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

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Epilepsy therapy is based on antiseizure drugs that treat the symptom, seizures, rather than the disease and are ineffective in up to 30% of patients. There are no treatments for modifying the disease—preventing seizure onset, reducing severity or improving 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, 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 status epilepticus. Oxidative stress was reduced in animals undergoing epileptogenesis by a transient treatment with *N*-acetylcysteine and sulforaphane, which act to increase glutathione 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 than either drug alone. The drug combination significantly delayed the onset of epilepsy, blocked disease progression between 2 and 5 months post-status epilepticus and drastically reduced the frequency of spontaneous seizures measured at 5 months without modifying the average seizure duration or the incidence of epilepsy in animals. Treatment also decreased hippocampal neuron loss and rescued cognitive deficits. Oxidative stress during epileptogenesis was associated with *de novo* brain and blood generation of high mobility group box 1 (HMGB1), a neuroinflammatory molecule implicated in seizure mechanisms. Drug-induced reduction of oxidative stress prevented 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 starting early after injury significantly improves long-term disease outcomes. This intervention may be considered for patients exposed to potential epileptogenic insults.

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Abbreviations: GSH = glutathione; NAC = *N*-acetylcysteine; ROS = reactive oxygen species; SFN = sulforaphane; SRS = spontaneous recurrent seizure

Introduction

Epilepsy is a brain disorder affecting over 50 million people worldwide and is associated with increased mortality, significant co-morbidities, unique stigmatization of affected individuals, and high societal cost. Current antiepileptic drugs mainly provide symptomatic relief from seizures, have multiple adverse effects, and fail to control seizures in up to 30% of people (Kwan *et al.*, 2010). Moreover, there are no drugs that prevent disease onset or its progression. New antiseizure 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) (Varvel *et al.*, 2015). To develop such drugs, we need to understand the pathological processes occurring during epileptogenesis in the brain of people exposed to injuries, or with an established diagnosis of epilepsy. Epileptogenesis is a complex multifactorial process, including both the development of an epilepsy condition and its progression after the condition is established (Pitkanen and Engel, 2014). It is characterized by various modifications in the brain, including neuroinflammation and oxidative stress. These two phenomena are rapidly and persistently induced in the injured brain; moreover, they are functionally interconnected and reinforce each other (Hsieh and Yang, 2013; Rowley and Patel, 2013; Vezzani *et al.*, 2015). Notably, neuroinflammation occurs in human epilepsy and peripheral markers of oxidative stress are increased in people with epilepsy (Aronica and Crino, 2011; Vezzani *et al.*, 2011*a*; Rowley and Patel, 2013; Cardenas-Rodriguez *et al.*, 2014; Puttachary *et al.*, 2015). They both contribute to neuropathology and behavioural deficits and play a role in determining seizure threshold in animal models (Rowley and Patel, 2013; Vezzani *et al.*, 2013; Pearson *et al.*, 2015).

A potential critical point of intersection between oxidative stress and neuroinflammation is the generation of the disulfide isoform of high mobility group box 1 (HMGB1), a protein crucially involved in the molecular cascade that contributes to seizure mechanisms (Vezzani *et al.*, 2011*b*; Balosso *et al.*, 2014). HMGB1 is a ubiquitous non-histone chromatin-binding protein that regulates gene transcription under physiological conditions. Following cell injury or pathophysiological cell activation, HMGB1 is hyperacetylated at two nuclear localization sequences, a step required for its translocation to the cytoplasm from where it can be released extracellularly upon inflammasome activation (Yang *et al.*, 2012; Lu *et al.*, 2013). Extracellular HMGB1 can be partially oxidized by formation of the disulfide bond between C23 and C45 (i.e. disulfide HMGB1); this isoform has pro-inflammatory, neuromodulatory and ictogenic activities by activating toll-like receptor 4 (TLR4) (Venereau *et al.*, 2012; Yang *et al.*, 2012; Balosso *et al.*, 2014). As reactive oxygen species (ROS) promote the stabilization of HMGB1 in its disulfide isoform (Yang *et al.*, 2012), there is a vicious cycle that links ROS production, the generation of the pathologic HMGB1 isoform and neuroinflammation, thus representing a potential mechanism of epileptogenesis.

Previous approaches to targeting oxidative stress with small molecules in animal models of epilepsy have met with some success in reducing neuronal damage following prolonged seizures but have notably failed to show a convincing effect on the development of epilepsy (Rong *et al.*, 1999; Liang *et al.*, 2000; Barros *et al.*, 2007; Rowley and Patel, 2013; Kovac *et al.*, 2014; Pearson *et al.*, 2015). We hypothesized that this is because the treatments given are either too short-lived or do not have a sufficiently rapid effect. We have overcome this hurdle by using a combination of two antioxidant drugs with complementary mechanism of action, *N*-acetylcysteine (NAC) acting as an acute antioxidant (Sun, 2010) and sulforaphane (SFN) increasing the longer-term endogenous antioxidant system (Houghton *et al.*, 2013). We show that this combination reduces oxidative stress in brain and blood during epileptogenesis in a rat model of acquired epilepsy induced by status epilepticus, and significantly improves pathological outcomes by providing blockade of spontaneous seizure progression, reduction of cell loss and rescue of comorbidities. Notably, although treatment was transiently given during epileptogenesis starting early after status epilepticus onset, it mediates long-term therapeutic effects.

Moroever, we provide the first evidence that links seizure-induced oxidative stress to the brain nucleus-to-cytoplasm translocation of the ictogenic and pathologic neuroinflammatory molecule HMGB1, and therapeutic outcomes of antioxidant drugs are associated with abrogation of this molecule translocation in brain and the increase of this molecule in blood. HMGB1 may therefore contribute to the pathogenic consequences of oxidative stress, and could act as a biomarker to determine efficacy of treatment.

