Epileptiform activity contralateral to unilateral hippocampal sclerosis does not cause the expression of brain damage markers

Francesco Noè *[1], Alessandro Cattalini *[1], Camilla Alessi*[1], Diogo Vila Verde[1], Matteo Figini[2], Ileana Zucca[2] and Marco de Curtis[1]

[1] Epilepsy Unit and [2] Scientific Direction Department, Fondazione IRCCS Istituto Neurologico *Carlo Besta*, via Celoria 11, Milano, Italy

(*)FN, AC and CA equally contributed to the realization of the manuscript

Running Title: Focal seizures and brain damage

Manuscript content: Title 108 characters; Running title: 31 characters; Abstract: 253 words; Body of manuscript: 6935 words (Intro 509); 50 references, 1 Table and 6 figures

Keywords: brain damage, epilepsy, focal seizures, non-convulsive status epilepticus.

Corresponding author: Marco de Curtis, MD - Epilepsy Unit, Fondazione IRCCS Istituto Neurologico *Carlo Besta*, via Celoria 11, 20133 Milano, Italy.
Tel. +39 0223942280; e-mail: marco.decurtis@istituto-besta.it

Abstract

Objective: Patients suffering from epilepsy often ask if recurrent seizures harm their brain and aggravate their epileptic condition. This crucial question has not been specifically addressed by dedicated experiments. We analyze here if intense bilateral seizure activity induced by local injection of kainic acid (KA) in the right hippocampus produces brain damage in the left hippocampus.

Methods: Adult guinea pigs were bilaterally implanted with hippocampal electrodes for continuous video-EEG monitoring. Unilateral injection of 1µg KA in the dorsal CA1 area induced non-convulsive status epilepticus (ncSE) characterized by bilateral hippocampal seizure discharges. This treatment resulted in selective unilateral sclerosis of the KA-injected hippocampus. Three days after KA injection, the animals were sacrificed, brains were submitted to *ex vivo* magnetic resonance imaging (MRI) and were processed for immunohistochemical analysis.

Results: During ncSE, epileptiform activity was recorded for 27.6 ± 19.1 hours in both the KA-injected and in the contralateral hippocampus. Enhanced T1-weighted MR signal due to gadolinium deposition, mean diffusivity reduction, neuronal loss, gliosis and blood-brain barrier permeability changes were observed exclusively in the KA-injected hippocampus. Despite the presence of a clear unilateral hippocampal sclerosis at the site of KA injection, no structural alterations were detected by MR and immunostaining in the hippocampus contralateral to KA injection three days and two months after ncSE induction.

Significance: We demonstrate that intense epileptiform activity during ncSE does not cause obvious brain damage in the hippocampus contralateral to unilateral hippocampal KA injection. These findings argue against the hypothesis that epileptiform activity *per se* contributes to focal brain injury.

Abbreviations: blood-brain barrier = BBB; dentate gyrus = DG; diffusion tensor imaging = DTI; fractional anisotropy = FA; glial fibrillary astrocytic protein = GFAP; kainic acid = KA; immunoglobulines = IgG; left hippocampus = LH; magnetic resonance = MR; mean diffusivity = MD; mesial temporal lobe epilepsy = mTLE; microtubule-associated protein 2 = MAP2; neuronal nuclei = NeuN; non-convulsive status epilepticus = ncSE; region of Interest = ROI; right hippocampus = RH.

Introduction

Patients suffering from epilepsy often ask if recurrent seizures harm their brain and aggravate epilepsy. Seizures and brain damage develop in parallel in many epilepsies and a definite answer to this question is missing [1,2]. In focal epilepsies due to structural and metabolic causes [3], the progression of brain damage and the organization of an epileptogenic network could either be the consequence of the acute brain injury (as in post-traumatic epilepsy [4]) or may result from a persistent pathogenic condition, as in epileptic encephalopathies [3,5]. Since seizure frequency usually intensifies in parallel with brain injury worsening, it has been suggested that seizure activity by itself may have a direct pathogenic action on tissue reorganization and may possibly worsen brain damage [6]. Nevertheless, a *correlation* between seizure activity and brain damage does not necessarily demonstrate a cause–effect relationship. It is not clear, indeed, whether the evolution of brain damage is either the natural consequence of the local structural changes triggered by the primary injury or is it due to seizure activity, or both.

Experimental studies do not help to clarify if simple seizures by themselves damage the brain. Brain injury progression has been documented during the life span of animals that develop mesial temporal lobe epilepsy (mTLE) with hippocampal sclerosis as consequence of status epilepticus [7-10], and was demonstrated in rodents after brain concussion [7,11]. The core of the question here addressed is to understand if brain damage is produced by the seizure activity *per se*. To this end, we utilized a guinea pig model of unilateral mTLE [12,13] induced by local injection of the excitatory glutamate receptor agonist, kainic acid (KA), in one hippocampus [14-16]. In this mTLE model, the unilateral intrahippocampal KA injection induces a non-convulsive status epilepticus (ncSE) characterized by bilateral epileptiform discharges and seizures. In a previous report, we observed that animals showed a clear damage in the KA-injected hippocampus, whereas the limbic regions contralateral to the KA-injected hemisphere were not apparently altered, despite their obvious epileptic activation during KA-induced ncSE [12]. These observations confirm the injuring capability of the neurotoxin KA, a drug known to induce within 24 h an acute neurodegeneration by triggering calcium-dependent neuronal apoptosis [17], and suggest that seizure activity does not damage the hippocampus contralateral to KA injection. We further investigated this aspect by quantifying brain changes in the hippocampi ipsi- and contralateral to KA injection with neurophysiological,

neuropathological and imaging techniques. It is worth to point out that we do not aim at investigating the role of seizure activity in the induction of brain damage at the site of KA injection. Since KA-induced brain damage is established within a few days after SE [18-20], we focused the analysis of brain tissue at three days after ncSE, when gliosis, neuroinflammation [21,21] and blood-brain barrier (BBB) permeability changes [23] are maximal, and other confounding factors (such as spontaneous seizures) are not established yet. We further validated the findings at a later time point, 2 months after ncSE, when an active epileptic condition associated with the unilateral KA-induced hippocampal sclerosis is established.

Methods

The study is based on a cohort of 32 adult male Hartley guinea pigs (200-250 g weight, 3 postnatal weeks of age; Charles River, Calco, Italy) housed in a 12 h light-dark controlled cycle environment with *ad libitum* food and water supply. Four different experimental groups were utilized: naïve guinea pigs (n=10; 6 for the acute experiments and 4 for the chronic condition – see below); sham-operated and sham-treated animals (n=5); guinea pigs unilaterally injected with KA in the right dorsal hippocampus sacrificed three days (n=11) and at 2 months (n=6) after ncSE. The experimental protocol was reviewed and approved by the Committee on Animal Care and Use and by the Ethics Committee of the Fondazione Istituto Neurologico and was approved by the Animal Welfare Office of the Italian Health Ministry (Authorization n. 36/2016-PR, released on January 18th, 2016), in accordance with the European Committee Council Directive (2010/63/EU). Efforts were made to minimize the number of animals used and their suffering. This study complies with the 3Rs principle recommended by the EU directives [24].

