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Blockade of the IL-1R1/TLR4 pathway mediates disease-modification therapeutic effects in a model of acquired epilepsy

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ABSTRACT

We recently discovered that forebrain activation of the IL-1 receptor/Toll-like receptor (IL-1R1/TLR4) innate immunity signal plays a pivotal role in neuronal hyperexcitability underlying seizures in rodents. Since this pathway is activated in neurons and glia in human epileptogenic *foci*, it represents a potential target for developing drugs interfering with the mechanisms of epileptogenesis that lead to spontaneous seizures. The lack of such drugs represents a major unmet clinical need. We tested therefore novel therapies inhibiting the IL-1R1/TLR4 signaling in an established murine model of acquired epilepsy. We used an epigenetic approach by injecting a synthetic mimic of micro(mi)RNA-146a that impairs IL1R1/TLR4 signal transduction, or we blocked receptor activation with antiinflammatory drugs. Both interventions when *transiently* applied to mice *after* epilepsy onset, prevented disease progression and dramatically reduced chronic seizure recurrence, while the anticonvulsant drug carbamazepine was ineffective. We conclude that IL-1R1/TLR4 is a novel potential therapeutic target for attaining disease-modifications in patients with diagnosed epilepsy.

1. Introduction

Epilepsy is a brain disorder affecting over 50 million people worldwide and is associated with increased mortality, significant comorbidities, unique stigmatization of affected individuals, and high societal cost (Duncan et al., 2006). Current antiepileptic drugs (AEDs) provide only symptomatic control of seizures, have multiple adverse effects, and are ineffective in up to 40% of patients (Weaver and Pohlmann-Eden, 2013). This represents a major unmet clinical need. To bridge the treatment gap, next generation therapies need to possess *disease-modifying* properties by targeting the mechanisms intimately involved in making the brain susceptible to generate spontaneous seizures. Such drugs are still lacking and they could potentially be used to halt or reverse the progression of epilepsy in patients with an established diagnosis, or delay or prevent the onset of epilepsy in susceptible individuals (Barker-Haliski et al., 2015).

Experimental evidence shows that the activation of the IL-1 receptor/Toll-like receptor (IL-1R1/TLR4) pathway is a major pathogenic factor in epilepsy since its pharmacological or genetic inactivation dramatically reduces seizure recurrence in experimental models of either acute seizures or established epilepsy (Ravizza et al., 2006; Vezzani et al., 2000; Vezzani et al., 2002; Balosso et al., 2008; Maroso et al., 2010; Maroso et al., 2011a; Vezzani et al., 2011b; Iori et al., 2013; Balosso et al., 2014). Notably, this pathway is activated in neurons and glia in epileptogenic *foci*

Abbreviations: AEDs, antiepileptic drugs; ANOVA, analysis of variance; AP-1, activator protein 1; BBB, blood brain barrier; CA, *Cornu Ammonis*; COX-2, cyclooxygenase-2; CBZ, carbamazepine; EEG, electroencephalography; GABA, gamma-aminobutyric acid; HMGB1, High Mobility Group Box 1; IL-1β, interleukin-1β; IL-1R1, interleukin-1 receptor type 1; intracerebroventricular, icv; IRAK-2, interleukin-1 receptor-associated kinase-like 2; LNA, lock-nucleic-acid; miRNA, microRNA; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NMDA, *N*-methyl-D-aspartate; TLR4, Toll-like receptor 4; TRAF-6, TNF receptor associated factor 6; SE, status epilepticus.

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surgically resected in patients affected by various forms of acquired pharmacoresistant epilepsy (Vezzani et al., 2011a).

Different epileptogenic insults imposed to mice or rats (*e.g.*, neurotrauma, stroke, infection, febrile and non-febrile status epilepticus) trigger a rapid and long-lasting IL-1R1/TLR4 activation in seizure-prone brain areas (Vezzani et al., 2011b; Vezzani et al., 2013) mediated by the release of interleukin(IL)-1β and *danger signals*, such as High Mobility Group Box 1 (HMGB1), from glia, neurons and cellular components of the blood brain barrier (BBB). The activation of IL-1R1/TLR4 pathway in receptor-expressing neurons promotes excitotoxicity and seizures by enhancing calcium influx *via N*-methyl-D-aspartate (NMDA) receptors (Viviani et al., 2003; Balosso et al., 2008; Pedrazzi et al., 2012; Iori et al., 2013; Balosso et al., 2014). Activation of IL-1R1/TLR4 in glial cells induces a neuroinflammatory cascade by transcriptional activation of NF-κB and AP-1 sensitive genes, including cytokines, chemokines, COX-2 and complement factors (Vezzani et al., 2011b; Vezzani et al., 2015b). The extent and persistence of these molecules in brain are key determinants of the switch from the homeostatic role of neuroinflammation to its contribution to cell damage and dysfunction (Heinemann et al., 2012; Devinsky et al., 2013). The link between IL-1R1/TLR4 signaling activation, neuronal hyperexcitability and reduction of seizure threshold may potentially contribute to the development of a chronic epileptogenic network ignited by different brain insults. This pathway therefore represents a potential target for attaining disease-modifications in epilepsy, thereby improving disease prognosis.

