive deficits and depression, that are often associated with epilepsy [7,8]. There is intense research to understand the mechanisms leading to comorbidities giving insights that they may share common molecular events with the hyperexcitability phenomena underlying seizure generation. Notably, animal models clearly show that these comorbid behaviors often arise before the development of spontaneous seizures indicating that they are not a mere consequence of seizures or the anticonvulsive treatments [9-16].

A consensus definition of epileptogenesis is crucial for designing therapeutic interventions based on specific targets and for searching mechanistic biomarkers of this process that are still lacking. In fact, the development of effective therapies to prevent or treat epileptogenesis remains an urgent unmet clinical need. Clinical trial designs for novel therapeutics able to prevent (anti-epileptogenic) or favorably change the disease course (disease-modifying) are likely to hinge on discovering non-invasive biomarkers that allow early identification of patients at high risk of developing the disorder, as well as patients with a progressive course of the disease, including the development of comorbidities and pharmacoresistance [17]. Both aspects are challenging and rely upon a better understanding of the mechanisms of epileptogenesis and their dynamics during disease development (Figure 1).

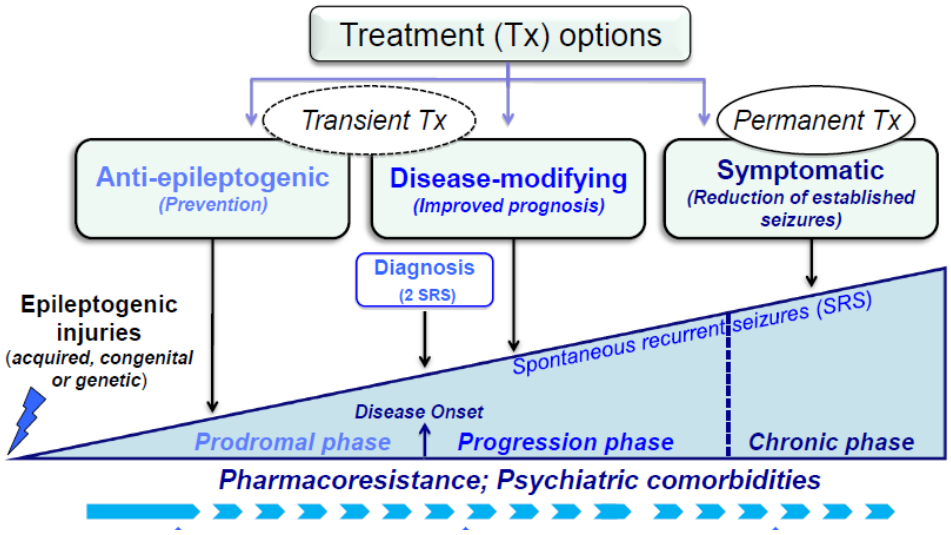

Figure 1. Mechanisms of treatment during epileptogenesis (from Terrone et al, 2016)

1.2 Animal models for studying epileptogenesis and anti-epileptogenic treatments

Animal models of pediatric and adult epilepsies have allowed to study the molecular mechanisms contributing to the complex process of epileptogenesis. The choice of adequate animal models is essential in order to test new treatments capable of preventing or modifying epilepsy after epileptogenic brain injuries. The most widely used models in this respect are kindling, post-SE models of TLE, and models of traumatic brain injury (TBI) [18-20]. In particular, we mainly studied new anti-epileptogenic treatments in two models of TLE obtained by either continuous electrical stimulation of hippocampus in adult male rat or intra-amygdala kainic acid injection in adult male mice.

In the first model, the CA3 area of ventral hippocampus is electrically stimulated unilaterally [21-23] until the develop of a convulsive self-sustained limbic status epilepticus (SSLSE). The following criteria allow the identification of rats experiencing SE and subsequently developing epilepsy: (i) an EEG pattern of uninterrupted bilateral spikes in the hippocampi in the absence of electrical stimulation (i.e. the "stimulus-off" period); -(ii) development of convulsive SE for 60 min during electrical stimulation; (iii)

development of self-sustained convulsive SE for at least 4 h after termination of electrical stimulation. SE remits spontaneously within 24 h from the initial stimulation. It may be not necessary to interfere pharmacologically to stop SE, since no mortality is observed in this model.

In this model, 100% of the animals conforming to the criteria reported above develop electroencephalographic and behavioral spontaneous recurrent seizures (SRS), leading to a diagnosis of epilepsy [24].

The prodromal phase of epileptogenesis lasts about 2 weeks on average; in fact, the onset of the first spontaneous seizure occurs about 14 ± 3 days after the induction of SE [23]. About 80% of epileptic rats develop an increase in SRS frequency during 5 months from epilepsy onset, while no progression in SRS is observed in the remaining 20% of epileptic rats. In the second model, the kainic acid is injected into the basolateral nucleus of the amygdala in adult male mice, leading to SE, lasting about 7 hours on average. After 40 minutes from SE onset, mice received diazepam intraperitoneally to improve their survival rate. The development of spontaneous recurrent seizures occurs after a seizure-free latent period of about 5 days [25]. In addition to these models in which brain injury is induced by SE, antiepileptogenic drug effects can also be studied in genetic animal models of epilepsy and in neonatal models.

However, none of the models used in the search for antiepileptogenic drugs has been validated as yet. In fact, one of the major challenges is represented by the validation of the anti-epileptogenic or disease modifying treatments identified in a specific animal model in an appropriately designed clinical trial.

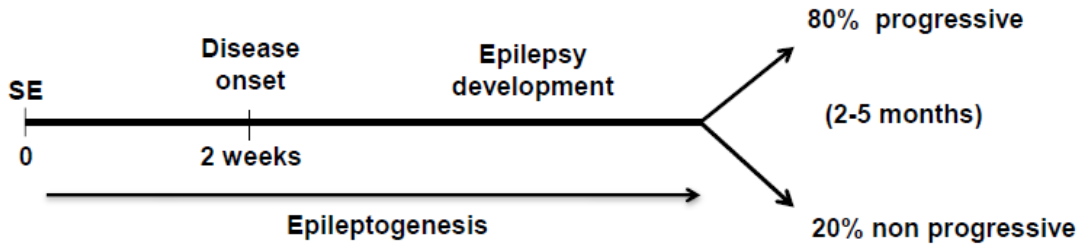

Figure 2. Experimental model of post-SE TLE in adult male rats

1.3 Mechanisms of epileptogenesis

Experimental data suggest that changes in specific molecules and cell signaling pathways after the inciting epileptogenic event show a specific temporal profile of induction, persistence and recovery. Many of these alterations continue to occur after the onset of the first spontaneous seizure and have been validated in human epileptic foci surgically resected from patients affected by various forms of drug-resistant epilepsy. Pharmacological or genetic interventions targeting such molecules and pathways can affect the onset and/or the development and severity of the ensuing epilepsy thus demonstrating their role in the process of epileptogenesis. The most studied and better validated molecules implicated in epileptogenesis include those related to immunity and inflammation [e.g. cytokines and danger signals such as interleukin-1 beta (IL-1β), high mobility group box 1 (HMGB1), cyclooxygenase-2 (Cox-2) and prostaglandins, complement system], trasforming growth factor-beta receptor (TGF-β) signaling activated by albumin in astrocytes following alterations in the blood brain barrier (BBB) permeability, extracellular matrix proteins, adenosine kinase, mTOR pathway (TSC1 and TSC2 gene), neurotrophic kinase receptors such as TrkB receptors activated by BDNF, JAK/STAT signaling pathways, and a variety of acquired and congenital channalopathies

and epigenetic factors. Functional interaction may exist among these molecular pathways suggesting that discovery of nodal points of intersection may facilitate the development of drugs controlling the broad cascade of pathologic events taking place during epileptogenesis [3-5].

This list of potential mechanisms underscores the importance of non neuronal cells such as astrocytes, microglia, and endothelial cells of the BBB, as key contributors to neuronal network dysfunctions leading to seizures and comorbidities [26-27]. Additionally, these molecules represent potential targets for anti-epileptogenesis interventions and potential sources of mechanistic biomarkers of epileptogenesis.

1.4 The role of the hippocampus in epilepsy and epileptogenesis

Most of current assumptions of neuronal mechanisms of epileptogenesis come from the epileptogenic changes that occur in the sclerotic hippocampus, created in animals and reported in patients with TLE [28].

Thus, even if other models of epilepsy, in particular genetic or post-TBI epilepsies, provided the proof that epileptogenic process is not confined to hippocampus, this region remains one of the most important brain area involved in the neuronal hyperexcitability underlying the seizures. Hippocampal region is located in the temporal lobe and consists of the hippocampal formation, which includes Ammon's horn (CA), dentate gyrus and subiculum, and parahippocampal region. The hippocampus receives informations from many associative neocortical areas. These informations come to hippocampus through structures of parahippocampal region, in particular the perirhinal cortex and parahippocampal cortex, which themselves project to the entorhinal cortex. After being processed in the hippocampus, these informations are relayed, via the entorhinal cortex to neocortical areas. Therefore, the entorhinal cortex represents the main input and output

way to the hippocampus. The hippocampus is divided into four sub-fields CA1, CA2, CA3, CA4 -according to density, size and branching of axons and dendrites of pyramidal cells-, and dentate gyrus. Hippocampus has a three-layered structure in contrast to six layers of neocortex, consisting of a molecular layer), cellular or pyramidal layer and polymorphic layer. The dominant neurons in the hippocampus are the pyramidal cells.

The different regions of hippocampus are interconnected through a tri-synaptic circuit, consisting of three defined synaptic relay stations (Figure 4).

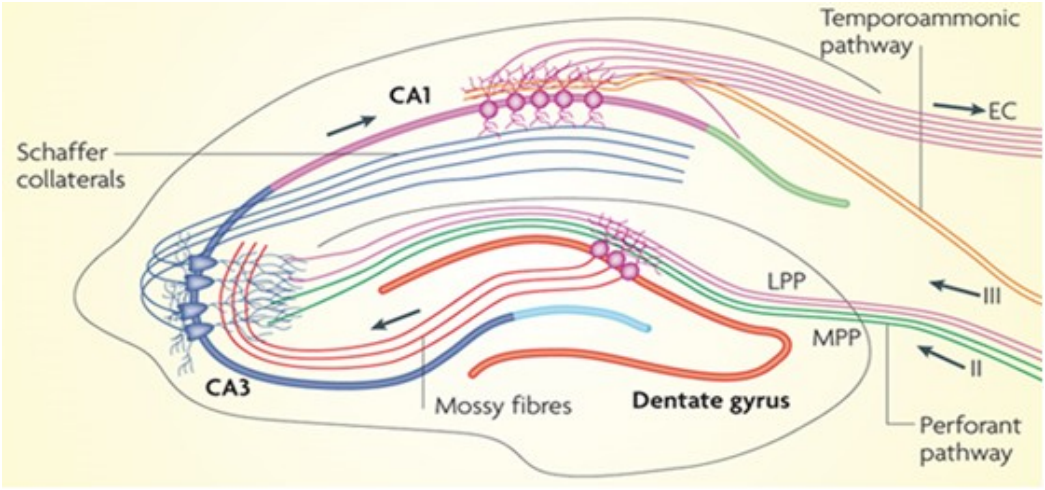

Figure 4. Hippocampal tri-synaptic circuit (Deng et al, 2010)

The information from the entorhinal cortex enters the hippocampus through the axons forming the “perforant pathway". The fibers of the performant pathway form excitatory synapses on the granular cells of the dentate gyrus. The axons of dentate granule cells ("mossy fibers") innervate CA3 pyramidal cells. CA3 neurons synapse onto CA1 pyramidal cells via the "Schaffer collateral pathway”. The output of CA1 goes to and from subiculum and, finally, back to the entorhinal cortex. The unidirectional flow of this circuit is broken only by the occasional synapses between the apical dendrites of CA3 and CA1 neurons, and fibers of the perforant pathway. The main neurotransmitter of the hippocampal formation is the glutamate, and the massive presence of excitatory synapses

explains the vulnerability of this brain area to seizures. In addition to glutamate, the inhibitory neurotransmitter acid-γ-aminobutyric acid (GABA) plays a pivotal role in the dentate gyrus, controlling the activities of the main excitatory cells. Several studies demonstrated the co-localization of the GABA with different calcium-binding proteins, such as parvalbumin (PV), calretinin (CR) and somatostatin (SST). In particular, the hilum of the dentate gyrus is characterized by the presence of three populations of inhibitory interneurons: the subgranular basketcells (parvalbumin-positive), the cells of the polymorphic layer (somatostatin-positive) and the mossy cells (calretinin-positive), GABAergic interneurons which establish numerous synaptic contacts axon-dendritic and dendro-dendritic with other inhibitory interneurons, which in turn communicate with excitatory cells of the granule layer [29]. The excitation level of granule cells is critical to determine the excitability of the hippocampus, and consequently of the limbic system. The degeneration of specific populations of hilar interneurons of the dentate gyrus that control the excitability of granule cells, and subsequent molecular, synaptic and cellular modifications, appear to significantly contribute to the development of epilepsy in experimental models. A wide range of molecular changes also independent of neuronal damage are involved in the epileptogenesis [30].

It still remains to be clarified which are the molecular and biochemical mechanisms necessary for the establishment of a neuronal hyperexcitability substrate and which is the cascade of events triggered by a epileptogenic event that is relevant to the generation and occurrence of spontaneous seizures.

1.5 Inflammatory mechanisms as potential targets of antiepileptogenic treatments

Whether different etiological factors induce distinct or overlapping epileptogenic mechanisms remains unknown, however, the search for such pathogenic mechanisms in

animal models has shown that neuroinflammation in seizure-prone brain regions is a common feature of various forms of drug-resistant symptomatic epilepsies in humans and animal models [31, 32]. Neuroinflammation is determined by the synthesis and release of pro-inflammatory molecules with neuromodulatory properties by glia, neurons and the BBB endothelium, in the context of innate immunity activation. *De novo* status epilepticus and other potential epileptogenic injuries (e.g. neurotrauma, stroke, CNS infections, gene mutations, i.e., GAERS rats with spike-and-waive discharges [33] or cystatin B mutation in a model of progressive myoclonus epilepsy [34]) trigger neuroinflammation, which therefore represents a consistent feature of epileptogenesis irrespective of the initial insult. Which are the triggering factors of neuroinflammation during epileptogenesis, and whether common factors ignite inflammation following differing epileptogenic injuries are still open questions. Basic knowledge of the mechanisms of inflammation by innate immunity activation, and experimental findings in models of seizures and epileptogenesis, suggest that one pivotal generator of neuroinflammation in epileptogenesis is likely to be the activation of toll-like receptors (TLR) by endogenous ligands, namely the alarmins/danger signals [35, 36].

These are endogenous molecules constitutively available that are released by brain cells and leukocytes upon tissue injury. One of such molecules is High Mobility group Box 1 (HMGB1) which is released by various epileptogenic injuries and is induced in human brain tissue from various forms of pharmacoresistant epilepsy [35, 36]. The activation and assembly of the inflammasome, a multiprotein complex which includes the cysteine protease ICE/caspase-1, is required for the biosynthesis and release of both the biologically active form of IL-1β and HMGB1. Extracellular ATP-mediated stimulation of P2X7 receptors and reactive oxygen species are powerful inducers of ICE/Caspase-1, and they are both commonly produced during epileptogenesis [35, 36].

Notably, it is well established that neuroinflammatory molecules such as IL-1beta, TNF-alpha, PGE2 and the complement system contribute to ictogenesis by promoting neuronal network hyperexcitability *via* activation of specific transcriptional and post-translational molecular mechanisms in neurons and glia, thereby reducing seizure threshold and promoting seizure generation and recurrence [35, 36]. In accord, anti-inflammatory treatments are anticonvulsive in some human pediatric and adult epilepsies [31, 37], and specific anti-inflammatory drugs significantly reduce acute symptomatic seizures and chronic spontaneous seizures in animal models of existing epilepsy [31, 37]. Additionally, target-specific anti-inflammatory treatments in SE models are providing increasing evidence of anti-epileptogenic and neuroprotective effects, thus supporting that neuroinflammation is also implicated in epileptogenesis [38, 39]. In general, anti-inflammatory drugs transiently given after the epileptogenic insult are unable to prevent the onset of epilepsy but significantly improve pathological outcomes. Typically, the frequency and severity of spontaneous seizures are significantly reduced, and in several instances anti-inflammatory drugs afford neuroprotection by decreasing the number of degenerating neurons, and comorbidities are often improved, in particular cognitive deficits. Interestingly, anti-inflammatory drug combinations showed improved therapeutic value as compared to drugs given alone [37]. This evidence suggests that the complex and reverberant inflammatory cascade activated during epileptogenesis may require a concomitant blockade of independent pathways for efficient interference with epileptogenesis. A few therapeutic attempts have been developed so far early after the onset of spontaneous seizures [40] providing proof-of-concept evidence that a delayed anti-inflammatory intervention may improve the disease prognosis.

1.6 Biomarkers of epileptogenesis

A biomarker for epileptogenesis is an objectively measurable characteristic of a biological process that reliably identifies the development, presence, severity, progression, or localization of an epileptogenic abnormality [1,17]. The validation of non-invasive, and easy to measure, biomarkers would be of great utility to monitor epileptogenesis, thus facilitating the screening of potential antiepileptogenic treatments in animal models.

Moreover, such biomarkers, particularly if measurable in blood or body fluids, may allow the stratification of patients at high risk of developing epilepsy after a potential epileptogenic injury thereby enriching the patient population eligible for clinical trials and making such trials affordable.

Research on basic mechanisms of epileptogenesis in animal models has provided a list of potential biomarkers ranging from those derived from genetic analyses to circulating, imaging (MRI, PET), electrophysiological or behavioral biomarkers [16, 17].

Biomarker discovery would strongly support the initiation of future clinical trials, and together with testing potential novel drugs would provide a major advance in the treatment of human epilepsy [41]

1.7 Conclusion

The development of new drugs preventing epilepsy and improving its prognosis requires a deep understanding of pathogenic mechanisms underlying the disease. Importantly, considering the complex pathophysiological underpinnings of the epileptogenesis, it is likely necessary to interfere with this process at multiple levels and in various cell types.

However, neuroinflammation demonstrated to have a pivotal role in seizure pathogenesis and so therapeutic approaches targeting inflammatory mechanisms could represent novel strategies for drug development in epilepsy.

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1.9 Publications

Pharmacological Research 110 (2016) 96–100

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Perspective

Preventing epileptogenesis: A realistic goal?

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ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form 2 May 2016
Accepted 5 May 2016
Available online 10 May 2016

Keywords:
Experimental models of epileptogenesis
Disease-modifications
Biomarkers

ABSTRACT

The definition of the pathologic process of epileptogenesis has considerably changed over the past few years due to a better knowledge of the dynamics of the associated molecular modifications and to clinical and experimental evidence of progression of the epileptic condition beyond the occurrence of the first seizures. Interference with this chronic process may lead to the development of novel preventive therapies which are still lacking. Notably, epileptogenesis is often associated with comorbid behaviors which are now considered primary outcome measures for novel therapeutics. Anti-epileptogenic interventions may improve not only seizure onset and their frequency and severity but also comorbidities and cell loss, and when applied after the onset of the disease may provide disease-modifying effects by favorably modifying the disease course. In the preclinical arena, several novel targets for anti-epileptogenic and disease-modifying interventions are being characterized and validated in rodent models of epileptogenesis. To move proof-of-concept anti-epileptogenesis studies to validation in preclinical trials and eventually to clinical translation is a challenging task which would be greatly facilitated by the development of non invasive biomarkers of epileptogenesis. Biomarker discovery together with testing potential novel drugs would provide a major advance in the treatment of human epilepsy beyond the pure symptomatic control of seizures.

1. Introduction

1.1. New definition of epileptogenesis

Epileptogenesis is characterized by the development and extension of brain tissue capable of generating spontaneous seizures, thus resulting in the development of an epileptic condition and/or progression of the epilepsy after it is established [1]. Epileptogenesis can be triggered by congenital, genetic or acquired insults, such as neurotrauma, stroke, infections, status epilepticus. The current definition takes into consideration the new knowlegde related to the cellular, molecular and functional changes occurring in seizure-prone brain areas both before and during the development of epilepsy, as emerging from clinical [2] and basic science investigations in animal models [3–5]. Consequently, the concepts related to epileptogenesis have evolved significantly over the past few years. The complexity of this process has been acknowledged as well as the evidence that epileptogenesis is not a stepwise process rather a *continuum* of modifications [6]. Moreover, epileptogenesis is not merely limited to the prodromal phase preceding the onset of spontaneous seizures, but also underlies the development of the disease after its diagnosis [1]. This concept has been perceived by William Gowers in 19th century that empirically set that "seizures beget seizures".

This novel understanding of epileptogenesis is compatible with clinical evidence reporting that human epilepsy has a progressive course in a significant percent of cases [2]. This concept is not only mechanistically but also therapeutically relevant since it means that epileptogenesis can potentially be targeted also after spontaneous seizures arise. In accord, anti-epileptogenic interventions could be designed not only for preventing the onset of the disease after risk factors are evaluated but also for improving the related pathological outcomes after the disease is diagnosed (i.e., disease-modifying treatments). Another relevant aspect emerging from basic science investigations relates to comorbidities such as anxiety, cognitive deficits and depression, that are often associated with epilepsy [7,8]. There is intense research to understand the mechanisms leading to comorbidities giving insights that they may share common molecular events with the hyperexcitability phenomena underlying seizure generation. Notably, animal models clearly show that these comorbid behaviours often arise before the development of spontaneous seizures indicating that they are not

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http://dx.doi.org/10.1016/j.phrs.2016.05.009
1043-6618/

a mere consequence of seizures or the anticonvulsive treatments [9–16].

1.2. Mechanistic and therapeutic implications

A consensus definition of epileptogenesis is crucial for designing therapeutic interventions based on specific targets and for searching mechanistic biomarkers of this process that are still lacking. In fact, the development of effective therapies to prevent or treat epileptogenesis remains an urgent unmet clinical need. Clinical trial designs for novel therapeutics able to prevent (anti-epileptogenic) or favorably change the disease course (disease-modifying) are likely to hinge on discovering non-invasive biomarkers that allow early identification of patients at high risk of developing the disorder, as well as patients with a progressive course of the disease, including the development of comorbidities and pharmacoresistance [17]. Both aspects are challenging and rely upon a better understanding of the mechanisms of epileptogenesis and their dynamics during disease development. In this frame, one should consider that comorbidity may not be eliminated by either the anti-epileptogenic or disease-modifying treatment unless the treatment is targeted against the common mechanistic pathway for the disease and comorbidities. Yet, once the mechanistic pathway of comorbidity (after being triggered) splits from that of the disease, no efficacy of either treatment should be expected.

2. Mechanisms of epileptogenesis and anti-epiletogenesis strategies

A large number of animal models of pediatric and adult epilepsies has become available for studying the molecular mechanisms contributing to epileptogenesis [18]. The complexity of epileptogenesis, and its potential heterogeneity in the human condition, make these investigations and their clinical translatability very challenging. Several recent reviews report in detail which mechanisms have been demonstrated to contribute to epileptogenesis in animal models [3–5,19]. These studies describe changes in specific molecules and cell signaling pathways after the inciting epileptogenic event with differing temporal profile of induction, persistence and recovery. Many of these alterations continue to occur after the onset of spontaneous seizures and have been validated in human epileptic foci surgically resected from patients affected by various forms of drug-resistant epilepsy. Pharmacological or genetic interventions targeting such molecules and pathways can affect either the onset and/or the development and severity of the ensuing epilepsy, thus demonstrating their role in the process of epileptogenesis. The most studied and better validated molecules implicated in epileptogenesis include those related to: immunity and inflammation (e.g. cytokines and danger signals such as IL-1beta, HMGB1 and TNF-alpha, Cox-2 and prostaglandins, complement system), TGF-beta signaling activated by albumin in astrocytes following alterations in the blood brain barrier (BBB) permeability, extracellular matrix proteins, adenosine kinase, mTOR, neurotrophic kinase receptors such as TrkB receptors activated by BDNF, JAK/STAT signaling pathways, and a variety of acquired and congenital channalopathies and epigenetic factors (Fig. 1). Some level of functional interaction exists among these molecular pathways suggesting that discovery of nodal points of intersection may facilitate the development of drugs controlling the broad cascade of pathologic events taking place during epileptogenesis. We refer to previous reviews for more details [3–5,19].

This incremental list of potential mechanisms underscores the importance of non neuronal cells such as astrocytes, microglia, and endothelial cells of the BBB, as key contributors to neuronal network dysfunctions leading to seizures and comorbidities [20,21]. Additionally, these molecules represent potential targets for anti-epileptogenesis interventions and potential sources of mechanistic biomarkers of epileptogenesis.

2.1. The focus on inflammatory mechanisms as potential targets

Because of our expertise in the field of immunity and inflammation, and considering that an exhaustive literature review of the preclinical anti-epiletogenesis interventions is not the focus of this article, we will report here about specific anti-inflammatory treatments in animal models of epileptogenesis, as an example of anti-epileptogenesis approaches [19]. Whether different aetiological factors induce distinct or overlapping epileptogenic mechanisms remains unknown, however, the search for such pathogenic mechanisms in animal models has shown that neuroinflammation in seizure-prone brain regions is a common feature of various forms of drug-resistant symptomatic epilepsies in humans and animal models [22,23]. Neuroinflammation is determined by the synthesis and release of pro-inflammatory molecules with neuromodulatory properties by glia, neurons and the BBB endothelium, in the context of innate immunity activation. *De novo* status epilepticus and other potential epileptogenic injuries (e.g. neurotrauma, stroke, CNS infections, gene mutations, i.e., GAERS rats with spike-and-waive discharges [24] or cystatin B mutation in a model of progressive myoclonus epilepsy [25]) trigger neuroinflammation, which therefore represents a consistent feature of epileptogenesis irrespective of the initial insult.

Which are the triggering factors of neuroinflammation during epileptogenesis, and whether common factors ignite inflammation following differing epileptogenic injuries are still open questions. Basic knowledge of the mechanisms of inflammation by innate immunity activation, and experimental findings in models of seizures and epileptogenesis, suggest that one pivotal generator of neuroinflammation in epileptogenesis is likely to be the activation of toll-like receptors (TLR) by endogenous ligands, namely the alarmins/danger signals [26,27]. These are endogenous molecules constitutively available that are released by brain cells and leukocytes upon tissue injury. One of such molecules is High Mobility group Box 1 (HMGB1) which is released by various epileptogenic injuries and is induced in human brain tissue from various forms of pharmacoresistant epilepsy [22,23,26,27]. The activation and assembly of the inflammasome, a multiprotein complex which includes the cysteine protease ICE/caspase-1, is required for the biosynthesis and release of both the biologically active form of IL-1beta and HMGB1. Extracellular ATP-mediated stimulation of P2X7 receptors and reactive oxygen species are powerful inducers of ICE/Caspase-1, and they are both commonly produced during epileptogenesis [26,27].

Notably, it is well established that HMGB1 as well as other neuroinflammatory molecules such as IL-1beta, TNF-alpha, PGE2 and the complement system, contribute to ictogenesis by promoting neuronal network hyperexcitability *via* activation of specific transcriptional and post-translational molecular mechanisms in neurons and glia, thereby reducing seizure threshold and promoting seizure generation and recurrence [26,27]. In accord, anti-inflammatory treatments are anticonvulsive in some human pediatric and adult epilepsies [23,28], and specific anti-inflammatory drugs significantly reduce acute symptomatic seizures and chronic spontaneous seizures in animal models of existing epilepsy [23,28]. Additionally, target-specific anti-inflammatory treatments in SE models are providing increasing evidence of anti-epileptogenic and neuroprotective effects, thus supporting that neuroinflammation is implicated in epileptogenesis [19,29–32]. Table 1 reports a list of successful anti-inflammatory interventions tested as preventive treatments in animal models of status epilepticus-induced epileptogenesis. In general,

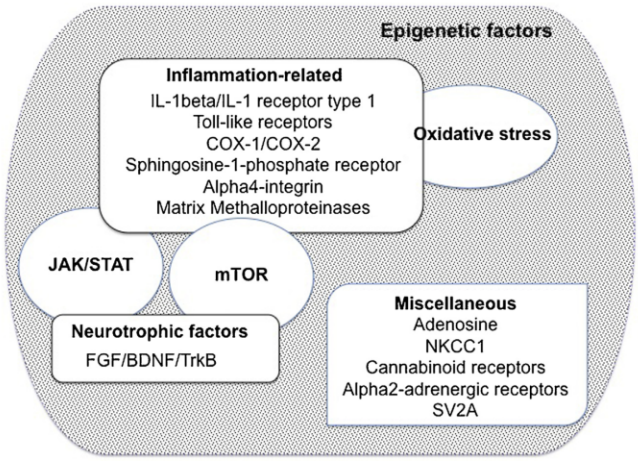

Fig. 1. Classes of molecular targets involved in the mechanisms of epileptogenesis in animal models.
Pharmacological or genetic interventions on these molecular pathways individually showed partial therapeutic effects on seizures, cell loss and comorbities in animal models of acquired or genetic epilepsies, thus highlighting their potential involvement in epileptogenesis. Some level of functional interaction exists among these molecular pathways suggesting that discovery of nodal points of intersection may allow the development of drugs controlling the broad cascade of pathologic events taking place during epileptogenesis. Combination of drugs rather than individual treatments might also maximize the chances of therapeutic success. Commonalities or divergence of mechanisms among different epileptogenesis insults in animal models may also help to optimize treatments for aetiology-specific anti-epileptogenesis treatments and biomarkers discovery.

Table 1
Treatments with anti-inflammatory properties inducing disease-modifications in animal models of epileptogenesis ensuing after status epilepticus.

Treatment (*action*):	Molecular target:	Reduction in:
Celecoxib (*enzyme inhibitor*)	COX-2	SRS frequency/duration, cell loss [42]; pharmacoresistance [43]
Parecoxib (*enzyme inhibitor*)	COX-2	Cell loss [44]
Aspirin (*enzyme inhibitor*)	COX1/2	SRS frequency/duration; cell loss [45]
α4-integrin-specific Ab (*receptor antagonist*)	Adhesion molecules; brain vessels	SRS frequency; anxiety [46]
Fingolimod (*receptor antagonist*)	S1P receptor	SRS frequency/duration/severity; cell loss [47]
Minocyclin (*microglia inhibitor*)	Cytokines	Cognitive deficits; cell loss [48] SRS frequency/duration/severity; cell loss [49]
Anakinra + VX-765 (*receptor antagonist + enzyme inhibitor*)	IL-1R1 + ICE	Cell loss [31]
Anakinra + COX-2 (*receptor antagonist + enzyme inhibitor*)	IL-1R1 + COX-2	SRS frequency; cell loss [29]
TLR4 + VX-765* (*receptor antagonist + enzyme inhibitor*)	TLR4 + ICE/caspase-1	SRS frequency & their progression; cognitive deficits; cell loss [33]
miRNA146a (*epigenetic antagonism*)	IL-1R1 + TLR4 signaling	SRS frequency & their progression; cognitive deficits; cell loss [33]
Nrf2 gene therapy* (*transcription factor*)	Oxidative stress	SRS frequency/duration; cell loss [34]
PGE2 (*antagonist*)	EP2 receptor	Cell loss [50]
Erythropoietin (*agonist*)	Erythropoietin receptor	SRS frequency/duration; cell loss; [51]; cognitive deficits; cell loss [52]

Treatments were given transiently after status epilepticus onset or after disease onset (*) (*refer to original manuscript reference for details of treatment schedule*). Main outcome measures showed reduction of spontaneous seizure burden and/or neuroprotection (reduced cell loss); comorbidities were also improved in some study. A COX-2 inhibitor, SC58236, was found to be ineffective on epileptogenesis [53]. Minozac, an inhibitor of astrocytes activation, has been shown to raise seizure threshold and improve comorbidites in a model of post-traumatic epilepsy [54]. In several instances the cell types potentially involved in the therapeutic effects have been identified as neurons, glia and endothelial cells of the BBB. ICE, interleukin converting enzyme; IL-1R1, IL-1 receptor type 1; SRS, spontaneous recurrent seizures; PGE2, prostaglandin E2; COX, cyclooxygenase; S1P, sphingosine-1 phosphate; TLR, toll-like receptor. *For details see* [1,55].

anti-inflammatory drugs transiently given after the epileptogenic insult are unable to prevent the onset of epilepsy but significantly improve pathological outcomes. Typically, the frequency and severity of spontaneous seizures are significantly reduced, and in several instances anti-inflammatory drugs afford neuroprotection by decreasing the number of degenerating neurons, and comorbidities are often improved, in particular cognitive deficits. Interestingly, anti-inflammatory drug combinations showed improved therapeutic value as compared to drugs given alone [29,33]. This evidence suggests that the complex and reverberant inflammatory cascade activated during epileptogenesis may require a concomitant blockade of independent pathways for efficient interference with epileptogenesis. A few therapeutic attempts have been developed so far early after the onset of spontaneous seizures [33,34] providing proof-of-concept evidence that a delayed anti-inflammatory intervention may improve the disease prognosis.

2.2. General considerations on preclinical anti-epileptogenesis therapies

In most instances, single treatments directed against differing targets when applied during the prodromal epileptogenesis phase in animal models may significantly improve the pathologic outcomes but do not prevent or cure the disease. Whether this is due to non optimal treatment schedules or the need of drug combinations for prevention and cure to be achieved is presently unknown. System biology approaches may help to determine the nodal points within the epileptogenic network which may be targeted for

controlling the broad cascade of pathologic events contributing to epileptogenesis [35,36].

In general, when targeting a potential epileptogenic mechanism, whether this is inflammatory or of different nature, it is important to understand how reverberant this pathway is, and its potential contribution to tissue homeostasis. Ideally, the intervention should be designed in order to preserve the homeostatic effects while preventing the deleterious ones from occurring. To deal with this level of complexity requires to gain a deep understanding of both dynamic changes of the target of interest and its pathophysiological role in the various epileptogenesis stages (before disease onset, early after diagnosis, during progression of the disease and at the chronic stage). This knowledge is instrumental for defining the best therapeutic window for intervention and maximize the chances of therapeutic success.

3. The need of biomarkers

A biomarker of epileptogenesis is defined as an objective measure of a specific underlying pathologic process, that could predict the development of an epileptic condition, or to identify the presence and extension of the epileptogenic tissue, or to measure the progression of the disease after its diagnosis, and possibly predict the therapeutic response to drugs [1,17].

The validation of non-invasive, and easy to measure, biomarkers would be of great utility to monitor epileptogenesis, thus facilitating the screening of potential anti-epileptogenic treatments in animal models. Moreover, such biomarkers, particularly if measurable in blood or body fluids, may allow the stratification of patients at high risk of developing epilepsy after a potential epileptogenic injury thereby enriching the patient population eligible for clinical trials and making such trials affordable. Objective surrogate markers (i.e., a laboratory measurement or physical sign that is used in therapeutic trials as a substitute for a clinically meaningful endpoint (epileptic seizures) and is expected to predict the effect of the therapy) may also be considered while establishing reliable biomarkers of the epileptic process.