Since we show that oxidative stress occurs in the human brain after status epilepticus and in chronic epilepsy, antioxidant interventions may represent a potential therapeutic strategy for improving disease prognosis in patients exposed to epileptogenic injuries.

Materials and methods

Animals

Adult male Sprague-Dawley rats (225–250 g; Charles-River) 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. In compliance with the ARRIVE guidelines, 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).

Electrical status epilepticus

Rats were implanted under 1.5% isoflurane anaesthesia with two 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) (Paxinos and Watson, 2005). 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 (neomycin; bacitracin; glycine; L-cysteine; DL-threonin) and injected with ampicillin (100 mg/kg, subcutaneous) 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 60–90 min to induce status epilepticus according to a well-established protocol (De Simoni *et al.*, 2000; Noé *et al.*, 2008). 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 first and the fourth epoch of stimulation onwards. These criteria selected rats developing status epilepticus that remitted spontaneously within 24 h from the initial stimulation then leading to subsequent epilepsy development (De Simoni *et al.*, 2000; Noé *et al.*, 2008). Status epilepticus 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 (Pitkanen *et al.*, 2005). The end of status epilepticus was defined by the occurrence of interspike intervals longer than 1 s. No pharmacological intervention was done to stop status epilepticus since no mortality is observed in this model. Status epilepticus was evaluated by EEG analysis and its total duration and the number of spikes were quantified during the first 24 h using Clampfit 9.0 program (Axon Instruments). Power spectral density (PSD) distribution of four 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 (Pitkanen *et al.*, 2005).

Spontaneous seizures detection and quantification

Rats exposed to status epilepticus were continuously video-EEG recorded (24 h/day) from status epilepticus induction until the first two spontaneous seizures occurred, at least 48 h apart from status epilepticus induction (epilepsy onset). All EEG seizures were associated with generalized motor seizures (forelimb clonus with or without rearing and/or falling) (Gorter *et al.*, 2001; Noé *et al.*, 2008). After epilepsy onset in each rat, video-EEG monitoring was discontinued and resumed at 2 and 5 months post-status epilepticus to determine spontaneous recurrent seizure (SRS) frequency by continuous EEG monitoring for 2 weeks (24 h/7 days). 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 (Noé *et al.*, 2008). 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; Grass-Telefactor). Digitized EEG data were processed using the TWin record and review software. EEG was visually inspected for seizure detection and quantification was performed by two independent expert operators blinded to the treatments. If there was lack of concordance then a third expert operator was consulted.

In vivo study design and drug treatment schedule

We investigated whether oxidative stress generated in the hippocampus during epileptogenesis in the electrical status epilepticus model was reduced by the antioxidant drugs NAC and SFN. Electrode-implanted rats not exposed to status epilepticus were used as controls (Sham); these rats were sacrificed at the corresponding time points of the respective status epilepticus-exposed rats after 10-day recovery period following surgery. Experimental rats were exposed to status epilepticus and sacrificed after 4 days ($n = 9$) or 14 days ($n = 5$) for HPLC analysis of reduced (GSH), oxidized (GSSG) glutathione and glutathionylated proteins (GS-Pro) and compared to sham rats ($n = 15$) (Figs 1A, 2A and 5A) (Pastore *et al.*, 1998; Liang and Patel, 2016; Supplementary material). A different group of rats was sacrificed 4 days post-status epilepticus ($n = 5$; sham = 4) for immunohistochemical analysis of markers of oxidative stress, such as inducible nitric oxide (iNOS),