Implantation of electrodes and injection cannula. Twenty-two animals were surgically implanted with bilateral depth (intrahippocampal) and superficial (epidural) EEG recording electrodes. The procedures for electrode implantation and KA injection have been previously published [12]. Briefly, 30 minutes before surgery, animals were subcutaneously treated with 4.2 mg/kg flunixin (Finadyne; Schering Ploug, Kenilworth, NJ, US) and 10 mg/kg dexamethasone (Soldesam; Laboratorio Farmacologico Milanese, Caronno, Italy). Thereafter, animals were deeply anesthetized with 5% isoflurane (Furane; Abbott Laboratories, Abbott Park, IL, US; flow rate of 1 L/min) and were fixed on a stereotaxic frame (David Kopf

Instruments, US). During surgery, isoflurane levels were maintained at 1.5-2.0 %. Two stainless steel screws of 1.1 mm diameter were implanted as epidural electrodes into the bone above the cerebellum, as reference and neutral electrodes, respectively. Two other epidural electrodes were bilaterally placed over the frontal neocortex. Two polyamide-coated stainless steel wires (0.175 mm diameter; Advent, Eynsham, Oxford, United Kingdom) were bilaterally positioned in the dorsal CA1 area of the hippocampus (stereotaxic coordinates relative to Bregma: anteroposterior -3 mm, mediolateral ± 3 mm, dorsoventral -3.25 mm). A stainless steel guide cannula (23 gauge; Cooper Needle Works Ltd, Birmingham, West Midland, UK) was glued to the right hippocampal electrode for intrahippocampal KA injection. The distance between the electrode tip and the cannula was 3.25 mm. All electrodes were inserted in a pedestal connector (Plastic One, Roanoke, VA, US) and were fixed on the skull with acrylic cement (Paladur; Heraeus, South Bend, IN, US). After surgery, animals were hosted in individual cages and were treated with subcutaneous injection of 5 mg/kg/die enrofloxacin (Baytril; Bayer, Leverkusen, Germany) for 5 days, and with intramuscular dexamethasone (1 mg/kg) and flunixin (2.2 mg/kg) every 12 hours for 48 hours. All implanted animals survived the surgery procedure.

Video-EEG recordings. Video-EEG monitoring started one week after surgery; implanted pedestals were connected to a cable mounted on a swivel coupled to the preamplifier stage of a Brain Quick EEG System (Micromed, Mogliano Veneto, Italy). After 24 h of adaptation, video-EEG was continuously recorded for 5 days, including 48 h before (baseline) and 3 days after either KA or NaCl hippocampal injection. EEG data were recorded wide-band (0.1-1.0 kHz at 2064 Hz sampling rate, with 16-bit precision) using the System Plus Evolution software (Micromed).

Unilateral intra-hippocampal KA and saline injection. Nine days after electrode implantation, 22 animals were injected in the CA1 area of the right hippocampus with either KA (n=11+6) or 0.9% NaCl saline solution (sham operated animals; n=5), respectively, under continuous video-EEG recording. A 30-gauge needle, connected to 5 µl Hamilton syringe via a polyethylene tube, was lowered through the guide cannula in the right hippocampus to slowly inject 1 µl of 1 µg KA (Sigma, St. Louis, MO, US) diluted in 0.9% NaCl solution. Within 15 minutes after KA injection, epileptiform activity was recorded in all animals. Video-EEG was monitored for 3 days after KA (or NaCl) injection. None of the sham operated/injected

guinea pigs showed epileptiform activity on the EEG. Six chronic mTLE animals were video-EEG recorded 7 days every other week for at least two months after ncSE to verify the presence of spontaneous epileptiform discharges and seizures [12,13]. Surgeries, animal shuffle in the different experimental groups, and intrahippocampal injections were performed by FMN (acute ncSE) and AC (chronic mTLE animals).

Video-EEG data analysis. Continuous 24-hour video-EEG recorded 2 days before and 3 days after KA (or saline) injection was analyzed off-line. Hippocampal EEG patterns during and after KA injection were identified and quantified for each animal. The EEG activity recorded in the frontal cortex was utilized to identify the presence of a diffuse EEG pattern and was not analyzed/quantified in the present study. KA-induced epileptiform activity was characterized by seizures combined with continuous rhythmic spiking activity at 1-3 Hz (Figure 1). Seizure events during KA-induced ncSE were defined by large amplitude spiking activity with clear tonic and bursting phases longer than 20 seconds, followed by post-ictal depression (Figure 1B). The time spent in seizure during ncSE was quantified for both right and left hippocampal recordings in the 11 animals sacrificed 3 days after KA. Seizure discharges were identified as focal unilateral or bilateral, based on the EEG pattern distribution. Bilateral epileptiform discharges during ncSE were previously described in the 6 chronic animals [13]. Extracellular population spike number and distribution (Figure 2) were quantified in both hippocampi by setting a threshold 2.5 standard deviation above the baseline amplitude, as calculated on the pre-KA injection EEG recording. Extracellular population spikes have been defined as sharp transient with a duration included between 70 and 200 msec. Seizure activity and spike counts during ncSE were blindly analyzed by CA and FMN. In the 6 chronic animals, spontaneous seizures during the chronic phase were identified with 7 days video-EEG recordings performed every other week for 2 months [12,13]. Details on the seizures and on the epileptic phenotype of the chronic epileptic animals are reported in previous manuscripts [12,13]. In these animals, ncSE duration values were similar to those calculated for the animals sacrificed at 3 days.

Brain preparation for MR imaging and anatomy. At the end of video-EEG recording sessions, animals were deeply anesthetized with sodium thiopental (125 mg/kg i.p., Farmotal; Pharmacia, Milano, Italy) and were trans-cardially perfused for 5 min with 0.9% NaCl solution, followed by 4% paraformaldehyde in phosphate buffer 0.1 M for 15 min. Four percent gadolinium (Magnevist, Bayer, Italy) was added to the perfusion

solution to evaluate the BBB integrity during later magnetic resonance (MR) analysis. After fixation, brains were carefully removed from the skull and were immersed in 4% paraformaldehyde for 24 hours at room temperature. Fixed brains were embedded in agar solution (4% in PBS) for the MR session. Naïve animals were perfused and post-treated following the same time course of the EEG-implanted and recorded animals.