Ideally biomarkers of epileptogenesis should be sensitive and specific, and in view of the complexity and possible heterogeneity of this process in epilepsies of differing aetiologies, it is likely that a combination of various biomarkers is required. Preclinical and clinical research have been characterizing and validating different types of biomarkers ranging from those derived from genetic analyses to circulating, imaging (MRI, PET) or electrophysiological biomarkers [17]. Behavioral biomarkers have also recently emerged as potential means to predict epilepsy in animal models [16,37]. In this context, the use of *in vivo* models where only a cohort of animals develop epilepsy, in spite of being all exposed to a similar injury, is instrumental for biomarker discovery and validation [16,38].

Importantly, the final validation will relay upon comparison of biomarker platforms between prospective clinical studies in cohorts of patients exposed to potential epileptogenic injures and the corresponding animal models.

4. Conclusions

Is prevention of epileptogenesis a realistic goal? Although the challenges to be faced at both preclinical and clinical level should not be underestimated, they should not preclude continuous research in this area. Current antiepileptic drugs are providing only symptomatic control of seizures, have multiple adverse effects, and fail to control seizures in up to 40% of people [39]. Therefore, next generation of therapies for epilepsy needs to target the mechanisms intimately involved in epileptogenesis to allow the development of preventive or disease-modifying treatments. Several preclinical proof-of-concept studies provided evidence for positive treatment effects on epileptogenesis (Fig. 1 and Table 1). In some instances, compound repurposing has also been proposed for targeting molecular pathways with molecules approved to treat other diseases or conditions (e.g., nonsteroidal anti-inflammatory drugs; Losartan, a TGF-beta receptor antagonist; Kineret, the human recombinant IL-1 receptor antagonist; VX-765, a ICE/caspase-1 inhibitor; Ifenprodil, a NR2B-NMDA receptor antagonist; Bumetanide, NKCC1 blocker; Levetiracetam, SV2A blocker; Fig. 1); in this scenario the translational path to the clinic may be facilitated. Several questions, however, remain open: for example, if novel drugs should target final common pathways in epileptogenesis (*master regulators*) or whether combinations of drugs (rather than monotherapy) should be considered for anti-epileptogenesis. The identification of the optimal time window for intervention is also crucial for therapeutic success.

One critical step is to move from proof-of-concept anti-epileptogenesis studies to validation in preclinical trials, and eventually to clinical translation. In this context, the ILAE and AES joint efforts of the Working Group on "Issues related to development of anti-epileptogenic therapies" has considered the possible problems that arise when moving from preclinical to clinical settings, and provided recommendations on how to best design the experimental studies for increasing result robustness [40,41].

Undoubtedly, biomarker discovery would strongly support the initiation of future clinical trials, and together with testing potential novel drugs would provide a major advance in the treatment of human epilepsy [17].

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

We thank European Union Seventh Framework Programme (FP7/2013-2017) under grant agreement no. 602102 (EPITARGET; AV), Fondazione Monzino (AV) and CURE (AV).

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Epilepsia
Official Journal of the International League Against Epilepsy

Neuroinflammatory targets and treatments for epilepsy validated in experimental models

Journal:	*Epilepsia*
Manuscript ID	EPI-00063-2017
Manuscript Type:	Supplement Article
Date Submitted by the Author:	20-Jan-2017
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Key Words:	immunity, anti-ictogenesis, anti-epileptogenesis, disease-modification, inflammation and drug development

Chapter 3

Neuroinflammatory targets and treatments for epilepsy validated in experimental models

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Abstract

A large body of evidence that has accumulated over the past decade strongly supports the role of inflammation in the pathophysiology of human epilepsy. Specific inflammatory molecules and pathways have been identified that influence various pathologic outcomes in different experimental models of epilepsy. Most importantly, the same inflammatory pathways have also been found in surgically resected brain tissue from patients with treatment-resistant epilepsy. New anti-seizure therapies may be derived from these novel potential targets. An essential and crucial question is whether targeting these molecules and pathways may result in anti-ictogenesis, anti-epileptogenesis and/or disease-modification effects. Therefore, preclinical testing in models mimicking relevant aspects of epileptogenesis is needed to guide integrated experimental and clinical trial designs. We discuss the most recent preclinical proof-of- concept studies validating a number of therapeutic approaches against inflammatory mechanisms in animal models that could represent novel avenues for drug development in epilepsy. Finally, we suggest future directions to accelerate preclinical to clinical translation of these recent discoveries.

KEY WORDS: Inflammation, Immune response, Drug development, Anti-ictogenesis, Anti-epileptogenesis, Disease-modification, Epilepsy

Introduction

There is an urgent need for the development of new drugs for patients with drug-resistant epilepsy. In particular, anti-epileptogenesis or disease-modifying therapies for preventing or delaying the onset of the disease are still missing. In the last decade an increasing body of clinical and experimental evidence supports the role of inflammation in the pathophysiology of epilepsy.[1] In particular, specific inflammatory molecules and pathways have been shown to significantly contribute to the mechanisms of seizure generation and progression in different experimental models.[2-6]

Here, we summarize presentations concerning targets and treatments validated in experimental models [i.e., Cyclooxygenase-2 (COX-2), Prostaglandin (PG) EP2 receptor, Monoacylglycerol lipase (MAGL), Interleukin-(IL)1β, High Mobility Group Box 1 (HMGB1)/Toll-like receptor (TLR) signaling, P2X7 receptor, Immunoproteasome, Mammalian target of rapamycin (mTOR), Transforming Growth Factor-β, Metalloproteinases, Chemokines; **Fig. 1**]. We will discuss strategies related to target validation, controversies and open questions that may hopefully help to improve research efforts for novel drug development and foster more coordinated experimental and clinical trial designs.

COX-2, PGE2 and MAGL

(W. Löscher, R. Dingledine, G. Terrone)

The enzyme COX-2, which catalyzes the production of prostaglandins, is induced rapidly and strongly in the brain after various insults, thus contributing to brain inflammatory processes involved in the long-term consequences of such insults. The use of selective COX-2 inhibitors has been considered as a novel approach for anti-epileptogenesis or disease-modification after brain injuries such as head trauma, cerebral ischemia or status epilepticus (SE).[7] Accordingly, conditional ablation of COX-2 limited to forebrain neurons is neuroprotective and reduces brain inflammation after SE.[8] Löscher reported the conflicting results related to prophylactic administration of different COX-2 inhibitors after SE in rodents. Jung et al. showed that prolonged administration of the COX-2 inhibitor celecoxib after pilocarpine-induced SE in rats

prevents neuronal damage in the hippocampus and reduces incidence, frequency and duration of spontaneous recurrent seizures, indicating an anti-epileptogenic or disease-modifying effect in this post-SE model of temporal lobe epilepsy.[9] In apparent contrast to the study of Jung et al.[9], Holtman et al.[10] did not find any evidence of anti-epileptogenic, disease-modifying or neuroprotective effects of prolonged treatment with another COX-2 inhibitor, SC58236, after electrically-induced SE. Similar experiments were performed by Polascheck et al.[11] , using parecoxib, a prodrug of the highly potent and selective COX-2 inhibitor valdecoxib, because celecoxib has been reported to exert also COX-2 independent actions. Using an electrical model of SE, Löscher and colleagues partially confirmed the findings of Jung et al.[9] in the pilocarpine model, thus allowing to evaluate which experimental factors may affect the outcome of such studies. Prophylactic treatment with parecoxib prevented the SE-induced increase in PGE2 and reduced neuronal damage in the hippocampus and piriform cortex. However, the incidence, frequency or duration of spontaneous seizures developing after SE or the behavioral and cognitive alterations associated with epilepsy was not affected by parecoxib. Only the severity of spontaneous seizures was reduced, suggesting a disease-modifying effect. These results substantiated that COX-2 contributes to neuronal injury developing after SE, but inhibition of COX-2 does not appear to provide an effective strategy to modify epileptogenesis. In view of the complexity of the inflammatory alterations after brain insults such as SE, it is more likely that a multi-targeted combination of different anti-inflammatory drugs is needed for efficient control of the pathologic inflammatory cascade. Indeed, Kwon et al. studied combinations of anti-inflammatory drugs in the pilocarpine model in developing rats (2-3 weeks of age) and found that none of these drugs affected epilepsy when administered alone, whereas the blockade of IL-1 receptor type 1 (IL-1R1) using anakinra and the concomitant COX-2 inhibition greatly reduced the development of spontaneous recurrent seizures and limited the extent of CA1 injury and mossy fiber sprouting.[12] However, treatment started shortly before SE induction, so that it is not clear whether the effects of treatment were due to initial insult modification or to genuine antiepileptogenesis. More recently, Citraro et al. reported that early long-term treatment with the COX-2 selective inhibitor etericoxib reduced by ~40% the

development of spontaneous absence seizures in WAG/Rij rats, a recognized animal model of absence epilepsy.[13] The latter finding could either indicate that COX-2 is more critical in epileptogenesis underlying absence epilepsy than in temporal lobe epilepsy or, more likely, post-SE models of epilepsy are too severe to disclose anti-epileptogenic effects of COX-2 inhibitors.

Notably, several COX-2 inhibitors have been withdrawn from the market due to cardiac events.[14] Therefore, an alternative strategy to increase target selectivity with milder adverse event profile, might be achieved by a drug acting on a downstream prostanoid receptor. Because PGE2 is a major product of COX-2 in the brain, and two of its receptors, EP1 and EP2, have been implicated in neuronal injury and inflammation in other disorders[15], Dingledine and colleagues focused on these two prostanoid receptors. A higher dose of systemic kainate was required to produce SE in EP1 knockout mice (KO) compared to wild-type (WT) controls, but EP1 KO mice that did experience typical SE had reduced hippocampal neurodegeneration and a blunted inflammatory response.[16] Co-expression of recombinant EP1 and kainate receptors in cell cultures demonstrated that EP1 receptor activation potentiates heteromeric but not homomeric kainate receptors via a second messenger cascade involving phospholipase C, calcium and protein kinase C. Turning to EP2, Dingledine and colleagues used high throughput screening and medicinal chemistry to develop a small molecule EP2 competitive antagonist that is potent (Schild Kb=18 nM for human EP2), selective (10 to >500-fold over other prostanoid receptors as well as 38 other receptors, enzymes and channels), brain-permeant (brain/plasma = 0.8-1.6), orally available and with sufficient plasma half-life (1.6-2.5 hr) in mouse and rat to be useful for *in vivo* studies.[17-19] Brief (2-3 days) treatment with the EP2 antagonist beginning 2-4 h after SE onset, during the time in which neuronal COX-2 is induced, appeared to fully replicate the effects of neuron-selective conditional COX-2 ablation. Thus, mice or rats treated with the EP2 antagonist showed neuroprotection, less neuroinflammation and blood-brain barrier (BBB) permeability function was preserved. Moreover, the typical development of a deficit in the novel object recognition test was prevented in rats treated briefly with the EP2 antagonist after SE onset.[20] This suggests that much of the morbidity associated with COX-2 induction after SE is

mediated by the activation of EP2, and possibly EP1, by COX-2 derived PGE2. Delayed inhibition of EP2 could therefore represent a viable adjunctive treatment for alleviating the deleterious consequences of SE. In studies of cultured microglia, EP2 has been shown to act as an immunomodulator of microglial activation, with much of its effects being mediated through ePAC (cAMP-regulated guanine nucleotide exchange factors) rather than protein kinase A.[21,22] Excessive EP2 activation can eventually kill activated microglia *in vitro* and thus might participate in resolution as well as generation of inflammation.

Terrone and colleagues provided evidence supporting that MAGL is a potential target for drug development in epilepsy, in particular for the treatment of drug-refractory SE.[23] MAGL is a key enzyme in the hydrolysis of the endocannabinoid 2-arachidonoylglycerol (2-AG) and represents the major brain source of arachidonic acid (AA) and eicosanoids.[24] They showed that the severity and duration of benzodiazepine-refractory SE, and the consequent cell loss and cognitive deficits, were significantly reduced in mice treated with a potent and selective irreversible MAGL inhibitor, and these therapeutic effects were potentiated by the ketogenic diet (KD). Notably, when MAGL was inhibited in KD-fed mice, SE was virtually abolished with immediate effect. Moreover, KD itself, although not affecting SE, prevented the hippocampal cell loss. Based on these positive results, they are further evaluating the mechanisms of therapeutic action of MAGL inhibition to determine if it is mainly mediated by 2-AG accumulation, thereby activating CB1 receptors, or by reduction of arachidonic acid availability to COX-2, thus providing anti-inflammatory effects (*unpublished observations*).

IL-1R1//TLR4 signaling, microRNAs, P2X7 receptors and immunoproteasome

(A. Vezzani, S. Bauer, B. A. Norwood, E. Aronica, D. Henshall, M. Mishto)

The activation of the IL-1R1 and TLR4 signaling pathways by their endogenous ligands IL-1β and HMGB1, respectively, is pivotal for the generation of neuroinflammatory responses during seizures or after epileptogenic injuries, and their pharmacological blockade or genetic inactivation results in powerful anticonvulsive effects.[5] These pathways are concomitantly activated during epileptogenesis, thus likely requiring a rational drug combination for their

effective blockade. Bauer and colleagues performed longitudinal cerebral microdialysis monitoring of hippocampal cytokine release in experimental epileptogenesis, detecting consistent elevations of IL-1β and Norwood provided evidence of endosomal Toll-like receptor dysregulation in experimental epilepsy (*unpublished observations*). Vezzani and colleagues studied whether a combination of anti-inflammatory drugs targeting the ictogenic IL-1β-IL-1R1 and HMGB1-TLR4 pro-inflammatory signals affects spontaneous seizure onset and disease progression in two rodent models of acquired epilepsy. They used two different schedules of intervention: male adult Sprague-Dawley rats exposed to electrical SE were given the drugs during the prodromal phase of epilepsy, while C57BL6 adult male mice exposed to intaamygdala kainate-induced SE were treated after the onset of the disease (i.e., after the first two unprovoked seizures). Rats were injected daily for 7 days with a combination of anakinra (IL-1 receptor antagonist), BoxA (HMGB1 antagonist) and ifenprodil (NR2B-NMDA receptor antagonist) starting 1 h post-SE. Mice were injected daily for 7 days with a combination of VX-765 (IL-1β biosynthesis inhibitor) and Cyanobacterial LPS (TLR4 antagonist) starting after the onset of epilepsy in each mouse. EEG recording (24/7) was performed from SE induction until the onset of spontaneous seizures, and for an additional 4 weeks in the chronic epilepsy phase. The combined treatments blocked seizure progression occurring in this model over 3 months post-SE leading to a reduction in the frequency of spontaneous seizures by 70-90% in the chronic epilepsy phase both in rats and mice as compared to corresponding vehicle-treated SE-exposed animals (Iori et al, *in press*). When drugs were given before the onset of epilepsy, they additionally reduced neurodegeneration in forebrain and improved memory deficits in epileptic animals. Similar therapeutic effects were attained when treating electrical SE-exposed rats with a combination of drugs targeting oxidative stress mechanisms (*unpublished observations*).

The targeting of IL-1R/TLR4 signaling has been attempted also by epigenetic intervention with microRNAs (miRNAs). Molecular biology has been revolutionized by the discovery of these small regulatory molecules that regulate gene expression. Several miRNAs have been found in human brain to have crucial roles in a wide range of normal biological processes, including inflammation.[25,26] In particular, Aronica and colleagues showed an upregulation of miRNAs

involved in the modulation of the IL-1R/TLR pathway, including miR146a, miR21 and miR155, during epileptogenesis.[27] These miRNAs were also shown to be deregulated in different human epilepsy-associated pathologies including hippocampal sclerosis, malformations of cortical development and glioneuronal tumors.[28-30] Interestingly, tumor and peritumoral miR146a expression was negatively correlated with frequency of seizures.[29] The expression of miR21, miR146a and miR155 in astrocytes within epileptogenic brain lesions points to the role of these cell types as both source and targets of these miRNAs. IL-1β stimulation strongly induced miR146a extracellular release in human astrocytes in culture and IL-1β signaling was differentially modulated by overexpression of miR155 or miR146a, resulting in pro- or anti-inflammatory effects, respectively.[30] It is likely that expression of miR146a in astrocytes may represent a homeostatic mechanism aimed at counteracting the inflammatory response triggered by either IL-1β or other pro-inflammatory miRNAs.

To provide a preclinical proof-of-concept evidence of a therapeutic treatment targeting this miRNA, Vezzani, Aronica and colleagues tested the effect of a synthetic oligonucleotide analog of miR-146a in murine models of acute seizures and chronic epilepsy. Intracerebroventricular injection of a synthetic miR-146a mimic significantly reduced neuronal excitability and acute seizure susceptibility. Moreover, the mimic stopped the disease development when injected in mice after the epilepsy onset, and reduced cell loss and improved memory deficit when given shortly after the epileptogenic event. These therapeutic effects were similar to those attained with anti-inflammatory drug intervention against the IL-1R1 and TLR4 receptors, and were associated with broad modifications in immune/inflammatory pathways in the brain (Iori et al, *in press*).[31]

Another key driver of neuroinflammation is the P2X7 receptor, an ATP-gated ionotropic purinergic receptor predominantly expressed in microglia, and also in presynaptic nerve terminals. Henshall reported that activation of these receptors results in cell depolarization and activation of signaling pathways that promote microglial activation and inflammasome-mediated IL-1β release leading to acute and chronic neuroinflammation.[32,33] Previous studies found increased expression of the P2X7 receptor in experimental and human epilepsy and antagonists

of this receptor have been reported to reduce seizure severity in certain models of SE.[34-36] In recent studies, a reporter mouse expressing enhanced green fluorescent protein driven by the *P2rx7* gene promoter has been used to identify the cell types involved. These studies show that the receptor is expressed by neurons as well as microglia in mice that develop epilepsy after intraamygdala kainic acid-induced SE.[37] Intracellular recordings and analysis of synaptoneurosomes indicated enhanced agonist-evoked and altered calcium responses in hippocampal neurons from epileptic mice. Increased P2X7 receptor levels were observed in hippocampus resected from patients with pharmacoresistant temporal lobe epilepsy, as well as in patients with focal cortical dysplasia.[37,38] Moreover, twice daily systemic injections of the centrally-available, potent and specific P2X7 receptor antagonist, JNJ-47965567 (30 mg/kg), significantly reduced spontaneous seizures during continuous video-EEG monitoring. Interestingly, the spontaneous seizure rate did not return to baseline during a relatively short monitoring after drug washout. The hippocampus of epileptic mice treated with the P2X7 receptor antagonist showed markedly reduced microgliosis and astrogliosis. These latest studies suggest that targeting the P2X7 receptor has anticonvulsant and possibly disease-modifying effects in experimental epilepsy.[37]

A potential novel target that was discussed by Mishto is the proteasome, the core of the ubiquitin proteasome system, which degrades the large majority of the cytoplasm proteins and thus it is involved in several metabolic pathways including synaptic protein metabolism.[39] The incorporation of the three inducible subunits β1i, β2i, and β5i into newly formed proteasomes results in the generation of the so-called immunoproteasome. This proteasome isoform is normally induced during inflammation, has altered polypeptide degradation dynamics compared to the standard proteasome isoform[40-42], and is endowed of pathophysiological functions related to immunity and inflammation responses.[39] In healthy young human brain, the expression of the immunoproteasome is almost absent.[43] On the contrary, it is expressed in the brain of elderlies and patients with different neurological diseases with an inflammatory component such as Huntington disease, Alzheimer disease, multiple sclerosis[43-46], as well as with temporal lobe epilepsy and malformations of cortical development.[47,48] In agreement with the latter

observations, the β5i immuno-subunit expression is induced also in experimental epilepsy, and its selective pharmacological inhibition reduced the incidence and delayed the occurrence of 4-aminopyridine-induced seizure-like events evoked in acute rat hippocampal/entorhinal cortex slices.[49] These effects were stronger in slices obtained from epileptic *vs* normal rat brain, likely due to the prominent β5i subunit induction in neurons and glia in diseased tissue. TLR4 signaling activation is likely involved in the transcriptional induction of β5i, an effect which was independent on promoter methylation.[49] The proteasome subunit expression is also induced by IL-1β in human astrocytes *in vitro* and is negatively regulated by treatment with the immunomodulatory drug rapamycin, an inhibitor of the mTOR pathway.[48] How the immunoproteasome could have a pro-ictogenic effect is unknown. It could have either a specific role in synaptic density and as enhancer of inflammatory milieu as demonstrated for other neurological diseases.[46,50] Of note, the immunoproteasome could regulate (neuro)inflammation not only as intracellular protease but also in the extracellular space. There, it could regulate pro-inflammatory functions of cytokines, as recently demonstrated for osteopontin in the multiple sclerosis context (Mishto et al., *personal communication*). Therefore, the immunoproteasome might be considered as therapeutic target for pharmacoresistant epilepsies as it is already under investigation for cancers, Alzheimer disease and other diseases.[46]

mTOR, TGF-beta and metalloproteinases as a targets for treatment

(J. A. Gorter, D. Kaufer, M.O. Poulter)

Recent observations show that inhibition of the mTOR pathway affects SE-induced epileptogenesis in rat models of temporal lobe epilepsy.[51,52] Gorter reported that a 6 week treatment with rapamycin (6 mg/kg, i.p, 1st injection starting at 4 h post-SE induction) reduced seizure frequency during treatment and resulted in decreased neuronal death, mossy fiber sprouting and BBB leakage. Remarkably, hippocampal microglia and astroglia activation, which was used as an indicator of inflammation, was not altered compared to vehicle-injected rats, suggesting that the suppressive effect on seizures was not directly linked to reduction of inflammation.[52] In order to get more insights into the time course of BBB damage and

hippocampal inflammation, magnetic resonance imaging (MRI) was performed in rats using gadobutrol as a contrast agent, and, in a separate group of rats, RT-qPCR of hippocampal tissue was performed to measure markers of inflammation.[52,53] These experiments showed that both BBB leakage and inflammation were initially increased during rapamycin treatment in SE-rats compared to vehicle-treated rats, but recovered to a greater extent after the first week following SE in drug- than in vehicle-treated rats. The fact that inflammation markers were not significantly altered at 6 weeks after SE in rapamycin-treated rats as compared to vehicle animals, implies that the drug reduces inflammation over time (*unpublished observations*). Moreover, the data suggest that rapamycin can modulate BBB integrity depending on the physiological condition of BBB during epileptogenesis.

In order to find out whether rapamycin reduces seizure frequency via an anti-seizure effect or an anti-epileptogenic effect, additional experiments were performed.[54] In the first experiment, rapamycin treatment was stopped 3 weeks post-SE and subsequent seizure development was monitored using hippocampal EEG recordings. It was found that the daily seizure reduction measured during treatment was lost after discontinuation of rapamycin. However, seizure frequency increased after drug withdrawal at a slower pace than was normally observed in untreated rats. In the second experiment, rats that were treated with rapamycin during the chronic epilepsy phase, showed a reduction in the number of daily spontaneous seizures. Accordingly, inhibition of mTOR by rapamycin has been shown to suppress established chronic seizures in the pilocarpine rat model of epilepsy.[55] Elimination of rapamycin from the bloodstream was extremely slow, with the drug being detectable still 1 month after the last injection in post-SE rats.[54] Interestingly, in mice, elimination of rapamycin from the brain has been shown to be much slower than in blood/plasma.[56]

Overall, these data suggest that the effect of rapamycin on seizure development could be due at least in part to a symptomatic seizure suppressing effect. During rapamycin treatment in post-SE rats, the rat's body weight was strongly reduced *vs* vehicle, also when rapamycin dose was reduced (3 mg/kg). Moreover, brain size was also reduced by rapamycin, as detected by MRI.

Thus, alternative mTOR inhibitors with a safer profile should be considered for therapeutic drug development in epilepsy.

Gorter, Kaufer, Friedman and colleagues have contributed to establish that BBB dysfunction occurs in epilepsy as in multiple neurological disorders including stroke and trauma. Consequently, blood-borne molecules enter the brain and induce an injury response leading to pathological changes.[57-59] In particular, Kaufer reported evidence in collaboration with Friedman that albumin, the most abundant protein in the blood, serves as a signaling molecule to trigger the inflammatory injury response.[57-60] Upon entering the brain after vascular damage, albumin is sensed by astrocytes, apparently via binding to transforming growth factor beta receptors (TGFβR), inducing the inflammatory TGFβ signaling pathway. This sets in motion a cascade of events that represent the earliest stages of post-traumatic pathology. Astrocytes become reactive and proliferate, releasing TGFβ1 and additional pro-inflammatory cytokines that amplify the injury response throughout the brain. In turn, subsequent widespread inflammatory signaling modulates neural physiology and network connectivity, causing changes that enhance neural excitability and ultimately lead to epilepsy. It has been shown that albumin activates TGFβR in astrocytes, causing a strong pro-inflammatory response and variety of changes within the neurovascular unit, including: reactive gliosis, reduced K^+ and glutamate buffering, increased excitatory synaptogenesis and aberrant neurogenesis. These changes recapitulate the major hallmarks of epileptogenesis that have been extensively documented across animal models of epilepsy and in tissue samples from patients, to provide a "missing link" for how injury triggers these pathological changes. Using bioinformatics transcriptome analysis following five diverse clinical scenarios where BBB is disrupted (stroke, trauma, chemically-induced BBB disruption, and infusion of serum albumin or TGFβ), a common transcriptional signature was discovered dependent on albumin-induced activation of TGFβ signaling. Extracellular matrix (ECM) related-genes emerged as a common transcriptional response, predicting consequent degradation of ECM.[60] Indeed, exposure of brain environment to albumin led to persistent degradation of perineuronal nets (PNNs, a protective ECM structure that provide synaptic stability and restrict reorganization of inhibitory interneurons) via activation of TGFβ signaling.

Exposure of the brain environment to either albumin or TGFβ1 was sufficient to induce both epileptiform activity and delayed spontaneous seizures. Importantly, blocking TGF signaling prevented most albumin-induced hallmarks of epileptogenesis including TGF signaling, transcriptome-wide changes, synaptogenesis, neurogenesis, perineuronal net (PNN) degradation, hyperexcitability and seizures.[57-59,61,62] Ongoing research evaluates the efficacy of multiple small molecules TGFβ signaling inhibitors in blocking or reversing the effects of BBB disruption on neuropathology, excitability, epilepsy development and cognitive functions.

The integrity and stability of PNNs in a cortical network is essential for proper network function.[63] Loss of interneuron synaptic stability and precise organization can lead to disruptions in the excitation/inhibition balance, a characteristic of epilepsy.[64,65] Recent research by Poulter's group supports the role of alterations to the GABAergic interneuron network in the piriform cortex after kindling-induced seizures.[66] Immunohistochemistry was used to mark PNNs and interneuron nerve terminals in control and kindled tissue. PNNs were found to be significantly decreased around parvalbumin-positive interneurons after the induction of experimental epilepsy. Additionally, a layer-specific increase was detected in GABA release sites originating from calbindin, calretinin, and parvalbumin interneurons, implying that there is a re-wiring of the interneuronal network. This increase in release sites was matched by an increase in GABAergic postsynaptic densities. The observations suggest that the breakdown of the PNN could be due to the activity of matrix metalloproteinases (MMPs) and that the prevention of PNN breakdown may reduce the rewiring of interneuronal circuits and suppress seizures. To test this hypothesis, doxycycline (DOX) a broad spectrum MMP inhibitor, was used to stabilize PNNs in kindled rats. DOX was found to prevent PNN breakdown, re-organization of the inhibitory innervation, and seizure genesis. These observations indicate that PNN degradation may be necessary for the development of seizures by facilitating interneuron plasticity and increased GABAergic activity. Another hypothesis is that activation of MMP after kindling may also be responsible for the conversion of pro-BDNF to BDNF. Furthermore, BDNF has been shown to induce the upregulation of the voltage gated potassium channel Kv1.6. This channel has also been shown to be upregulated in a kindling model causing a profound change in the excitability of

parvalbumin positive interneurons.[67] In order to see if MMP inhibition altered expression of BDNF, immunohistochemistry was performed in brain slices from kindled rats treated with DOX. Interestingly, BDNF expression was significantly less in this group in comparison to saline-treated kindled rats. It has also been observed that BDNF treatment of cultured cortical neurons increased the expression of Kv1.6. In kindled tissue DOX also normalized Kv1.6 expression.

In sum, these data show that upregulation of MMP activity after kindling has wide ranging effects that alter innervation of interneurons as well as the excitability of a subpopulation neurons that are important regulators of network activity.

Chemokines

(P. Louboutin, M. Caleo, Y. Bozzi, E. Palma)

These inflammatory molecules play a pivotal role in leukocyte migration across the BBB during neuroinflammation and other neuropathological processes.[68,69] When interacting with endothelial surfaces, some chemokines initiate integrin clustering, arrest leukocytes at sites of injury and guide them from vascular lumen into the brain. Once within the brain, these cells, together with microglia and astrocytes, contribute to endothelial cell activation and further chemokine secretion. CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) induce chemotaxis of T lymphocytes and macrophages. *In vitro* CCL4 and CCL5 increase T cell adhesion to endothelial cells. CCL2 binding to CCR2 receptor stimulates efficient migration of monocytes/macrophages across brain vasculature, and CCR1 and CCR5 receptors facilitate CCL5-driven movement of blood mononuclear cells (PBMC) across brain endothelium. CCR5 is a member of the CC-chemokine receptor family that binds several chemokines, including CCL3, CCL4 and CCL5. It is reportedly increased in epilepsy, as are its ligands. CCR5 is also expressed by microglia.

Louboutin and colleagues[69-71] investigated the role of CCR5 in a rat model of seizures provoked by intraperitoneal administration of kainic acid (KA). Four months before KA injection, adult rats were given femoral intramarrow inoculations of SV(RNAiR5-RevM10.AU1), which carries an interfering RNA (RNAi) against CCR5, plus a marker epitope (AU1), or its monofunctional RNAi-

carrying homolog, SV(RNAiR5). This treatment lowered expression of CCR5 in circulating PBMC. Controls received unrelated SV(BUGT) vector. In control rats, seizures increased the expression of CCR5 ligands MIP-1α and RANTES in the microvasculature and induced BBB leakage as well as CCR-positive cells, inflammation, neuronal loss and gliosis in the hippocampi. Animals given either the bi- or the monofunctional vector were largely protected from KA-induced seizures, neuroinflammation and BBB damage, and exhibited a decreased production of MIP-1α and RANTES, as well as reduced neuron loss. Brain CCR5 mRNA was reduced in these rats. Rats receiving RNAiR5-bearing vectors showed far greater repair responses with increased neuronal proliferation. Thus, inhibition of CCR5 in circulating cells strongly protected rats from seizures, BBB leakage, CNS injury and inflammation, and facilitated neurogenic repair. These results suggest that inhibition of CCR5 in circulating PBMC can decrease their interaction with endothelial cells, thus reducing leukocyte migration across the BBB, and consequently neuroinflammation and deleterious related events.

The chemokine CCL2 is elevated in brain tissue from patients with pharmacoresistant epilepsy.[72,73] Caleo and colleagues[72,74] showed a crucial role for CCL2 and its receptor CCR2 in seizure control. Mice with spontaneous seizures and neuropathology resembling mesial temporal lobe epilepsy were systemically injected with lipopolysaccharide (LPS) to mimic a peripheral inflammatory challenge. LPS was found to increase seizure frequency and up-regulate the brain expression of many inflammatory proteins, including CCL2. To test the potential role of CCL2 in seizure exacerbation, either a CCL2 transcription inhibitor (bindarit) or a selective antagonist of the CCR2 receptor (RS102895) was administered systemically. Interference with CCL2 signaling potently suppressed LPS-induced seizure worsening.[74] Intracerebral administration of anti-CCL2 antibodies also abrogated LPS-mediated seizure enhancement in chronically epileptic animals. These results reveal that CCL2 is a key mediator that link peripheral inflammation with neuronal hyperexcitability.

Palma reported that cytokines and chemokines can both influence $GABA_A$ receptor function, although they can exert opposite effects on neuronal synaptic transmission. For example the chemokine fractalkine (CX3CL1) is responsible for a positive modulation of $GABA_A$ receptor in

human TLE brain because it slows the $GABA_A$ use-dependent desensitization (i.e. rundown).[75] Differently, IL-1β was found to reduce $GABA_A$-mediated current amplitude by activating IRAK1 and protein kinase C.[76] In both cases, the effects on GABA currents were intrinsic characteristic of the epileptic brain tissue, since such effects were absent in non-epileptic controls. This observation is explained, at least in part, by an upregulation of the cytokine/chemokine receptors. Interestingly, while in the fractalkine case the receptor's upregulation may represent an attempt to reduce changes induced by epileptic insults, the IL-1β pathway actively contributes to ictogenesis through the impairment of GABA-mediated neurotransmission.[75,76] Altogether, these data indicate that GABAergic system is significantly modulated by inflammatory mediators released in epileptic foci, thus opening a wide scenario of novel therapeutic opportunities for controlling neuronal network hyperexcitability in epileptic patients.

The role of peripheral immune cells

(S. Koh, R. Dingledine)

Koh's laboratory has pioneered the characterization of inflammatory cell infiltrates in the surgically removed fresh brain samples from pediatric patients in search for novel therapeutic targets. They used an unbiased flow cytometric analysis of inflammatory leukocytes in resected brain tissues from pediatric patients with genetic (focal cortical dysplasia, FCD) or acquired (encephalomalacia) epilepsy. They detected functionally activated lymphocytes within the epileptogenic lesion of both patients and experimental animals. Brain infiltration of inflammatory myeloid cells and memory CD4+ and CD8+ T cells was observed (*unpublished observations*).[77] This is in line with previous observations in both focal cortical dysplasia type IIb and cortical tubers in Tuberous Sclerosis Complex patients.[78-81] In particular, T cells are concentrated in the epileptogenic lesion and their numbers positively correlate with disease severity, whereas numbers of regulatory T cells (Tregs) inversely correlate (*unpublished observations*). T cell-deficient mice systemically injected with KA showed reduction in SE severity while Treg depletion heightened seizure severity. These data support a role for peripherally-derived innate

and adaptive immune responses in the pathogenesis of intractable pediatric epilepsy favoring the clinical development and testing of novel immunotherapeutic drugs.