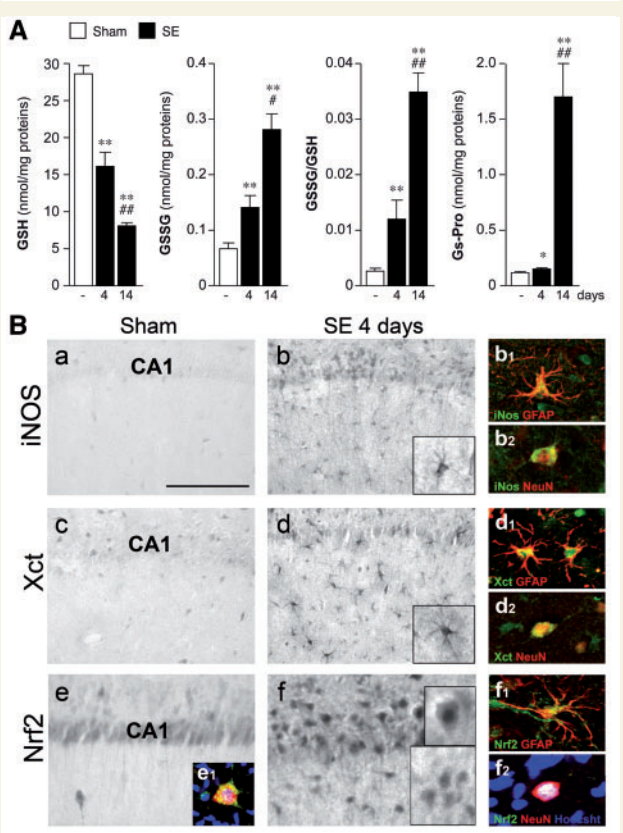

Figure 1 Generation of oxidative stress in hippocampal tissue of rats exposed to electrical status epilepticus. (**A**) HPLC analysis of reduced (GSH) and oxidized (GSSG) glutathione levels, and their ratio, and the level of glutathionylated proteins (GS-Pro) in the rat hippocampus at Days 4 ($n = 9$) and 14 ($n = 5$) after status epilepticus (SE) onset compared to corresponding baseline levels in sham rats (electrode-implanted but not stimulated, $n = 15$). Sham values are pooled (since they did not differ) from rats sacrificed at 4 days ($n = 10$) or 14 days ($n = 5$) after 10-day recovery period following surgery to match the same time points of the corresponding status epilepticus-exposed rats. Data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ versus sham by Kruskal-Wallis followed by Dunn's *post hoc* test; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus Day 4 by Mann-Whitney test. (**B**) Representative immunohistochemical micrographs of the CA1 region depicting the expression of iNOS, the Xct and Nrf2 in sham (**a**, **c** and **e**) and 4 days post-status epilepticus (**b**, **d** and **f**) ($n = 4$–5). (**b**, **d** and **f**) Show the increase in the respective marker expression in GFAP-positive activated astrocytes (**b1**, **d1** and **f1**) and in neurons (**b2**, **d2** and **f2**). Activated astrocytes are defined by hypertrophic cell body with thick processes. Nrf2 expression is increased in neuronal nuclei (**f2** versus **e1**) indicating increased transcriptional activation of detoxifying enzymes. Scale bars: **B**(**a–f**) = 25 μm; inset in **B**(**b**, **d and f**) = 15 μm; immunofluorescence inset = 10 μm.

the cysteine transporter (Xct), the transcriptional nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Fig. 1B) and HMGB1 (Fig. 5B) (Supplementary material).

In parallel experiments, status epilepticus-exposed rats were treated with NAC (Sigma-Aldrich; 500 mg/kg dissolved in H_2O, pH 7.4) and SFN (LKT Laboratories; 5 mg/kg dissolved in 0.1% DMSO in buffered saline, pH 7.4) intraperitoneally (i.p.) either alone or in combination, or their vehicles. For determining the effect of each drug alone, or their combination, on oxidative stress (Fig. 2A), a cohort of rats ($n = 5$ rats in each group) was treated with either NAC alone (twice daily 6 h apart) for 7 days (protocol in Supplementary Fig. 1A) or SFN alone (5 mg/kg, i.p., once daily) for 14 days (Supplementary Fig. 1A), or NAC + SFN combination for 7 days (same schedule as each drug given alone) followed by SFN alone for an additional 7 days (Supplementary Fig. 1A). In each treatment schedule, the first drug dose was injected 1 h after status epilepticus 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 releasable HMGB1 (Fig. 5 and Supplementary Fig. 1B), a group of status epilepticus-exposed rats was treated with NAC + SFN (same schedule as each drug given alone) or their vehicles for 4 days ($n = 9$–11 each group), and compared to time-matched sham rats ($n = 9$). Both glutathione form levels were measured in the hippocampus; HMGB1 was also measured in corresponding venous blood in each animal (Supplementary material). Additionally, to verify whether oxidative stress promotes HMGB1 translocation, a rat cohort ($n = 5$) was treated with the drug combination (NAC + SFN) for 4 days and their brain analysed by immnohistochemistry (Fig. 5B).

All rats used for immunohistochemical or biochemical analysis in cross-sectional studies were EEG recorded from status epilepticus induction until 4 days post-status epilepticus, and no SRS were observed in the various experimental groups.

To assess NAC + SFN therapeutic effects on spontaneous seizures, cognitive deficits, and cell loss, the drugs (same dose of each drug given alone) were co-administered for 7 days post-status epilepticus followed by SFN administered alone for an additional 7 days (Figs 3, 4 and Supplementary Fig. 1C). Rats were randomly assigned 1 h after status epilepticus onset to either drug ($n = 9$) or vehicle groups ($n = 9$), and longitudinally followed by video-EEG recording as described above. At the end of EEG recording, rats underwent the T-maze test, then they were sacrificed and brains were collected for subsequent histological analysis.

Fluoro-Jade® analysis of cell loss was done to determine the effect of the first day drug treatment on status epilepticus-induced acute injury (Ravizza *et al.*, 2008) (Supplementary material). An additional group of rats ($n = 6$) was injected 1 h after status epilepticus onset with NAC (500 mg/kg, i.p.) followed by a second NAC dose 6 h apart. One hour after the first NAC dose, rats were injected with SFN (5 mg/kg; i.p.), then they were killed 24 h post-status epilepticus onset (protocol in Supplementary Fig. 1B). Rats exposed to status epilepticus and injected with corresponding vehicles and sham rats ($n = 5$ each group) were used as controls.

Finally, we determined whether a delayed treatment schedule also reduced hippocampal oxidative stress and cell loss in rats injected with NAC + SFN until Day 4 and starting treatment 24 h post-status epilepticus (protocol in Supplementary Fig. 1B). For measuring GSH and GSSG, we prepared new cohorts of NAC + SFN treated rats (24 h post-status epilepticus until Day 4; $n = 8$–10) and time-matched status epilepticus + vehicle and sham rats ($n = 9$–10) (Supplementary Table 1) and compared them with NAC + SFN rats depicted in Fig. 5A (1 h post-status epilepticus). For measuring hippocampal cell loss,