Magnetic resonance imaging. MR imaging was performed on isolated and fixed guinea pig brains using a 7T horizontal-bore scanner (BioSpec 70/30 USR; Bruker, Ettlingen, Germany), equipped with actively shielded gradient/shim coil with a maximum gradient strength of 440 mT/m, and a 38 mm transmit/receive birdcage volume quadrature coil. The magnetic field homogeneity was optimized by a localized second-order shimming procedure featured on a volume of interest covering the whole field of view. Axial T1-weighted images were acquired with the following parameters: field of view = 35x35 mm^2; in-plane resolution = 137x137 μm^2; slice thickness = 400 µm; 17 slices; echo Time = 7 ms; repetition time = 619 ms; number of averages = 200; number of repetitions = 1; acquisition time = 1h 39min. This sequence was repeated three times to cover the whole brain. Diffusion tensor imaging (DTI) was acquired by using an echo planar imaging sequence with the following parameters: field of view = 30x30 mm^2; in-plane resolution = 333x333 μm^2: slice thickness = 328 µm; 60 slices; echo time = 60 ms; repetition time = 6000 ms; number of averages = 40; number of repetitions = 1; δ = 5 ms; Δ = 12 ms; 5 b = 0 volumes; 30 diffusion weighted volumes acquired in non-collinear directions with the following diffusion weightings: b = 500, 1000, 1500, 2000, 2500 s/mm^2; acquisition time = 10h 20min. On T1-weighted images, Regions of Interest (ROIs) were manually delineated on the whole hippocampi using ITK-SNAP (www.itksnap.org) and their volume was computed. ROIs were also drawn on the dorsal part of the hippocampi and the ratio between their mean signal intensity and the mean signal in a reference ROI placed in the unaffected cortex was considered as an index of gadolinium enhancement. DTI images were corrected for motion and eddy current distortions with FMRIB's Linear Image Registration Tool in FSL (http://www.fmrib.ox.ac.uk/fsl/). The diffusion tensor in each voxel was estimated from the DTI raw data, and Mean Diffusivity (MD) and Fractional Anisotropy (FA) maps [25] were computed using Diffusion Toolkit (http://www.trackvis.org [26]). The

mean MD and FA were calculated in ROIs manually delineated in the hippocampi and frontal cortices. Chronic mTLE animals were not submitted to MRI. MRI data were blindly analyzed by MF and IZ.

Immunohistochemical study. After the completion of the MRI study, brains were cut into 50 µm coronal sections for immunohistochemical processing. The following histological parameters were investigated: (a) hippocampal neurodegeneration using neuronal nuclei NeuN immunostaining and thionine staining; (b) reactive astrogliosis by glial fibrillary acid protein (GFAP) staining; (c) acute neuronal injury by microtubule associated protein 2 (MAP2) staining, that concentrates in soma in the course of cytotoxic edema [27] and during reversible neuronal suffering [28,29]; (d) BBB permeability alteration by evaluating the presence of endogenous guinea pig IgG that are not present in the brain tissue in normal conditions. Serial coronal sections rostral and caudal to the KA injection local damage were selected and were blindly analyzed by AC and CA.

A standardized protocol has been used for immunohistochemical staining. Briefly, after endogenous peroxidase inactivation (3% H_2O_2 in PBS) and non-specific antigen binding sites blocking (1% BSA/0.2% Triton-X 100 in PBS), free-floating sections were incubated overnight at 4° C with the following primary antibodies in 0.1% BSA/0.2% Triton-X 100 at 4° C: (a) monoclonal mouse anti-neuronal nuclei (NeuN, 1:1000 - Millipore, Darmstadt, Germany); (b) polyclonal rabbit anti-glial fibrillary acid protein (GFAP, 1:500 - DAKO, Glostrup, Denmark); (c) mouse anti-microtubule associated protein 2 (MAP2, 1:1000 - Neomarker, CA, US). On the following day, sections were incubated for 75 min in biotinylated secondary antibody (1:200) diluted in 0.1% BSA. The tissue was washed in PBS 3 times and then processed for 75 min with avidin-biotin-peroxidase protocol (ABC; Vector Laboratories, Burlingame, CA, US). Visualization of labeling was achieved using 3,3'-diaminobenzidine tetra hydrochloride (DAB, 0.075% in 0.05 M Tris-HCl/ 0.02% H_2O_2; Sigma, Milano, Italy). Slices were rinsed, mounted, dehydrated and cover-slipped with distyrene plasticizer xylene. For endogenous guinea-pig IgG immunostaining (d), slices were treated only with the secondary antibody. The IgG immunostaining and the amplification of the primary-antibody labeling were obtained by 75 min incubation with biotinylated goat anti-guinea pig IgG diluted 1:200 (Vector Laboratories). Thionine and GFAP staining was performed in the 6 chronic mTLE guinea pigs and in additional 4 naïve guinea pigs.

Immuno-stained sections were visualized using the Scanscope software (Aperio Technologies, CA, US). Hippocampal and cortical staining for NeuN, GFAP, MAP2 and IgG were analyzed in the three different experimental conditions (naïve, sham-treated and KA-injected animals). Quantitative field fraction estimates of NeuN, GFAP, MAP2 and IgG immunostaining was carried out in both hippocampi using Image-Pro Plus 7 software (Media Cybernetics, Inc. MD, US). The percentage of neuronal occupancy (specific immunostaining density) had been estimated in previously determined regions of interest (ROIs) positioned in CA1 and CA3 hippocampal subfields, in the granular layer of DG and in the hilus (see upper left panel in Figure 4). The size of NeuN and thionine measurements ROI was arbitrary established at 0.043 mm^2 for CA1, 0.086 mm^2 for CA3, 0.035 mm^2 for DG and 0.35 mm^2 for the hilus region. For GFAP, MAP2 and IgG, measurements ROIs of 0.078 mm^2 were positioned on the different subfields in each analyzed section magnified at 5x; a threshold of staining intensity was defined with respect to background signal. Neuronal density in each ROI was automatically calculated by the software on two adjacent slices in each hippocampal subfield, after symmetry between right and left hippocampi was verified. For each slice in each experimental group, mean NeuN density was calculated on 4 ROIs in CA1, 2 in CA3, 4 in DG and 1 in hilus for each slice. To avoid inclusion in the analyses of electrode-related tissue alterations, densitometric ROIs in KA-injected and sham-treated guinea-pigs were positioned at least 0.5 mm away from the electrode tracks (arrows in the lower middle panel in Figure 4). CA and AC performed GFAP, MAP2-immunostaining analysis by blindly evaluating two ROIS on two different sections per animals in CA1, CA3 and hilus, using Image-Pro Plus 7 software. A single ROI in CA1 was analyzed on two sections from the same brain to evaluate IgG staining. Densitometric data from the above mentioned ROIs were calculated in both right and left hippocampi in naïve and sham-operated animals and were pooled together (black columns in Figures 5 and 6).

Statistical analysis. Surgeries and KA-injections were performed by FMN and CA; MF and AC analyzed raw EEG, MRI and IHC data in a blinded manner. Data were normally distributed and statistically analyzed with Graph-Pad Prism 3.0 (GraphPad Software Inc., San Francisco, CA, US). Results are expressed as means ± standard deviation (SD). Differences in number of seizures, time spent in seizures and number of spikes between the different experimental groups were evaluated using Student *t*-test for unpaired data and

Tukey post hoc test. For each MRI measure (hippocampal volume, dorsal hippocampus signal intensity, hippocampus MD, cortex MD, hippocampus FA and cortex FA) the difference between the left and right (KA-injected) side and between RH(KA) from KA-injected animals and hippocampal values from control animals (sham-operated and naïve) was evaluated using a non-parametric Wilcoxon signed-rank test; differences between experimental groups of animals were assessed using Wilcoxon rank-sum test. For densitometric immunohistochemical and thionine analysis, after running a Kolmogorov-Smirnov Normality test, *ANOVA One Way* coupled with Tukey's post-hoc test was used to compare multiple groups (RH(KA), LH and controls), as reported in Table 1. The tests are two-sided and significance was set at $p<0.05$. The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Results