Steroids that are known for their anti-inflammatory and immuno-suppressive properties have shown efficacy in many types of drug-resistant epilepsies. However, the severe side effects of steroids have prevented long-term or widespread use of these drugs. Koh and colleagues used a two-hit model of epileptogenesis to design novel therapies to treat epilepsy using immunomodulatory approaches independent of broadly-acting immunosuppressive agents. They hypothesize that dampening ongoing inflammation in the brain could effectively reduce unprovoked recurrent seizures in the absence of systemic immunosuppression. Previous work by Miller's lab documented the success of using biodegradable nanoparticles formulated from the FDA-approved biopolymer poly(lactide-*co*-glycolide) (PLG) to treat a variety of inflammatory immune-mediated disease in animal models.[82] Miller's lab has also demonstrated that supplementation of autologous natural regulatory T cells (nTregs) significantly reduces disease severity in multiple animal models of multiple sclerosis.[83,84] In the two-hit model of early-life seizures, treatment with PLG nanoparticles induced leukocyte sequestration in the spleen, thereby reducing their brain infiltration, and prevented the priming effect of early-life seizures for heightened seizure susceptibility in adulthood. Similarly, infusion of nTregs after first seizure blocked the priming effect of early-life seizures (*unpublished observations*). Inhibition of long-term effects of early seizures priming was attained also by directly blocking inflammatory microglia in the hippocampus with minocycline.[85] The characterization of the adaptive immunity responses in epilepsy and the effects of restriction of brain infiltration by inflammatory leukocyte subsets on seizures represent major steps forward in our understanding of epilepsy pathophysiology.

Dingledine and colleagues examined the cellular components of innate immune inflammation in the early days following kainate- or pilocarpine-induced SE in adult mice by discriminating microglia versus brain-infiltrating monocytes.[86] CCR2+ monocytes invade hippocampus between one and three days after SE. In contrast, at odds with early-life seizures only occasional CD3+ T-lymphocytes were encountered three days post-SE in adult animals. The

chemokine CCL2, a ligand for CCR2, was expressed in perivascular macrophages and microglia one day after SE. Four days after SE the induction of the pro-inflammatory cytokine IL-1β was greater in FACS-isolated microglia than in brain monocytes. However, in WT naive mice the mRNA levels of IL-1β were 125-fold higher in circulating blood monocytes than in resting microglia, and TNFα levels were 486-fold greater in blood monocytes. *Ccr2* KO mice displayed greatly reduced monocyte recruitment into brain and reduced levels of the pro-inflammatory cytokine IL-1β in hippocampus after SE. This was explained by high level of the cytokine in circulating monocytes in wild-type mice which were prevented from entering the brain after SE in *Ccr2* KO mice. These findings indicate that microglia and monocytes respond differently to SE. Whereas brain-invading monocytes show little induction of IL-1β and TNFα mRNA in response to SE, both IL-1β and CCL2 are significantly induced in activated microglia. However, these results also indicate that the level of IL-1β and TNFα in circulating monocytes is much higher than that of activated microglia. Therefore, preventing monocyte recruitment into the brain may reduce the extent of neuroinflammation. Mice with impaired monocyte recruitment showed accelerated weight regain, reduced BBB leakage, and attenuated neuronal damage following SE.[86] These findings identify brain-infiltrating monocytes as a myeloid cell subclass that contributes substantially to the extent of neuroinflammation and morbidity after SE. Inhibiting brain invasion of CCR2+ monocytes could represent a viable method for alleviating the deleterious consequences of SE.

Conclusion and Future directions

Several inflammatory targets and pathways discussed above provide examples of new potential avenues for future therapeutic approaches in epilepsy. Importantly, these studies indicate that immunomodulatory treatments alone might not be sufficient to counteract epileptogenesis efficiently, rather that it is likely necessary to interfere with the complex pathophysiological underpinnings of epileptogenesis at multiple levels and in various cell types (e.g., with drug combinations or complementary treatment approaches). miRNAs as master regulators of immune responses could also represent interesting targets for therapy. miRNA formulation,

delivery methods into the CNS for bypassing the BBB and specifically targeting the seizure focus remain major challenges in the clinical translation.

Despite the achievements and significant progress reported during the IIE2016 meeting, a major challenge is represented by the translation of these experimentally successful anti-ictogenic or disease modifying targets into clinically effective human therapies. This translation requires intense and collaborative efforts by both preclinical and clinical scientists as well as industry partners. Future success of drug development should be based on a more coordinated experimental and clinical trial designs guided by relevant biomarkers that may be used to monitor epileptogenesis.

Acknowledgements

Additional contributors to the studies described in this manuscript are: Valentina Iori and Teresa Ravizza (IRCCS-Mario Negri Institute for Pharmacological Research, Milano, Italy); Jackelien van Scheppingen, Cato Drion and Erwin van Vliet (Amsterdam Medical Center, Amsterdam, The Netherlands); Tobias Engel (Royal College of Surgeons in Ireland, Dublin, Ireland).

The authors acknowledge their sources of support: Epitarget (FP7/2007-2013) under grant agreement n°602102 (AV and EA); EPISTOP (grant agreement no. 602391; EA); The National Epilepsy Fund "Power of the Small" and the Hersenstichting Nederland (EF-13-1); Health Research Board Ireland (HRA-POR/2010/123, HRA-POR/2012/56) and Science Foundation Ireland (13/IA/1891) (to D.C.H.); AICE-FIRE and UCB Pharma Bruxelles (EP). NIH awards UO1 NS058158, R21 NS093364 and R01 NS097776 (RD).

Disclosure

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

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Table 1. Overview of targets and treatments validated in experimental models

Target/pathway	Experimental models	Treatment/drugs	Effect	References
Cox-2	Pilocarpine-induced SE Electrically-induced SE Electrically-induced SE Pilocarpine (+LPS)-induced SE Absence seizures in WAG/Rij rats Pilocarpine-induced SE	COX-2 inhibitor celecoxib COX-2 inhibitor SC58236 COX-2 inhibitor parecoxib COX-2 inhibitor CAY 10404 COX-2 inhibitor etericoxib COX-2 neuronal conditional KO	Antiepileptogenic, disease-modifying No antiepileptogenic Disease-modifying No antiepileptogenic Disease-modifying	(9) (10) (11) (12) (13) (8)
Cox 2 + IL-1β	Pilocarpine (+LPS)-induced SE	COX-2 inhibitor CAY 10404+ recombinant Interleukin-1 antagonist	Disease-modifying	(12)
EP2/ PGE2 receptor	Organophosphorus induced SE Pilocarpine-induced SE	EP2 competitive antagonist EP2 competitive antagonist	Disease-modifying Disease-modifying	(19, 20) (18)
Monoacylglycerol lipase	Intraamygdala kainic acid induced SE (C57/BL6N)	CPD-4645 (MAGL inhibitor)	Disease-modifying	Terrone, Vezzani et al., unpublished observations
IL-1β	Bicuculline induced seizures Pilocarpine-induced SE Rapid kindling model Electrically-induced SE/ pilocarpine-induced SE Genetic absence epilepsy (GAERS); kainic acid–induced seizures; electrically-induced SE/ pilocarpine-induced SE; kindling model	Human recombinant IL-1 receptor antagonist (anakinra) VX-765 (caspase-1 inhibitor)	Anti-convulsive Anti-convulsive Antiepileptogenic No antiepileptogenic or disease-modifying Anti-convulsive/ Antiepileptogenic	(87) (88) (89) (90) (90-94)
TLR4 or HMGB1	Kainic acid–induced seizures	HMGB1 and TLR4 antagonists	Anti-convulsive	(95)
IL-1β/HMGB1		Anakinra (IL-1 receptor antagonist), BoxA (HMGB1 antagonist)	Disease-modifying	Vezzani et al., unpublished observations.

IL-1β/miR146	Kainic acid–induced seizures Intraamygdala kainic acid induced SE	miR146a-mimic	Anti-convulsive and Disease-modifying	(31, 96) unpublished observations.
P2X7 receptor	intra-amygdala kainic acid-induced status epilepticus	P2X7 receptor antagonist JNJ-47965567	Disease-modifying	(37)
Immunoproteasome	4-aminopyridine-induced seizure-like events	β5i immuno-subunit inhibitor	Anti-convulsive	(49)
mTOR	Kainic acid–induced SE Electrically-induced SE/ Kainic acid–induced SE	Rapamycin	Anti-convulsive/ Antiepileptogenic Anti-convulsive and Disease-modifying	(97) (52-54)
TGF β	rat model of vascular injury/ albumin-induced seozures	Losartan (TGF-β signaling blocker)	Anti-convulsive/ Antiepileptogenic	(62)
Metalloproteinases	kindling-induced seizures	Doxycycline (broad spectrum MMP inhibitor)	Anti-ictogenic	(66)
Chemokines	Kainic acid–induced SE Kainic acid–induced SE + LPS	RNAi against CCR5 CCR2 antagonist (RS102895); transcription inhibitor (bindarit)	Disease-modifying Anti-convulsive	(69) (74)
Peripheral immune cells	Acquired and genetic epilepsy models	PLG nanoparticles combined with autologous natural regulatory T cells Inhibiting brain invasion of CCR2+ monocytes	Effects on epileptogenesis: to be determined Effects on epileptogenesis: to be determined	Koh et al., unpublished observations (86)

SE, status epilepticus.

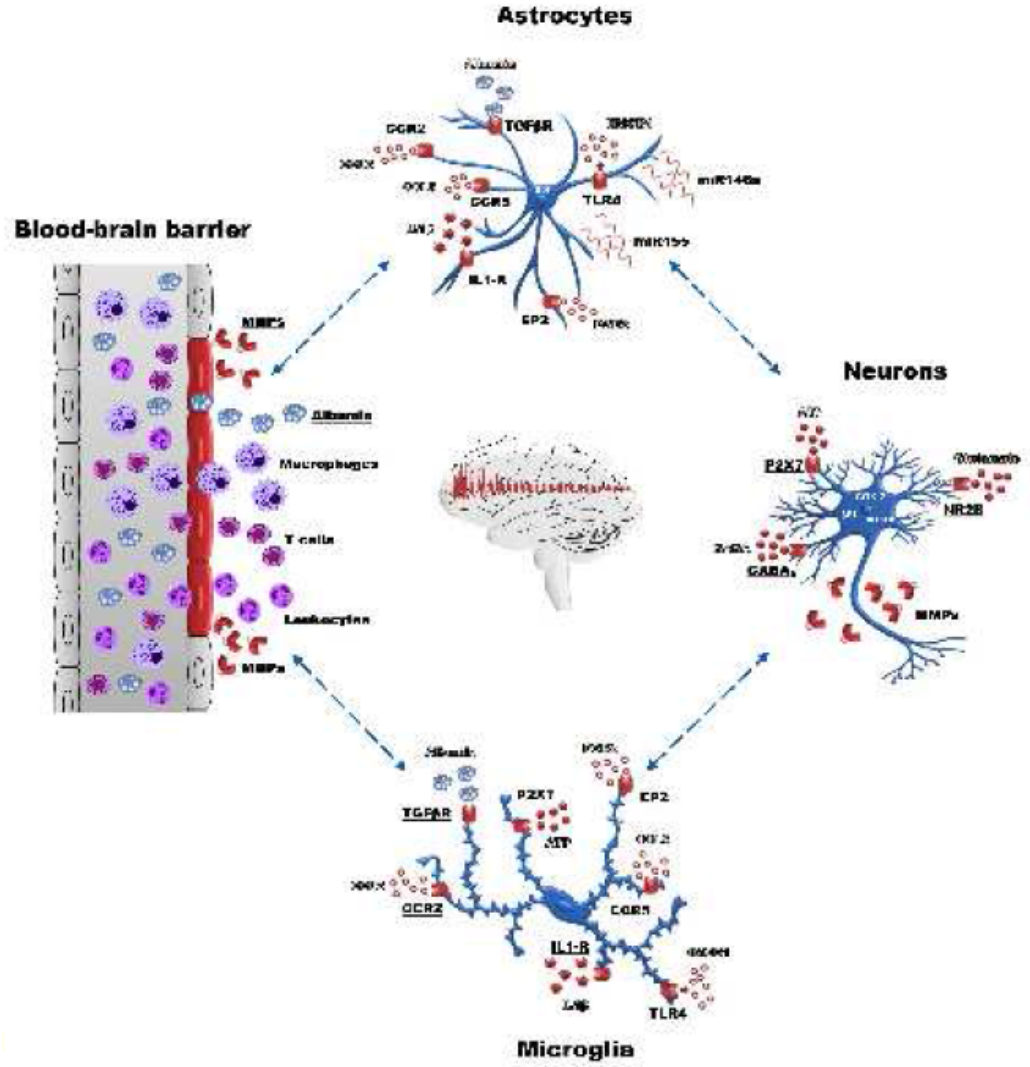

Figure 1. *Schematic representation of the cascade of inflammatory processes in which neurons, astrocytes, microglia and endothelial cells are involved*

Several inflammatory targets have been validated in experimental models (i.e. cyclooxygenase-2, prostaglandin EP2 receptor, Interleukin-1β, HMGB1/Toll-like receptor signaling, P2X7 receptor, immunoproteasome, mTOR, TGF-beta, metalloproteinases (MMP), chemokines). The complement activation (*not shown*) could also contribute to a sustained inflammatory response[98,99], deserving further investigation as potential target of therapy.

CHAPTER II

"STUDY OF A NOVEL COMBINATION OF DRUGS WITH ANTIOXIDANT EFFECTS IN AN EXPERIMENTAL MODEL OF ACQUIRED EPILEPTOGENESIS"

2.1 INTRODUCTION

2.1.1 Oxidative stress

Oxidative stress is a pathological condition caused by impaired balance between the production of reactive oxygen and nitrogen species (ROS, RNS) and antioxidant defense systems promoting their elimination.

Disruption of this balance may occur following: an overproduction of ROS and RNS, decreased functionality of the anti-oxidant systems in the cells or a combination of these two factors (Fig.1) [1,2] Experimental evidence demonstrated that free radicals have a dual function: at low concentrations, they have important physiological functions (i.e. regulation of the vascular tone and erythropoietin production, control of oxygen tension, homeostatic maintenance of the redox state tissue, defense against pathogens), whereas at high concentrations they induce tissue damage and dysfunction [3].

There are endogenous anti-oxidant systems that can counteract the increased production of ROS and RNS. However, following an excessive production of free radicals, these defense mechanisms are insufficient, resulting in the development of oxidative stress.

Since free radicals have an unpaired electron, they are very unstable and highly reactive molecules. Therefore, they react with other substrates such as nucleic acids (nuclear and mitochondrial), lipids, proteins and carbohydrates, causing their oxidation, with consequent alteration / loss of their function [4].

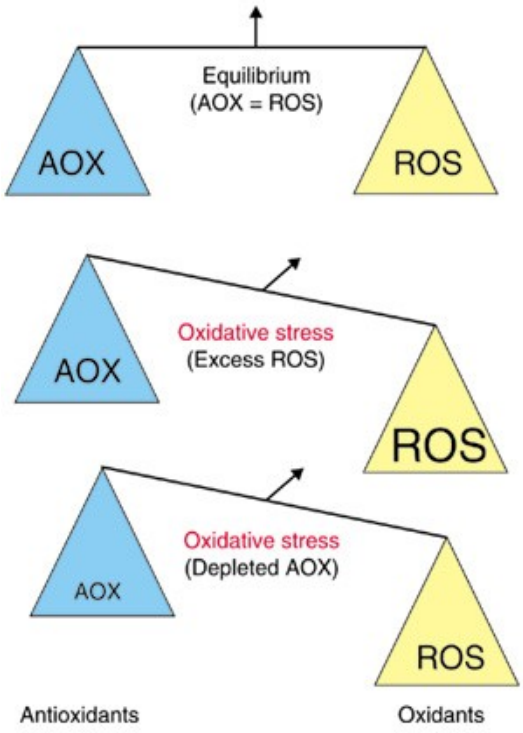

Figure 1. Schematic representation of oxidative stress (from Scandalios et al, 2002)

2.1.2 Oxidative stress and Central Nervous System (CNS)

The brain is particularly susceptible to the harmful effects of free radicals, because:

• it is characterized by a high aerobic metabolism; the high consumption of oxygen (about 20% oxygen inhaled) required to fulfill the energy request of neurons, increases the production of free radicals [5];

• the levels of the anti-oxidant enzymatic (catalase and glutathione peroxidase) and non-enzymatic (reduced glutathione and vitamin E) systems in neurons are relatively low; therefore, these cells are more vulnerable to the action of free radicals [6];

• brain contains a high number of mitochondria, required to support the high aerobic metabolism. Mitochondrial DNA is particularly exposed to the harmful action of free radicals since it is not protected by histones and is localized close to the inner mitochondrial membrane, where the free radicals are produced [7];

• brain has a high concentration of polyunsaturated fatty acids contained in the cell membrane phospholipids. These molecules are particularly susceptible to peroxidation [4,8].

2.1.3 Oxidative stress and epilepsy: experimental and clinical evidence

Oxidative stress has been implicated in the pathogenesis of various neurodegenerative diseases and epilepsy [8-12].

Clinical studies in brain tissue and plasma of patients with temporal lobe epilepsy detected the presence of oxidative stress, characterized by morphological alterations of the mitochondria [13], increase in lipid peroxidation [14-15], increase of some anti-oxidant enzymes (superoxide dismutase and catalase) [15,16], and reduced concentrations of vitamin C and A (two anti-oxidant molecules) [14].

Data supporting the involvement of oxidative stress in epilepsy derived from experimental models. In particular, biochemical studies conducted in the hippocampus of rats and mice exposed to epileptogenic injury (status epilepticus, cerebral trauma, viral infections, hypoxia and cerebral ischemia) revealed higher levels of ROS and RNS, oxidized lipids and proteins, hydrogen peroxide, and presence of mitochondrial dysfunction (ultrastructural changes and DNA damage) [8-9, 17-19].

The most important anti-oxidant system is represented by the non-enzymatic reduced glutathione (GSH). In presence of oxidative stress, GSH is oxidized in its disulfide form (GSSG). The increase in the GSSG/GSH ratio is commonly used as a marker of oxidative stress. In rats exposed to status epilepticus it was observed an increase in the GSSG/GSH ratio in hippocampal mitochondria [12,19]. These changes occur within 24-48 hours after the onset of status epilepticus and persist during epileptogenesis and in the chronic phase of epilepsy [12,19]. These evidences indicate that oxidative stress is an active process in

the stages preceding the development of spontaneous seizures and thus, it could contribute to epileptogenesis.

The treatment with anti-oxidant agents (such as vitamin C and E, and coenzyme Q10), reduces in rats neurodegeneration associated with status epilepticus [20,21], indicating a role of the oxidative stress in neuronal death associated to seizures.

Moreover, oxidative stress may contribute to neuronal damage and onset of hyperexcitability phenomena through the modification of the redox state of specific proteins due to increased production of free radicals. For example, ROS can increase the extracellular concentration of glutamic acid, an excitatory neurotransmitter that contributes to the genesis of seizures, through two mechanisms: promoting the inactivation of glutamine synthetase, an enzyme that converts glutamic acid into glutamine [5]; reducing the activity of GLT-1 and GLAST, two transporters of glutamic acid. These transporters remove glutamic acid from the synapses, preventing its extracellular accumulation [22]. Finally, High Mobility Group Box-1 is a nuclear protein that contributes to seizure recurrence and precipitation only when it is oxidized in the extracellular space [23].

2.1.4 High Mobility Group Box-1 (HMGB1)

HMGB1 has emerged as one of the main mediators in both acute and chronic inflammation and play a key role in seizure mechanisms.

HMGB1 is a ubiquitous nuclear protein of 216 amino acid, highly conserved among mammals (100% homology to the rat-men, 98% mouse-man) and belongs to the group of the *Damage Associated Molecular Patterns* (DAMPS) molecules that alerts nearby cells and the immune system to immediate danger, triggering inflammation [24,25].

HMGB1 contains two HMG DNA-binding domains, called A and B boxes, (Fig.2), and an acidic tail comprising exclusively glutamic and aspartic acids [26].

A and B boxes have two nuclear localization sites (NLSs) directing the traslocation of the protein into the nucleus and are organized into α-helix structures with a high affinity for the non-specific DNA minor groove. Thus, HMGB1 promotes conformational alterations of chromatin facilitating gene transcription [24,28].

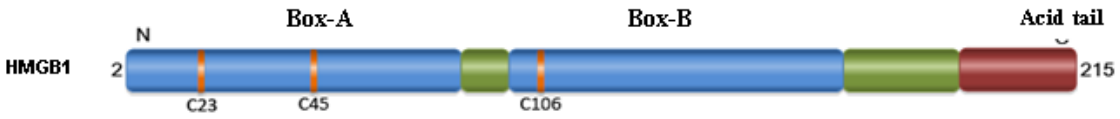

Figure 2. HMGB1 structure (from Antoine et al, 2014)

HMGB1 can exit the cell through two different pathways: passive or active release. In central nervous system, HMGB1 is actively secreted by monocytes/macrophages and neuronal and glial cells in response to Pathogen-Associated Molecular Patterns (PAMPs) or pro-inflammatory cytokines such as IL-1β, TNF-α or lipopolysaccharide (LPS, a component of the Gram negative bacterial wall) or passively released by necrotic cells that lost their membrane integrity [28,29]. Following its release, HMGB1 acts as a pro-inflammatory molecule, and assumes pleiotropic functions including the recruitment of inflammatory cells into the site of damage, cell mitosis to compensate the loss of necrotic cells, the promotion of angiogenesis, the production of cytokines and the activation of metalloproteases by contributing to the degradation of the extracellular matrix [27,28].

The acetylation of NLS lysines is crucial for nucleus-to-cytoplasm translocation of HMGB1, reducing the affinity of this molecule for DNA [24,25].

The nucleus-to-cytoplasm translocation of HMGB1 is a necessary condition for its subsequent release.

HMGB1 exerts its effects by interacting with Toll-like receptors (TLR), in particular TLR4, RAGE (Receptor for Advanced Glycation End products) and the chemokine receptor CXCR4 [30,31].

TLRs are a family of pattern-recognition receptors with well-established roles in the host's immune response to infection. RAGE behaves as a pattern recognition receptor involved in the recognition of endogenous molecules, such as advanced glycation end products (AGEs) or S100 proteins, released in the context of infection, physiological stress or chronic inflammation [32,33].

2.1.5 HMGB1 and neuronal hyperexcitability

HMGB1 is released by neurons and glia following a pro-convulsive stimulation [34]. In particular, in experimental models of seizures, it has been observed an increase of the levels of HMGB1 in the cytoplasm of astrocytes and microglia, indicating that the stimulus induced by pro-convulsant agents causes the translocation of HMGB1 from the nucleus to the cytoplasm, and consequently its extracellular release [34].

HMGB1, administered before the pro-convulsant agents, anticipates the onset of seizures and increases the number of seizures and the time spent in seizures, while drugs that antagonize the effects (Boxa), or antagonists of the TLR4 receptor (LPS-RS, Cyp), drastically reduce seizure activity [34], indicating a contribution of HMGB1-TLR4 axis in the mechanisms of seizure precipitation and recurrence. In addition to TLR4 receptors, RAGE is involved in the ictogenesis mediated by HMGB1, and the activation of both these receptors contributes to the development of spontaneous epileptic activity following the induction of status epilepticus [35].

However, RAGE and TLR4 play distinct roles in mediating the neuropathological processes (neuronal damage and neurogenesis) developing during epileptogenesis [35].

These findings suggest that HMGB1 produced after an epileptogenic insult could contribute to the seizure precipitation and recurrence.

The relevance of preclinical data for human epilepsy is supported by evidence that the "pattern" of HMGB1 expression described in experimental models has also been observed in epileptic tissue of patients with drug-resistant epilepsy (temporal lobe epilepsy and epilepsy associated with malformations of cortical development) [34,36,37].

2.1.6 HMGB1 isoforms

Experimental evidence demonstrated that the redox state of HMGB1 is crucial for the interaction with its receptors and for its biological effects [38-40]. In particular, the redox state of the three cysteines modulates the extracellular activities of HMGB1.

HMGB1 contains three cysteines: C23, C45 and C106 according to their position in the amino acid sequence of the molecule. The use of mass spectrometry allowed to identify three isoforms of HMGB1: disulfide HMGB1, reduced HMGB1 and sulfonyl-HMGB1.

The cysteines C23 and C45, contained in BoxA domain, can be oxidized to form a disulfide bridge [41]. In these conditions the cysteine C106, which is contained in BoxB domain, remains unpaired and contributes to bind the TLR4 [31]. This isoform is defined disulfide HMGB1 and has pro-inflammatory properties, promoting the production and release of cytokines by immunocompetent cells [31,40].

The fully reduced HMGB1 isoform in which all three cysteine residues are reduced, has chemoattractant properties. Notably, fully reduced HMGB1 forms a complex with CXCL12, which binds to the CXCR4 receptor. The chemoattractant activity of HMGB1 is dependent on this axis, and is suppressed by the oxidation of HMGB1 cysteines [39].

The biological activities mediated by reduced or disulfide HMGB1 are inhibited by the simultaneous sulphonation of all three cysteines C23, C45 and C106. This condition leads to the formation of sulfonyl-HMGB1 which has neither pro-inflammatory activity, nor chemoattractant, and probably represents a physiological mechanism activated to block the biological effects mediated by HMGB1 [40].

The non acetylated and fully reduced HMGB1 is the isoform present in the nucleus of cells under physiological conditions [42].

Disulfide extracellular HMGB1 is the predominant isoform released in conditions of oxidative stress [38]. After cell damage or epileptogenic insult, fully reduced HMGB1 present in the nucleus, is acetylated and translocates into the cytoplasm, where interacts with free radicals, produced by oxidative stress, and is oxidized into disulfide HMGB1.

This mechanism creates a vicious circle that links cell damage or epileptogenic event, oxidative stress and the production of disulfide HMGB1 (Fig.3).

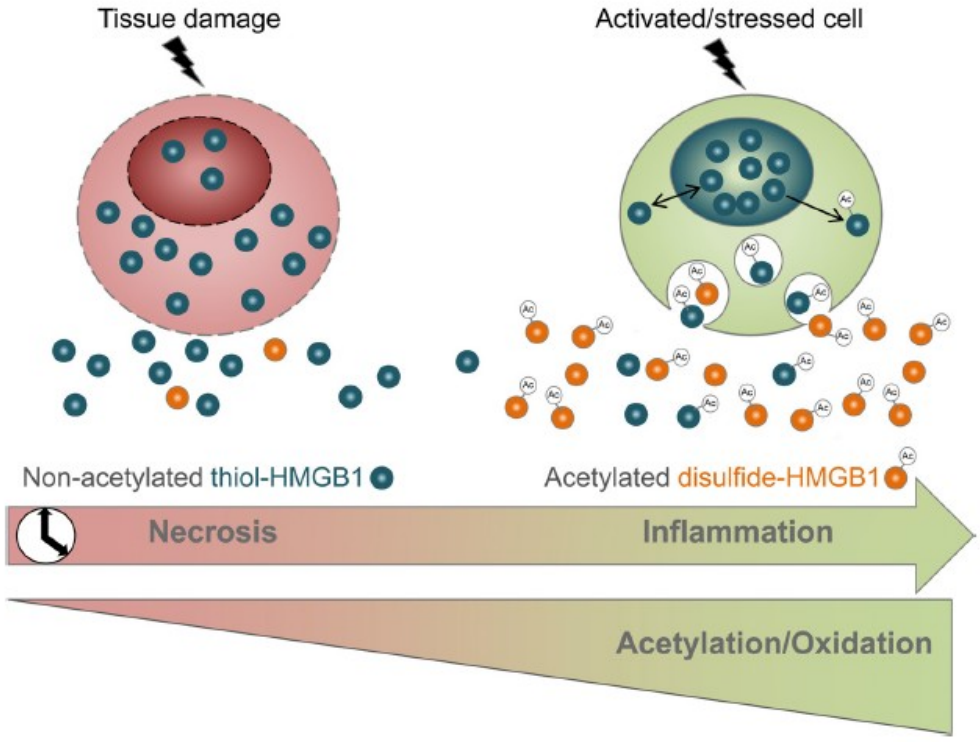

Figure 3. Post-transcriptional modifications of HMGB1 (from Venereau et al, 2016)

Recent pharmacological studies demonstrated that disulfide HMGB1 isoform contributes to seizure recurrence [43]. The mechanism that mediates the effects of disulfide HMGB1 is the same activated by IL-1β [23] and involves an increased activation of the N-Methyl-D-Aspartate (NMDA) receptors that mediate the excitatory glutamatergic transmission during seizure [43]

2.2 AIMS OF STUDY

Epilepsy therapy is based on antiseizure drugs which treat the symptom, seizures, rather than the disease and are ineffective in up to 30% of patients. There are no treatments for preventing the onset of epilepsy or improving disease prognosis.

In this study, we investigated whether oxidative stress occurs in brain during epileptogenesis in a rat model of acquired epilepsy induced by electrical status epilepticus (SE) and if this pathological process is also present in hippocampus of humans who died following SE.

Then, we studied whether HMGB1 traslocation, and its disulfide isoform that promotes seizures and cell loss, are generated by ROS during oxidative stress.

Finally, we investigated if oxidative stress can be efficiently resolved by a transient treatment with N-acetyl-cysteine (NAC) and sulforaphane (SFN), two drugs known to raise the levels of the antioxidant glutathione (GSH) and already used in humans for other therapeutic indications.

The main scope was testing their therapeutic effects on spontaneous seizures, cell loss and comorbidities.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Adult male Sprague-Dawley rats (225–250 g; Charles-River, Calco, Italy) were housed at constant temperature (23 ± 1°C) and relative humidity (60 ± 5%) with free access to food and water and a fixed 12 h light/dark cycle. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n.26, G.U. March 4, 2014) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) and were reviewed and approved by the intramural ethical committee.

2.3.2 Electrical status epilepticus

Rats were implanted under 1.5% isofluorane anesthesia with 2 bipolar Teflon-insulated stainless-steel depth electrodes placed bilaterally into the temporal pole of the hippocampus (*from bregma*, mm: AP −4.7; L ± 5.0; −5.0 below dura) [44]. Two screw electrodes were positioned over the nasal sinus and the cerebellum, and used as ground and reference electrodes, respectively. Electrodes were connected to a multipin socket and secured to the skull by acrylic dental cement. After surgical procedures, rats were treated locally with Cicatrene powder (Neomicyn; Bacitracin; Glicyne; L-Cysteine; DL-Threonine) and injected with Ampicillin (100 mg/kg, i.p.) for 4 days to prevent infections. Rats were allowed to recover from surgery in their home cage for 10 days. Before electrical stimulation, EEG baseline hippocampal activity was recorded in freely-moving rats for 24 h. Then, rats were unilaterally stimulated (50 Hz, 400 μA peak-to-peak, 1 ms biphasic square waves in 10 s trains delivered every 11 s) in the CA3 region of the ventral hippocampus for 90 min to induce SE according to a well established protocol [45,46].

EEG was recorded in each rat every 10 min epoch for 1 min in the absence of electrical stimulation, i.e., the "stimulus-off" period. All rats used for subsequent analysis showed an EEG pattern of uninterrupted bilateral spikes in the hippocampi during the "stimulus-off" period, starting between the 1st and the 4th epoch of stimulation onwards. These criteria selected rats developing SE that remitted spontaneously within 24 h from the initial stimulation then leading to subsequent epilepsy development [45,46]. SE was defined by the appearance of continuous spike activity with a frequency >1.0 Hz intermixed with high amplitude and frequency discharges lasting for at least 5 s, with a frequency of ≥8 Hz. Spikes were defined as sharp waves with amplitude at least 2.5-fold higher than baseline and duration lower than 100 ms, or as a spike-and-wave with duration lower than 200 ms [47]. The end of SE was defined by the occurrence of inter-spike intervals longer than 1 s. No pharmacological intervention was done to stop SE since no mortality is observed in this model. SE was evaluated by EEG analysis measuring its total duration and the number of spikes during the first 24 h using Clampfit 9.0 program (Axon Instruments, Union City, CA, U.S.A.). Power spectral density (PSD) distribution of 4 frequency bands (delta: 1–4 Hz; theta: 4–8 Hz; alpha: 8–13 Hz; beta-gamma: 13–40 Hz) was calculated during 9 h segmented in temporal windows of 1 h each. Fast Fourier transforms (FFTs) were computed by 50% overlapping sliding windows (1024 data-point each) with Hanning windowing function. EEG data were normalized by dividing the EEG power density at each frequency with the EEG power density averaged across all frequencies [47].

2.3.3 Spontaneous seizures detection and quantification

Rats exposed to electrical SE were continuously video-EEG recorded (24 h/day) from SE induction until the first 2 spontaneous seizures occurred, at least 48 h apart from SE

induction (*epilepsy onset*). All EEG seizures were associated with generalized motor seizures (forelimb clonus with or without rearing and/or falling) [46,48]. After epilepsy onset in each rat, video-EEG monitoring was discontinued and resumed at 2 months and 4.5 months post-SE to determine spontaneous seizure frequency by continuous EEG monitoring for 2 weeks (24/7). Spontaneous seizures were discrete EEG ictal episodes lasting on average 60 s, characterized by high-frequency and high-voltage synchronous spike activity and/or multi-spike complexes [46]. EEG was recorded using the TWin EEG Recording System connected with a Comet AS-40 32/8 Amplifier (sampling rate 400 Hz, high-pass filter 0.3 Hz, low-pass filter 70 Hz, sensitivity 2000 mV/cm; Grass-Telefactor, West Warwick, R.I., U.S.A.). Digitized EEG data were processed using the TWin record and review software. EEG was visually inspected for seizure detection and quantification by two independent expert operators blinded to the treatments.

2.3.4 Drug treatment

To maximize the chances of therapeutic outcomes, we used two anti-oxidant drugs with complementary mechanisms of action that should reinforce each others.

The first was the N-acetyl-cysteine (NAC), the precursor of GSH which represents the major non-enzymatic antioxidant pathway of the body. The second was the sulforaphane (SFN), a natural isothiocyanate derived from a glucosinate found in cruciferous vegetables, especially broccoli. SFN is a potent activator of the nuclear factor (erythroid-derived 2)-like 2 (Nrf-2), a transcription factor that regulates the anti-oxidant response by promoting the activation of *Antioxidant Responsive Elements* (ARE). AREs are present in the promoters of different genes encoding for enzymes with detoxifying and anti-oxidant activity as glutathione s-transferase, glutathione peroxidase, NADPH: quinone oxidoreductase 1, heme-oxygenase 1, thioredoxin, and others [49].

2.3.5 In vivo study design

The first set of experiments was done to investigate the level of oxidative stress in the electrical model of SE-induced epileptogenesis. In each experiment, electrode-implanted rats not exposed to SE were used as controls (Sham). In one experiment, rats were exposed to SE and sacrificed 4 days (n=9) or 14 days (n=5) later for HPLC analysis of reduced (GSH), oxidized (GSSG) glutathione and glutathionylated proteins (GS-Pro) in the hippocampus *vs* sham rats (n=15) (Fig.4A). A different group of rats was sacrificed 4 days post-SE (n=5 each group; Sham=4) for immunohistochemical analysis of inducible nitric oxide (iNOS), the cysteine transporter (Xct), the Nrf2 and HMGB1 (Fig.4B).