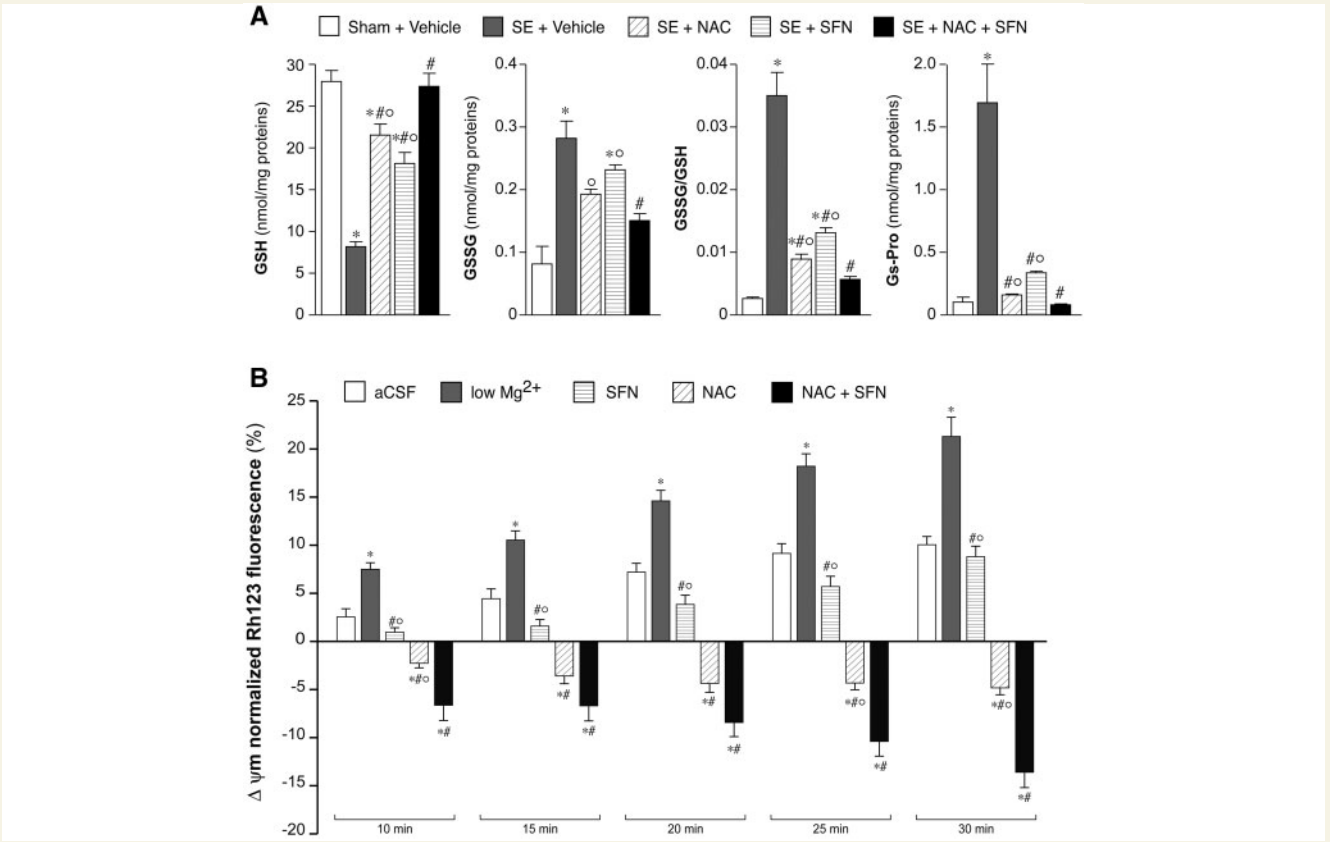

Figure 2 Effect of drug combination versus single drug alone on oxidative stress markers. (**A**) GSH and GSSG levels, and their ratio, and GS-Pro levels in the hippocampus of rats exposed to status epilepticus versus sham rats as assessed by HPLC analysis. Status epilepticus-exposed rats (n = 5 each 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). Controls were sham rats injected with vehicle (n = 5). The drug combination reduced oxidative stress to a greater extent than each drug given alone. Data are mean ± SEM. $^{*}P < 0.01$ versus Sham; $^{\circ}P < 0.01$ versus status epilepticus + NAC + SFN; $^{\#}P < 0.01$ versus status epilepticus + vehicle by Kruskal-Wallis followed by Dunn's *post hoc* test. (**B**) Bargrams depict low Mg^{2+}-induced mitochondrial membrane potential changes of neocortical cell cultures (Supplementary Fig. 3B–E). Preincubation of neurons with SFN (5 μM, 24 h) decreased the rate of depolarization at all time points. Addition of NAC (10 mM, acutely) evoked hyperpolarization in the mitochondrial membrane potential. Pretreatment with SFN (5 μM, 24 h) with addition of NAC (10 mM, acutely) showed similar effect as NAC alone, with significantly higher hyperpolarization after 25 and 30 min. Data are mean ± SEM. $^{*}P < 0.01$ versus artificial CSF (aCSF); $^{\#}P < 0.01$ versus low Mg^{2+}; $^{\circ}P < 0.01$ versus NAC + SFN by Kruskal-Wallis followed by Dunn's *post hoc* test. SE = status epilepticus.

two new rat cohorts were treated with NAC + SFN starting either 1 h or 24 h (n = 4–5 each group) post-status epilepticus and compared with time-matched status epilepticus + vehicle and sham rats (n = 5 each group) (Supplementary Table 2).

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 five hippocampal specimens (removed from patients undergoing surgery for drug-resistant epilepsy) and 11 hippocampal specimens obtained at autopsy from patients who died after status epilepticus were examined. Control material was obtained at autopsy from age-matched control patients, without a history of seizures or other neurological diseases. All autopsies were performed within 24 h after death. 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 (Blumcke *et al.*, 2013). The clinical features of the cases analysed are reported in Supplementary Table 3.