In this study, we tested the potential therapeutic effects, based on disease modifications, of epigenetic or pharmacological interventions designed for inhibiting the IL1R1/TLR4 pathway activation in a widely used mouse model of acquired epilepsy (Shinoda et al., 2004; Li et al., 2008; Mouri et al., 2008; Jimenez-Mateos et al., 2012; Liu et al., 2013; Gu et al., 2015). Epigenetic intervention was based on micro(mi)RNA brain delivery. miRNAs are small non-coding RNA that represent key epigenetic posttranscriptional regulators of cellular protein levels (Jimenez-Mateos and Henshall, 2013). Specifically, we selected to enhance the negative feed-back regulation of the IL-1R1/TLR4 signaling mediated by miR-146a (Taganov et al., 2006; O'Neill, 2008; Boldin et al., 2011; Quinn and O'Neill, 2011; Iyer et al., 2012; Zeng et al., 2013; van Scheppingen et al., 2016) using a synthetic oligonucleotide mimic. Notably, miR146a is induced in neurons and glia in both experimental and human epilepsy (Aronica et al., 2010; Omran et al., 2012; Prabowo et al., 2015; van Scheppingen et al., 2016). In complementary studies, we used a combination of antiinflammatory drugs for effectively blocking IL-1R1/TLR4 activation. We applied these agents for a limited period of time after the onset of epilepsy in mice to simulate a clinical intervention in patients with diagnosed epilepsy.

2. Materials and methods

2.1. Animals

We used 8 week-old C57BL6N male mice (~23–30 g) in all experiments, except for electrophysiological recordings that were done in 21 day-old C57BL6N male mice (Charles River, Calco, Italy). 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 standard food pellet (2018S, Envigo, Udine, Italy) or to CBZ-in-food and its control pellet (BioServe, F05572; Frenchtown, NJ, USA; Grabenstatter et al., 2007) and water, and with a fixed 12 h light/dark cycle. Mice were housed 5 animals per cage. After experimental manipulations (as reported below) each mouse was individually housed in the presence of environmental enrichment (i.e. toilet paper, straw).

2.2. Study design

In this study we investigated the potential therapeutic effects of epigenetic or pharmacological targeting of the IL-1R1/TLR4 pathway in an established mouse model of acquired epilepsy. Drugs were therefore *transiently applied* after the onset of epilepsy in each mouse, as determined by the occurrence of the first two video-EEG recorded spontaneous seizures at least 48 h after status epilepticus (SE) was elapsed. Treatment schedule was determined according to pharmacokinetic and pharmacodynamic data, as specified in each treatment protocol.

Data are presented as mean ± SEM and are inclusive of all mice that were randomized in the biochemical or therapeutic studies. No animal was excluded from the study except for mice which did not develop SE (8 out of 88) due to kainate injection misplacement. The number of mice in each experiment is indicated by n values in the figure legends, methods and supplementary materials. In each experiment, simple randomization was used as treatment allocation rule; blinding was applied to treatment administration and data analysis. In the proof-of-concept studies in Figs 1, 2 and 3, animal sample size was estimated empirically on the basis of our previous experience with the epilepsy models and the therapeutic effects of anti-inflammatory drugs (Vezzani et al., 2000; Balosso et al., 2008; Maroso et al., 2010; Maroso et al., 2011a; Iori et al., 2013; Balosso et al., 2014). Our primary endpoint was ≥50% reduction in the frequency of seizures at the end stage of the disease (*i.e.* 2.5 months after epilepsy onset) in the treated group compared to the respective control group. We also took into careful consideration the principles of the 3 Rs (Replacement, Reduction and Refinement; https://www.nc3rs.org.uk/the-3rs). 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.

2.3. Intracerebroventricular injections of oligonucleotides

Mice were surgically implanted under general gas anesthesia (1–3% isoflurane in O_2) and stereotaxic guidance (Maroso et al., 2010; Iori et al., 2013) with a guide cannula positioned on top of the *dura mater* (from bregma, mm: nose bar 0; anteroposterior 0, lateral 0.9) (Franklin and Paxinos, 2008) one week before the injections. miR-146a mimic (Applied Biosystems, Carlsbad, CA, USA), antagomiR LNA (Superior probes, RiboTaskApS, Odense, Denmark) or their respective controls (specific random sequence for mimic or negative control with no effects on known miRNA function; Applied Biosystems; see table below) were dissolved in sterile PBS and injected intracerebroventricularly (icv, 0.25 μl/min) in freely moving mice over 4 min using a 30-gauge injection needle connected to a 10.0 μl Hamilton microsyringe *via* PE20 tubing, according to convection-enhanced delivery method (Gasior et al., 2007). At the end of infusion, the needle was left in place for one additional minute to avoid backflow through the guide cannula, then gently removed.

The mimic or its negative control was administered icv as single injection (5 or 10 μg in 1 μl; Fig. 1A,B; Figs. S1, S2, panels a–f) or repetitively (10 μg in 1 μl; one injection every three days for a total of five injections; Fig. 2; Figs. S2, panels g–o, S3D) while the antagomiR or its negative control was injected icv twice a day for six consecutive days (Krutzfeldt et al., 2005) (1 μg in 1 μl; Fig. 1C,D).

Oligonucleotide	sequence
Mimic	UGAGAACUGAAUUCCAUGGGUU, Cat# MC10722
AntagomiR	AacCcaTggAauTcaGuuCucA, custom made (Capital letters: LNA modification; small letters: 2-o-methyl modification)
Mimic negative control	Cat# 4464059
AntagomiR negative control	Cat# 4464077