Non-convulsive SE activity after unilateral intrahippocampal KA injection. Seizure-like EEG discharges appeared in the injected hippocampus within 15 min from unilateral intra-hippocampal KA injection both in the 11 guinea pigs sacrificed 3 days after KA and in the 6 chronic epileptic animals (for the latter group, see [12, 13]). In the 11 acute animals, epileptiform activity propagated to both the ipsilateral neocortex and the contralateral left hippocampus either immediately (n=9; Figure 1A and B, right panel) or within 10 min (n=2). The recorded epileptiform activity correlated with recurrent non-convulsive seizures [13] integrated in a continuous, irregular spiking activity (Figure 1C) that rapidly evolved in bilateral rhythmic spiking at 1-3 Hz (Figure 1D). Non-convulsive seizures typically correlated with explorative behavior, tonic immobility, lateralized facial and head myoclonias (for details, see [13]). KA-induced non-convulsive SE (ncSE) lasted from a minimum of 6 to a maximum of 70 hours (average 27.63 ± 19.17 hours – mean ± SD), and remitted spontaneously. The number of seizures recorded during ncSE varied from a minimum of 10 to a maximum of 263 (Figure 1E), with an average of 5.45 ± 1.82 seizures per hour. As previously reported [13], the large majority of non-convulsive seizures engaged both hippocampi (Figure 1E and F). The unilateral EEG involvement at ncSE onset (left panel in Figure 1B) exclude the possibility that the bilateral activation

observed later during the ncSE is due to activity volume-conducted from the KA-injected hippocampus. None of the 5 sham-operated animals injected with 0.9% NaCl solution showed EEG abnormalities during the three days of video-EEG monitoring.

To verify the involvement of the KA-injected right hippocampus [RH(KA)] and of the contralateral left hippocampus (LH) during ncSE, we quantified the number of EEG seizures (Figure 2A), the time spent in seizures (Figure 2B) and the number of spikes (Figure 2C). As illustrated in the box plots on the right of each panel in Figure 2, both RH(KA) and LH were equally involved in the generation of epileptiform activity. This is the pre-condition for the evaluation of the effects of epileptiform activity in the LH contralateral to KA injection. Six animals were video-EEG monitored for two months, when hippocampal sclerosis and chronic seizures develop, as described and characterized in two previous manuscripts [12,13].

Magnetic resonance imaging (MRI) ipsi- and contralateral to KA injection. Three days after ncSE induction, animals were injected with a bolus of 4% gadolinium just before the transcardiac perfusion of paraformaldehyde (see Methods). MR imaging was performed on fixed brains (9 KA-treated, 5 sham-operated and 6 naïve) one week after sacrifice, to verify both brain damage and BBB permeability changes to gadolinium. MRI was not performed in two guinea pigs due to an error in the gadolinium administration protocol and was not implemented in the 6 chronic animals. The normalized T1-weighted signal measured on manually delineated ROIs of whole hippocampi in both RH and LH was 1.21 ± 0.14 and 1.31 ± 0.16 in KA-treated animals, 1.29 ± 0.21 and 1.27 ± 0.16 in naïve animals and 1.25 ± 0.13 and 1.26 ± 0.13 in sham-operated animals. It was significantly higher in RH(KA) than in the LH in all KA-treated animals ($p<0.05$), whereas in the sham-operated and in the naïve groups no significant differences were found between the left and the right hippocampi. The difference in T1-weighted signal between RH and LH was 7.95 $\pm$ 3.11 % in the KA-injected group, as shown in Figure 3A. No significant difference in hippocampal volume was observed between experimental groups or between the LH and RH within each group. The mean diffusivity (MD) measures on whole hippocampal ROIs in RH and LH were 3.53 ± 0.35 x 10^{-4} mm^2/s and 4.09 ± 0.26 x 10^{-4} mm^2/s in KA-treated animals, 3.98 ± 0.19 x 10^{-4} mm^2/s and 3.99 ± 0.21 x 10^{-4} mm^2/s in naïve animals and 4.08 ± 0.18 x 10^{-4} mm^2/s and 4.07 ± 0.22 x 10^{-4} mm^2/s in sham-operated animals. The difference between RH and LH was significantly higher in KA-treated than in naïve or sham-operated animals ($p <$

0.0005). MD values in the RH (KA) were significantly lower than in the contralateral LH of the same animals ($p = 0.001$), and also compared to the hippocampal measurements of naive and sham animals ($p < 0.005$; Figure 3B). No statistical difference was found by comparing LH in KA-treated animals with naïve/control MD measurements. MD reduction was also observed in the right frontal cortex in most KA-injected animals, and the difference between the right and the left cortex was significant in the KA group ($p<0.05$) but not in the sham-operated and naïve groups. The analysis of FA values in the same hippocampal ROIs did not show any statistically significant difference between hemispheres and between animal groups (not shown).

Immunohistochemical analysis in the hippocampi ipsi and contralateral to KA injection. One week after MRI scans, the brains were cut and sections including hippocampi were processed for NeuN, GFAP, MAP2 and IgG immunostaining (Figures 4 and 5) to retrospectively evaluate neuronal loss, astrogliosis, neuronal suffering and BBB permeability alterations. In both naïve (left column in Figure 4) and sham-operated/treated controls (middle column in Figure 4) no sign of brain damage was observed, with the exception of GFAP and IgG staining in the tissue surrounding the hippocampal electrode tracks (arrows in the lower middle photo in Figure 4), likely due to a local inflammatory reaction. Interestingly, MAP2 staining in control animals (both naïve and sham-operated) showed a peculiar pattern with no staining in the pyramidal cell/radiatum layers of CA2 and CA3/4 regions (Figures 4 and Supplementary Figure 1B and C). In KA-treated animals (right column in Figure 4), neuronal cell loss and astrogliosis were observed in KA-injected hippocampi. A reduction of dendritic MAP2 signal in CA1-CA3 and hilus was observed, with a typical staining of the neuronal soma at the boundaries of the lesional tissue. IgG staining was observed exclusively around the KA injection site (lower right photo in Figure 4).

The qualitative evaluation suggested that the hippocampus contralateral to KA injection did not show obvious expression of brain damage markers. To quantify this observation, we analyzed the density of immunostaining in different hippocampal areas (CA1, CA3, DG and hilus) in the RH(KA) and in the contralateral (LH) hippocampi (Figure 5) and compared these values with densitometric measurements obtained from equivalent hippocampal subfields in both hemispheres of naïve and sham-operated control animals. As illustrated in Figure 5A, the LH of KA-treated animals (n=11) showed NeuN densitometric

values in CA1 (44.04 ± 5.06 % density), CA3 (31.14 ± 3.35 %), DG (73.06 ± 4.59 %) and hilus (20.23 ± 3.52 %) similar to control animals (43.84 ± 4.21 % in CA1; 36.12 ± 4.77 % in CA3, 66.02 ± 4.24 % in DG and 22.79 ± 3.77 % in the hilus; n=11 for each subfield – measured in both right and left hippocampi), suggesting that NeuN immunostaining in non-KA-injected LH subfields were comparable to normal tissue. As expected, all RH(KA) (left grey columns in Figure 5A) showed a clear cell loss in CA1 (19.94 ± 10.80 % density), CA3 (17.30 ± 11.25 %), DG (63.23 ± 12.25 %) and hilus (9.46 ± 7.27 %) compared to both controls and non-injected LH (not significant for paired DG values). The statistical significance of NeuN findings is reported in the first row of Table 1.