In the subsequent set of experiments, SE-exposed rats were treated with NAC (Sigma-Aldrich, St. Louis, MO, USA; 500 mg/kg dissolved in H_2O, pH 7.4) and SFN (LKT Laboratories, St Paul, MN, USA; 5 mg/kg dissolved in 0.1% DMSO in buffered saline, pH 7.4) either alone or in combination, or their vehicles.

For determining the effect of each drug alone, or their combination, on oxidative stress (Fig. 5A), a cohort of rats (n=5 rats in each group) was treated with either NAC alone (twice/daily 6 h apart) for 7 days (Fig.15A, *panel a*) or SFN alone (5 mg/kg, i.p., once daily) for 14 days (Fig.15A, *panel b*), or NAC+SFN combination for 7 days (same schedule as each drug given alone) followed by SFN alone for additional 7 days (Fig. 15A, *panel c*). In each treatment schedule, the first drug dose was injected 60 min after SE onset. In the combination protocol, SFN was injected 1 h after the first NAC administration. Rats were sacrificed at the end of each treatment for HPLC analysis of glutathione forms.

For determining if oxidative stress was associated with the generation of disulfide HMGB1 (Fig.10; Fig.15B), a group of SE-exposed rats was treated with NAC+SFN (same

schedule as each drug given alone) or their vehicles for 4 days (n=11 each group), and compared to sham rats (n=15). Both glutathione forms and HMGB1 levels were measured in the hippocampus; HMGB1 was also measured in corresponding venous blood in each animal.

To assess their therapeutic effect on spontaneous seizures, cell loss and cognitive deficits, NAC+SFN (same doses of each drug given alone) were co-administered for 7 days post-SE followed by SFN administered alone for additional 7 days (Fig.15C; Figs.8 and 9). Rats were randomly assigned 1 h after SE onset to either drug (n=9) or vehicle groups (n=9), and EEG was recorded as previously described. At the end of EEG recording, rats underwent the T-Maze test, then they were sacrificed and blood and brain were collected for subsequent analysis.

2.3.6 Blood collection

In the cross-sectional study (Fig.10), blood was collected by the heart atrium at the time of sacrifice, i.e., 4 days after SE onset. In the longitudinal study (Fig.11), blood was drawn by the tail vein at the end of treatment (i.e., 14 days post-SE) in animals under light isofluorane anaesthesia placed on a warming pad to avoid hypothermia. Blood was drawn (~500 µl) using a butterfly (21G needle) and collected in VACUTAINER test tubes. Plasma was isolated according to standard procedures, aliquoted and stored at -80°C until assay.

2.3.7 Rat brain histology and immunohistochemistry

Rats were deeply anaesthetized using ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) then perfused *via* the ascending aorta [50,51]. The brains were removed from skull and post-fixed for 90 min at 4°C, the transferred to 20% sucrose in phosphate buffered saline

(PBS) for 24 h at 4°C, frozen in n-pentane for 3 min at −50°C and stored at −80°C until assay. Serial horizontal sections (40 μm) were cut on a cryostat throughout the temporal extension of the hippocampus (7.6 to 4.6 mm from bregma) [44]. All evaluations were done by an experienced investigator blinded to the treatment.

Nissl staining. Cell loss was measured as previously described [52,53]. Briefly, images of the whole hippocampus in each hemisphere were captured at 20X magnification using a BX61 microscope equipped with motorized platform (Olympus, Germany) and digitized. Quantification was done in 4 Nissl-stained horizontal sections of the stimulated temporal hippocampus in each rat brain. Neuronal cell loss was quantified by reckoning the number of Nissl-stained neurons in CA1 and CA3/CA4 pyramidal cell layers, the hilar interneurons and the entorhinal cortex. Nissl-positive cells were marked by one investigator blinded to the identity of the samples, and an automated cell count was generated using Fiji software. Data obtained in each section/area/rat were averaged, thus providing a single value for each area/rat, and this value was used for the statistical analysis. Although this cell counting method has some limitations as compared to design-based stereological analysis [54], the occurrence of any bias in counting neurons should similarly affect control and experimental samples since these samples underwent the same methodological procedures in parallel.

Immunohistochemistry. Inducible nitric oxide (iNOS), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and system xc- cystine/glutamate antiporter (Xct) were assessed as established cellular markers of oxidative stress. For each rat brain, we used one set of 12 consecutive slices, using 4 alternate slices for each marker. The effect of anti-oxidant drug combination on intracellular HMGB1 localization was evaluated (4 slices/rat).

<u>iNOS:</u> slices were incubated at 4°C for 10 min in 1% H_2O_2 in PBS followed by 1 h incubation in 10% fetal bovine serum (FBS) in 0.4% Triton X-100 in PBS. Then, they

were incubated at 4°C with a primary anti-iNOS antibody (1:250, Abcam) in 10% FBS in 0.2% Triton X -100 in PBS for 24 h.

Nrf2: slices were incubated at 4°C for 30 min in 10% FBS in 0.4% Triton X-100 in PBS. Then, slices were incubated with a primary anti-Nrf2 antibody (1:1500, Santa Cruz, La Jolla, CA, USA) at 4°C in 10% FBS and 5% bovine serum albumin (BSA) in 0.1% Triton X-100 in PBS for 24 h.

Xct: slices were incubated at 4°C for 30 min in 10% FBS in 0.2% Triton X-100 in PBS. Then, they were incubated at 4°C with a primary anti- Xct antibody (1:350; donated by Prof. La Bella) in 10% FBS and 5% BSA in 0.1% Triton X-100 in PBS for 24 hours.

HMGB1: slices were incubated at 4°C for 1 h in 10% FBS in 0.1% Triton X-100 in PBS, followed by an overnight incubation with the primary antibody against HMGB1 (1:1000; Abcam, Cambridge, UK) at 4°C in 10% FBS in 0.1% Triton X-100 in PBS [34].

All sections were reacted using DAB. For the co-localization experiments, we used 6 additional slices per animal for each oxidative stress marker. After incubation with the primary antibody against iNOS or Nrf2 or Xct slices were incubated at 4°C with the secondary anti-rabbit antibody conjugated with Alexa488 (1:500; Molecular Probes) in 0.4% Triton X-100 in PBS for 30 min. For HMGB1, slices were incubated with an anti-rabbit biotinylated secondary antibody (1:200, Vector Labs, Burlingame, CA, USA), then in streptavidin–HRP and the signal was revealed with tyramide conjugated to Fluorescein using TSA amplification kit (NEN Life Science Products, Boston, MA, USA). The slices were then incubated at 4°C for 24 h in 3% FBS in 0.1% Triton X-100 in PBS with an antibody directed against the fibrillary acidic protein (GFAP, 1:3500, Chemicon), selective marker of astrocytes, or with an anti-OX-42 (1:100 antibody, Serotec), marker of microglial cells, or with an antibody against the neuronal nuclear protein (NeuN, 1:1000, Chemicon), or with mouse anti-rat endothelial protein (EBA, 1:10,000, Sternberger,

Lutherville, MD, USA) to identify microvessels. The fluorescence was detected using secondary antibodies conjugated with Alexa546 (1:250; MolecularProbes). Each slice was additionally incubated in Hoechst 33258 (1:500; Molecular Probes) in PBS to make the cell nuclei visible. The sections were then examined with an Olympus confocal microscope (BX61 microscope and confocal FV500) system using the 488 nm excitation waves (laser Ar) for fluorescein, of 546 nm (He-Ne Laser green) to Alexa546, and 350 nm (UV).

Using 4 slices/rat/marker, calretinin and somatostatin immunostaining was performed to label hippocampal interneurons. For calretinin immunostaining, slices were incubated at room temperature (RT) for 30 min in 0.4% Triton X-100 in TBS, then for 90 min in 10% FBS followed by 24 h incubation with the primary antibody against calretinin (1:1000; Swant 7698) at RT in 10% FBS in 0.4% Triton X-100 in TBS. For somatostatin immunostaining, slices were incubated at RT for 2 h in 10% FBS, 0.1% BSA, 0.4% Triton X-100 in TBS, followed by 24 h incubation at RT with the primary antibody against somatostatin (1:2000; donated by Prof. Sperk) in 10% FBS, 0.1% BSA, 0.4% Triton X-100 in TBS. The sections were reacted using DAB and the signal was amplified by nichel ammonium. Images of the whole hippocampus in each hemisphere were captured at 20X magnification using a BX61 microscope equipped with motorized platform (Olympus, Germany) and digitized. Calretinin and somatostatin immunopositive cells were identified in the hilus by one investigator blinded to the identity of the samples, and an automated cell count was generated using Fiji software. Data obtained in each of the 4 sections/marker/rat were averaged, thus providing a single value for each marker in each rat, and this value was used for the statistical analysis.

2.3.8 Human subjects

The cases included in this study were obtained from the archives of the departments of neuropathology of the Academic Medical Center (AMC, Amsterdam, The Netherlands) and the VU University medical center (VUmc, Amsterdam, The Netherlands). A total of 5 hippocampal specimens (removed from patients undergoing surgery for drug-resistant epilepsy) and 11 hippocampal specimens obtained at autopsy from patients that died after SE were examined. Control material was obtained during autopsy of age-matched individuals without a history of seizures or other neurological diseases. Tissue was obtained and used in accordance with the Declaration of Helsinki and the AMC Research Code provided by the Medical Ethics Committee. All cases were reviewed independently by two neuropathologists and the classification of hippocampal sclerosis was based on analysis of microscopic examination as described by the International League Against Epilepsy [54]. The clinical features of the cases analysed are reported in Table 1.

	Control (n=7)	Hippocampal Sclerosis (n=5)	Post-SE (n=11)
Male/Female	4/3	2/3	4/7
Mean age of surgery or death after SE (years)	46 (25-76)	43.6 (35-62)	64 (31-87)
Mean duration of epilepsy (years)	Not applicable	17 (11-32)	Not applicable
Time of death after SE (days)	Not applicable	Not applicable	1-49

Table 1. Summary of clinical features of cases according to pathology.

2.3.9 Immunohistochemistry in human brain

Human brain tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 5 μm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel, Braunschweig, Germany) and processed for immunohistochemical staining. Sections were deparaffinated in xylene, rinsed in ethanol (100%, 95%, 70%) and incubated for 20 minutes in 0.3% hydrogen peroxide diluted in methanol. Antigen retrieval was performed using a pressure cooker in Tris-EDTA buffer (10mM Tris + 1mM EDTA, pH 9) at 120°C for 10 minutes. Slides were washed with phosphate-buffered saline (PBS; 0.1 M, pH 7.4) and incubated overnight with primary antibody (anti-iNOS antibody, 1:250, Abcam; anti-Nrf2 antibody, 1:500, Santa Cruz ; anti-Xct antibody, 1:250) in PBS at 4°C. For single labeling, after washing with PBS, sections were stained with a polymer based peroxidase immunohistochemistry detection kit (Brightvision plus kit, ImmunoLogic, Duiven, The Netherlands) according to the manufacturer's instructions. Staining was performed using Bright DAB substrate solution (1:10 in 0.05 M Tris-HCl, pH 7.6; ImmunoLogic) with 0.015% H_2O_2. Sections were dehydrated in alcohol and xylene and coverslipped.

For double labeling, sections with primary antibodies were incubated with Brightvision poly-alkaline phosphatase (AP)-anti-rabbit (Immunologic) and horseradish peroxidase labelled (HRP)- goat anti-mouse IgG (1:100, Southern Biotech, Birmingham, USA) for 1 hour at room temperature. Primary antibodies for NeuN (neuronal nuclear protein, mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:200) and GFAP (monoclonal mouse, Sigma, St. Louis, Mo, USA; 1:4000), were incubated overnight at 4°C. NeuN and GFAP staining was visualized with 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich, Zwijndrecht, Netherlands).

2.3.10 Analysis of HMGB1 isoforms by electrospray ionization liquid chromatography mass spectrometry

Rats were deeply anesthetized using ketamine (75 mg/kg) and medetomidine (0.5 mg/kg), then rapidly perfused via the ascending aorta with 50 mM ice-cold PBS (pH 7.4) for 1 min to remove blood from brain vessels. Then, rats were decapitated, brain removed and the ventral hippocampi were dissected out at 4°C, immediately frozen in liquid nitrogen and stored at −80 °C. The analysis of the isoforms of HMGB1 was performed as previously described in detail [56]. Non-identifiable samples were analyzed by an investigator blinded to the identity of the samples. All chemicals and solvents were of the highest available grade (Sigma Aldrich, Dorset, UK). Samples were precleared with 50 µl protein G-Sepharose beads for 1 h at 4°C. Supernatant HMGB1 was immunoprecipitated with 5 µg rabbit anti-HMGB1 (Abcam; ab18256) for 16 h at 4°C as previously described [57]. Free thiol groups within HMGB1 were alkylated for 90 min with 10 mM iodoacetamide at 4°C. Cysteine residues in disulfide bonds were then reduced with 30 mM DTT at 4°C for 1 h followed by alkylation of newly exposed thiol groups with 90 mM NEM at 4°C for 10 min. Samples were subjected to trypsin (Promega, Southampton, UK) or GluC (New England Biolabs, Hitchin, UK) digestion according to manufacturer's instructions and desalted using ZipTip C18 pipette tips (Merck Millipore). Characterization of whole protein molecular weights, acetylated lysine residues, or redox modifications on cysteine residues within HMGB1 were determined as described previously by whole protein electrospray ionization or tandem mass spectrometry [56,57] using either an AB Sciex QTRAP 5500 or an AB Sciex TripleTOF 5600 (Sciex Inc., Warrington, UK). Peptide analysis was determined using an AB Sciex QTRAP 5500 equipped with a NanoSpray II source by in-line liquid chromatography using a U3000 HPLC System (Dionex, Thermo Fisher UK Ltd., Hemel Hempstead, UK), connected to a 180 µm × 20 mm nanoAcquity

UPLC C18 trap column and a 75 μm × 15 cm nanoAcquity UPLC BEH130 C18 column via reducing unions. A gradient from 0.05% TFA (v/v) to 50% ACN/0.08% TFA (v/v) in 40 min was applied at a flow rate of 200 nl/min. The ionspray potential was set to 2200–3500 V, the nebulizer gas to 19, and the interface heater to 150°C.

The proteolitic digestions of HMGB1 required for LC/MS-MS analysis generates peptide fragments that either contain the acetylation sites or the redox sensitive sequence. Therefore, the results shown in Fig.10C,D and Fig.11B depict acetylated HMGB1 separately from the reduced and disufide isoforms. Since reduced and disulfide HMGB1 are mutually exclusive isoforms, their amounts account for total HMGB1 levels. Finally, HMGB1 isoforms in serum have been determined by a patented (USA US8748109 B2, Europe EP2449378, Japan 5721707) absolute quantification method [56,57] which has been extensively validated to bioanalytical guidelines set out by the SAFE-T IMI funded biomarker consortium (http://www.imi-safe-t.eu/). Since a method for absolute quantification of HMGB1 isoforms has not been extensively validated for brain tissue as yet, HMBG1 isoforms in brain are expressed as fold-change of respective basal values.

Total HMGB1 was measured by a commercially available ELISA kit in accordance with manufacturer's instructions (Shino-test Corp, Sagamihara, Japan).

2.3.11 HPLC analysis of GSH, GSSG, GS-Pro

The ventral hippocampus in each rat was longitudinally divided into two parts which were randomly used for the quantification of free GSH or GSSG, GS-Pro and glutathione precursors, respectively. The sample preparation and HPLC analysis were performed as previously described in detail [58]. The various molecules were identified in the chromatogram according to their different retention time, and subsequently quantified. Values were expressed as nmol of compound/mg tissue protein measured using the

bicinchoninic acid protein assay (BCA, Pierce, Rockford, Illinois, USA). The amount of total glutathione was calculated by summing free GSH, GSSG and GS-Pro. Samples were analyzed by an investigator blinded to their identity.

The levels of GSH and GSSG in plasma were measured with an ESA (Chelmsford, MA) 5600 CoulArray HPLC equipped with eight electrochemical cells following the method described previously [59].

2.3.12 Behavioral test

Hippocampal-dependent spatial memory was measured in a standard two arms T-maze apparatus (50x40x10 cm each arm) in rats undergoing the longitudinal study for seizure assessment at the end of EEG recording (i.e., 5 months post-SE). Animals were tested by an investigator blinded to their identity. Animals were placed into the starting arm of the T-maze and allowed to freely choose to enter one of the two arms in each of the seven trials. A successful alternation consisted of alternate arm entries while unsuccessful alternation occurred when the rat returned to the most recently explored arm. Total arm entries and sequence of entries were recorded for each rat and as percent of correct choice was reckoned, i.e., the number of correct alternations/the maximum number of alternations × 100. Rats with intact hippocampal-dependent spatial memory will remember the arm that was previously visited and will prefer to enter a new, unexplored arm (alternation rate ≥ 60%).

2.3.13 Cortical cell cultures

Mixed cortical neurons and glial cells cultures were prepared from postnatal (P0-P1) Sprague-Dawley rat pups (UCL breeding colony) using an enzymatic procedure according to a modified protocol described by Haynes [60,61]. The pups were sacrificed by cervical

dislocation, and rat brains were quickly removed and neocortical tissue was isolated and submerged in ice-cold HBSS (Ca^{2+}, Mg^{2+}-free, Gibco-Invitrogen, Paisley, UK). The tissue was treated with 1% trypsin for 10 minutes at 37°C to dissociate cells. The final neuronal cell suspension was plated on 25 mm round coverslips coated with poly-L-lysine (1 mg/ml, Sigma), and cultured in Neurobasal A medium (Gibco-Invitrogen) supplemented with B-27 (Gibco-Invitrogen) and 2 mM L-glutamine. Neocortical cultures were fed once a week and maintained in a humidified atmosphere of 5% CO2 and 95% air at 37 °C in a tissue culture incubator. The cultures were used for experiments at 13-17 DIV. Neurons were distinguished from glia by their typical appearance using phase-contrast imaging with smooth rounded somata, bright-phase and distinct processes.

2.3.14 Imaging of intracellular Ca^{2+} ($[Ca^{2+}]_c$) and mitochondrial membrane potential ($\Delta\psi m$)

Preincubations and experiments were performed at room temperature in an HEPES-buffered salt solution (aCSF), composition in mM: 125 NaCl, 2.5 KCl, 2 $MgCl_2$, 1.25 KH_2PO_4, 2 $CaCl_2$, 30 glucose and 25 HEPES, pH adjusted to 7.4 with NaOH.

Experiments were carried out in either the HEPES buffered salt solution including (aCSF) or excluding $MgCl_2$ (low Mg^{2+}) [60]. Before recording, neocortical neuronal cultures were incubated for 30 minutes with 5 μM Fura-2-AM (Invitrogen, Paisley, UK), and 0.005% pluronic acid in aCSF.

For simultaneous measurement of $[Ca^{2+}]_c$ and $\Delta\psi m$, Rhodamine123 (Rh123) (Sigma, UK) (2 μM) was added into the culture dishes during the last 15 minutes of the Fura-2-AM loading period. Cells were then washed 3 times prior to recordings. Fluorescence images were made on an epifluorescence inverted microscope equipped with a 20X fluorite objective. Measurements of $[Ca^{2+}]_c$ and mitochondrial membrane potential were

performed in single cells using excitation light provided by a xenon arc lamp, the beam passing through a monochromator at 340, 380, and 490 nm with bandwidth of 10 nm (Cairn Research, Faversham, UK). Emitted fluorescent light was reflected through a 515 nm long-pass filter to a cooled CCD camera (Retiga; QImaging) and digitised to 12-bit resolution. Imaging data were analysed using software from Andor (Belfast, UK). Traces are presented as the ratio of excitation at 340 and 380 nm, both with emission at >515 nm. We acquired fluorescent data with a frame interval of 10 seconds. $[Ca^{2+}]_c$ was expressed by the Fura ratio and was not calibrated because of inaccuracies arising from different calibration methods. An increase of Rhodamine123 signal indicates depolarisation of mitochondria. Rhodamine123 signals were normalised to the baseline level (set 0) and maximum signal produced by mitochondrial oxidative phosphorylation uncoupling with carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP, 1 μM; set to 100). Each experiment was repeated 2-3 times using 3-4 different cultures.

2.3.15 Statistical analysis

Sample size was *a priori* determined based on previous experience with the epilepsy model as well as following the principles of the 3 Rs (Replacement, Reduction and Refinement; https://www.nc3rs.org.uk/the-3rs). Endpoints (outcome measures) and statistical tests were prospectively selected. A simple random allocation was applied to assign a subject to a particular experimental group. All efforts were made to minimize the number of animals used and their suffering. Data acquisition and analysis was done blindly.

Statistical analysis was performed by GraphPad Prism 7 (GraphPad Software, USA) for Windows using absolute values. Data are presented as mean ± s.e.m. (n = number of individual samples). Mann–Whitney test for two independent groups and Kruskal-Wallis

followed by Dunn's post-hoc test for more than two independent groups were used for statistical analysis of data. In the longitudinal study, changes in time to seizure onset were analyzed by Log-rank (Mantel Cox) test. The temporal distribution of spikes during SE was analysed by two-way ANOVA followed by Bonferroni's multiple comparisons test. Differences were considered significant with a $p<0.05$.

2.4 RESULTS

2.4.1 Assessment of oxidative stress during epileptogenesis

We studied whether oxidative stress was generated during epileptogenesis in SE-exposed rats by measuring the hippocampal levels of oxidized (GSSG) and reduced glutathione (GSH) and their ratio (GSSG/GSH) which is an established indicator of reactive oxygen species (ROS) production [12,63]. We choose two time points post-SE reflecting early epileptogenesis (before the onset of epilepsy, i.e. 4 days) and shortly after disease onset (when the treatment was stopped, i.e., 14 days). As previously reported in other rodent models of epileptogenesis, the levels of both GSH and GSSG significantly changed ($p<0.01$) between 4 days and 14 days post-SE (n=5-9) resulting in a progressive 3- to 14-fold increase in GSSG/GSH ratio above control values (in sham rats not exposed to SE, n=15) (Fig. 4A). A concomitant increase in glutathionylated proteins (Gs-Pro) was measured in the same hippocampal tissue (Fig. 4A). To determine which cell types were undergoing oxidative stress, immunohistochemical analysis was done in a different cohort of rats, 4 days post-SE. Fig. 4B depicts the cellular expression of molecular markers of oxidative stress, namely iNOS (a,b), the cystine transporter (Xct; c,d) and the transcriptional factor Nrf2 (e,f). These molecules were induced in activated GFAP-positive astrocytes (b_1,d_1,f_1) as well as in NeuN-positive neurons (b_2,d_2,f_2) but not in OX-42-positive microglia (*not shown*). In particular, we found increased Nrf2 staining in neuronal nuclei (f_2 vs e_1) likely reflecting transcriptional activation of anti-oxidant enzyme genes in response to ROS production [64-66].

2.4.2 Anti-oxidant drug combination vs single treatment

In order to design the optimal treatment protocol for the therapeutic study (Fig. 15C), we tested whether a combination of NAC and SFN, two anti-oxidant drugs with

complementary mechanism of action, was more effective in reducing oxidative stress than each drug given alone. We designed a treatment schedule of 14 days to encompass the time window between the epileptogenic injury (i.e., SE) and the early phase after disease onset. Then, treatment was stopped for determining whether antiepileptogenic or disease-modification therapeutic effects occur after drug withdrawal. Based on the available PK and PD information [67-72] we treated different cohorts of rats with either NAC (500 mg/kg, i.p., twice/day for 7 days) or SFN alone (5 mg/kg, i.p., once daily for 14 days), or their combination (NAC+SFN injected for 7 days followed by SFN injected alone for additional 7 days). Treatment began 1 h after the onset of SE (Fig. 15A). NAC was administered to rats for 1 week to attain a *rapid scavenging* action of ROS during SE [73]. SFN was administered for one additional week after NAC withdrawal to provide a *sustained anti-oxidant effect* [73]. Fig. 5A shows that GSH and GSSG were modified by SE resulting in a significant increase in GSSG/GSH ratio ($p<0.01$ vs sham, n=5 each group). Each drug alone increased GSH and reduced GSSG compared to SE-exposed rats receiving vehicles ($p<0.01$, n=5 each group). However, the combination of NAC and SFN showed a greater effect *vs* single drugs ($p<0.01$) in normalizing both GSH and GSSG levels and their ratio. Similarly, Gs-Pro level returned to sham value after the drug combination whereas it was still significantly elevated in rats treated with each drug alone. We further tested whether the drug combination was more effective than the individual drugs in preventing mitochondrial dysfunction using primary neuronal cortical cultures where epileptiform activity was induced by removing extracellular Mg^{2+} (Fig.6). We measured the changes in the mitochondrial inner membrane potential evoked by epileptiform activity and the effects of drugs. Fig.5B shows a progressive increase in mitochondrial membrane depolarization which was positively correlated with the time of exposure to low Mg^{2+}-induced epileptiform activity (from 10 to 30 min; Fig. 6B). This

effect was significantly reduced by preincubation with SFN or NAC alone; notably, NAC induced membrane hyperpolarization ($p<0.01$ *vs* respective aCSF in Fig. 5B; Fig. 6C,D). The combination of NAC+SFN was more effective in preventing inner membrane depolarization and increasing membrane hyperpolarization than each drug alone (at 25 and 30 min; $p<0.01$ *vs* each drug alone; Fig. 5B; Fig. 6E).

2.4.3 Therapeutic effects of antioxidant drug combination in SE-exposed rats

The combined treatment protocol was applied starting 1 h after SE onset (Fig. 15C). The drug combination did not attenuate the overall severity of the initiating injury, namely the duration of SE, or the frequency of spikes and their total number, as quantified by continuous EEG analysis for 24 h from SE onset. No difference was detected between the treatment and vehicle groups in the relative power distribution for each band during SE (Fig. 7B,C).

2.4.4 Spontaneous seizures onset and their progression

Rats exposed to SE and injected with vehicle developed spontaneous recurrent seizures (SRS) 8.6 ± 0.7 days (n=9) after SE onset (Fig.8A,B). Rats treated for 2 weeks during epileptogenesis with NAC+SFN showed a significant delay in the time to seizure onset (11.7±1.1 days, n=9, $p<0.01$) compared to vehicle-injected controls (Fig.8B). This epilepsy model is characterized by an average 5-fold increase in SRS frequency between 2 months (5.1 ± 1.8 SRS/2 weeks) and 5 months (24.2 ± 7.7 SRS/2 weeks) post-SE. Although the number of seizures was not significantly modified by the drugs during treatment (3.6 ± 0.9, n=9) as compared to their vehicle controls (2.4 ± 0.5, n=9), SRS progression was prevented in drug-exposed rats after treatment withdrawal (Fig.8C, $p<0.01$ *vs* vehicle). Overall, drug-treated rats showed ~70% SRS reduction at 5 months

post-SE compared to vehicle-injected rats ($p<0.05$; Fig.8C). Total SRS duration during 2-week EEG recording at 5 months was significantly reduced by drugs compared to vehicle controls ($p<0.05$; Fig.8D).

2.4.5 Cognitive deficits

Rats were tested in the T-maze at the end of EEG recordings (i.e. 5 months post-SE). SE-exposed animals treated with vehicles showed an impairment of spatial memory in the T-maze, as shown by failure of correct alternation in the entry arm of the maze (40.5 ± 2.8% correct alternation, n=9, $p<0.01$) compared to sham rats (66.8 ± 3.9% correct alternation, n=9) (Fig.8E). The drug combination rescued this behavioral deficit as shown by the correct alternation rate (68.4 ± 5.0%, n=9) of treated rats which was similar to sham rats (Fig.8E). No differences in locomotion were detected among the experimental groups in the open field task (*not shown*). All rats were confirmed to be epileptic before the T-maze test (Fig.8C) but they did not show behavioral seizures during the test.

2.4.6 Neurodegeneration

At the end of behavioral test, rats were killed for quantitative analysis of cell loss in Nissl-stained forebrain sections of the ventral pole of the stimulated hippocampus (Fig.9; rats are the same reported in Fig.8). Vehicle-injected rats showed significant neuronal cell loss in CA1 and CA3 pyramidal cell layers and hilar interneurons (about 40% decrease *vs* sham rats; $p<0.01$; Fig.9A,B). The anti-oxidant treatment reduced cell loss by half in CA1 ($p<0.05$; panel A) and virtually prevented the neurodegeneration of hilar interneurons ($p<0.01$; panel B). In particular, hilar calretinin- (but not somatostatin, *not shown*) positive cells were significantly protected by the drug combination (panel C). No significant

neuroprotection was observed in CA3, the region of electrical stimulation, or in adjacent entorhinal cortex (*not shown*).

2.4.7 Effects of the anti-oxidant drug combination on the redox state of HMGB1

We tested the novel hypothesis that reduction of oxidative stress during epileptogenesis prevents the generation of the pathologic disulfide HMGB1 isoform in the brain. We found that NAC+SFN decreased oxidative stress already 4 days post-SE (Fig. 10A), and this effect was similar when treatment was started 1 h (Fig.10A) after SE onset. At the same time point, we found immunohistochemical evidence of nucleus-to-cytoplasm translocation of HMGB1 in astrocytes, microglia and brain endothelium of SE-exposed rats (Fig. 10B, panel b *vs* a) which is indicative of its extracellular release. Accordingly, LC-MS/MS measurements of HMGB1 isoforms in the hippocampus showed a 10-fold increase of the acetylated (*releasable*) HMGB1 isoform 4 days post-SE compared to sham rats (Fig. 10C; $p<0.01$ vs sham; n=9-11). We also found that the pathologic disulfide HMGB1 isoform, which is absent in control brain tissue, is generated during epileptogenesis (Fig. 10C; $p<0.01$ vs sham; n=9-11). The increase in both acetylated and disulfide HMGB1 (Fig. 10C), as well as the cytoplasmatic translocation of HMGB1 (Fig. 10B, panel c *vs* b), were abolished by NAC+SFN. We also detected a minor but significant increase ($p<0.01$) in reduced HMGB1 during epileptogenesis which is the constitutive isoform bound to nuclear chromatin (Fig. 10C). NAC+SFN blocked also the reduced and total HMGB1 increase (Fig. 10C). Notably, the changes in brain HMGB1, and the effects of treatment, were similarly detected in the blood of the same animals (Fig. 10D).

Based on these findings, we measured the GSSG/GSH ratio as well as total HMGB1 and its isoforms in the blood of SE-exposed rats undergoing the therapeutic trial (same rats as

in Fig.8). Blood was drawn by tail vein at the end of treatment (i.e., 14 days post-SE). GSSG/GSH ratio (Fig. 11A; $p<0.05$), total HMGB1 and its isoforms (Fig. 11B; Fig. 2; $p<0.01$) were increased in blood of SE-exposed rats compared to sham rats. All these effects were prevented by drug treatment (Fig. 11A,B).

2.4.8 Oxidative stress in brain specimens from patients with status epilepticus and in temporal lobe epilepsy

We used immunohistochemistry to analyze the presence of oxidative stress markers in autoptic hippocampal specimens from patients experiencing SE as well as in surgically resected human hippocampal tissue from temporal lobe epilepsy (TLE). Fig. 12 shows increased expression of iNOS, Xct and Nrf2 in NeuN-positive neuronal cells and GFAP-positive astrocytes in a patient who died 49 days after SE. Nrf2 signal was increased prominently in cell nuclei, an indication of its nuclear translocation. A similar pattern of cellular expression of these markers was observed in SE patient specimens evaluated between 1 and 49 days post-SE and in chronic epilepsy hippocampal tissue from TLE patients (Fig. 13). Notably, HMGB1 cytoplasmatic staining was increased in both neurons and astrocytes in adjacent slices (Fig. 14), in accordance with previous finding in human TLE [34].

2.5 DISCUSSION

After brain injury and during seizures, mitochondrial dysfunction and increased NADPH oxidase and xanthine oxidase activities lead to excessive generation of ROS, thereby contributing to neuropathology [11,62]. Indeed, animal models of acquired epilepsy provide evidence of profound changes in mitochondrial function and ROS production as a result of various epileptogenic injuries. These alterations occur rapidly after the inciting event, persist during epileptogenesis and are still observed in the chronic epilepsy phase [12,19,75]. It is likely that the mechanisms leading to oxidative tissue damage vary in the different phases of the epileptic process [11]. It remains to be determined if, and by which mechanisms, ROS generation contribute to the onset and recurrence of spontaneous seizures. Importantly, whether targeting oxidative stress has any effect on epileptogenesis is still unresolved.

Our novel findings show that a transient post-injury intervention with a specific combination of antioxidant drugs, namely NAC and SFN, mediates clinically relevant therapeutic effects in a rat model of acquired epilepsy. This combined treatment was more effective in rescuing mitochondrial dysfunction and reducing oxidative stress during epileptogenesis than the single drugs alone. Both NAC and SFN were previously shown to provide neuroprotection in various brain injury models [65,76-78] and displayed anticonvulsive properties in animal models of acute seizures [65,78,79]. Notably, NAC showed anticonvulsive effects in human progressive myoclonus epilepsy [80].

Oxidative stress has been implicated in cell loss and cognitive dysfunctions developing during epileptogenesis in different animal models [81-84]. Accordingly, our drug combination afforded neuroprotection and rescued cognitive deficits in a reference/working memory test by preventing the persistence of oxidative stress during epileptogenesis. Neuroprotection is compatible with the role of ROS in glutamate

excitotoxicity as well as in apoptotic cell death [85-87]. Additionally, we show that calretinin-positive cells in dentate hilus are particularly sensitive to neuroprotection mediated by our drug combination. These hilar interneurons form a subpopulation of GABAergic cells with frequent axo-dendritic and dendro-dendritric contacts with other inhibitory interneurons. This unique connectivity may enable them to play a crucial role in the generation of synchronous, rhythmic hippocampal activity by controlling other interneurons terminating on dendritic and somatic compartments of principal cells [88], therefore they are suggested to play a key role in the hippocampal inhibitory network. Notably, the density of calretinin-immunopositive cells is decreased significantly in the sclerotic hippocampus from human TLE, a phenomenon that may contribute to seizure generation and recurrence [89]. Whether neuroprotection plays a role in the rescue of cognitive deficit in the T-maze remains speculative. Similar positive effects on cognitive dysfunctions were recently reported using SFN in a model of okadaic acid-induced memory impairment [90] or using a metalloporphyrin catalytic antioxidant in a rat model of pilocarpine-induced epileptogenesis [84]. However, at variance with the lack of effect of antioxidant intervention on SRS reported in a previous study [84], our combined treatment significantly delayed epilepsy onset and induced a long-term reduction in spontaneous seizure frequency and duration compared to vehicle-injected rats. It is possible that our drug combination is particularly effective in antagonizing oxidative stress damage contributing to SRS. One major difference may be due to the mechanism of antioxidant action of direct antioxidants used in previous studies vs Nrf2 inducer used in this study. The latter induces multiple genes many of which encode endogenous antioxidants resulting in longer-lasting effects [74]. Another factor possibly explaining the difference in results is the longer video-EEG monitoring of our study until the late phases of disease development which allowed to appreciate the effect of antioxidant treatment on

seizure progression. Our study is therefore the first report showing that antioxidant intervention applied for a limited time post-injury arrests the progression of epilepsy resulting in a strong reduction of SRS in the chronic epilepsy phase. Since these therapeutic effects outlasted by several weeks the end of treatment, they indicate that our antioxidant drugs mediate disease-modifying effects. Similar effects on spontaneous seizures progression were recently reported using an inducible nitric oxide inhibitor reducing reactive nitrogen species [91].