Statistical analysis

Sample size was *a priori* determined based on previous experience with the rat epilepsy model as well as following the principles of the three 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 (rat) to a particular

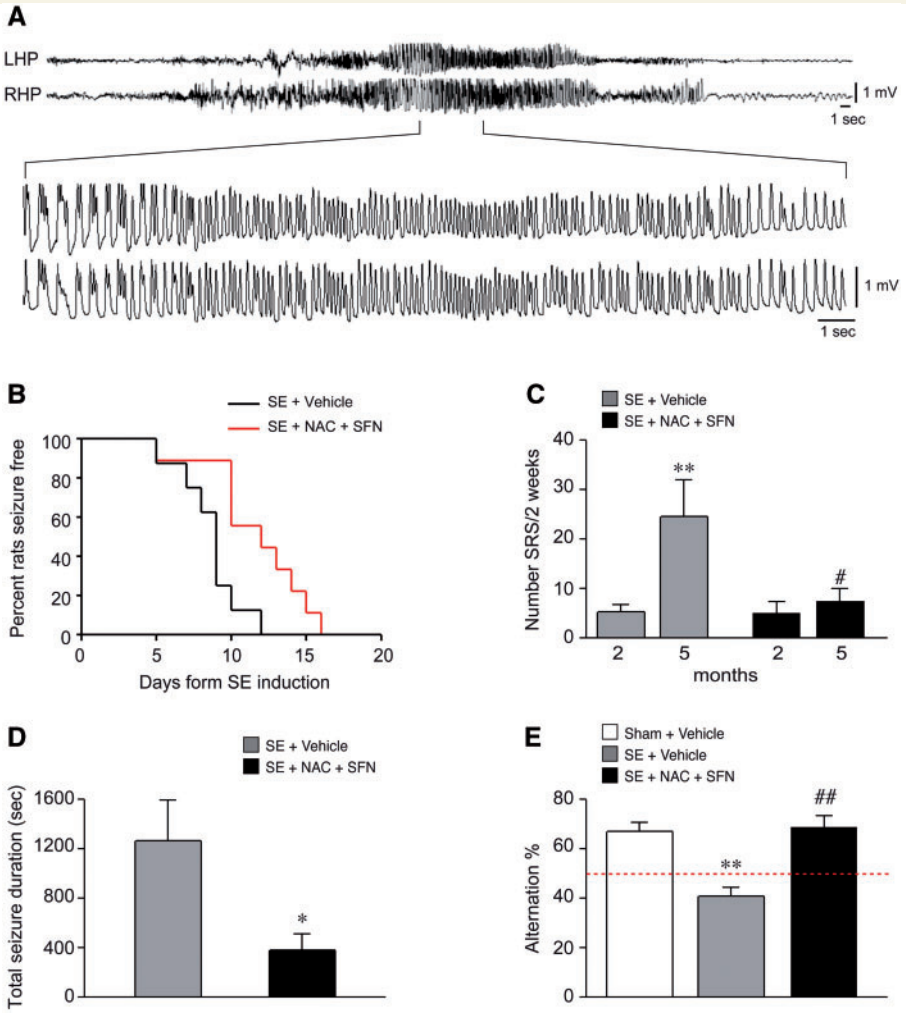

Figure 3 Therapeutic effects of antioxidant drug combination in status epilepticus-exposed rats. (**A**) A typical EEG recorded spontaneous seizure in a chronically epileptic rat injected with vehicle. LHP and RHP are left and right hippocampus, respectively. (**B**) Kaplan-Meier survival curve reporting the per cent of rats developing the first spontaneous seizure as a function of days post-status epilepticus: status epilepticus + vehicle, 100% rats developed the first spontaneous seizure by Day 12 versus status epilepticus + NAC + SFN by Day 16 ($P < 0.01$ by Log-rank test, $n = 9$ each group). (**C**) The number of SRS during 2-week EEG recording at 2 and 5 months post-status epilepticus in vehicle- and drug-treated rats. The data show that seizure progression was prevented by the treatment resulting in 70% SRS reduction at 5 months versus vehicle injected status epilepticus-exposed rats (**$P < 0.01$ versus 2 months; $^{\#}P < 0.05$ versus status epilepticus + vehicle by Mann-Whitney test). (**D**) Depicts the average cumulative duration of SRS recorded by EEG for 2 weeks at 5 months post-status epilepticus; this parameter was reduced by treatment versus vehicle (*$P < 0.05$ versus status epilepticus + vehicle by Mann-Whitney test). (**E**) The rat performance in the T-maze showing the average per cent of correct alternation in the each arm in the various experimental groups. The drug combination rescued the behavioural deficit in the epileptic rats. Data are mean ± SEM. **$P < 0.01$ versus Sham ($n = 9$); $^{\#\#}P < 0.01$ versus status epilepticus + vehicle by Kruskal-Wallis followed by Dunn's *post hoc* test. SE = status epilepticus.

experimental group. All efforts were made to minimize the number of animals used and their suffering. Data acquisition and analysis were done blindly.

Statistical analysis was performed by GraphPad Prism 6 (GraphPad Software, USA) for Windows using absolute values. Data are presented as mean ± standard error of the mean (SEM) (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 analysed by Log-rank (Mantel Cox) test. The temporal distribution of spikes during status epilepticus was analysed by two-way ANOVA followed by Bonferroni's multiple comparisons test. Differences were considered significant with a $P < 0.05$.