Densitometric measurements were also performed for GFAP, MAP2 and IgG immunostaining. Hippocampal subfields densities in KA-treated guinea pigs (grey columns in Figure 5B-D) were compared to control immunodensity values obtained by merging measurements from left and right hippocampi of sham-operated and naïve animals (black columns). The GFAP staining intensity measured on two ROIs on two slices per region in 8 KA-treated animals and in 11 controls (naïve and shams) showed a statistically significant density increase in RH(KA) CA1 and hilus (3.35 ± 2.43 % and 1.36 ± 0.75 %, respectively) compared to controls (0.56 ± 0.53 % and 0.08 ± 0.12 %, respectively). No difference was observed between controls and LH of KA-treated animals in CA1 and hilus (1.03 ± 0. 85 % and 0.44 ± 0.52 %, respectively; Figure 5B). The low densitometric values (< 0.1%) measured in CA3 did not consent a statistical comparison between RH(KA), LH and controls (data rejected to Kolmogorov-Smirnov Normality test). MAP2 immune-signal was measured in 10 KA-injected animals and in 11 control guinea pigs (Figure 5D). MAP2 density was significantly lower in RH(KA) (14.48 ± 10.16 % in CA1; 20.18 ± 17.35 % in CA3 and 41.58 ± 21.11 % in the hilus) compared to controls (72.20 ± 12.21 % in CA1, 57.25 ± 12.77 % in CA3 and 62.00 ± 25.12 % in the hilus) and to the contralateral LH for CA1 (70.65 ± 20.09 %) and CA3 (44.69 ± 8.78 %); no significant difference was observed by comparing RH(KA) and LH measurements in the hilus (52.96 ± 17.99 % in LH). IgG immunostaining density was 31.33 ± 29.08 % in the CA1 region of RH(KA) and was 0.64 ± 0.18 % in the LH CA1 area of KA-injected animals (n=8; Figure 5C). No IgG staining was found in CA1 areas of naïve animals. The statistical significance of GFAP, MAP2 and IgG densitometric measurements is reported respectively in the second, third and fourth rows of Table 1. These findings demonstrated that BBB

damage, cell loss and gliosis are not detectable in the hippocampus contralateral to KA injection, despite the occurrence of intense epileptiform activity during the ncSE.

Densitometric measurements were also performed on brain sections obtained from the 6 KA-injected animals recorded for two months after ncSE, when spontaneous seizures associated to unilateral hippocampal sclerosis in the KA-injected hippocampus developed [12,13]. Thionine density in CA1 and CA3 was 4.42 ± 3.95 % and 15.37 ± 11.67 % in RH(KA) and 15.05 ± 3.67 % and 24.34 ± 4.49 % in LH of KA-treated guinea pigs; 15.40 ± 1.03 % in CA1 and 25.48 ± 3.95 % in CA3 of naïve animals (n=6; measured in 2 sections per animal). As illustrated in Figure 6B, mean densities were not significantly different when LH values were compared to control animal hippocampi (p values in the fifth row in Table 1). GFAP intensity staining measured on two slices in 6 KA-treated animals (Figure 6C and D) showed a statistically significant increase in CA1 and CA3 density in RH(KA) (16.13 ± 6.01 % and 8.29 ± 6.28 %, respectively) compared to LH (1.83 ± 2.00 % and 3.41 ± 3.52 %, in CA1 and CA3) and to naïve controls (2.12 ± 4.09 % and 3.51 ± 4.54 % in CA1 and CA3, respectively; 2 sections each in 4 animals). No difference was observed between controls and LH of KA-treated animals (Figure 6D). Statistical significance is reported in the bottom row in Table 1.

Discussion

The present study demonstrates that sustained recurrent seizure activity during non-convulsive status epilepticus does not induce the expression of brain damage markers associated to neuronal death, astrogliosis and altered BBB permeability in the hippocampus contralateral to KA injection in a model of unilateral mTLE [14-16] applied to guinea pigs [12,13]. KA induces rapid excitotoxic neuronal death [22,30,31] in the CA1 region of the injected hippocampus and promotes epileptiform discharges that equally involve both the KA-injected and the contralateral hippocampi. The absence of gliosis and cell loss contralateral to KA injection is demonstrated three days after KA treatment and is confirmed two months after ncSE in chronic mTLE guinea pigs.

In the unilateral KA mTLE model, acute focal seizures are characterized by immobility, followed by facial clonus, masticatory movements and head nodding, and could develop into bilateral forelimb clonus and rearing/falling [12,13,32-34]. Seizure activity in the unilateral KA model is characterized by acute bi-hippocampal

discharges superimposed to a condition of continuous large-amplitude slow spikes at 1-3 Hz [12,13,14] that involve the injected hippocampus and propagate to the contralateral amygdala, hippocampus and frontal cortex [12,13,14,16]. Interestingly, periodic EEG slow spikes associated with focal seizures have been described during ncSE induced by acute brain injury in humans [35].

Our study confirms that intracerebral KA establishes a focal unilateral hippocampal sclerosis [12] in the KA-injected hippocampus characterized by neuronal loss, astrogliosis and altered BBB permeability. Enhenced GFAP, MAP2 and IgG immunostainings are restricted to the ipsilateral CA1-CA3 region and to the DG. In line with our findings, a rapid increase in GFAP-immunoreactivity was detected in the KA-injected hippocampus in the mouse [22]. MAP2 staining typically observed in large dendrites is abolished and/or transferred to neuron soma in acutely injured brain tissue during excitotoxic damage [27] and ischemia [28]. In our experiments, MAP2 staining relocation to the soma at the boundary of the damaged areas was limited to the KA-injected CA1/DG.

We utilize the unilateral KA injection model as an instrument to test whether epileptiform activity in regions remote from the KA injection area is sufficient to induce brain damage. The correlation between seizures and tissue damage is controversial and can be verified exclusively in animal models characterized by focal unilateral epileptogenic lesions, such as intracerebral KA injection and the local electrical stimulation models; it cannot be analyzed in epilepsy models that result from systemic pilocarpine or KA treatments, because these procedures induce diffuse bilateral alterations. The study by Arabadzisz and colleagues [36] demonstrated that unilateral KA injection into one dorsal hippocampus induced ncSE followed by ipsilateral focal seizures and damage, whereas the contralateral hippocampus remained structurally unaffected and seizure-free. In two studies, contralateral hippocampal alterations were observed when a convulsive status epilepticus was promoted using either high doses of unilaterally injected KA [37] or repeated unilateral hippocampal stimulation [38]. In a recent report, unilateral hippocampal electrical stimulation in rats demonstrated cell loss and changes in the expression of N-cadherin in the hippocampus contralateral to stimulation [39] exclusively in a subgroup of animals that developed seizures four weeks after ncSE. Interestingly, contralateral neuronal loss was not observed in rats submitted to ncSE that did not develop spontaneous seizures in the chronic phase. The study showed also a specific increase

in Iba and GFAP (but not S100) protein levels in the hippocampi of animals with and without chronic spontaneous seizures; glial changes contralateral to the stimulated hippocampus are mentioned in the study, but were not quantified. This report supports the evidence that ncSE activity does not induce obvious damage markers in rats that do not develop a chronic spontaneous seizures. The differences between these studies and our findings could be due to the very mild chronic epileptic phenotype observed in guinea pigs two months after ncSE [12] , characterized by an average of 1 behavioral seizure per week. Unilateral mTLE models data suggest that the contralateral hippocampus is not obligatorily altered after acute ncSE and support our findings that seizure and epileptiform activity during ncSE may not damage regions far away from the original epileptogenic focus. In the rat kindling model, apoptosis and neuronal loss were demonstrated in the DG, hilus, CA1 and CA3 and in the entorhinal cortex during kindling acquisition [40,41]. Nevertheless, spontaneous and stimulus-evoked seizures can reliably be induced without detectable cell loss in adult [42,43] kindled rats, suggesting that in this focal seizure model a consistent correlation between seizure activity and tissue damage is not demonstrated.