Disturbances in the normal redox state of the cells may contribute to epileptogenesis in various manners. There is evidence of at least two potential links between mitochondrial oxidative stress and increased neuronal excitability, namely bioenergetic failure due to increased demand for neuronal mitochondria to produce cellular energy during hyperexcitability phenomena, and metabolic fuel utilization [11]. Moreover, neuronal excitability is controlled by glutamate and GABA, the biosynthesis of which depends on mitochondria [92]. ROS have the potential to influence epileptogenesis also *via* oxidative damage to macromolecules including proteins, lipids, and DNA. We tested the novel hypothesis that a pathological switch in the redox state of brain tissue during epileptogenesis leads to the generation of disulfide HMGB1, a proinflammatory molecules with neuromodulatory and ictogenic properties [34,35,43,93]. Studies in literature showed that disulfide HMGB1 contributes to seizure generation and excitotoxic cell loss by activation of TLR4 and RAGE [34,35] and mice lacking either one of these receptors develop a milder form of epilepsy following SE [35]. Moreover, HMGB1 by activating TLR4 and RAGE mediated cognitive dysfunctions in mice [94]. Overall, this set of evidence supports the novel concept that disulfide HMGB1 may be a key mediator of the pathological effects of oxidative stress during epileptogenesis. In accordance, we found that the antioxidant effects of our drug combination were associated with prevention of

disulfide HMGB1 generation and extracellular release in brain tissue. Notably, the brain changes measured during epileptogenesis in total HMGB1 and its isoforms, as well as in oxidative stress indicators, were mirrored by similar changes in blood, and the blood levels of these molecules were modified by the antioxidant intervention similarly to the brain. Thus, these molecules may be potential biomarkers for determining the efficacy of the anti-oxidant drugs on their targets and possibly predicting their therapeutic effects.

The translation of our findings to the clinical setting is supported by our fresh evidence that oxidative stress occurs in brain of patients experiencing SE, as well as in patients with drug-resistant TLE, and this phenomenon is associated with cytoplasmatic translocation of HMGB1 in neurons and glia. Moreover, both NAC and SFN have been used in human clinical trials at doses comparable with the effective doses in our study. In particular, after extrapolating the human equivalent dose [95], we found that NAC and SFN doses in rats correspond to 5 g twice/daily and to 48 mg/daily for a 60 kg person, respectively. Interestingly, an intravenous infusion of 150 mg/kg NAC (corresponding to 9 g in a 60 kg person) in healthy individuals or Parkinson's and Gaucher's disease patients was well tolerated and resulted in increased brain GSH as assessed by magnetic resonance spectroscopy [96]. Moreover, NAC doses up to 3.6 g/day for several weeks have been used in neurological and psychiatric disorders [97]. In epilepsy clinical studies, NAC was used up to 6 g/daily for several months in progressive myoclonus epilepsy, in particular in Unverricht–Lundborg Disease with evidence of seizure improvement [97]. NAC seemed to be fairly well tolerated with no significant between group differences in most of the controlled trials. As far as SFN is concerned, clinical studies in cancer used daily doses of 60 mg [97], and up to 27 mg were administered daily in autism spectrum disorders [99] with signs of improvement and a safety profile.

In summary, our findings have high translational value since we report novel evidence that: 1. oxidative stress markers occur in the hippocampus of humans who died following SE or with chronic pharmacoresistant epilepsy; 2. the drug doses we used in animals are compatible with human doses in the therapeutic range given for protracted treatment periods.

Noteworthy, symptomatic (structural/lesional) epilepsies are often associated with a worse prognosis, therefore providing an ideal patients population for testing antioxidant drugs with potential disease-modifying properties [100,101].

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2.6 FIGURES AND LEGENDS

Figures 1-3 (included in the main text)

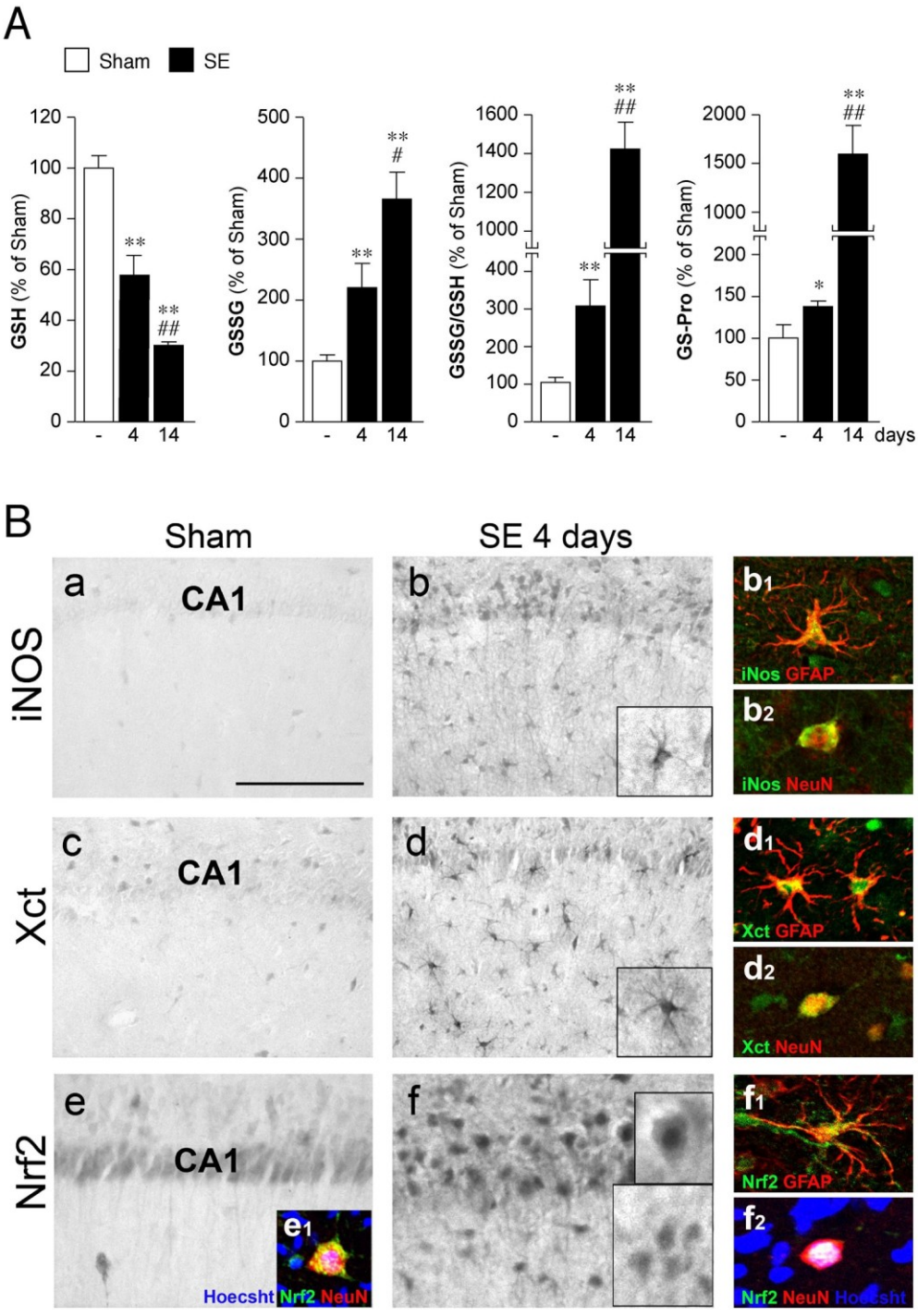

Figure 4. Generation of oxidative stress in hippocampal tissue of rats exposed to electrical SE

Panel A: HPLC analysis of reduced (GSH) and oxidized (GSSG) glutathione levels, and their ratio, and the level of glutathionylated proteins (GS-Pro) in the rat hippocampus at day 4 (n=9) and

14 (n=5) after SE onset compared to corresponding baseline levels in sham rats (electrode-implanted but not stimulated, n=15). Data (mean ± SEM) represent the percent changes compared to sham. *p<0.05; **p<0.01 vs sham by Kruskal-Wallis followed by Dunn's post-hoc test; #p<0.05; ##p<0.01 vs day 4 by Mann-Whitney test. Statistical analysis of data was done using absolute values.

Panel B: Representative immunohistochemical micrographs of the CA1 region depicting the expression of inducible nitric oxide (iNOS), the cysteine transporter (Xct) and the transcriptional nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in sham (a,c,e) and 4 days post-SE (b,d,f) (n=4 each experimental group). Panels b,d,f show the increase in the respective marker expression in GFAP-positive astrocytes (b1,d1,f1) and in neurons (b2,d2,f2). Nrf2 expression is increased in neuronal nuclei (f2 vs e1) indicating increased transcriptional activation of detoxifying enzymes. *Scale bar*, a-f 25 μm; insert in b,d,f 15 μm; immunofluorescence insert, 10 μm.

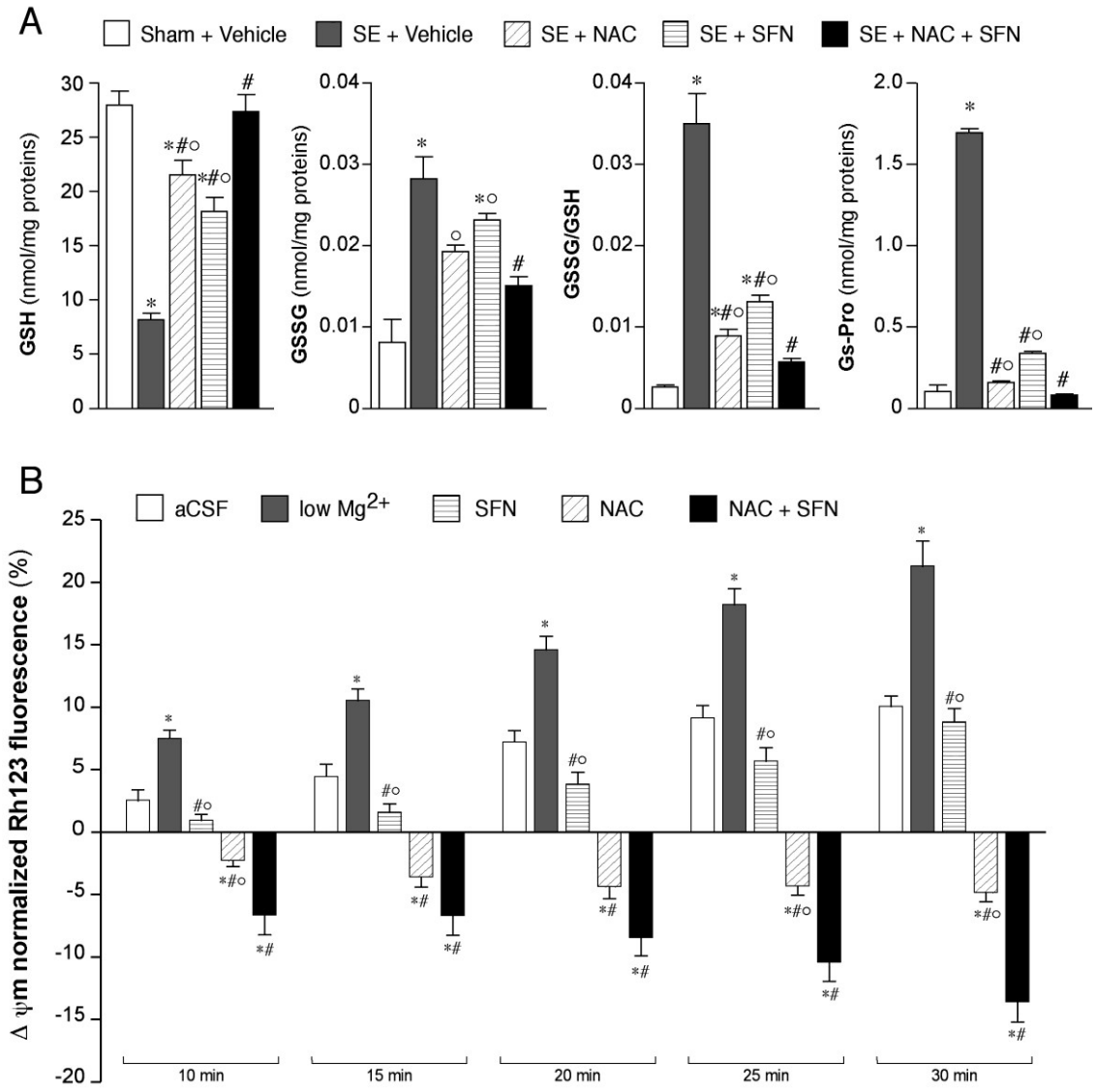

Figure 5. Effect of drug combination vs single drug alone on oxidative stress markers

Panel (A) reports GSH and GSSG levels, and their ratio, and GS-Pro levels in the hippocampus of rats exposed to SE vs sham rats as assessed by HPLC analysis. SE-exposed rats (n= 5 each group) received either vehicle combination, or NAC (500 mg/kg, i.p., twice daily for 7 days) or SFN (5 mg/kg, i.p., daily for 14 days) or their combination (NAC+SFN for 7 days followed by SFN alone for additional 7 days; Fig. 15A). Controls were sham rats injected with vehicle (n= 5). The drug combination reduced oxidative stress to a greater extent than each drug given alone. *p<0.01 vs

Sham; °p<0.01 vs SE+ NAC+ SFN; #p<0.01 vs SE+vehicle by Kruskal-Wallis followed by Dunn's post-hoc test.

Panel B: Bargrams depict low Mg^{2+}-induced mitochondrial membrane potential changes of neocortical cell cultures (Fig. 6B-E). Preincubation of neurons with SFN (5 μM, 24h) decreased the rate of depolarization at all time points. Addition of NAC (10 mM, acutely) evoked hyperpolarization in the mitochondrial membrane potential. Pre-treatment with SFN (5 μM, 24h) with addition of NAC (10 mM, acutely) showed similar effect as NAC alone, with significantly higher hyperpolarization after 25 and 30 min. *p<0.01 vs aCSF; #p<0.01 vs low Mg^{2+}; °p<0.01 vs NAC+SFN by one-way ANOVA followed by Kruskall-Wallis test.

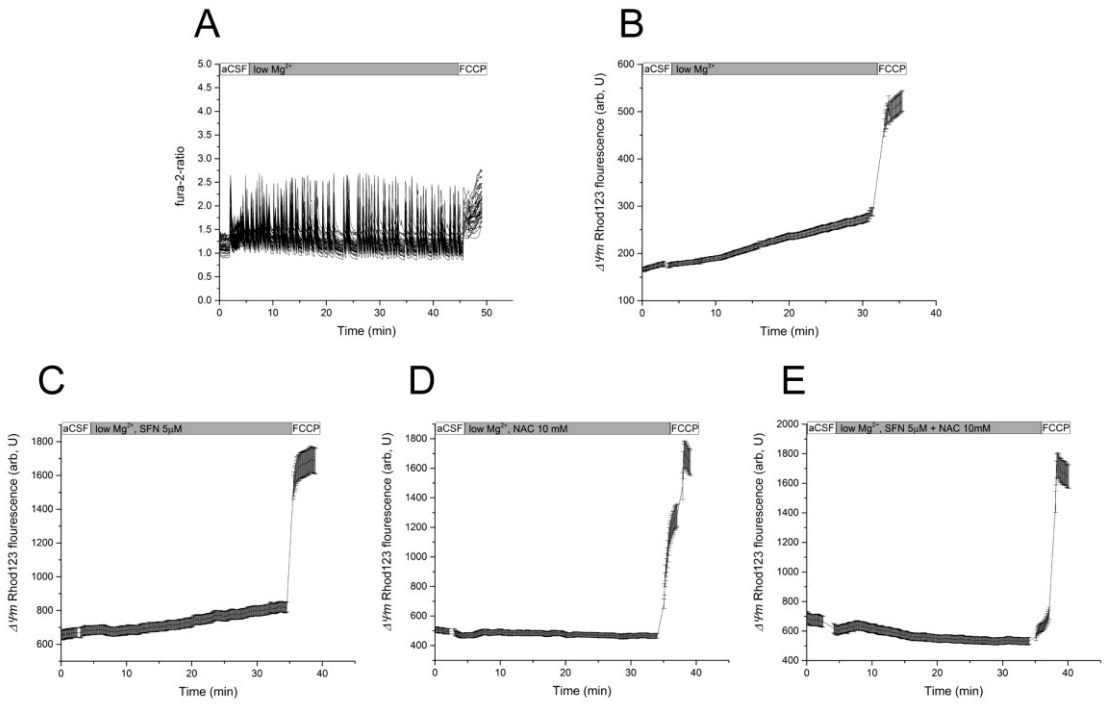

Figure 6. Low-Mg2+-induced Ca2+ signals and mitochondrial depolarization in neurons

Replacement of aCSF with low-Mg2+ aCSF induced synchronous oscillatory Ca2+ signals (A) and mitochondrial depolarization (i.e., increase of Rhodamine123 fluorescence) (B). Treatment with SFN (5 μM, 24h) induces a significant decrease of the effect of low-Mg2+ aCSF on mitochondrial membrane potential in neurons (C). NAC (10 mM, acutely) produced hyperpolarization in the mitochondria (D) and this effect was also observed when neurons were pre-treated with SFN (5 mM, 24h; E). Data are mean ±SEM of 80-100 neurons of one coverslip.

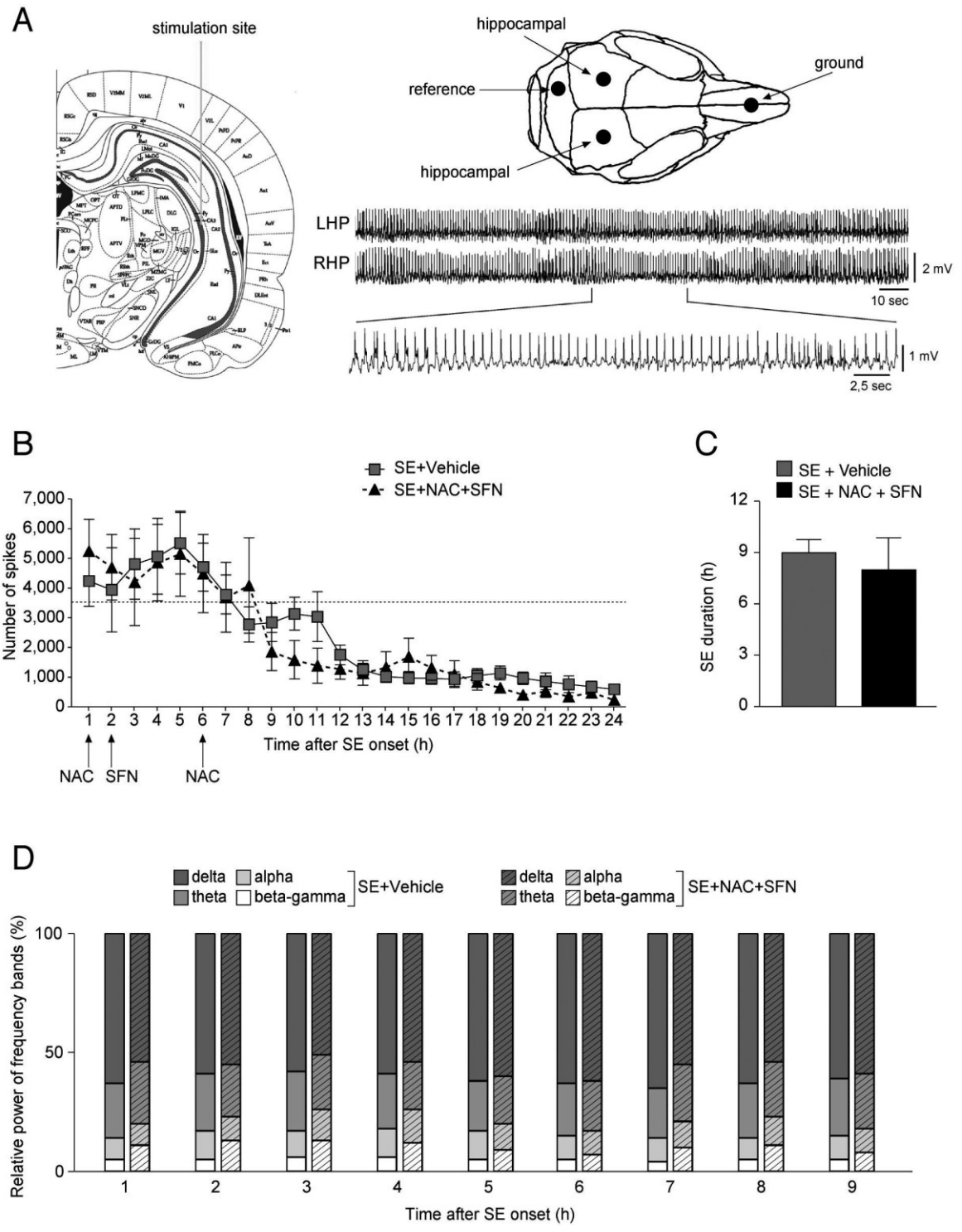

Figure 7 . Experimental model of SE-induced epilepsy and related injection protocol

Panel (A). Brain atlas plate depicting electrode stimulation site in the CA3 area of temporal hippocampus. The schematic skull reproduction shows surface reference and ground electrode

placement and the position of the depth hippocampal electrodes. Representative EEG tracing depicting spike activity occurring during SE as recorded in the left (LHP) and right (RHP) hippocampi. Panel (B). Temporal spike distribution during SE in rats subsequently randomized 1 h after SE onset in vehicle or treatment groups. Each point represents the cumulative number of spikes during progressive 1 h intervals. Curves did not differ by two-way ANOVA followed by Bonferroni's multiple comparisons test. NAC (500 mg/kg, i.p.) was given 1 h after SE onset followed 1 h later by SFN (5 mg/kg, i.p.), then a second NAC injection was done 6 h after the first NAC injection. Rats were treated in the following days as depicted in Fig. 15C. Panel (C) depicts the total duration of SE in the two experimental groups. The end of SE was defined by the occurrence of inter-spike intervals longer than 1 sec. Panel (D) depicts frequency bands (delta, theta, alpha, beta-gamma) expressed as percent relative power for each hour of SE in vehicle- and drug-treated rats. Data were analyzed by two-way repeated measures ANOVA. Data are mean±SEM (n=9 each group).

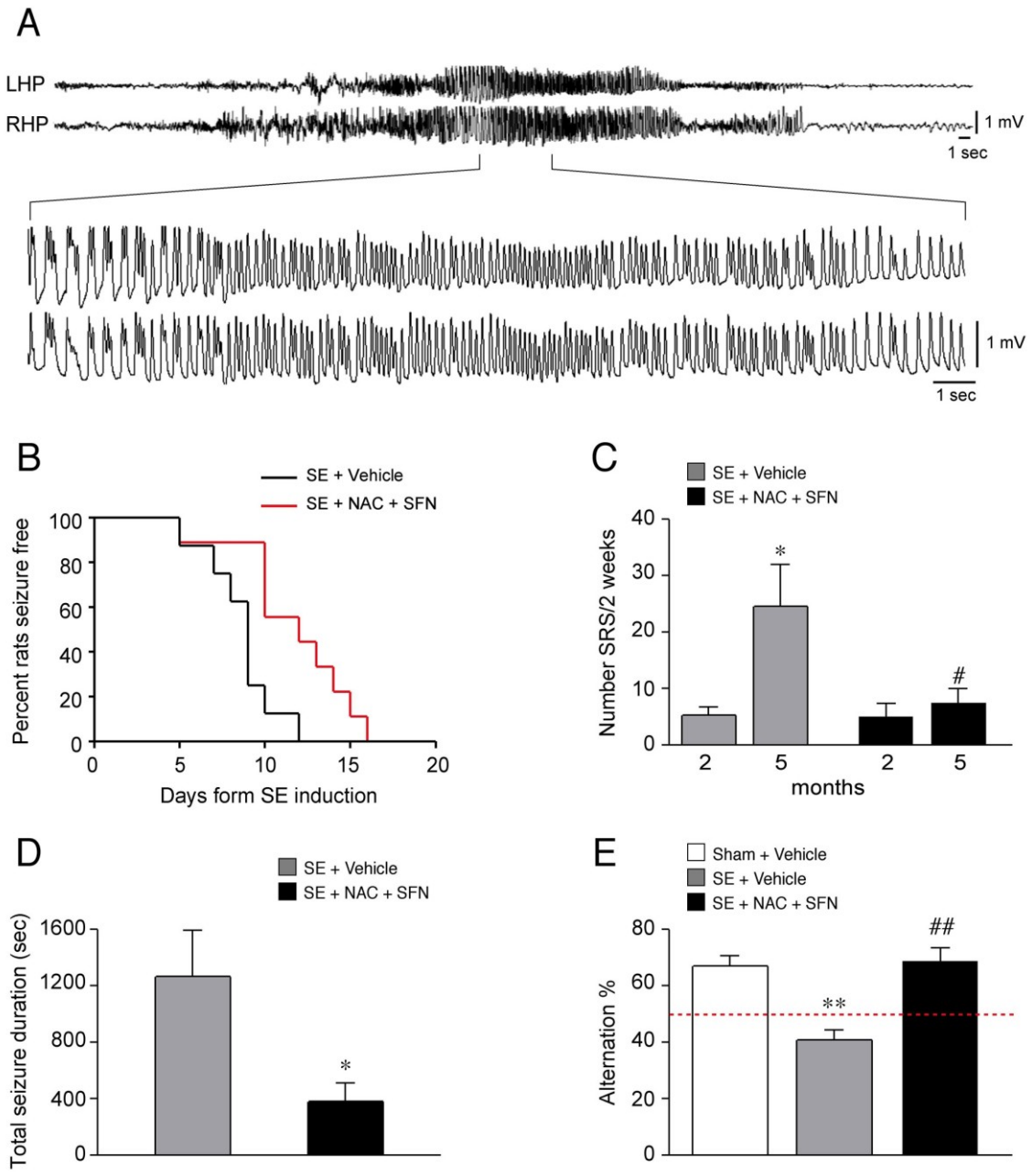

Figure 8. Therapeutic effects of antioxidant drug combination in SE-exposed rats

Panel A depicts a typical EEG recorded spontaneous seizure in a chronically epileptic rat injected with vehicle. LHP and RHP are left and right hippocampus, respectively. *Panel B* shows the onset of epilepsy (first spontaneous seizure) which was significantly delayed by the treatment ($p<0.01$ by long-rank test, n=9 each group). *Panel C* reports the number of seizures during 2-week EEG recording at 2 months and 5 months post-SE in vehicle- and drug-treated rats (Fig. 15C). The data show that seizure progression was prevented by the treatment resulting in 70% SRS reduction at 5

months vs vehicle injected SE-exposed rats (**p<0.01 vs 2 months; #p<0.05 vs SE+Vehicle by Mann-Whitney test). Panel (D) depicts the average total duration of spontaneous seizures (SRS) recorded by EEG for 2 weeks at 5 months post-SE; this parameter was reduced by treatment *vs* vehicle (*p<0.05 vs SE+Vehicle by Mann-Whitney test). Panel (E) reports the rat performance in the T-maze showing the average percent of correct alternation in the each arm in the various experimental groups. The drug combination rescued the behavioral deficit in the epileptic rats. **p<0.01 vs Sham (n=8); ##p<0.01 vs SE+vehicle by Kruskal-Wallis followed by Dunn's post-hoc test.

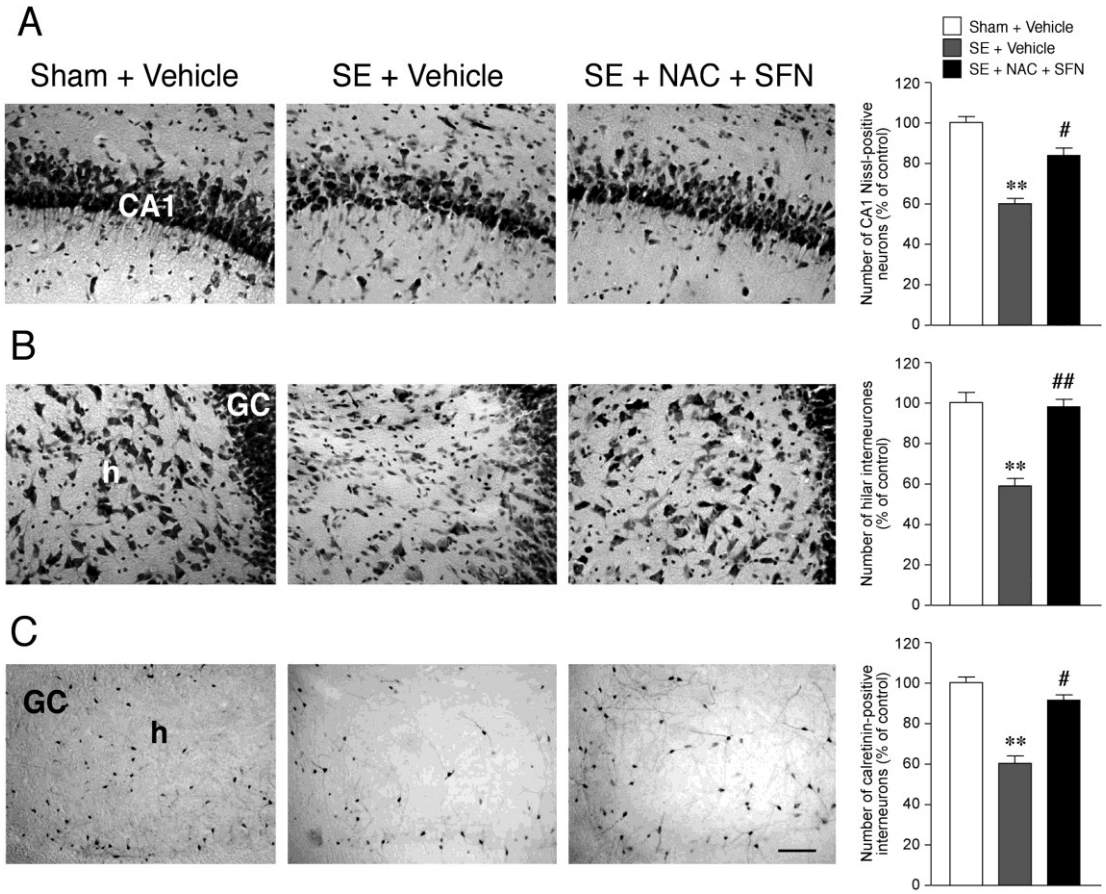

Figure 9. Histological analysis and quantification of cell loss in the hippocampus of SE--exposed rats treated with the anti-oxidant drugs vs vehicles

Panels depict representative microphotographs of Nissl-stained neurons in CA1 pyramidal layer (A) and in the hilus (B) and calretinin-stained hilar interneurons (C) in control (Sham+Vehicle) and epileptic rats treated with vehicle (SE+Vehicle) or the anti-oxidant drugs (SE+NAC+SFN; same rats of Fig. 8). Bargrams (mean ± SEM) report the correspondent quantification of cell loss. Data show the neuroprotective effect of the treatment. *Scale bar*: A,B 100 µm; C 50 µm. GC: granule cell layer; h: hilus; CA1: CA1 pyramidal cell layer of the hippocampus**p<0.01 *vs* Sham+Vehicle; #p<0.05, ##p<0.01 *vs* SE+Vehicle by Kruskall-Wallis followed by Dunn's post-hoc test.

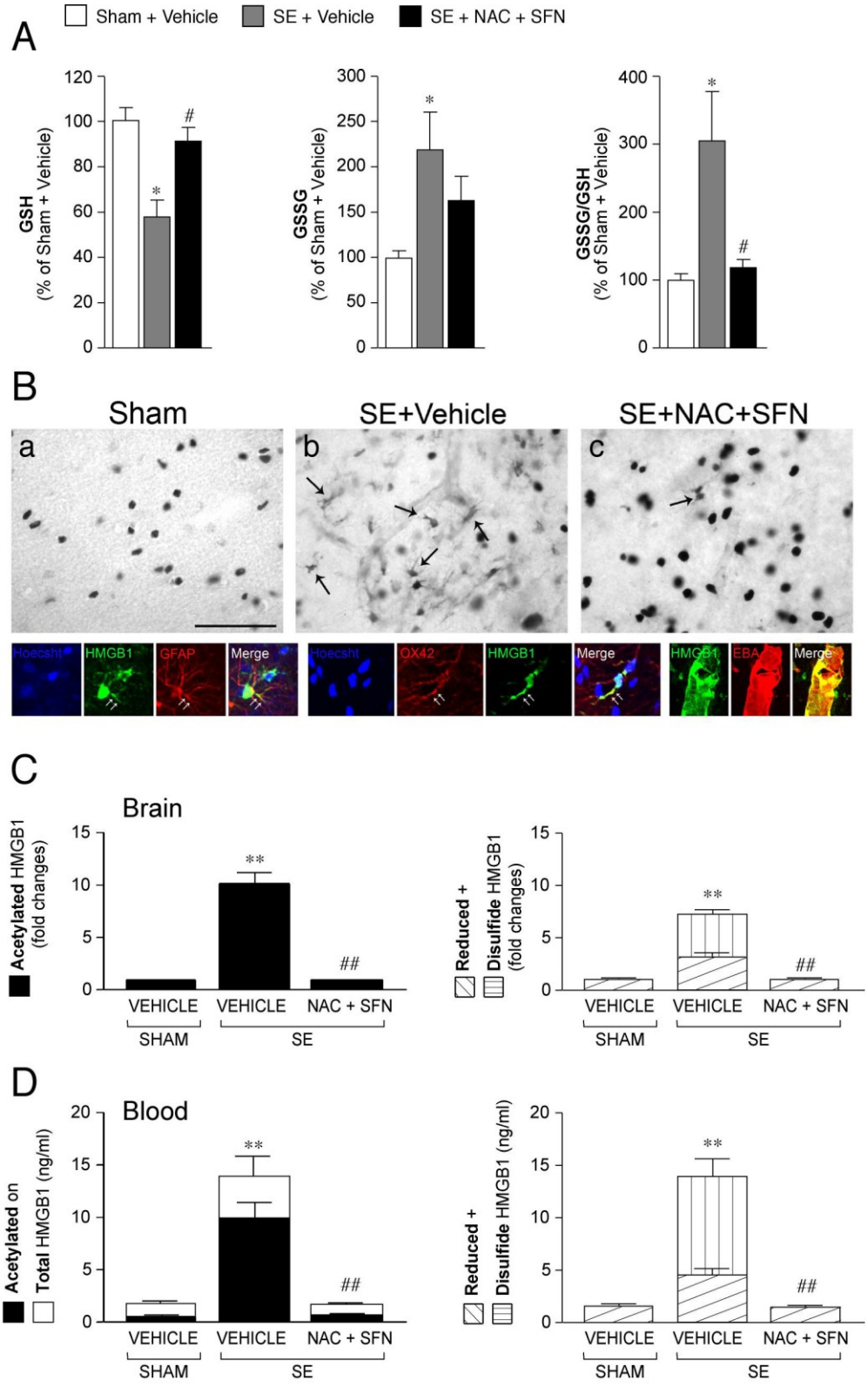

Figure 10. Effect of anti-oxidant treatment on HMGB1 isoforms in brain and blood

Bargram in (A) shows the increase in GSSG/GSH ratio during epileptogenesis (i.e., 4 days post-

SE) and its reduction to baseline (Sham+vehicle, n=15) by 4 day treatment with NAC (5 mg/kg, i.p. twice daily) + SFN (5 mg/kg, i.p., once daily) (n=11) (Fig. 15B; treatment was started 1 h post-SE). **p<0.01 vs Sham+vehicle; ##p<0.01 vs SE+vehicle by Kruskal-Wallis followed by Dunn's post-hoc test.