Results

Assessment of oxidative stress during epileptogenesis

We studied the generation of oxidative stress during epileptogenesis in status epilepticus-exposed rats by measuring

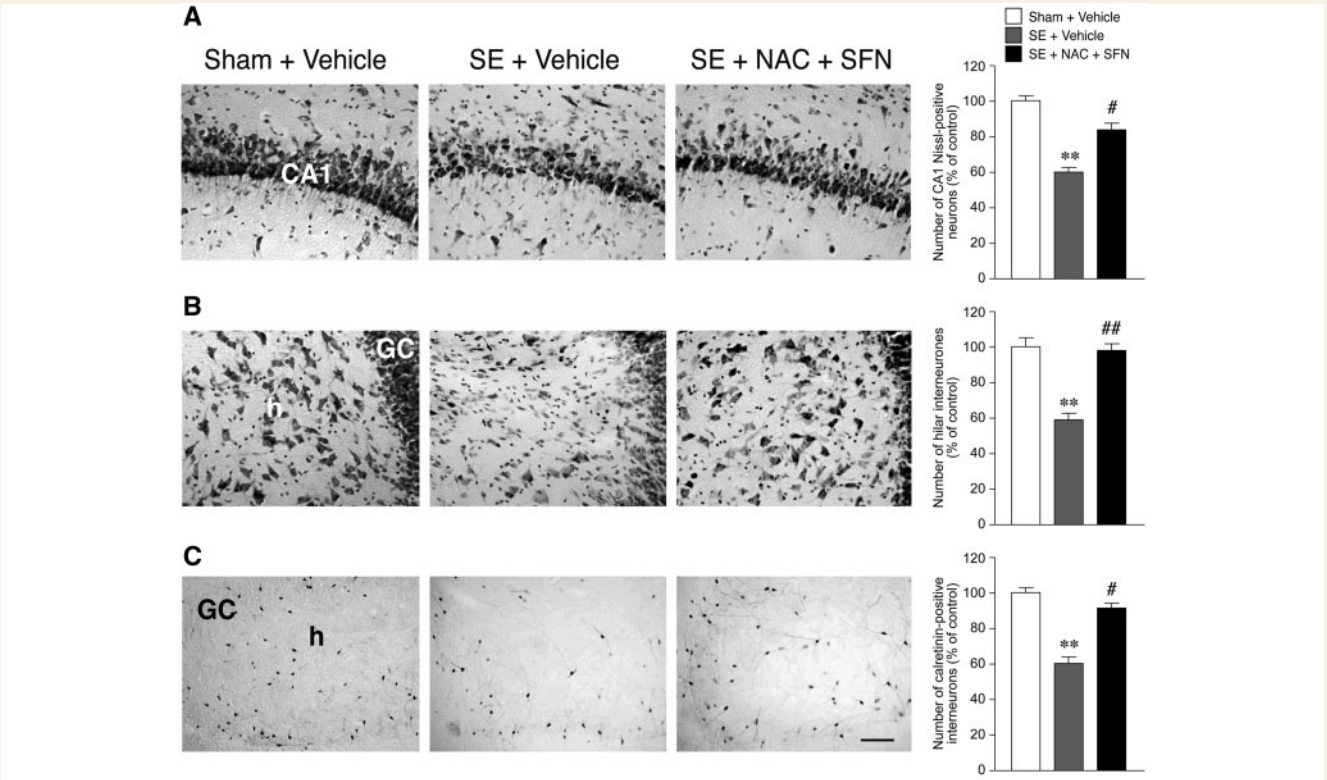

Figure 4 Histological analysis and quantification of cell loss in the hippocampus of status epilepticus-exposed rats treated with the antioxidant drugs versus vehicles. Panels depict representative microphotographs of Nissl-stained neurons in CA1 pyramidal layer (**A**) and in the hilus (**B**) and calretinin-stained hilar interneurons (**C**) in control (Sham + vehicle) and epileptic rats treated with vehicle (status epilepticus + vehicle) or the antioxidant drugs (status epilepticus + NAC + SFN; same rats of Fig. 3). Bargrams (mean ± SEM, $n = 9$ each group) report the correspondent quantification of cell loss showing the neuroprotective effect of the treatment. Scale bars: **A** and **B** = 100 μm; **C** = 50 μm. GC = granule cell layer; h = hilus; CA1 pyramidal layer. $^{**}P < 0.01$ versus Sham + vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus status epilepticus + vehicle by Kruskall-Wallis followed by Dunn's *post hoc* test. SE = status epilepticus.

the hippocampal levels of GSSG and GSH and their ratio (GSSG/GSH), which is an established indicator of ROS production (Liang and Patel, 2006; Ryan *et al.*, 2014). We chose two time points post-status epilepticus reflecting early epileptogenesis before the onset of epilepsy (4 days) and shortly after disease onset (14 days, the time at which the treatment was stopped). In accordance with other rodent models of epileptogenesis, the levels of both GSH and GSSG significantly changed ($P < 0.01$) between 4 days ($n = 9$) and 14 days post-status epilepticus ($n = 5$) resulting in a progressive 3- to 14-fold increase in GSSG/GSH ratio above control values (in sham rats not exposed to status epilepticus, $n = 15$) (Fig. 1A). A concomitant increase in glutathionylated proteins (Gs-Pro), another indicator of oxidative stress, was measured in the same hippocampal tissue (Fig. 1A).