Our MRI study on fixed brain tissue confirmed that brain damage is lateralized exclusively to the KA-injected hippocampus. These data confirmed the results of the above mentioned study on ncSE model induced by unilateral electrical stimulation of one hippocampus [39]. We utilized post-fixed tissue because it allows longer MR scanning times that improve imaging definition without altering the significance of the recorded signals; apparent diffusion coefficient (ADC) measurements in fixed brains are characterized by lower values compared to *in vivo* condition [44,45]. T1-weighted MRI images showed signal changes suggesting BBB disruption only in KA-injected hippocampus, although we cannot exclude minor, undetectable BBB permeability changes on the contralateral side. DTI data showed a prominent MD decrease in all the KA-injected hippocampi and in the ipsilateral frontal cortex. These changes are probably caused by a reduction of the extracellular space due to cytotoxic edema, as suggested by DTI findings in the acute stage of KA lesions [46,47]. The IgGs extravasation observed exclusively in the tissue surrounding the KA injection site confirmed the MRI findings. BBB permeability changes during KA-induced seizures are expected to promote IgG extravasation three days after intense epileptiform activity, when neurons and astrocytes incorporate BBB-leaked IgGs [23]. The absence of IgG extravasation in the hippocampus

423 contralateral to KA injection contributes additional evidence on the absence of seizure-mediated damage
424 in areas contralateral to KA injection. The lack of contralateral seizure-induced BBB alterations conflicts
425 with the report that both permeability changes and inflammatory molecules expression is associated with
426 seizure activity [21,48,49]. We can hypothesize that if seizure-dependent BBB permeability changes occur in the
427 hippocampus contralateral to KA injection, they are not severe enough to induce extravasation of IgGs and
428 other factors that are required to initiate the epileptogenic process that produces tissue damage.
429 The possibility that seizures induce alterations that are not detectable by the immunohistochemistry and
430 MRI utilized in our study should be considered. A molecular expression analysis could improve the
431 identification and the quantification of changes that occur contralateral to KA-induced mTLE.
432
433 In conclusion, our study utilized a ncSE model as an instrument to evaluate the effect of epileptiform
434 activity on brain tissue far from the primary epileptogenic lesion (the KA injection site). We provide
435 experimental evidence that markers commonly utilized for the neuropathological identification of
436 neuronal cell loss, gliosis and BBB permeability changes are not stimulated by seizure activity *per se*. These
437 findings are in line with our recent report that recurrent seizures do not induce the expression of brain
438 damage markers in peri-lesional epileptogenic cortex studied in postsurgical tissue from cryptogenic and
439 FCD patients [50]. The demonstration that seizures as such do not contribute to brain damage does not imply
440 that seizures are not potentially dangerous events and that their control should not be pursued. It is
441 obvious, and it has to be clearly stated here, that the need to treat seizures in clinical setting is mandatory
442 because of the cognitive and functional impairment that seizures produce and imply.
443
444
445 **Acknowledgments**
446 The Authors are grateful to Barbara Cipelletti, Gloria Milesi and Patrizia Aracri for help with
447 immunostaining analysis and statistics. The work has received funding from the European Union's by
448 grants JTC2014-ERANET-Neuron 0004 (BrIE) and by Horizon 2020 research and innovation programme

449 under the Marie Slklodowska-Curie grant agreement No 722053 (EUGliaPhD), from the Italian Health
450 Ministry and from a grant of the *Fondazione Banca del Monte di Lombardia*.

451

452 **Author's contributions**

453 MdC and FMN elaborated the conception and design of the study; FMN, CA, DVV, AC, MF and IZ
454 contributed to the acquisition and analysis of data; MdC, CA, FMN, MF and AC drafted a significant portion
455 of the manuscript and figures.
456 The Authors have no conflicts of interest or competing financial and non-financial interests to declare.
457 We comply to Data Availability rules of the Journal.

458

References

1. Haut SR, Veliskova J, Moshe SL Susceptibility of immature and adult brains to seizure effects. Lancet Neurol. 2004;3(10):608-17.

2. Avanzini G, Depaulis A, Tassinari A, et al. Do seizures and epileptic activity worsen epilepsy and deteriorate cognitive function? Epilepsia. 2013;54 Suppl 8:14-21.

3. Berg AT, Cross JH Towards a modern classification of the epilepsies? Lancet Neurol. 2001;9(5):459-61.

4. Annegers JF, Hauser WA, Coan SP, et al. A population-based study of seizures after traumatic brain injuries. N Engl J Med. 1998;338(1):20-24.

5. Korff CM, Brunklaus A, Zuberi SM Epileptic activity is a surrogate for an underlying etiology and stopping the activity has a limited impact on developmental outcome. Epilepsia. 2015;56(10):1477-81.

6. Pitkanen A, Sutula TP Is epilepsy a progressive disorder? prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet Neurol. 2002;1(3):173-81.

7. Pitkanen A, Schwartzkroin PA, Moshe SL Models of seizures and epilepsy. Burlington, MA 01803, USA Elsevier Academic Press (2006).

8. Bouilleret V, Boyet S, Marescaux C, et al. Mapping of the progressive metabolic changes occurring during the development of hippocampal sclerosis in a model of mesial temporal lobe epilepsy. Brain Res. 2000;852(2):255-62.

9. Roch C, Leroy C, Nehlig A, et al. Predictive value of cortical injury for the development of temporal lobe epilepsy in 21-day-old rats: An MRI approach using the lithium-pilocarpine model. Epilepsia. 2002;43(10):1129-36.

10. Sutula TP Mechanisms of epilepsy progression: Current theories and perspectives from neuroplasticity in adulthood and development. Epil Res. 200460(2-3):161-71.

11. Pitkanen A, Immonen RJ, Grohn OH, et aI. From traumatic brain injury to posttraumatic epilepsy: What animal models tell us about the process and treatment options. Epilepsia 50 Suppl. 2009;2:21-29.

12. Carriero G, et al. A guinea pig model of mesial temporal lobe epilepsy following nonconvulsive status epilepticus induced by unilateral intrahippocampal injection of kainic acid. Epilepsia. 2012;53(11):1917-27.