Panel (B) depicts representative photomicrographs of *stratum lacunosum-moleculare* of hippocampi from control rats (Sham) or SE-exposed rats treated with vehicle or NAC+SFN (n=5 each group; treatment protocol is the same as in panel A). (a) HMGB1 immunoreactivity is localized in cell nuclei in sham rats; (b) HMGB1 immunoreactivity is increased in cytoplasm of glial cells (arrows, b) following SE; (c) reduced cytoplasmatic staining in SE-exposed rats treated with NAC+SFN denoting inhibition of HMGB1 nuclear-to-cytoplasm translocation. *Second row* shows HMGB1 signal (green) in OX-42-positive microglia (red), GFAP-positive astrocytes (red) and EBA-positive endothelial cells (red); co-localization signal is depicted in yellow (merge). White arrows represent cytoplasmic staining. Hoechst-positive nuclei are shown in blue. Scale bar: first row 25 μm; second row 12 μm.

Bargrams in (C,D) show levels (mean ± SEM, n=9-11) of HMGB1 (acetylated, disulfide and reduced) isoforms in brain tissue (hippocampus, C) and corresponding blood (D) of rats during epileptogenesis (i.e., 4 days post-SE), and the effect of treatment. NAC+SFN abolished the increase in acetylated (releasable) and disulfide (pathogenic) HMGB1 isoforms in brain (B) and in blood (C). Total and reduced HMGB1 were also decreased by the treatment. **p<0.01 vs Sham+vehicle; ##p<0.01 vs SE+vehicle by Kruskal-Wallis followed by Dunn's post-hoc test.

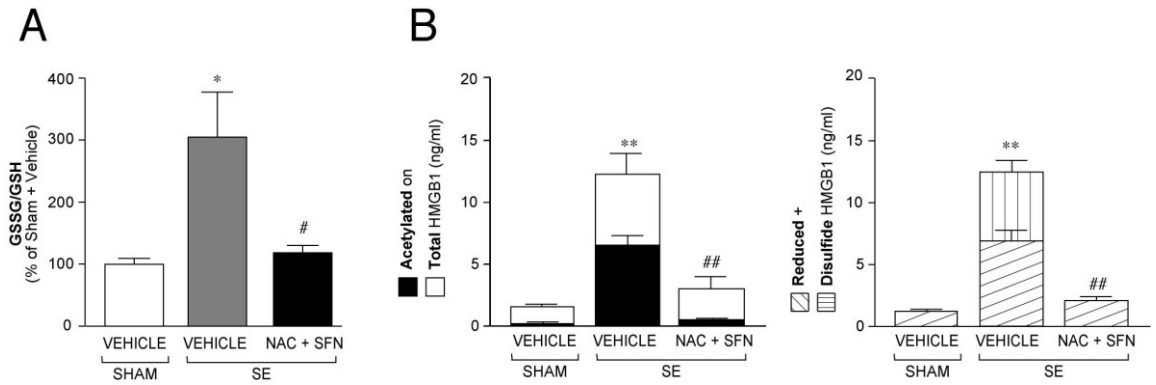

Figure 11. Oxidative stress markers and HMGB1 isoforms in blood

Bargrams (mean ± SEM, n=9-10) reports GSSG/GSH ratio (A) and total HMGB1 and its isoforms (B) in blood of SE-exposed rats injected with either vehicle or antioxidant drug combination (NAC+SFN) (same rats as in Fig. 8) compared to baseline values in sham rats (Sham+Vehicle). Blood was drawn by the tail vein at the end of treatment (i.e., 14 days post-SE) then rats were followed up for monitoring SRS at 2 and 5 months post-SE (Fig. 15C). Notably, the blood levels of the molecules reflect their brain changes (Figs. 1, 2 and 5) and, as in brain, they were normalized by the treatment. **p<0.01 *vs* Sham; #p<0.05 *vs* SE+Vehicle by Kruskal-Wallis followed by Dunn's post-hoc test.

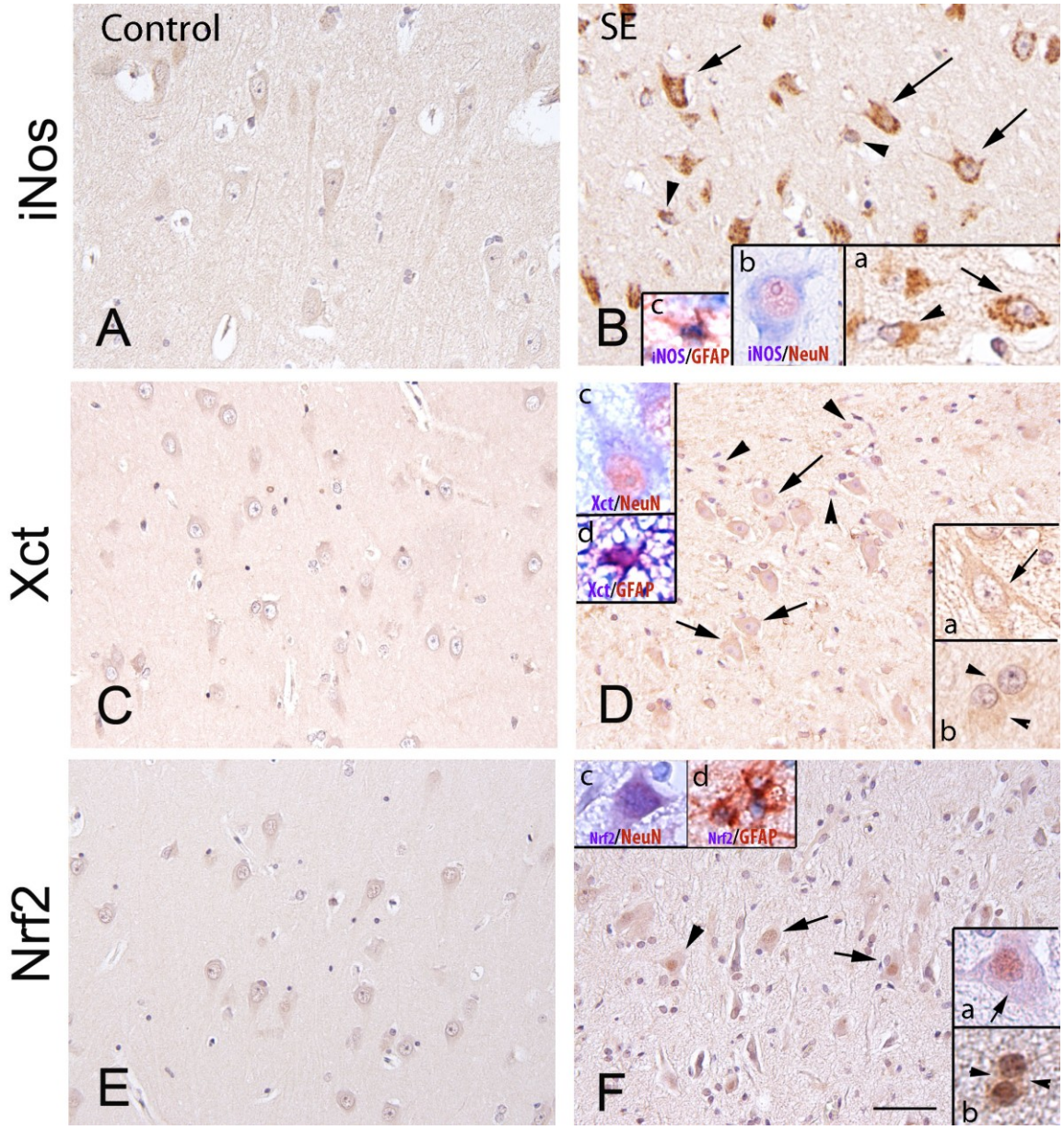

Figure 12. Oxidative stress in the hippocampus of patients experiencing SE.

Representative immunohistochemical micrographs of the CA1 region showing the expression of inducible nitric oxide (iNos; A-B), the cysteine transporter (Xct, C-D) and the transcriptional nuclear factor (erythroid-derived 2)-like 2 (Nrf2; E-F) in control hippocampus (A,C,E) and in a patient who died after SE (7 weeks post-SE; B,D,F). Increased expression of these markers was observed in the hippocampus of patients post-SE in cells with neuronal (arrows in B,D,F and inserts) and glial morphology (arrowheads in B,D,F and inserts), as compared to controls. Nrf2 expression shows nuclear expression (F), denoting an increase in the transcription of detoxifying

enzymes. Inserts in B show iNOS positive neurons (arrow in a, and co-localization with NeuN in b) and astrocytes (arrowhead in a, and co-localization with GFAP in c). Inserts in D show Xct positive neurons (arrow in a, and co-localization with NeuN in c) and astrocytes (arrowhead in b, and co-localization with GFAP in d). Inserts in F show NrF2 positive neurons (arrow in a, and co-localization with NeuN in c) and astrocytes (arrowhead in b and co-localization with GFAP in d). Scale bar in F: A-F: 80μm; insert in B: 40μm; inserts in B,D,F: 25μm.

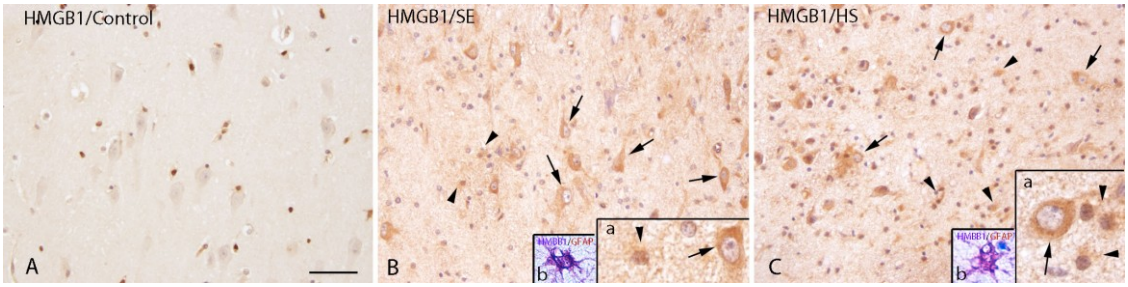

Fig. 13. Expression of HMGB1 in the hippocampus of patients with SE and in temporal lobe epilepsy with hippocampal sclerosis (HS).

Representative immunohistochemical micrographs of HMGB1 signal in the CA1 region of control hippocampus (A) and the hippocampus of a patient who died after SE (7 weeks post-SE, B) and in epileptic patients (C), showing increased HMGB1 expression in post-SE and HS specimens, as compared to controls, with cytoplasmatic staining in cells with neuronal (arrows in B, C and inserts a) and glial morphology (arrowheads in B, C and co-localization with GFAP in inserts b). In control hippocampus (A), HMGB1 is exclusively localized in cell nuclei. Scale bar in: A-C: 80µm; inserts in B and C 25µm.

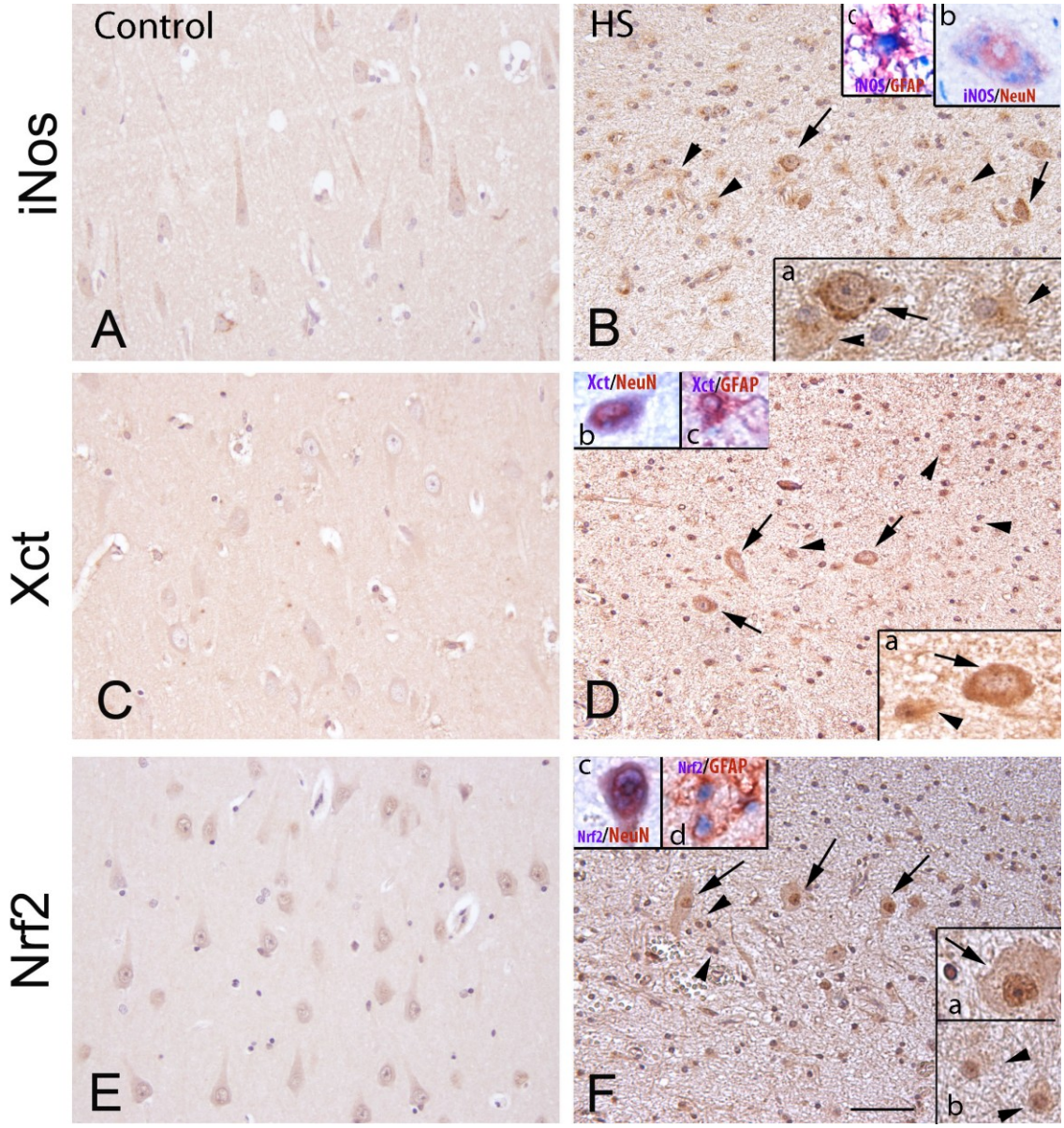

Fig. 14 Oxidative stress in the hippocampus of temporal lobe epilepsy patients with hippocampal sclerosis (HS).

Representative Immunohistochemical micrographs of the CA1 region showing the expression of inducible nitric oxide (iNos; A, B), the cysteine transporter (Xct; C, D) and the transcriptional nuclear factor (erythroid-derived 2)-like 2 (Nrf2; E-F) in control hippocampus (A, C, E) and in TLE-HS (B, D, F). In epileptic specimens, an increased expression of these markers was observed in cells with neuronal (arrows in B, D, F and inserts), and glial morphology (arrowheads in B, D, F and inserts), as compared to controls. In epileptic tissues, Nrf2 expression shows nuclear

expression (F), indicating increased transcriptional activation of detoxifying enzymes. Inserts in B show iNos-positive neurons (arrow in a, and co-localization with NeuN in b) and astrocytes (arrowheads in a, and co-localization with GFAP in c). Inserts in D show Xct-positive neurons (arrow in a, and co-localization with NeuN in b) and astrocytes (arrowhead in a, and co-localization with GFAP in c). Inserts in F show Nrf2-positive neurons (arrow in a, and co-localization with NeuN in c) and astrocytes (arrowheads in b, and co-localization with GFAP in d). Scale bar in F: A-F: 80µm; inserts in B, D, F: 25µm.

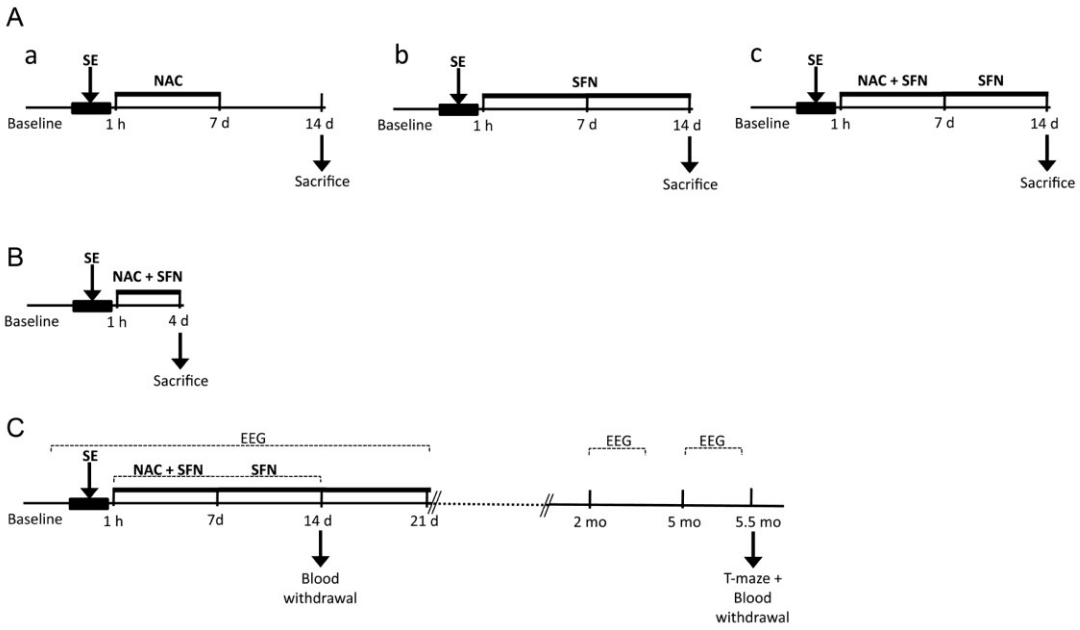

Figure 15. Experimental design of rats exposed to electrical status epilepticus (SE) and treatment schedule.

Panel A refers to rats exposed to SE and used to test whether the combination of NAC and SFN was more effective in reducing oxidative stress than each drug given alone. Three different cohorts of SE-exposed rats were used, one cohort was treated with NAC (500 mg/kg, i.p., twice/day for 7 days, panel a) or with SFN alone (5 mg/kg, i.p., once daily for 14 days, panel b), or with their combination (NAC+SFN for 7 days, then SFN alone for additional 7 days, panel c). Treatment began 60 min after SE onset. Rats were killed 14 days post-SE to assess oxidative stress markers in the hippocampus by HPLC. Data relate to Figure 5A. Panel B refers to rats treated for 4 days with NAC+SFN (doses as above), then killed for measurements of either oxidative stress markers by HPLC (Fig. 10A) or HMGB1 levels (Fig. 10B) in the hippocampus. Panel C refers to rats exposed to SE and used to study the therapeutic effects of the combined anti-oxidant drugs treatment (NAC+SFN for 7 days, then SFN alone for additional 7 days, see panel c). Treatment began 60 min after SE onset. Video-EEG recordings was done continuously from SE induction until the onset of the first two spontaneous seizures (epilepsy onset), and for 2 consecutive weeks (24/7) at 2 and 5 months post-SE. One h after SE induction, rats were randomized into treatment

(NAC+SFN) and vehicle groups (n=9 rats each group). At the end of EEG recording at 5 months post-SE, rats were tested in the T-maze, and then sacrificed for assessment of neuronal cell loss. Blood was withdrawn from the tail vein at the end of treatment (14 days post-SE) and at the time of rat sacrifice (5 months). Data relate to Figures 10 and 11.

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2.8 Publication

OXFORD UNIVERSITY PRESS | Brain

Targeting oxidative stress improves disease outcomes in a rat model of acquired epilepsy

Journal:	*Brain*
Manuscript ID	Draft
Manuscript Type:	Review Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Pauletti, Alberto; Mario Negri Institute for Pharmacological Research, Neuroscience Terrone, Gaetano; Mario Negri Institute for Pharmacological Research, Neuroscience SHEKH-AHMAD, Tawfeeq; University College London Salamone, Alessia; Mario Negri Institute for Pharmacological Research, Neuroscience Ravizza, Teresa; Mario Negri Institute for Pharmacological Research, Neuroscience Rizzi, Massimo; IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Neuroscience Pastore, Annamaria; Children's Hospital and Research Institute "Bambino Gesù" , Metabolomics and Proteomics Pascente, Rosaria; Mario Negri Institute for Pharmacological Research, Neuroscience liang, li-ping; University of Colorado Denver Villa, Bianca; IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Neuroscience Balosso, Silvia; IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Neuroscience Abramov, Andrey; UCL institute of Neurology, Molecular Neuroscience van Vliet, Erwin; Academic Medical Center, University of Amsterdam, (Neuro)pathology; Del Giudice, Ennio; University of Naples Federico II, Department of Paediatrics Aronica, Eleonora; Academic Medical center, University of Amsterdam, (Neuro)Pathology Antoine, Daniel; University of Liverpool, Patel, Manisha; University of Colorado Denver, Department of Pharmaceutical Sciences Walker, Matthew; UCL Institute of Neurology, Department of Clinical and Experimental Epilepsy; National Hospital of neurology and Neurosurgery, Clinical Neurophysiology Vezzani, Annamaria; Mario Negri Institute for Pharmacological Research, Neuroscience
Subject category:	Epilepsy and sleep

Targeting oxidative stress improves disease outcomes in a rat model of acquired epilepsy

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Short title: Anti-oxidant intervention inhibits epileptogenesis

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Abstract

Epilepsy therapy is based on antiseizure drugs which treat the symptom, seizures, rather than the disease and are ineffective in up to 30% of patients. There are no treatments for preventing the onset of epilepsy or improving disease prognosis. Among the potential molecular targets for attaining these unmet therapeutic needs, we focused on oxidative stress since it is a pathophysiological process commonly occurring in experimental epileptogenesis and observed in human epilepsy.

Using a rat model of acquired epilepsy induced by electrical status epilepticus (SE), we show that oxidative stress occurs in both neurons and astrocytes during epileptogenesis, as assessed by measuring biochemical and histological markers. This evidence was validated in the hippocampus of humans who died following SE.

Oxidative stress was reduced in animals undergoing epileptogenesis by a transient treatment with N-acetylcysteine (NAC) and sulforaphane (SFN), which act to increase glutathione (GSH) levels through complementary mechanisms. These antioxidant drugs are already used in humans for other therapeutic indications. This drug combination transiently administered for 2-weeks during epileptogenesis inhibited oxidative stress more efficiently that either drug alone. The drug combination significantly delayed the onset of epilepsy, blocked disease progression and drastically reduced spontaneous seizures. Treatment also decreased hippocampal neuron loss and rescued cognitive deficits.

Oxidative stress during epileptogenesis was associated with *de novo* brain and blood generation of disulfide High Mobility Group Box 1 (HMGB1), a neuroinflammatory molecule implicated in seizure mechanisms. Drug-induced reduction of oxidative stress prevented disulfide HMGB1 generation, thus highlighting a potential novel mechanism contributing to therapeutic effects.

Our data show that targeting oxidative stress with clinically used drugs for a limited time window post-injury significantly improves long-term disease outcomes. This intervention may be considered for patients exposed to potential epileptogenic insults.

Key words: cognitive deficit, neuronal cell loss, HMGB1, spontaneous seizures, neuroinflammation

CHAPTER III

"NEW THERAPEUTIC STRATEGIES IN A MICE MODEL OF REFRACTORY STATUS EPILEPTICUS"

3.1 INTRODUCTION

3.1.1 Status Epilepticus: definition and classification

Status Epilepticus (SE) is a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms, which lead to abnormally, prolonged seizures [1]. SE is a common life-threatening condition with a prevalence of 10-41/100,000 people [2,3]. Approximately 50% of these cases present with no *prior* diagnosis of epilepsy [4]. About 20% of patients will die as a result of SE [5].

According the last classification of ILAE, SE can be defined by two operational dimensions: the first is the length of the seizure and the time point (t1) beyond which the seizure should be regarded as "continuous seizure activity." The second time point (t2) is the time of ongoing seizure activity after which there is a risk of long-term consequences including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizures. In the case of convulsive (tonic–clonic) SE, the time points are 5 min for t1 and 30 min for t2, respectively and are based on animal experiments and clinical research [1].

From a taxonomical point of view, SE is a very heterogeneous condition; there are many forms of SE and causes prove to be extremely varied.

SE can be classified following four diagnostic axes: *semiology, etiology, EEG correlates* and *age*. In terms of *semiology*, the paramount dichotomous categorization occurs between forms with or without prominent motor signs, which are modulated, in parallel, by the occurrence of focal versus generalized clinical and electrographic features, as well

as by the degree of consciousness impairment [1].

Etiologies are classically divided into acute symptomatic, which account for over the half of all cases, remote symptomatic, progressive symptomatic and idiopathic/unknown [6]. The etiology of SE is also influenced by the *age* of onset (neonatal, infancy, childhood, adolescence/adulthood, elderly).

In particular, while pediatric SE is more often caused by infections and genetic/congenital disorders [7], in adults antiepileptic drug (AED) withdrawal, cerebrovascular disorders and tumors predominate [7,8].

Focusing on inflammatory SE etiologies, CNS or severe systemic infections (viral, bacterial or parasitic) may account for 3–35% of cases, according to the geographical location: patients in developing countries are indeed more prone to have an infective etiology of SE [9,10]. Autoimmune etiologies globally seem rarer, accounting for only about 2–3% of SE episodes [9]. In patients with autoimmune SE, most of the episodes are related to anti-NMDA-receptor antibodies, anti-glutamic acid decarboxylase antibodies or multiple sclerosis, while other antibodies, including those associated with paraneoplastic syndromes, as well as Rasmussen encephalitis seem rarer [11]. SE episodes with potentially, yet unproven, inflammatory origin, often presenting in the context of a febrile illness without any previous history of seizures, account for about 5% of SE cohorts [7,8]. In adults, such forms are called ‘new-onset refractory SE’ (NORSE) [12], while in children the acronym ‘febrile infection-related epilepsy syndrome’ (FIRES) has been proposed [13].

The exact incidence of these entities is still unclear, nevertheless, they may account for a significant proportion of super-refractory SE episodes. Finally, SE can be described according to the electroencephalographic patterns even if none of these ictal patterns is specific for any type of SE.

3.1.2 Treatment and Prognosis:

Current standard of care includes three treatment lines: the first line agents represented by benzodiazepines (lorazepam, midazolam, clonazepam), second line agents by intravenously AEDs (levetiracetam, valproate, phenytoin) and third line agents by general anesthetics (Figure 1).

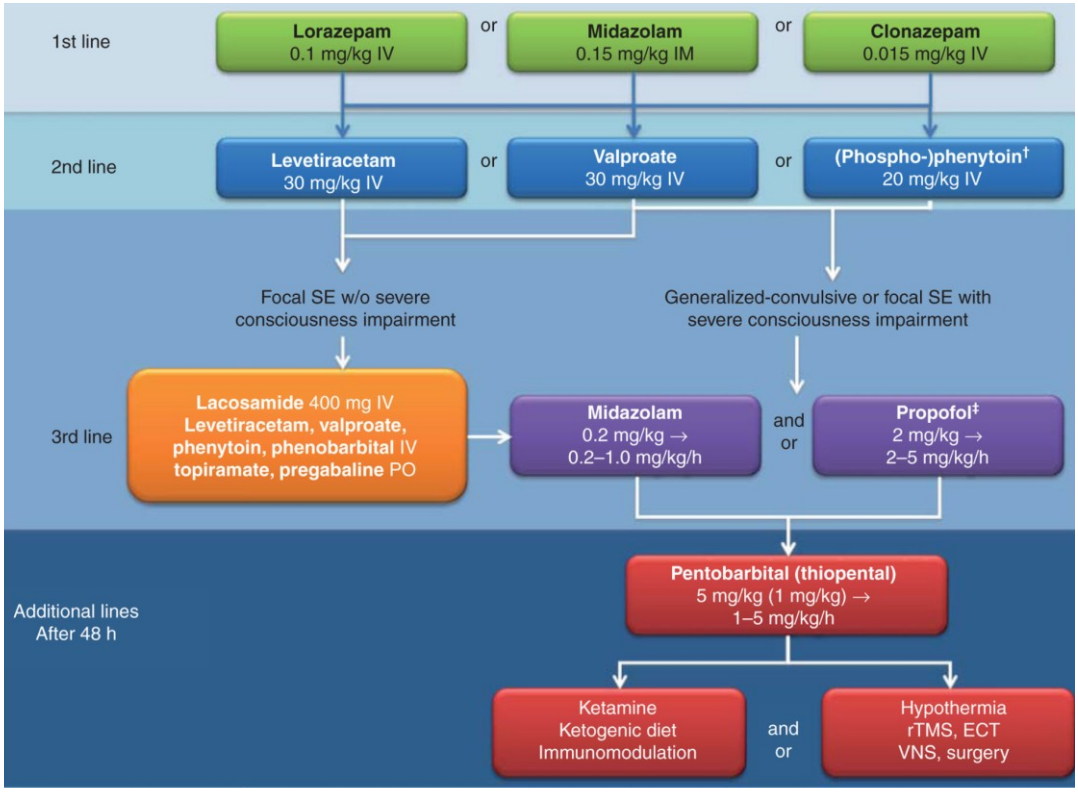

Figure 1. Therapeutic management of Status epilepticus (from Vezzani et al, 2015)

About one third of patients still continue seizing despite the first two treatment lines thus evolving to refractory SE, and half of these subsequently develop super-refractory SE, resistant to a first anesthetic treatment [14]. In these cases, alternative treatments as other anesthetics and AEDs, vagal nerve stimulation, ketogenic diet (KD), transcranial magnetic stimulation, electroconvulsive treatment, mild hypothermia or resective surgery can be

considered [14].

Furthermore, refractory SE is linked to a worse prognosis, both in terms of mortality and morbidity. Decline in intellectual functions and cognitive deficits have been reported particularly in new onset refractory SE in both children and adults [13]. Moreover, chronic epilepsy development occurs in up to 50% of adults after *de novo* refractory SE [15,16]. AEDs have not shown a major impact on SE prognosis. Achieving control of SE without requiring prolonged drug-induced coma or severe EEG suppression correlate with better prognosis and improved functional outcome [17].

SE in preclinical models is typically induced by a chemoconvulsant or electrical stimulation, mirroring *de novo* SE in humans [18]. Moreover, since experimental studies in multiple mammalian species demonstrated that SE itself is sufficient to induce neurological co-morbidities and epilepsy [19], the occurrence of prolonged unremitting seizures is likely to contribute to pathological outcomes in humans independently of underlying pathologies. This correlation between SE preclinical models and human pathology provides an ideal platform for developing new treatments that can move from pre-clinical testing to a clinical population with high unmet need.

The search for potential therapeutic targets led to the identification of molecular mechanisms underlying SE development in rodents. In this context, endocannabinoid system and the proinflammatory arachidonic acid (AA)-derived eicosanoid cascade appear to play a significant role in the incidence of SE in animals, as well as for cell loss and co-morbidities [20,21]. These two pathways are functionally linked in the brain by the activity of the key enzyme monoacylglycerol lipase (MAGL).

3.1.3 Role of MAGL pathway in epilepsy:

MAGL is a serine hydrolase that preferentially hydrolyzes monoacylglycerols to glycerol

and fatty acid, with highest expression in brain, white adipose tissue, and liver in mice and serves as a critical node for regulating these lipid signaling pathways in brain both in physiological and pathological conditions [22,23]. In brain, MAGL is expressed on presynaptic terminals and hydrolyzes the endocannabinoid 2-arachidoylglycerol (2-AG) into arachidonic acid (AA), the precursor for pro-inflammatory prostaglandin synthesis [24,25]

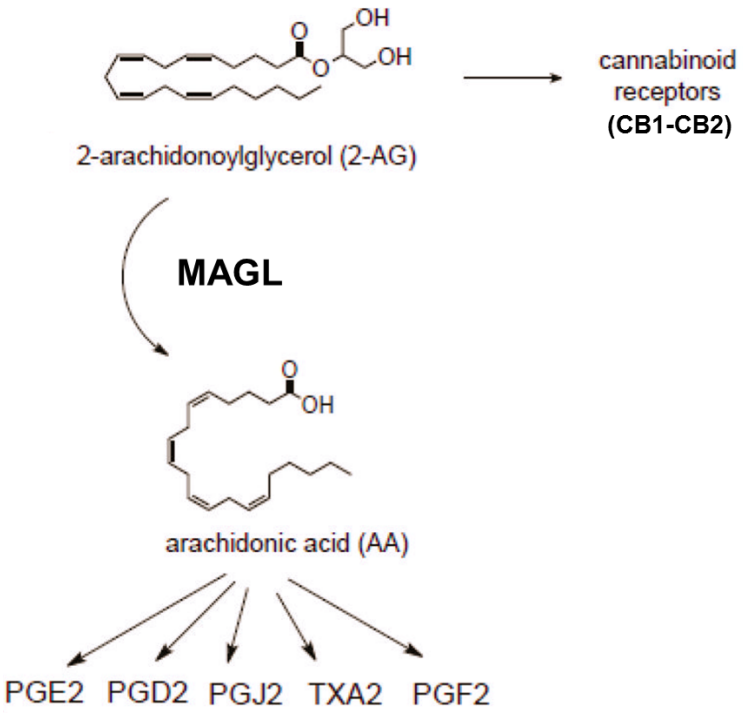

Figure 2. MAGL pathway in brain (modified from Mulvihill and Nomura, 2013)

The endocannabinoid 2-AG is produced and released from postsynaptic neurons and then retrogradely binds presynaptic CB1 receptors, modulating presynaptic or interneuron release of excitatory or inhibitory neurotransmitters [26-28].