To determine which cell types underwent oxidative stress, immunohistochemical analysis was done in the hippocampus in a group of rats killed 4 days post-status epilepticus. Figure 1B depicts the cellular expression of molecular markers of oxidative stress: iNOS [Fig. 1B(a,b)], the cystine transporter [Xct; Fig. 1B(c,d)] and the transcriptional factor Nrf2 [Fig. 1B(e,f)] in the representative CA1 hippocampal region. These molecules were induced in activated GFAP-positive astrocytes [Fig. 1B(b1,d1,f1)] as well as in NeuN-positive neurons [Fig. 1B(b2,d2,f2)] but they were not detected in OX-42-positive microglia (not shown). Nrf2 staining was increased in neuronal nuclei [Fig. 1B (f2 versus e1)] reflecting transcriptional activation of antioxidant genes in response to ROS production (Abbas *et al.*, 2011; Mazzuferi *et al.*, 2013; Steele *et al.*, 2013). These changes were similarly observed in the various CA3/CA4 hippocampal subfields, in the hilus, subiculum/parasubiculum and entorhinal cortex (not shown).

Antioxidant drug combination versus single treatment

To design the optimal treatment protocol for the therapeutic study, we tested whether a combination of NAC and SFN, two antioxidant drugs with complementary mechanism of action, was more effective in reducing oxidative stress than each drug given alone. We designed a treatment schedule lasting 14 days to encompass the epileptogenesis phase

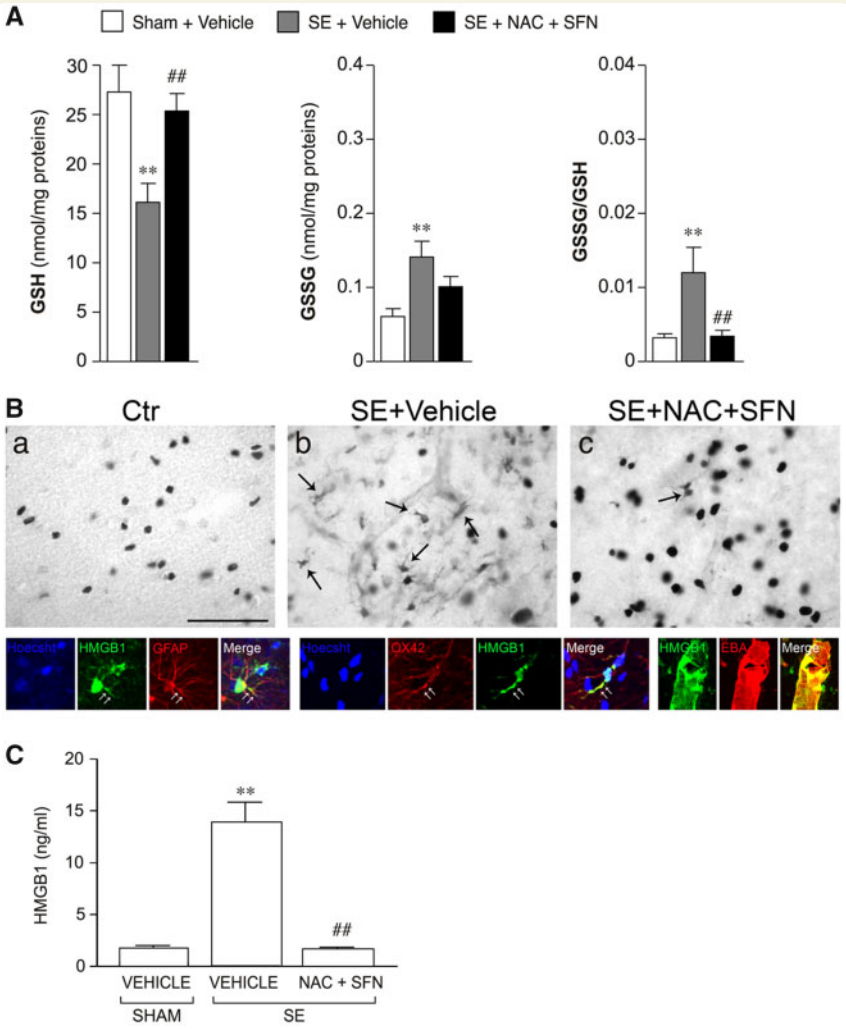

Figure 5 Effect of antioxidant treatment on HMGB1 in brain and blood. Histogram in **A** shows the increase in GSSG/GSH ratio during epileptogenesis (4 days post-status epilepticus, $n = 9$) and its reduction to baseline (Sham + vehicle, $n = 10$) by 4 day treatment with NAC (5 mg/kg, i.p. twice daily) + SFN (5 mg/kg, i.p., once daily) ($n = 11$) (treatment was started 1 h post-status epilepticus). A similar antioxidant effect was observed when treatment was started 24 h after status epilepticus (Supplementary Table 1). Data are mean ± SEM. $^{**}P < 0.01$ versus Sham + vehicle; $^{\#\#}P < 0.01$ versus status epilepticus + vehicle by Kruskal-Wallis followed by Dunn's *post hoc* test. (**B**) Representative photomicrographs of CA1-CA3 stratum lacunosum-moleculare of hippocampi from control rats (Sham) or status epilepticus-exposed rats treated with vehicle or NAC + SFN ($n = 4$–5; treatment protocol as in **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 status epilepticus; (**c**) reduced cytoplasmatic staining in status epilepticus-exposed rats treated with NAC + SFN denoting inhibiton of HMGB1 nuclear-to-cytoplasm translocation. *Second row:* 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 bars: first row = 25 μm; second row = 12 μm. Histograms in **C** show levels (mean ± SEM, $n = 9$–11) of HMGB1 in blood of rats during epileptogenesis (i.e. 4 days post-status epilepticus), and the effect of treatment. NAC + SFN abolished the increase in HMGB1 in blood. $^{**}P < 0.01$ versus Sham + vehicle; $^{\#\#}P < 0.01$ versus status epilepticus + vehicle by Kruskal-Wallis followed by Dunn's *post hoc* test. SE = status epilepticus.