13. Arcieri S, et al. Variable electrobehavioral patterns during focal nonconvulsive status epilepticus induced by unilateral intrahippocampal injection of kainic acid. Epilepsia. 20115;55(12):1978-85.

14. Bouilleret V, et al. Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: Electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience. 1999;89(3):717-29.

15. Bragin A, et al. Electrophysiologic analysis of a chronic seizure model after unilateral hippocampal KA injection. Epilepsia. 1999;40(9):1210-21.

16. Riban V, et al. Evolution of hippocampal epileptic activity during the development of hippocampal sclerosis in a mouse model of temporal lobe epilepsy. Neuroscience. 2002;112(1):101-11.

17. Rao MS, Hattiangady B, Reddy DS, et al. Hippocampal neurodegeneration, spontaneous seizures, and mossy fiber sprouting in the F344 rat model of temporal lobe epilepsy. J Neurosci Res. 2006;83(6):1088-105.

18. Schwob JE, Fuller T, Price JL, et al. Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: A histological study. Neuroscience. 1980;5(6):991-1014.

19. Fujikawa DG The temporal evolution of neuronal damage from pilocarpine-induced status epilepticus. Brain Res. 1996;725(1):11-22.

20. Jarvela JT, et al. Kainic acid-induced neurodegeneration and activation of inflammatory processes in organotypic hippocampal slice cultures: Treatment with cyclooxygenase-2 inhibitor does not prevent neuronal death. Neuropharmacology. 2011;60(7-8):1116-25.

21. Ravizza T, et al. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: Evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis. 2008;29(1):142-60.

22. Pernot F, et al. Inflammatory changes during epileptogenesis and spontaneous seizures in a mouse model of mesiotemporal lobe epilepsy. Epilepsia. 2011;52(12):2315-25.

23. van Vliet EA, da Costa Araújo S, Redeker S, et al. Blood-brain barrier leakage may lead to progression of temporal lobe epilepsy. Brain. 2007;130(Pt 2):521-34.

512 24. Lidster K, et al. Opportunities for improving animal welfare in rodent models of epilepsy and seizures. J
513 Neurosci Methods. 2016;260:2-25.
514 25. Basser PJ, Pierpaoli C Microstructural and physiological features of tissues elucidated by quantitative-
515 diffusion-tensor MRI. J Magn Reson. 1996;111(3):209-19.
516 26. Wang R, Benner T, Sorensen AG, et al. Diffusion toolkit: A software package for diffusion imaging data
517 processing and tractography. Proc Intl Soc Mag Reson Med. 2007;15:3720.
518 27. Pierpaoli C, et al. Histopathologic correlates of abnormal water diffusion in cerebral ischemia: Diffusion-
519 weighted MR imaging and light and electron microscopic study. Radiology. 1993;189(2):439-448.
520 28. Pastori C, Regondi MC, Librizzi L, et al. Early excitability changes in a novel acute model of transient
521 focal ischemia and reperfusion in the in vitro isolated guinea pig brain. Exp Neurol. 2007;204(1):95-
522 105.
523 29. Breschi GL, et al. Penumbra region excitability is not enhanced acutely after cerebral ischemia in the in
524 vitro isolated guinea pig brain. Epilepsia. 2012;53(3):448-58.
525 30. Nadler JV, Perry BW, Cotman CW Intraventricular kainic acid preferentially destroys hippocampal
526 pyramidal cells. Nature. 1978;271(5646):676-7.
527 31. Levesque M, Avoli M The kainic acid model of temporal lobe epilepsy. Neurosci Biobehav Rev.
528 2013;37(10 Pt 2):2887-2899.
529 32. Raedt R, et al. Seizures in the intrahippocampal kainic acid epilepsy model: Characterization using long-
530 term video-EEG monitoring in the rat. Acta Neurol Scand. 2009;119(5):293-303.
531 33. Leite JP, et al. Neuron loss, mossy fiber sprouting, and interictal spikes after intrahippocampal kainate in
532 developing rats. Epil Res. 1996; 26(1):219-31.
533 34. Groticke I, Hoffmann K, Loscher W. Behavioral alterations in a mouse model of temporal lobe epilepsy
534 induced by intrahippocampal injection of kainate. Exp Neurol. 2008;213(1):71-83.
535 35. Ruiz R, Vlachy J, Lee JW, et al. Association of Periodic and Rhythmic Electroencephalographic Patterns
536 With Seizures in Critically Ill Patients. JAMA Neurol. 2017; 74:181-8.

537 36. Arabadzisz D, Antal K, Parpan F, et al. Epileptogenesis and chronic seizures in a mouse model of
538 temporal lobe epilepsy are associated with distinct EEG patterns and selective neurochemical
539 alterations in the contralateral hippocampus. Exp Neurol. 2005;194(1):76-90.
540 37. Magloczky Z, Freund TF Selective neuronal death in the contralateral hippocampus following unilateral
541 kainate injections into the CA3 subfield. Neuroscience. 1993;56(2):317-35.
542 38. Gorter JA, Gonçalves Pereira PM, Van Vliet EA, et al. Neuronal cell death in a rat model for mesial
543 temporal lobe epilepsy is induced by the initial status epilepticus and not by later repeated
544 spontaneous seizures. Epilepsia. 2003;44(5):647-58.
545 39. Avdic U, Ahl M, Chugh D, et al. Nonconvulsive status epilepticus in rats leads to brain pathology.
546 Epilepsia. 2018;59:945-58.
547 40. Bengzon J, Kokaia Z, Elmer E, et al. Apoptosis and proliferation of dentate gyrus neurons after single and
548 intermittent limbic seizures. Proc Natl Acad Sci USA. 1997;94(19):10432-37.
549 41. Cavazos JE, Das I, Sutula TP Neuronal loss induced in limbic pathways by kindling: Evidence for induction
550 of hippocampal sclerosis by repeated brief seizures. J Neurosci. 1994;14(5 Pt 2):3106-21.
551 42. Michalakis M, Holsinger D, Candace J, et al. Development of spontaneous seizures over extended
552 electrical kindling. I. electrographic, behavioral, and transfer kindling correlates. Brain Res. 1998;793(1-
553 2):197-211.
554 43. Brandt C, Ebert U, Loscher W Epilepsy induced by extended amygdala-kindling in rats: Lack of clear
555 association between development of spontaneous seizures and neuronal damage. Epil Res. 2004;62(2-
556 3):135-56.
557 44. Zucca I, Milesi G, Medici V et al. Type II focal cortical dysplasia: Ex vivo 7T magnetic resonance imaging
558 abnormalities and histopathological comparisons. Ann Neurol. 2016;79(1):42-58.
559 45. D'Arceuil HE, Westmoreland S, de Crespigny AJ An approach to high resolution diffusion tensor imaging
560 in fixed primate brain. Neuroimage. 2005;35(2):553-65.
561 46. Tokumitsu T, et al. Metabolic and pathological effects of temporal lobe epilepsy in rat brain detected by
562 proton spectroscopy and imaging. Brain Res. 1997;744(1):57-67.