2-AG can also binds CB2 receptors on microglia cells [29], reducing neuronal firing frequency [30] and providing antinflammatory effects.

Prostaglandin E2 (PGE2) is the major product of COX-2 in the brain, and two of its receptors, EP1 and EP2, have been implicated in neuronal injury and inflammation in

CNS disorders [31]. Pharmacological blockade of MAGL leads to an enhancement of 2-AG signaling and a reduction of AA availability to COX-2, thus providing antinflammatory effect. The 2-AG has a crucial role for suppressing seizures in experimental model of epilepsy. In particular, in a kindling model of temporal lobe epilepsy, inhibition of MAGL by JZL184 provided a significant delay in the development of generalized seizures and a decrease in seizure thresholds. These effects were mediated by the action of 2-AG on CB1 receptors [32]. Moreover, kainite-induced seizures in mice lacking the 2-AG synthesizing enzyme were much more severe compared with those in CB1-receptor knock out mice and were comparable to those in mice lacking both cb1 and CB2 mediating signaling. This study confirmed the importance of 2-AG action on both CB1 and CB2 receptors [33].

On the other hand, the inhibition of MAGL also decreases the levels of AA, needed for prostanoids synthesis. The antagonism of EP2 receptor has been shown to mediate neuroprotection and less neuroinflammation [34]. Moreover, the cognitive deficit developed after SE onset was prevented in rats treated briefly with the EP2 antagonist [34]. This suggests that much of the morbidity associated with COX-2 induction after SE is mediated by the activation of EP2, and possibly EP1, by COX-2 derived PGE2. Delayed inhibition of EP2 could therefore represent a viable adjunctive treatment for alleviating the deleterious consequences of SE.

3.1.4 Role of ketogenic diet in epilepsy

Ketogenic diet (KD) is a high-fat low-carbohydrate diet used as an effective treatment in patients with medically intractable epilepsy, including inflammation-induced epileptic encephalopathies as FIRES [35].

In clinical settings KD has been shown to stop refractory SE and improve cognitive deficit

[36]; while in experimental models, KD prevents epileptogenesis and disease progression and provides significant neuroprotection [37]. At present, there are many hypotheses regarding KD effects, and it is becoming more apparent that the KD likely works through multiple mechanisms that target fundamental biochemical pathways linked to cellular substrates (e.g., ion channels) and mediators responsible for neuronal hyperexcitability.

Among these several mechanisms, KD seems to provide anti-inflammatory effects. In particular, in a lipopolysaccharide (LPS)-induced fever model in rats KD reduces AA levels in blood and IL-1β in brain and blood, providing anti-inflammatory effects [38].

Moreover, KD or beta-hydroxybutirrate (BHB), one of the ketone bodies produced in liver during the diet, decreased caspase-1 activation and IL-1β secretion in mouse models of NLRP3-mediated diseases as familial cold autoinflammatory syndrome [39]. This anti-inflammatory effect of the KD [40] may be due in part to BHB's specific effects on the NLRP3 inflammasome, and further underscore the importance of inflammation in epilepsy [41].

3.2 AIMS OF STUDY

As status epilepticus is a commonly drug-refractory condition, novel therapies are greatly needed to rapidly terminate ongoing seizures for preventing mortality and morbidity.

In this study, we investigated if MAGL inhibition using the brain penetrant selective and irreversible inhibitor CPD-4645, affects diazepam-refractory SE in mice, and reduces pathological sequelae such as cognitive deficits and cell loss.

To further characterize the mechanism of action, we investigated whether CPD-4645 effects were mediated by a reduction of excitatory synaptic transmission due to 2-AG modulation of CB1 receptors or by reduction of AA availability for the eicosanoid cascade and 2-AG activation of microglial CB2 receptors, thereby inducing antinflammatory effects.

Finally, we studied the effect of MAGL inhibition on SE in mice fed with a ketogenic diet (KD) for assessing a potential synergy between these two treatments since the KD is one therapeutic option for controlling refractory SE in humans.

3.3 MATERIALS AND METHODS

3.3.1 Animals

We used 8 week-old C57BL6N male mice (~20 g; Charles River, Calco, Italy).

CB1 receptor knock-out (*Cnr1*$^{-/-}$) mice and their respective wild-type (Wt) littermate controls were bred and acquired from the Translational Animal Research Centre of Mainz University in Germany. These mice were allowed to habituate to the new environmental conditions for at least 3 weeks. Mice were maintained in SPF facilities at the Mario Negri Institute and housed at a constant room temperature (23°C) and relative humidity (60 ± 5%) with free access to food and water and a fixed 12 h light/dark cycle. All experimental procedures were conducted in conformity with institutional guidelines that are in compliance with national (D.L. n.26, G.U. March 4, 2014) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996), and were reviewed and approved by the intramural ethical committee.

3.3.2 Drug Formulation, Dosing and Tissue Collection

CPD-4645 was dissolved in vehicle (5:5:90; DMSO:cremophor:saline) and administered subcutaneously to mice at a dose of 10 mg/kg. Plasma and brain samples were collected at 0.5, 1, 2, 4, 8, 12, and 24 hours post dose (3 mice per time point). Plasma and brain samples of vehicle treated mice were collected at 1 hour post dose and were pooled across multiple experiments. Blood was collected by first anesthetizing mice with isoflurane followed by cardiac puncture. Whole blood was placed into tubes containing NaF and K_2EDTA (0.5 M, 20 μL per 1 mL blood) to inhibit plasma hydrolase activity and as an anti-coagulant, respectively. Blood was centrifuged at 4,000 rcf for 5 min to harvest plasma. Plasma sample was then split into two aliquots. The plasma sample used for

pharmacodynamic measurement was diluted into a 20% v:v assay buffer (100 mM NH_4OAc in water (pH 2.0 adjusted by formic acid) and 5 mM PMSF) in order to inhibit plasma hydrolase activity. The plasma sample was then frozen at -80°C until analysis. The plasma sample used for pharmacokinetic measurement was spiked with IS (internal standard) solution (100 ng/mL Labtalol, 400 ng/mL diclofenac and 200 ng/mL tolbutamide in ACN/MeOH (v:v, 50:50) with 0.1% formic acid), the mixture was vortexed and centrifuged at 15,700 rcf for 5 min at 4°C. Supernatant was harvested and then frozen at -80°C until analysis. Immediately following blood sampling mice were euthanized by cervical dislocation and their brains were rapidly removed (less than 30 seconds) and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

3.3.3 Pharmacokinetic Measurement

Plasma samples were prepared for CPD-4645 measurement on wet ice. Briefly, samples were spiked with IS solution followed by extraction in water/MeOH (v:v 75:25) with 0.1% formic acid. Samples were then centrifuged at 4,000 rcf for 10 min at 4°C. Supernatant was removed and directly injected for LC-MS/MS analysis. Cerebellum samples were prepared on wet ice. Briefly, cerebellums were homogenized with 3 volumes (w:v) of PBS. The homogenates were then spiked with IS solution followed by centrifugation at 15,700 rcf for 15 min at 4°C. Supernatant was removed and directly injected for LC-MS/MS analysis.

3.3.4 Pharmacodynamic Measurement

Brain samples were prepared on wet ice. Briefly, brain homogenates were prepared by homogenizing brain with 3 volumes (w:v) of homogenization buffer (1% PMSF and

5% 100 mM NH_4OAc in water pH 2.0, adjusted with formic acid). The brain homogenates were then spiked with IS2 [1 μg/mL d_5-2-AG or d_8-AA (deuterated 2-arachidonoylglycerol, deuterated anandamide or deuterated arachidonic acid) (Cayman Chemical Ann Arbor, Michigan) in ACN] solution followed by precipitation and organic extraction in ACN. Samples were then centrifuged at 15,700 rcf for 15 min at 4°C. Supernatant was removed from each sample directly injected for LC-MS/MS analysis. Calibration standards and quality control samples were prepared using the same methodology. The 2-AG calibration curve consisted of 50-50,000 ng/mL 2-AG in homogenization buffer. The AA calibration curve consisted of 50-50,000 ng/mL AA in homogenization buffer. Samples were directly injected onto a C-18 UPLC column held at 50°C at a flow rate of 0.5 mL/min. For 2-AG measurement mass spectrometry was run in positive ESI mode with SRM detection. The 2-AG m/z was 379.4/287.3. The d_5-2-AG m/z was 384.4/287.4. For AA measurement mass spectrometry was run in negative ESI mode with SRM detection. The AA m/z was 303.1/205.2. The d_5-2-AG m/z was 311.2/267.0.

3.3.5 Mouse model of status epilepticus

Mice were surgically implanted under general gas anesthesia (1-3% isoflurane in O_2) and stereotaxic guidance. A 23-gauge guide cannula was unilaterally positioned on top of the *dura mater* for the intra-amygdala injection of kainic acid (*from bregma*, mm: nose bar 0; anteroposterior-1.06, lateral -2.75) [44]. In mice a nichrome-insulated bipolar depth electrode (60 μm OD) was implanted in the dorsal hippocampus (*from bregma*, mm: nose bar 0; anteroposterior –1.8, lateral 1.5 and 2.0 below *dura mater*) [44] ipsilateral to the

injected amygdala and a cortical electrode was placed onto the somatosensory cortex in the contralateral hemisphere.

Kainic acid (KA) (0.3 μg in 0.2 μl) was injected in the basolateral amygdala in freely moving mice (n=70) using a needle protruding of 3.9 mm below the implanted cannula. Status epilepticus (SE) developed after approximately 10 min from KA injection, and was defined by the appearance of continuous spikes with a frequency >1.0 Hz. Spikes were defined as sharp waves with an amplitude at least 2.5-fold higher than the baseline and a duration of <20 msec, or as a spike-and-wave with a duration of <200 msec. The end of SE was determined by inter-spike interval >2 sec. After 40 min from SE onset, mice received a sedative dose of diazepam (10 mg/kg, i.p.) for decreasing motor seizures and improving mice survival. However, electrographic SE was unaltered by diazepam. Power spectral density (PSD) distribution of five frequency bands (delta: 1–4 Hz; theta: 4–8 Hz; alpha: 8–13 Hz; beta: 13-30 Hz; gamma: 30–40 Hz) was calculated during the first 4 h of SE. Fast Fourier transforms (FFTs) were computed by 50% overlapping sliding windows (1.024 data-point each) with Hanning windowing function. EEG data were normalized by dividing the EEG power density at each frequency with the EEG power density averaged across all frequencies. SE was successfully evoked in **65** out of **70** mice, of which 10 mice died, therefore **55** mice were used for the experiments. The total number of spikes was measured during 12 h after KA administration (Clampfit 9.0, Axon Instruments, Union City, CA, U.S.A). The effect of CPD-4645 vs its vehicle was tested in **32** mice fed with a standard diet and **23** mice fed for 4 weeks with a ketogenic diet (KD; #F3666, Bio-serv, Frenchtown, NJ, USA). At the end of SE, mice were tested in a spatial memory task, in particular Novel Object recognition test and, then, they were killed for histological analysis of cell loss. At the time of killing, blood was withdrawn from heart atrium and serum separated and aliquoted for analysis of beta-hydroxybutyrate. Two additional

experimental groups consisted of mice undergoing the same surgical procedure as described above and fed either with standard (n=12) or KD (n=7). These mice were used as sham controls (not exposed to SE) in the novel object recognition test and for histological analysis.

Cnr1*$^{-/-}$ *mice: *Cnr1*$^{-/-}$ mice (n=**18**) and their respective WT littermate controls (n=**16**) underwent the same protocol of status epilepticus, described above. SE was successfully evoked in **28** out of **34** mice and these animals were used for the experiments.

3.3.6 CPD-4645 treatment

Mice were fed *ad libitum* with standard diet (SD) or ketogenic diet (KD) for 4 weeks in order to induce ketosis. A cohort of randomized mice in each diet group (SD or KD) received intraamygdala KA injection and 1 h after SE onset, animals were randomized to either CPD-4645 [10 mg/kg, i.p., dissolved in DMSO:cremophor:saline (0.5% DMSO:0.5% cremophor)] or vehicle injection. A second injection of either drug or vehicle was done in the same mice 7 h post-SE onset to maintain therapeutic drug levels as indicated by PK analysis, and then mice were treated twice a day for the following week until completion of behavioral test.

Cnr1*$^{-/-}$ *mice: *Cnr1*$^{-/-}$ and Wt mice were fed *ad libitum* with standard diet. They were randomized to either CPD-4645 [10 mg/kg, i.p., dissolved in DMSO:cremophor:saline (0.5% DMSO:0.5% cremophor)] or vehicle injection. For this experimental group, we followed the same treatment schedule described above.

3.3.7 Novel object recognition test (NORT)

All experiments began between 9:00 and 10:00 am. Two days after the end of SE, spatial memory was tested in mice in NORT where the ability of rodents to recognize a set of novel objects in an otherwise familiar environment is taken as a measure of recognition memory [45]. Although this task is associated with the perirhinal cortex function, we used a 24 h intertrial interval from familiarization to test phase, a condition which also involves hippocampal activity [46, 47]. The test was performed in the open-square gray arena (40 x 40cm) surrounded by 30 cm high wall, with the floor divided into 25 equal squares by black lines. Mouse behavior was remotely monitored via video camera. Twenty four hours prior to the test, mice were allowed to habituate in the arena for 5 min. The proper test began on the next day with the familiarization phase, when mice were placed into the open field for 10 min in the presence of two identical objects positioned in internal non-adjacent squares. The following objects were randomly used: black plastic cylinders (4 x 5 cm); transparent scintillation vials with white caps (3 x 6 cm); metal cubes (3 x 5 cm); plastic black pyramids (4 x 5 cm). Cumulative exploration time of both objects and of each object separately was recorded. Exploration was defined as sniffing, touching, and stretching the head toward the object at a distance not more than 2 cm. Twenty four hours after familiarization, the recognition phase of the test was performed: mice were placed for 10 min in the open field, which contained one object presented during the familiarization phase (familiar object, F), and a novel unfamiliar object (N). The time spent exploring N versus F represents a measure of trial unique recognition memory. As the recognition phase was performed 24 h after the familiarization phase, the procedure can be regarded as a test of long-term memory. Time spent (s) exploring F and N was expressed as a discrimination index: (N - F)/(N + F).

3.3.8 Fluoro-Jade staining

At the end of behavioural tests, mice were deeply anaesthetized using 10% ketamine+ 10% medetomidine + 80% saline; 10 ml/kg, i.p.) then perfused by the ascending aorta with cold PBS (50 mM, pH 7.4). After decapitation, brains were rapidly removed and the hemisphere ipsilateral to KA injection was post-fixed in 4% paraformaldehyde (PAF) solution at 4°C for one week, then transferred to 20% sucrose in PBS for 24 h at 4°C. Tissue was rapidly frozen in -50°C isopentane for 3 min and stored at -80°C until assayed. Serial cryostat coronal sections (40 μm) were cut throughout the septal extension of the hippocampus (−1.22 to −2.54 mm from bregma) [44] and slices were collected in 0.1 M PBS. Four sections for each animal were stained with Fluoro-Jade to detect degenerating neurons [48,49]. Sections from control and experimental mice were matched for antero-posterior location. High-power non-overlapping fields of the whole hippocampus (20X magnification; Olympus) were acquired to measure the Fluoro-Jade-stained neurons along the CA1, CA3, CA4 pyramidal cell layers and amygdala. Data obtained in each image within the same hippocampal subfield were added together providing one single value *per* slice in each mouse. Data obtained in each of the 4 slices per brain were averaged, providing a single value for each brain, and this value was used for statistical analysis.

3.3.9 Real-time quantitative polymerase chain reaction analysis

The contralateral hemisphere in each brain was snap frozen and kept for mRNA isolation and qRT-PCR analysis. Total RNA was isolated from frozen tissues using Qiazol lysis reagent and RNeasy columns following manufacturer directions. cDNA was synthesized using high capacity RNA to cDNA master mix (Applied Biosystems). Reactions for quantitative TaqMan PCR contained 1x primer/probe sets, cDNA and gene expression master mix in a 10 uL final volume (Applied Biosystems). PCR was performed for 40

cycles using a Viia 7 machine. Relative quantitation (2^-ΔΔCT) of gene expression was determined using β-actin (Actb) as the endogenous control gene. Primer probe sets were purchased from Applied Biosystems [Il1β: Mm01336189_m1; Ptgs2: MmXXXXX_m1; Mgll: Mm00449274_m1].

3.3.10 β-hydroxybutyrate measurement in plasma

Blood was collected for mice at indicated timepoints post dosing via cardiac puncture into K2-EDTA vacutainers and kept on ice. Plasma was isolated by centrifugation at 4,000 rcf for 5 minutes. β-hydroxybutyrate was measured using a colorimetric assay kit (Cayman Chemical, No700190). Kit was performed according to manufacturers directions at a sample dilution of 1:5.

3.3.11 Randomization and statistical analysis of data

Sample size was *a priori* determined based on previous experience with the animal model and the respective behavioral analyses. Simple randomization was applied to assign a subject to a specific experimental group (vehicle or drug) using a web site randomization program (http://www.randomization.com). After vehicle or drug injection, rats were linked to numerical identifiers by an external investigator not involved in the study and unaware of the treatment. This procedure ensured that animal handling and data acquisition during the analyses were done by investigators blinded to the identity of the animals. Data are the mean ± s.e.m. (n=number of individual samples). Data were analysed by Mann-Whitney test for two independent groups and Kruskal-Wallis or one-way ANOVA followed by Dunn' or Bonferroni's post-hoc tests for more than two independent groups. Data obtained from experimental groups which differ for two independent variables were analysed by two-way ANOVA followed by Sidak's post-hoc

test. Survival rates between *Cnr1*$^{-/-}$ mice and their respective Wt were analysed by Log-rank test. Statistical tests were done using GraphPad Prism 7 (GraphPad Software, USA) for Windows. Differences were considered significant with $p<0.05$.

3.4 RESULTS

3.4.1 Pharmacokinetic and pharmacodynamic analysis of CPD-4645

The selective MAGL inhibitor CPD-4645 (Fig.3A) is a hexafluoroisopropyl (HFIP) carbamate identified from published patent literature, analogous to the previously reported SAR127303 (Patent # WO11151808). CPD-4645 is a potent and irreversible MAGL inhibitor (Human IC_{50} = 1 nM, Mouse IC_{50} = 3.25 nM) and selective over FAAH (Human IC_{50} = 2.1 μM, 2100-fold). The compound exhibits adequate pharmacokinetic and pharmacodynamic profile for in vivo evaluation in mice, including brain penetration, as evidenced by neuropharmacokinetic assay and a half-life of 3.7 hours (Fig.3B). A single 10mg/kg subcutaneous dose is sufficient to elevate brain 2-AG levels ~4 fold for 8 hours. Consistent with the MAGL mechanism of action we found a concomitant ~4 fold decrease in levels of arachidonic acid (Fig.3C).

3.4.2 Effect of CPD-4645 on status epilepticus and the associated cognitive deficit in standard diet (SD)-fed mice

The MAGL inhibitor CPD-4645 blunted the development of SE in mice fed with a standard diet. In vehicle-injected mice, spiking activity increased in frequency during the first 3 h from SE onset reaching a *plateau* between 4 h and 7 h, then spike frequency progressively declined until SE spontaneously elapsed (Fig.4A, n=21). CPD-4645 reduced spike frequency by 60% on average starting from 3 h after drug injection as compared to spike frequency in time matched vehicle-injected mice ($p<0.01$; n=21) (Fig.4A,B).

The drug significantly reduced the alpha band frequency (8–13 Hz; $p<0.01$; n=6) during the first 3 h from injection vs vehicle (n=5) as assessed by Power Spectral Density (PSD) analysis (Figure 4A, inset). No significant differences of PSD (μV2/Hz) for the delta: 1–4 Hz (SE+Vehicle 9.06 ± 0.7; SE+drug 11.06 ± 0.6); theta: 4–8 Hz (SE+Vehicle 4 ± 0.4; SE+drug 3.6±0.3); beta: 13-30 Hz (SE+Vehicle 0.52 ± 0.03; SE+drug 0.4 ± 0.01); gamma: 30–40 Hz (SE+Vehicle 0.05 ± 0.005; SE+drug 0.04 ± 0.002) bands were found. Total duration of SE was 9.2 ± 0.4 h in vehicle-injected mice which was shortened by 4 h in drug-treated mice (4.9 h ± 0.5, $p<0.05$) (Fig.4B).

To determine whether CPD-4645 rescues impaired memory following SE, we compared NORT performance among five experimental cohorts of mice as follows: 1) naïve mice; 2) sham mice injected with vehicle, 3) sham mice injected with CPD-4645; 4) SE-exposed mice injected with vehicle; 5) SE exposed mice injected with CPD-4645. Naïve (n=14) and sham mice, treated either with vehicle (n=13) or drug (n=8), spent more time exploring the novel object compared to the familiar one (average discrimination index= 0.37± 0.05; 0.4 ± 0.05 and 0.28 ± 0.03 respectively), indicating that they remembered the familiar object from the object familiarization phase and thus had a preference for the novel object (Fig. 4C).

SE-exposed mice injected with vehicle (n=10) failed to remember the familiar object and spend more time exploring this object as the novel one (average discrimination index = -0.24 ± 0.07; $p<0.001$ vs naïve; $p<0.001$ vs drug-injected sham mice; $p<0.05$ vs drug-injected sham mice) (Fig.4C).

CPD-4645 rescued this behavioral deficit as shown by the average discrimination index of 0.28± 0.05 ($p<0.05$ vs vehicle-injected SE-exposed mice) which is similar to naïve and sham mice. No difference in locomotor activity was detected among the experimental groups in the first day of NORT. Mice did not show behavioral seizures during the test.

3.4.3 Effect of CPD-4645 on status epilepticus in Cnr1$^{-/-}$ and related Wt mice and survival curves

In order to better characterize the mechanisms of CPD-4645 effects on SE, the same treatment protocol was applied in Cnr1-/- and related Wt mice. The drug significantly decreased the number of spikes as compared to vehicle in *Cnr1*$^{-/-}$ mice (n=7/group; p=0,005, two-way ANOVA, F 8.082 between 1 and 144 degrees of freedom), starting from 3 h after drug injection (Fig.5A,B). Total duration of SE was significantly shortened by 4 h in drug-treated mice (6.4 ± 1.3 h) as compared to control mice (10± 0.5 h; $p<0.05$). As far as *Cnr1*$^{-/-}$ sensitivity to SE, *Cnr1*$^{-/-}$ mice developed a more severe SE in terms of spike number as compared to respective Wt mice (Fig.5A,4A). The drug confirmed its therapeutic effects on SE also in *Cnr1*Wt related mice (Fig. 5A,B).

Cnr1$^{-/-}$ mice showed a higher mortality rate during the days after SE onset as compared to Wt controls ($p< 0.05$ by Kaplan–Meier survival analysis). This mortality rate was unaffected by the drug in each strain, therefore all data were put together independently on vehicle or drug treatment (Fig.6C).

3.4.4 MAGL inhibition attenuates neuroinflammation following SE

To assess the expression of inflammatory mediators in the brain following SE, the contralateral hippocampus of SE-exposed mice was harvested (the ipsilateral hippocampus was used for 2-AG measurement at 1 h while the whole ispilateral hemisphere was used for Fluoro-Jade analysis of cell loss at 7 days) at 1 h and 7 days after CPD-4645 or vehicle injection. IL-1β (*Il-1b*) and COX-2 (*Ptgs2*) transcripts were induced by 4.3 ± 0.8- and 8.4 ± 2.4-fold respectively following SE and this effect was progressively reduced by CPD-4645 ($p<0.05$ at 7 days; Figure 4). MAGL expression (*Mgll*) was unaltered by SE or drug treatment which is consistent with MAGL being a constitutive (non-inducible) enzyme.

3.4.5 Effect of CPD-4645 on status epilepticus in ketogenic diet (KD)-fed mice

In vehicle-injected mice fed with KD (n=12), spiking activity pattern, frequency of spikes and SE duration were similar to standard diet-fed mice, except for the first hour after SE. However, when CPD-4645 was injected in KD-fed mice (n=11), SE was virtually abrogated and the drug's effect was immediate, starting at 1 h from injection (Fig.8A). Total duration of SE was significantly reduced by 7 h in drug-treated (3.4 h ± 0.7 h) as compared to vehicle-injected mice (10.0 ± 1.0 h) ($p<0.01$) (Fig.8B).

All mice fed with KD, regardless of treatment with CPD-4645, showed high levels of β-hydroxybutyrate in plasma as compared to mice fed with SD, confirming they were in ketosis (Fig.9).

3.4.6 Effect of CPD-46454 on neuronal cell loss

At the end of behavioral testing, mice were killed for quantitative analysis of cell loss in Fluoro-jade sections of the dorsal hippocampus and amygdala, ipsilateral to the KA injection. In CPD-4645 treated SD-fed mice, CA1 pyramidal cell loss was significantly reduced (number of FJ-positive cells/slice, 1.8 ± 1.2, n=8) compared to vehicle-injected mice (199.8 ± 43.0, n=12) (Fig.10A). CPD-46454 did not afford neuroprotection in the hilus while a 34% decrease in cell loss was found in CA3/CA4 (SE+Vehicle, 52.8 ± 10.5; SE+CPD-4645, 34.8 ± 7.7) (Fig.10A). The KD itself greatly reduced the number of Fluoro-Jade-positive CA1 pyramidal neurons in SE-exposed mice (0.75 ± 0.5; Fig.10B). In CPD-4645 treated KD-fed mice, virtually no cell loss was observed in CA1 while degenerating neurons in the amygdala were reduced by half vs vehicle-injected mice independently on the diet (Fig.10B). A 33% reduction was observed in CA3/4 (SE+Vehicle, 33.8 ±17.0; SE+CPD-4645, 22.8 ± 11.0) although this difference was not statistically significant.

3.5 DISCUSSION

Studies in several animal models demonstrate that SE induced either by chemoconvulsant drugs or electrical stimulation causes a rapid and intense inflammatory cascade in the forebrain involving interactions among neurons, reactive astrocytes, activated microglia, vascular endothelial cells and infiltrating monocytes from the blood [50].

Prostaglandins produced by cyclooxygenase 1 and 2 (COX-1/2) [51] expressed in neurons and glial cells [52] play an important role in inflammatory cascade generated in the brain during SE. Experimental studies demonstrated that rodents treated with COX-1/2 inhibitors or knock-out (KO) mice for these enzymes show neuroprotection in models of neurodegenerative disorders with a neuroinflammatory component such as Parkinson's and Alzheimer's [53, 54] and also in status epilepticus models [55].

MAGL is a constitutively active enzyme and is mainly expressed in the hippocampus, amygdala, cerebellum and prefrontal cortex [56] and plays a crucial role in the synthesis of prostanoids [57].

Recent experimental studies showed that genetic or pharmacological inactivation of MAGL causes a significant reduction in AA levels in the brain and a consecutive increase of 2-AG pool that selectively acts on CB1 or CB2 receptors, providing antiexcitatory and antiinflammatory effects [58-60]. Interestingly, the 2-AG can suppress seizures and provides anti-epileptogenic effects by reducing excitatory synaptic inputs in the dentate gyrus through CB1 and CB2 receptors [32,33].

Our data confirmed that MAGL inhibition mediated by CPD-4645 increases the levels of 2-AG and reduces the biosynthesis of AA, essential for prostanoids synthesis (Fig.3). However, treatments targeting the pathway of AA and prostaglandins (i.e. EP2 receptor antagonists) provided neuroprotection, reduced inflammation and SE-related mortality, but failed to interfere with SE [34, 61].

Notably, CPD-4645 is the first "anti-inflammatory" treatment able to dramatically reduce the development of SE in animals resistant to diazepam, which is one of the first line agents for SE in clinical practice. The drug administration time (i.e. 60 min) after SE onset is also compatible with the proposed treatment algorithm for novel interventions for human refractory SE (i.e., there is no clear evidence to guide therapy after 40-60 min phase of unremitting SE) [62].

Moreover, MAGL inhibition rescues the cognitive deficit in NORT (Fig.4), confirming recent data of Dingledine *et al*. which demonstrated that EP2 antagonism blocks long term memory impairment in rats expiriencing organophosphorus-induced SE [63].

Interestingly, CPD-4645 also affords neuroprotection in the hippocampal CA1 subregion and amygdala, ipsilateral to KA injection (Fig.10). The neuroprotective effects in CA1 are also provided by KD itself, confirming experimental studies in which ketone bodies, especially β-hydroxybutyrate conferred neuroprotection against different types of cellular injury [64].

Thus, MAGL inhibition may mediate therapeutic effects on SE and related pathological consequences by two main mechanisms: 1. accumulation of 2-AG which activates the presynaptic CB1R, thereby reducing glutamate-mediated excitatory neurotransmission [60]; 2. antiinflammatory effects resulting from lowering of AA and reduction of eicosanoids synthesis as well as 2-AG mediated activation of CB2 receptors expressed by activated glial cells, thereby reducing their pro-inflammatory phenotype [65,66].

Our findings support that MAGL inhibition reduces SE mainly via an antiinflammatory mode of action since its therapeutic effect was unaltered in the absence of CB1R.

The CB2-mediated antinflammatory action is also supported by the reduction of proinflammatory cytokines, in particular IL-1β and COX-2, in the hippocampus of CPD-4645-treated mice (Fig.7).

Infact, the IL-1β- IL-1 receptor type 1 axis and the PGE2 activating EP1 receptors play a key role in the pathological sequaele related to SE (i.e cognitive deficits, neuronal cell loss) [34,62,67].

The abrogation of SE in a short time is critical for reducing mortality and preventing the long-term pathological consequences in clinical settings (including neuronal injury, neuronal death, functional deficits)[1].

Despite the maximal MAGL inhibition occurs within 30 minutes from drug injection, as indicated by the pharmacokinetic and pharmacodynamic data, the CPD-4645 maximum effect on SE occurs from the 3rd hour after injection. The delayed therapeutic effect of CPD-4645 may depend on the availability of a pre-existing pool of AA for proinflammatory eicosanoid synthesis. The anticipation of the drug effects on SE in KD-fed mice may be due to the reduction of this pool of AA as suggested by evidence from literature [38]. Moreover, KD is also able to decrease inflammatory mediators as IL-1β in brain and blood, providing anti-inflammatory effects [38]. The diet's anti-inflammatory effect can be mediated by dietary polyunsaturated fatty acids (PUFAs): in particular, N-3 PUFAs can decrease the production of inflammatory eicosanoids, cytokines, and reactive oxygen species. They can inhibit the Nuclear Factor Kappa B-mediated transcriptional activation of inflammatory genes by activating peroxisome proliferator-activated receptors or exert anti-inflammatory actions by promoting the generation of resolvins and docosanoids [68,69].

Notably, beta-hydroxybutyrate decreased caspase-1 activation and IL-1β secretion through specific effects on the NLRP3 inflammasome [39,40].

Thus, we demonstrate for the first time that the antinflammatory effects of MAGL pharmacological blockade are potentiated by KD and the combination of these two treatments is able to block SE.

Our results suggest that MAGL may be a promising therapeutic target for refractory SE and for preventing the associated pathological long term consequences.

Searching for a clinical translation of these results, MAGL inhibitor might represent a new interesting drug to stop benzodiazepine-resistant SE arising in patients with severe immune-mediated pharmacoresistant epileptic encephalopathies (i.e. FIRES) already treated with KD.

3.6 FIGURES AND LEGENDS

Figures 1-2 (included in the main text)

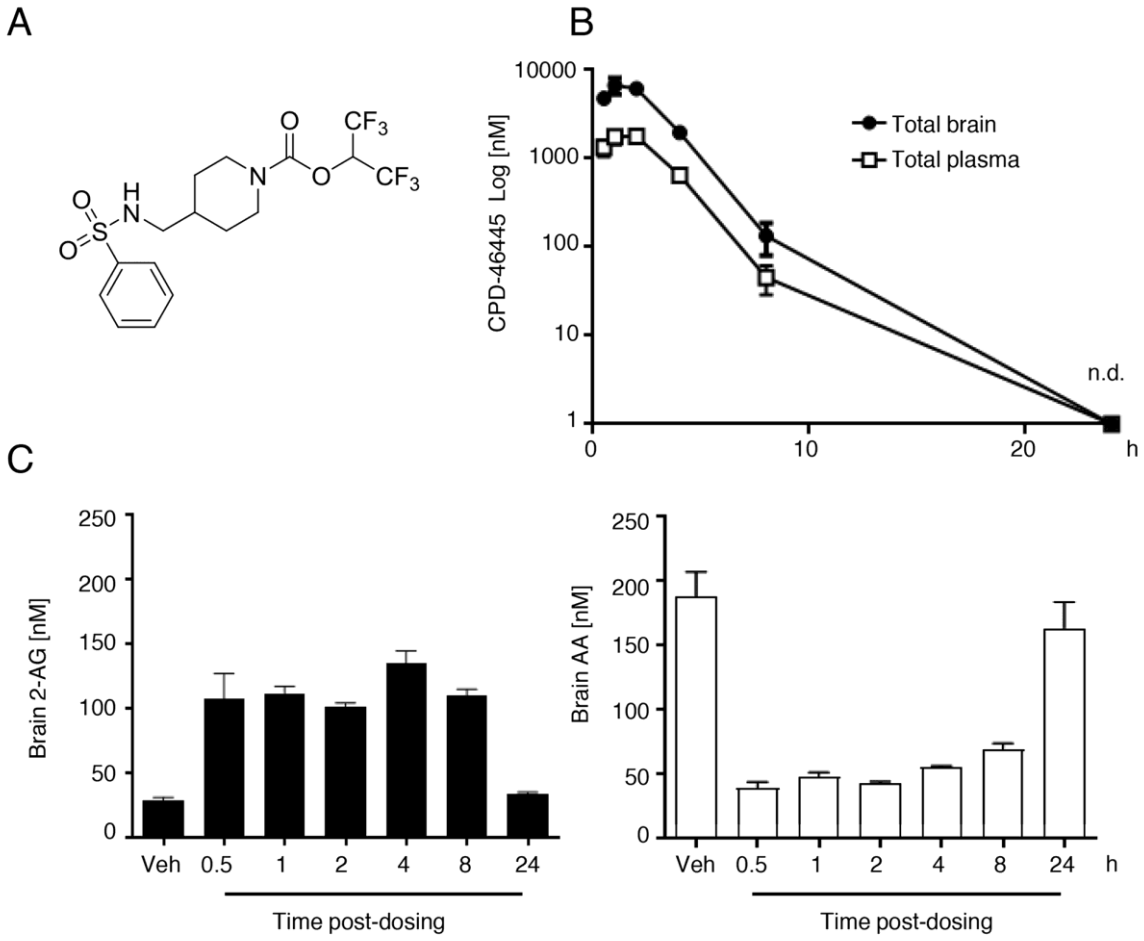

Figure 3. Pharmacokinetic and pharmacodynamic profile of CPD-4645

Panel A: Chemical structure of CPD-4645. *Panel B*: Total concentrations of CPD-4645 in plasma and brain following subcutaneous (sc) administration of compound (10 mg/kg) in vehicle (5:5:90 DMSO:Cremophor:Saline). *Panel C*: The resulting brain concentrations of 2-arachidonoylglycerol (2-AG) and arachidonic acid (AA) determined at indicated time points by LC/MS/MS. Data are means $\pm$ SEM (n=3 mice/time point; n=12 vehicle treated mice).

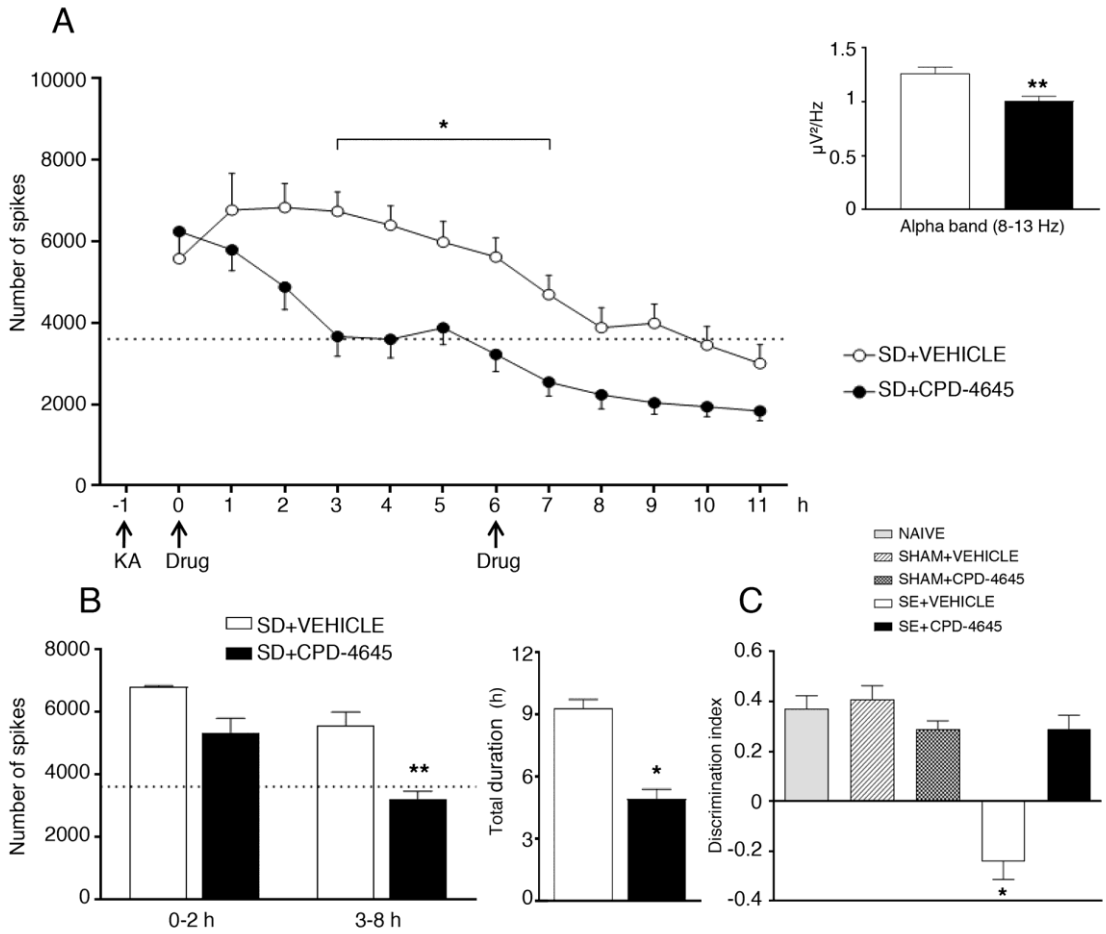

Figure 4. Effect of CPD-4645 on status epilepticus (SE) and the associated cognitive deficit in standard diet (SD)-fed mice

Panel A: Number of spikes during SE in mice treated with CPD-4645 (10 mg/kg, sc) or its vehicle (n = 21 each group). CPD-4645 was injected 1 h and 7 h after SE onset according to PK and PD data. *p<0.05 vs VEHICLE by two-way ANOVA followed by Bonferroni's multiple comparisons test. Inset shows the CPD-4645 induced reduction of the frequency of the alpha band measured by Power Spectral Density analysis during the first 3 h of treatment (i.e. 1-4 hour after the beginning of SE); **p<0.01 vs VEHICLE by Mann-Whitney test, n=5-6.

Panel B: bargrams represent the average number of spikes in the first 2 h after drug or vehicle injection, and in the subsequent 3-8 hours, and the effect of treatment on total SE duration. CPD-4645 abolished SE by z<3 h after injection. *p<0.05, **p <0.01 vs VEHICLE by Mann-Whitney

test. The dotted line represents the cut-off of 3.600 spikes/h (1Hz) below which SE elapses (inter-spike interval >1 s).

Panel C: discrimination index in SE-exposed mice treated with CPD-4645 or vehicle (same mice as in panel A) vs corresponding Sham mice (implanted with electrodes, vehicle-injected but not exposed to SE, n=13). Naïve mice (no surgery and no injections, n= 14) were used as additional controls.*$p<0.05$ vs NAIVE and SHAM mice by Kruskall-Wallis followed by Dunn's post hoc test. Data are mean ± SEM (n= number of mice).

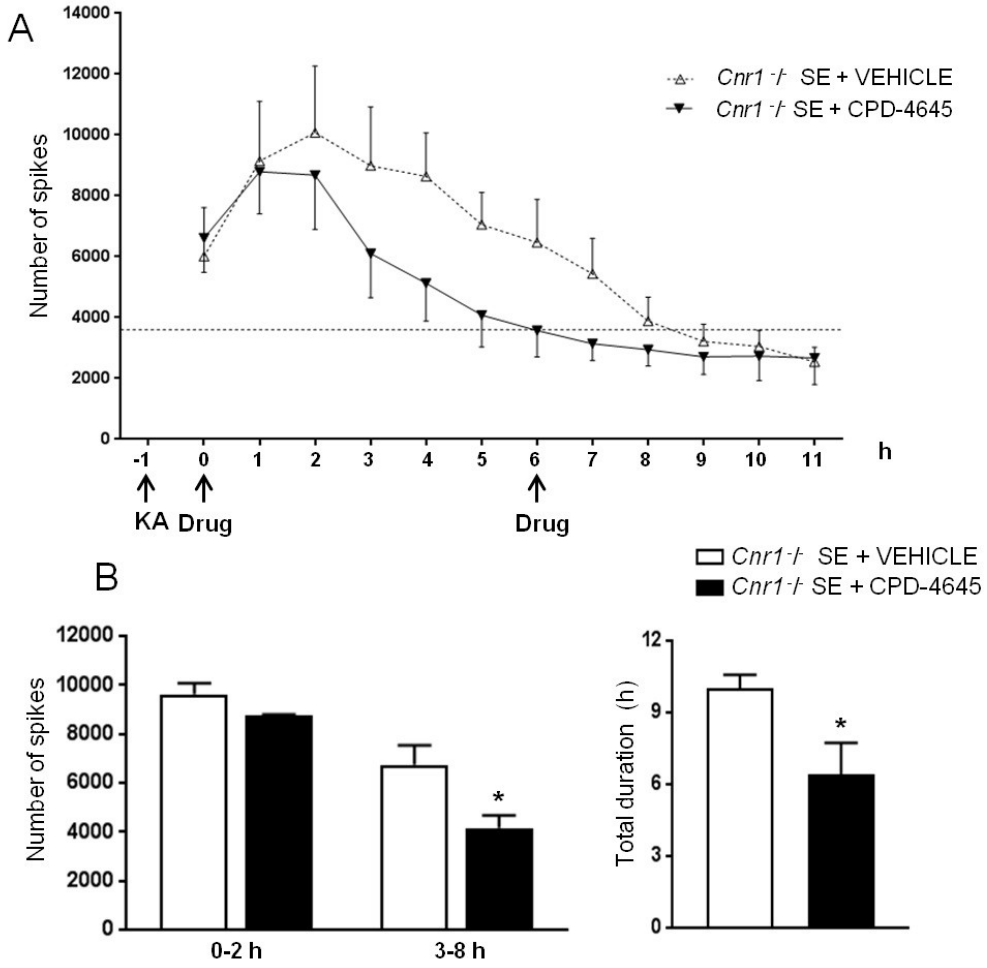

Figure 5. Effect of CD-4645 on status epilepticus (SE) in *Cnr1*$^{-/-}$ mice

Panel A: Number of spikes in *Cnr1* $^{-/-}$ mice treated with CPD-4645 or vehicle (n=7 each group) at 1 hour and 7 hours after the SE induction. CPD-4645 significantly reduced spike frequency compared to vehicle injected mice; *p<0.05; $F=8.082_{(DF1,144)}$ by two-way ANOVA. The dotted line represents the cut-off of 3600 spikes/h (1Hz). *Panel B*: Bargrams represent the effect of treatment on the number of spikes during the first 3 hours after drug or vehicle injection and in the subsequent 4-9 hours, and the effect of treatment on total SE duration. *p<0.05 vs SE+VEHICLE by Mann-Whitney test.

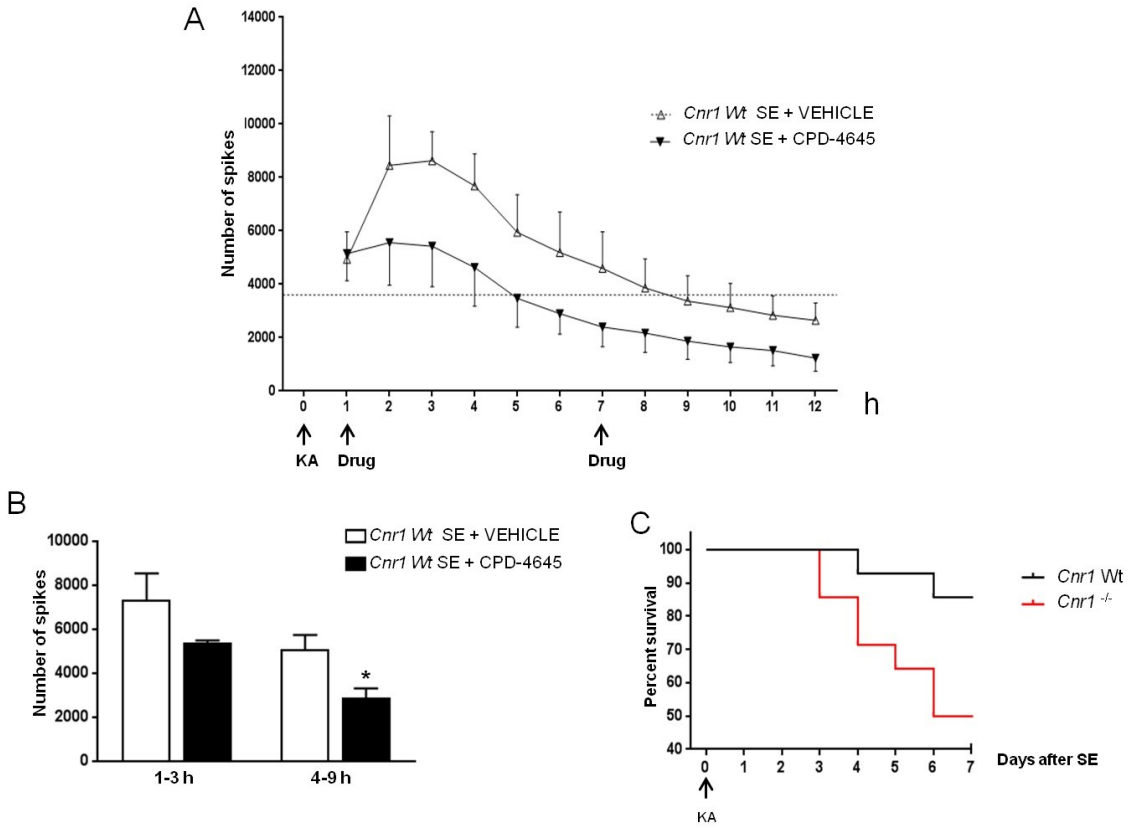

Figure 6. Effect of CD-4645 on status epilepticus (SE) in *Cnr1*$^{-/-}$ related Wt mice and survival curves

Panel A: Number of spikes in *Cnr1*$^{-/-}$ related WT mice treated with CPD-4645 or vehicle (n = 7 each group) at 1 hour and 7 hours after the SE induction. Spike frequency was significantly reduced by CPD-4645 compared to vehicle similarly to mice in Figure 2A. **p<0.01 vs SE+VEHICLE; $F=18.43_{DF1,143}$ by two-way ANOVA. The dotted line represents the cut-off of 3600 spike / hour (1Hz) below which SE ends (inter-spike intervals longer than 1 s). *Panel B*: Bargram represents the effect of treatment on the number of spikes in the first three hours after drug or vehicle injection, and in the subsequent 4-9 hours. CPD-4645 abolished SE starting from 4 hours after injection. *p<0.05 vs VEHICLE by Mann-Whitney test. *Panel C*: Survival curves showing the mortality rate in *Cnr1-/-* mice and their respective WT controls exposed to SE. CPD-4645 did not affect these curve compared to VEHICLE in each strain, therefore, data were pooled together independently on treatment; *p<0.05 *Cnr1-/-* vs WT by Kaplan–Meier survival analysis.

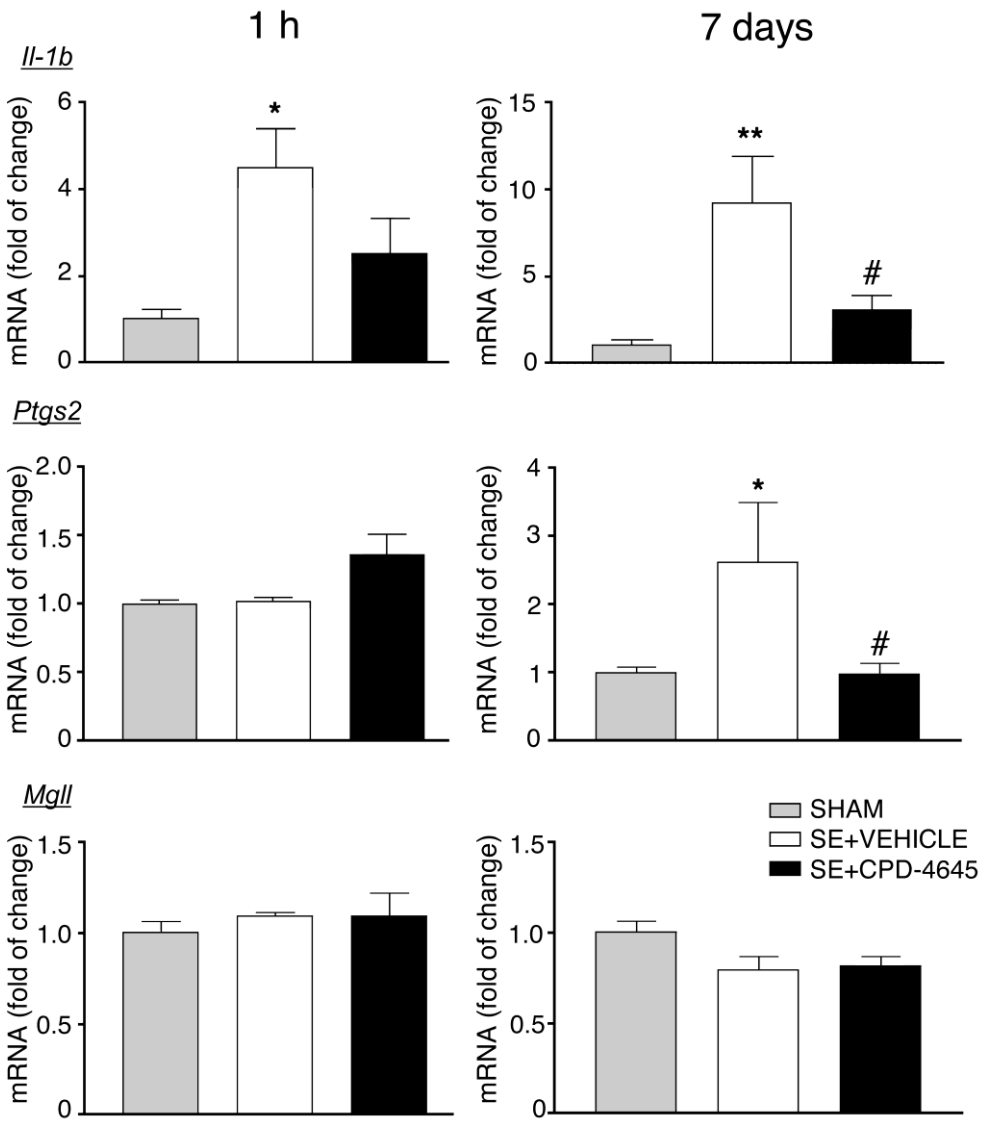

Figure 7. Inhibition of MAGL attenuates neuroinflammation following status epilepticus (SE)

Il-1b, *Ptgs2* and *Mgll* gene expression in the hippocampus contralateral to injected amygdala of mice treated with CPD-4645 vs vehicle measured at 1 hr and 7 days post-SE. CPD-4645 did not change the expression of the genes in SHAM mice (SHAM+VEHICLE, *Il-1b*: 1.05 ± 0.2; *Ptgs2*, 1.0 ± 0.03; *Mgll*, 1.0 ± 0.06; SHAM+CPD-4645, *Il-1b*: 0.75 ± 0.2; *Ptgs2*, 1.1 ± 0.09; *Mgll*, 1.0 ± 0.08). Data are mean ± SEM (n=5-6 mice/group). *p<0.05;**p<0.01 vs SHAM; # p<0.05 vs SE+VEHICLE by mixed model repeated measures ANOVA followed by Sidak post-hoc test. Outliers were identified as data points falling outside 2x the interquartile range above the upper

quartile or below the lower quartile, separately for each treatment group: *Il-1b* at 1 h: one value in SE+CPD-4645; *Ptgs2* at 1 h: one value in SE + Vehicle; at 1 h: one value in SE + Vehicle.

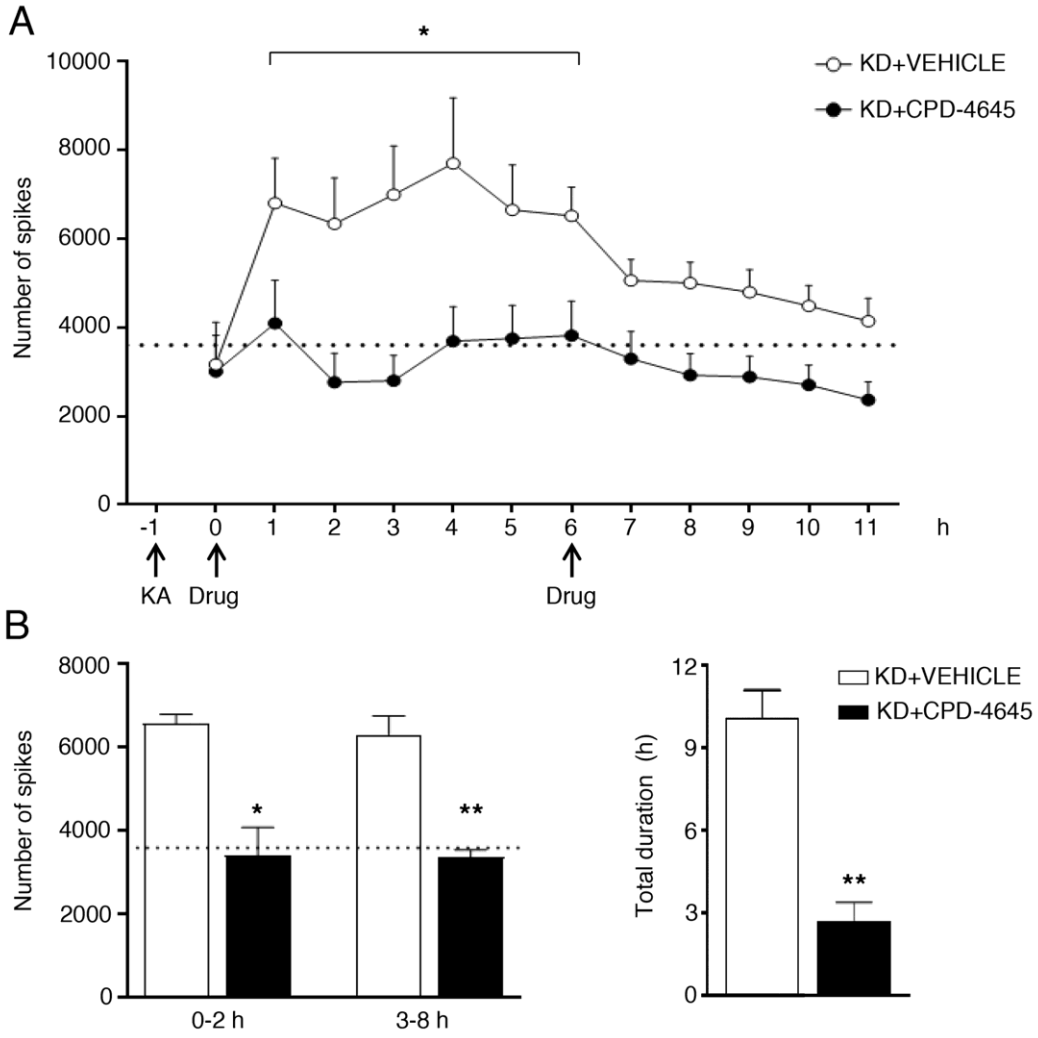

Figure 8. Effect of CPD-4645 on status epilepticus (SE) in ketogenic diet (KD)-fed mice

Panel A: Number of spikes in SE-exposed mice fed with KD for 4-weeks. Mice were treated with CPD-4645 or its vehicle (n = 11-12) at 1 h and 7 h after the SE induction. *p<0.05 vs KD+VEHICLE by two-way ANOVA followed by Bonferroni's multiple comparisons test.

Panel B reports the number of spikes in the first 2 h after drug or vehicle injection and the following 3-8 h, and the effect of treatment on total SE duration. CPD-4645 abolished SE with immediate effect in KD-fed mice (compare with Figure 2A,B). The dotted line represents the cut-off of 3600 spikes/h (1Hz). Data are mean ± SEM (n=number of mice). *p<0.05, **p <0.01 vs KD+VEHICLE by Mann-Whitney test.

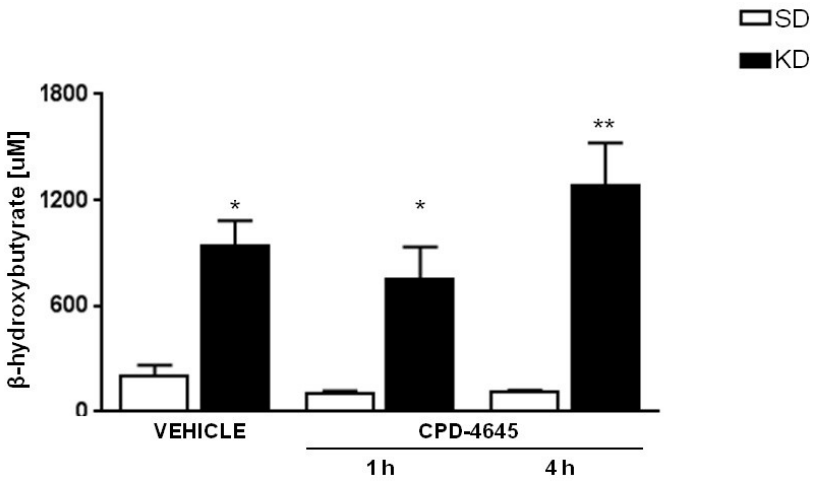

Figure 9. Ketogenic diet elevates levels of β-hydroxybutyrate

β-hydroxybutyrate level (means ± SEM, n=8-9 mice/group) in plasma of naive mice fed with standard (SD) or ketogenic diet (KD) for 4 weeks. Mice were killed 1 hour or 4 hours after CPD-4645 or VEHICLE injection. *p<0.05, **p<0.01 vs VEHICLE by one-way ANOVA followed by Bonferroni's multiple comparisons.

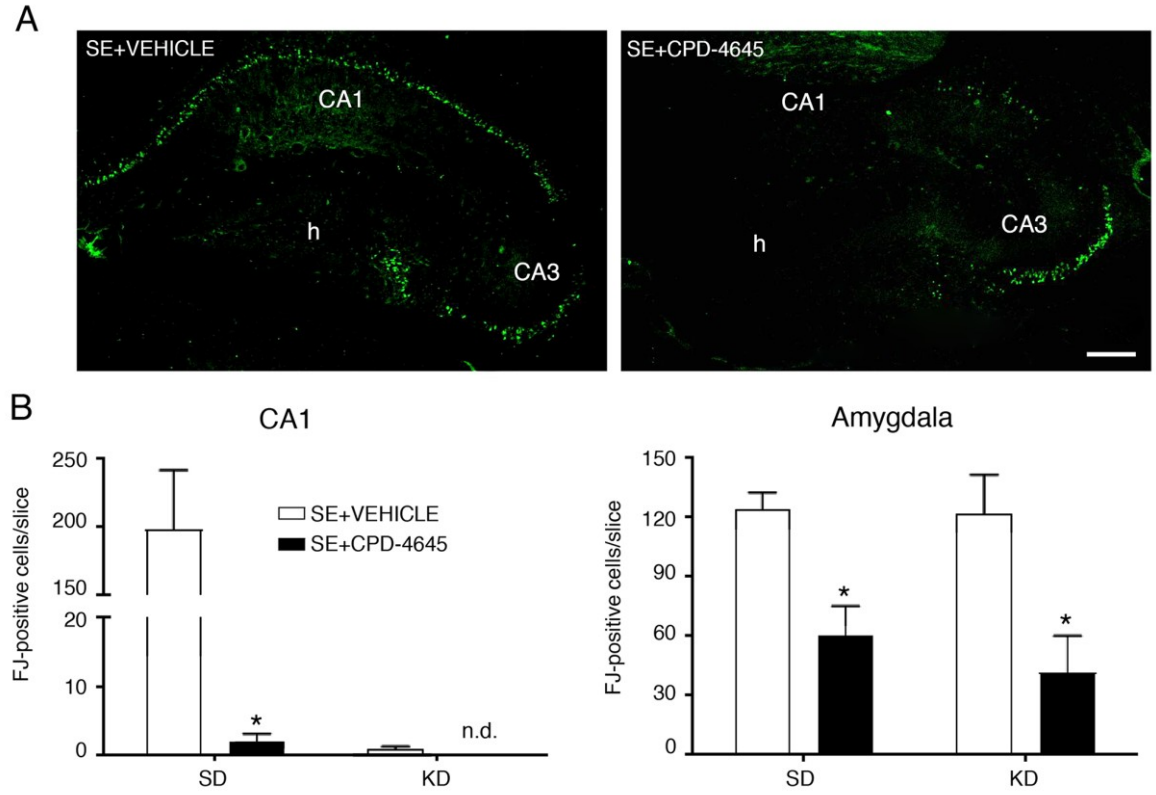

Figure 10. Effect of CPD-4645 on neuronal cell loss

Panels A: Photomicrographs depicting Fluoro-Jade (FJ)-positive degenerating neurons in the hippocampus of standard diet (SD)-fed mice exposed to status epilepticus (SE) and treated with vehicle (n=12) or CPD-4645 (10 mg/kg, sc, n=8) (B). Scale bar: 5 mm.

Panel B: Bargrams report the number of FJ-positive forebrain neurons in SD- or 4-week ketogenic diet (KD)-fed mice exposed to SE and treated with vehicle (n=9) or CPD-4645 (n=7). In the hippocampus, the KD itself drastically reduced the number of CA1 degenerating pyramidal neurons. CPD-4645 reduced neurodegeneration in CA1 under both SD and KD and no cell loss was observed in CPD-4645 treated mice under KD. In the amygdala, CPD-4645 reduced degenerating neurons independently on the diet. *$p<0.05$ vs SE+VEHICLE by two-way ANOVA followed by Sidak's *post hoc* test.

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CONCLUSIVE REMARKS

To date, targeting pharmacoresistant epilepsies represents an unmet clinical need in the field of experimental and clinical research on epilepsy. The next generation of therapies for epilepsy needs to target the mechanisms intimately involved in the process of epileptogenesis to allow the development of preventive or disease modifying treatments. The identification of the optimal time window for intervention is crucial for therapeutic success. One critical step is to move from proof-of-concept anti-epileptogenesis studies in animal models to validation in preclinical trials, and eventually to clinical translation.

In fact, anti-epileptogenesis or disease-modifying therapies for preventing or delaying the onset of the epilepsy are still missing in clinical setting.

In this PhD thesis, I pointed my attention on the role of neuroinflammation in the mechanisms of epileptogenesis. Specific inflammatory molecules and pathways have been shown to significantly contribute to the mechanisms of seizure generation and progression in different experimental models.

In particular, I have contributed to demonstrate for the first time that targeting oxidative stress with clinically tested drugs, for a limited time window post-injury, significantly delayed the onset of epilepsy, blocked disease progression and drastically reduced spontaneous seizures and long-term pathological consequences (cell loss, cognitive deficit). This intervention may be considered for patients exposed to potential epileptogenic insults as status epilepticus. Drug-induced reduction of oxidative stress prevented disulfide HMGB1 generation, thus highlighting a potential novel mechanism contributing to therapeutic effects.

Moreover, my research work provided evidence supporting that monoacylglycerol lipase (MAGL) is a new potential target for drug development in epilepsy, in particular for the treatment of drug-refractory status epilepticus (SE). Inhibition of MAGL by a new potent and selective irreversible inhibitor (CPD-4645) protects mice against refractory SE and its therapeutic effects are potentiated by the ketogenic diet.

In conclusion, all these experimental data contribute to better clarify the complex mechanisms underlying the pathophysiology of epilepsy, in order to identify new potential therapeutic targets. However, the major challenge will be represented by the translation of these experimental results into clinically effective human therapies.

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List of publications:

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22. “ Le neuroscienze nell’area napoletana: prima giornata d’incontro”. Naples, 15 December 2016.

- **Terrone G**, Pauletti A, Villa BR, Salamone A, Guilmette E, Piro J, Samad T, Vezzani A. Inhibition of monoacylglycerol lipase by PF-4645 protects mice against refractory status epilepticus. 12th European Congress on Epileptology, Prague, 11-15 September 2016.

- **Terrone G,** Pauletti A, Pastore A, Antoine DJ, Balosso S, Pascente R, Villa BR, Ravizza T, Del Giudice E, Vezzani A. Ruolo dello stress ossidativo e della neuroinfiammazione nell’epilettogenesi: nuovi target terapeutici nell’epilessia. 39° Congresso Nazionale LICE Roma, 8-10 Giugno 2016.

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- Turco R, Ummarino D, Miele E, **Terrone G,** Del Giudice E, Staiano A. Esophageal high resolution manometry in neurologically impaired children and gastro-oesophageal reflux disease. 48th Annual Meeting of The European Society for Paediatric Gastroenterology, Hepatology and Nutrition 6-9 May 2015, Amsterdam, Netherlands.

- **Terrone G**, Esposito M, Giardino G, Del Giudice E. Associazione fra disturbo del movimento di tipo coreico, mioclono noncorticale multifocale, epilessia e disabilità intellettiva: descrizione di due casi familiari. Riunione Policentrica LICE, Roma, 29-30 Gennaio 2015.

- Giardino G, **Terrone G**, Raimondi F, Iorio R, Parente I, Clayton P, Del Giudice E. Epilessia da deficit di PNPO: follow-up di 5 anni. XL Congresso Nazionale SINP-27-29 Novembre, Palermo 2014.

- Giardino G, Tufano M, Cappuccio G, **Terrone G**, Manganelli F, Pisciotta C, Geroldi A, Capponi S, Del Giudice E. Deficit di mitofusina associata a grave ritardo mentale e note dismorfiche. XL Congresso Nazionale SINP-27-29 Novembre, Palermo 2014.

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- **Terrone G**, Raimondi F, Iorio R, Parente I, Mills PB, Clayton PT, Del Giudice E. Epilessia da deficit di PNPO: follow-up di 5 anni. 37° Congresso Nazionale LICE Trieste, 4-6 Giugno 2014.

Awards:

- Best poster for Basic Science with the abstract "Inhibition of monoacylglycerol lipase by PF-4645 protects mice against refractory status epilepticus." at the 12th European Congress on Epileptology, Prague, 11-15 September 2016.

Grant:

- Research gran fromt AICE (Associazione Italiana contro l'Epilessia) 2016-2017 for the project: **"Role of oxidative stress and neuroinflammation in an infant rat model of early-life status epilepticus: implications for novel therapeutic strategies in pediatric pharmacoresistant epilepsy."**

Technical expertise:

- In vivo models of seizures and epilepsy using mice and rats including stereotactic electrode and injection cannula implantation procedures
- Acute and long-term video-EEG recordings
- EEG analysis: quantification of seizures, spikes and power spectrum density
- Experience with models of electrical and chemical status epilepticus (i.e. *self-sustained limbic status epilepticus, intra-amygdala kainic acid-induced status epilepticus*)
- In vivo pharmacology
- Immunohistochemistry (NISSL, Fluoro-jade)
- Behavioral tests of spatial and non spatial memory, motor activity and anxiety-like behaviors (T-maze, Water Morris maze test, Novel object recognition test, Open field).
- Experience with optic and fluorescence microscopy

Clinical activities:

- Diagnostic and therapeutic management of paediatric patients with genetic or metabolic epileptic encephalopathies.
- Video-EEG study of paediatric patients with different neurological disorders, ranging from epilepsy to sleep disorders or headache.
- Diagnostic and clinical follow-up of patients with isolated or syndromic corpus callosum abnormalities.
- Diagnostic and clinical management of patients with Intellectual Disability of unknown origin.
- Clinical follow-up of patients with severe cerebral palsy and feeding disorders.
- Clinical monitoring of adverse effects of antiepileptic drugs in paediatric epileptic population

Professional affiliations

Member of Italian League Against Epilepsy (LICE)

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