563 47. Hasegawa D et al. Seizure-induced brain-borne inflammation sustains seizure recurrence and blood-
564 brain barrier damage. Ann Neurol. 2012;72(1):82-90..
565 48. Vezzani A, Friedman A. Brain inflammation as a biomarker in epilepsy. Biomark Med. 2011;5:607-14.
566 49. Librizzi L, Noè F, Vezzani AM et al. Seizure-induced brain-borne inflammation sustains seizure
567 recurrence and blood-brain barrier damage. Ann Neurol. 2012;72(1):82-90.
568 50. Rossini L, Garbelli R, Gnatkovsky V et al. Seizure activity per se does not induce tissue damage markers
569 in human neocortical focal epilepsy. Ann Neurol 2017;82:331-41.

Figure Legends

Figure 1. Status epilepticus activity induced by unilateral intrahippocampal KA injection. A: compressed EEG 12 h recording in right (RH-KA) and left (LH) CA1 areas of the hippocampi and in the right frontal neocortex (RFNc). KA was injected in the right hippocampus (vertical arrow on RH(KA) trace). **B:** the first recorded focal EEG seizure (left panel) and the second bilateral EEG seizure (right panel), observed after KA injection illustrated in panel A, are shown with expanded time scale. **C:** irregular spiking involving both hippocampi. **D:** regular, rhythmic spikes at 1-3 Hz in the two hippocampi, which inconstantly project to the RFNc. **E:** number of seizures characterized by unilateral (white columns) and bilateral (black columns) hippocampal EEG discharge, for each of the 11 KA-treated guinea pigs sacrificed 3 days after KA treatment (gp1-11). **F:** mean number of unilateral or bilateral EEG seizures observed in all animals. (*): $p < 0.05$ (by T-test).

Figure 2. Quantification of hippocampal EEG epileptiform activity ipsi and contralateral to KA injection. Comparison of the number of seizures (**A**), the time spent in seizures (**B**) and the number of spikes (**C**) recorded in the KA-injected right hippocampal CA1 [RH(KA)] and in the contralateral left CA1 (LH) in the 11 guinea pigs during the three days following ncSE induction. On the right column of each panel, box plots represent the distribution of the mean values for the same parameters, as analyzed in each KA-injected guinea pig sacrificed 3 days after KA treatment (n=11).

Figure 3. Magnetic resonance features 3 days after KA-induced status epilepticus. **A:** On the top, representative T1-weighted images of a KA-treated (left) and a sham-operated (right) guinea pig brains. Images were obtained with a 7T MR instrument in fixed brains from animals perfused with gadolinium during sacrifice (see Methods). On the bottom: percentage T1-weighted signal differences between RH(KA) and LH for each KA-injected animal (left panel); averaged values obtained in KA treated, sham and naive animal groups are shown on the right panel. Positive values represent a higher T1-weighted signal in RH(KA) due to the presence of gadolinium. **B:** Representative MD map in a KA-treated brain (left) and

597 difference between the MD in the RH(KA) and LH averaged in each group (right). (*): p< 0.05 by non-
598 parametric Wilcoxon test.
599
600 **Figure 4. Immunohystochemical features of naïve (left column), sham-operated (middle column)**
601 **and KA-injected guinea pigs (right column), three days after KA-induced ncSE.** KA was injected in the right
602 hippocampal CA1 region. For each panel, neuronal density (NeuN; first row), astrogliosis (GFAP, second
603 row), reversible neuronal sufferance (MAP2, third row) and blood-brain barrier permeability changes (Ig,
604 bottom row) are illustrated. Arrows mark the intrahippocampal recording electrode tracks. Calibration bar
605 = 1 mm. Left and right (KA-injected) hemispheres are marked by L and R. ROI samples utilized for the
606 densitometric quantification of immunostaining in CA1, CA3, DG and hilus (Figure 5) are illustrated in the
607 top left panel.
608
609 **Figure 5. Semi-quantitative analysis of NeuN, GFAP, MAP2 and IgG densitometry ipsi and**
610 **contralateral to KA injection, three days after status epilepticus. A:** Comparison between densitometric
611 measurements of NeuN (A). The grey columns illustrate the average percentage of densitometric changes
612 measured in CA1, CA3, DG and hilus in the LH and in the RH(KA) of KA-treated animals (n=11);
613 densitometric values obtained from the same areas in control animals (sham and naïve) are illustrated by
614 black columns (n=11). For the control group, the densitometric measures were performed in both the right
615 and left hippocampi (R+LH). (*): p<0.05; and (**): p<0.001 (by ANOVA One Way; see Table 1)**.** Comparison
616 between densitometric GFAP (**B**), IgG (**C**) and MAP2 (**D**) measurements in KA-injected RH(KA) and
617 contralateral LH (grey columns) and in sham-operated and naïve guinea pigs (black columns; n=11). For
618 GFAP and MAP2 staining, ROIs were positioned in CA1, CA3 and hilus; for IgG staining, only CA1 ROIs were
619 analyzed. Eight animals were utilized for GFAP measurements in CA1, CA3 and hilus; 10 animals for MAP2
620 counts in CA1, CA3 and hilus and 8 guinea pigs for IgG staining in CA1. (*): p<0.05; and (**): p<0.001 (by
621 ANOVA One Way; see Table 1). As mentioned in the text, CA3 data were rejected to the Kolmogorov-
622 Smirnov Normality test.
623

Figure 6. Semi-quantitative analysis of thionine and GFAP staining ipsi and contralateral to KA injection in guinea pigs that developed mesial temporal lobe epilepsy (mTLE), sacrificed 2 months after the induction of ncSE. A: representative microphotographs of thionine-stained coronal sections of the left (LH) and the KA-injected right RH(KA) hippocampi and of naïve control animals. Higher magnification of the boxed area in the upper small photograph are illustrated in the lower panels. **B:** The grey columns illustrate the average percentage of densitometric thionine staining changes measured in CA1 (left panel) and CA3 (right panel) of 6 chronically epileptic guinea pigs; densitometric values obtained from the same areas in control naïve animals are illustrated by black columns (n=4; calculated on both hemispheres). For the control group, the densitometric measures were performed in both the right and left hippocampi (R+LH). (*): $p<0.05$; and (**): $p<0.001$ (by ANOVA One Way; see fifth row in Table 1). **C:** representative microphotographs of GFAP immunostained coronal sections of the left (LH) and the KA-injected right RH(KA) hippocampi. Higher magnification of the boxed area in the upper small photograph are illustrated in the lower panels. **D:** Comparison of densitometric GFAP measurements in RH(KA) and LH (grey columns; n=6), and in naïve guinea pigs (black columns; n=4). GFAP staining was measured in CA1 and CA3. (*): $p<0.05$; and (**): $p<0.001$ (by ANOVA One Way; see bottom row in Table 1).

Supplementary Figure 1. Immunohystochemical markers of damage in the KA-injected hippocampi, three days after KA-induced status epilepticus. Details of GFAP (**A**) and MAP2 (**B** and **C**) staining in KA-injected RH(KA). CA = Cornu Ammonis; DG = dentate gyrus. Calibration bars = 100 µm (30 µm in the insert in **A**).

Table 1. Statistical significance of densitometric values measured by ANOVA One Way in hippocampal areas of both acutely sacrificed guinea pigs (3 days after KA treatment; top 4 rows) and of chronic animals evaluated 2 months after KA treatment (bottom two rows), as illustrated in Figures 5 and 6.