between the acute injury (status epilepticus) and the aftermath of disease onset. Based on the available pharmacokinetic and pharmacodynamic information (Holdiness, 1991; Farr *et al.*, 2003; Hu *et al.*, 2004; Arakawa and Ito, 2007; Harvey *et al.*, 2008; Wang *et al.*, 2011), we treated different cohorts of rats with either NAC (500 mg/kg, i.p., twice a 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 an additional 7 days). Treatment began 1 h after the onset of status epilepticus (protocol in Supplementary Fig. 1A). NAC, the precursor of GSH, which represents the major non-enzymatic antioxidant pathway of the body, was administered to rats for 1 week to attain a rapid scavenging action of ROS during status epilepticus (Sun, 2010). SFN, an activator of *Nrf2*-dependent transcription of detoxification enzymes, was

administered for one additional week after NAC withdrawal to provide a sustained antioxidant effect (Houghton *et al.*, 2013). Figure 2A shows that GSH and GSSG were modified by status epilepticus resulting in a significant increase in GSSG/GSH ratio ($P < 0.01$ versus sham, $n = 5$ each group). Each drug alone increased GSH and reduced GSSG compared to status epilepticus-exposed rats receiving vehicles ($P < 0.01$, $n = 5$ each group). However, the combination of NAC and SFN showed a greater effect versus 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+} (Supplementary Fig. 2 and Supplementary material) (Haynes, 1999). We measured the changes in the mitochondrial inner membrane potential evoked by epileptiform activity and the effects of drugs. Figure 2B 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; Supplementary Fig. 2B). This effect was significantly reduced by preincubation with SFN or NAC alone; notably, NAC induced membrane hyperpolarization ($P < 0.01$ versus respective artificial CSF in Fig. 2B and Supplementary Fig. 2D and E). 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; $P < 0.01$ versus each drug alone; Fig. 2B and Supplementary Fig. 2E). These compounds were not found to have an anti-seizure effect *in vitro* (not shown).

Therapeutic effects of antioxidant drug combination in status epilepticus-exposed rats

The combined treatment protocol was applied starting 1 h after status epilepticus onset for 14 days, then treatment was stopped to determine if therapeutic effects occurred after drug withdrawal (protocol in Supplementary Fig. 1C). The drug combination did not attenuate the overall severity of the acute injury, namely the duration of status epilepticus or the frequency of spikes and their total number, as quantified by continuous 24 h EEG analysis from status epilepticus onset (Supplementary Fig. 3B and C). No difference was detected between the treatment and vehicle groups in the relative power distribution for each frequency band during status epilepticus (Supplementary Fig. 3D). Moreover, the treatment did not affect the acute cell loss induced by status epilepticus assessed by quantification of Fluoro-Jade-positive cells in the stimulated hippocampus 24 h post-status epilepticus (Supplementary Fig. 3E).

Spontaneous seizures onset and their progression

Rats treated for 14 days during epileptogenesis with NAC + SFN showed a significant delay in the time to spontaneous seizure onset (11.7 ± 1.1 days, $n = 9$, $P < 0.01$) compared to status epilepticus-exposed rats injected with vehicle (8.6 ± 0.7 days, $n = 9$). According to the Kaplan-Meier survival curve, 100% rats developed the first spontaneous seizure by Day 12 post-status epilepticus in the status epilepticus + vehicle group versus Day 16 in the drug-treated status epilepticus rats (Fig. 3B). 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, $P < 0.01$) post-status epilepticus while the average seizure duration did not change (2 months, 46.8 ± 3.5 s; 5 months, 56.0 ± 4.7 s). Although the number of seizures was not significantly modified by the drugs during the 14 days of treatment (status epilepticus + vehicle, 2.4 ± 0.5; status epilepticus + NAC + SFN, 3.6 ± 0.9, $n = 9$/each group), or at 2-month follow-up after treatment withdrawal (SRS/2 weeks, status epilepticus + vehicle, 5.1 ± 1.8; status epilepticus + NAC + SFN, 4.9 ± 2.5), the SRS progression between 2 and 5 months was prevented (Fig. 3C, $P < 0.01$ versus vehicle). Overall, drug-treated rats showed ~70% SRS reduction at 5 months post-status epilepticus compared to vehicle-injected rats ($P < 0.05$; Fig. 3C) although the proportion of rats with epilepsy did not change. Cumulative SRS duration during 2-week EEG recording at 5 months was significantly reduced by drugs compared to vehicle controls ($P < 0.05$; Fig. 3D) while the average duration of seizures was not affected by the treatment (status epilepticus + vehicle, 56.0 ± 4.7 s; status epilepticus + NAC + SFN, 49.5 ± 3.3 s, $n = 9$/each group). Similarly, drugs did not affect average seizure duration at 2-month follow-up (status epilepticus + vehicle, 46.8 ± 3.5 s; status epilepticus + NAC + SFN, 55.1 ± 5.8 s, $n = 9$/each group).

Cognitive deficits

Rats were tested in the T-maze at the end of EEG recordings (i.e. 5.5 months post-status epilepticus). Status epilepticus-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 ($40.5 \pm 2.8\%$ correct alternation, $n = 9$, $P < 0.01$) compared to sham rats ($66.8 \pm 3.9\%$ correct alternation, $n = 9$) (Fig. 3E). The drug combination rescued this behavioural deficit as shown by the correct alternation rate ($68.4 \pm 5.0\%$, $n = 9$) of treated rats, which was similar to sham rats (Fig. 3E). No difference in locomotion was detected among the experimental groups in the open field task

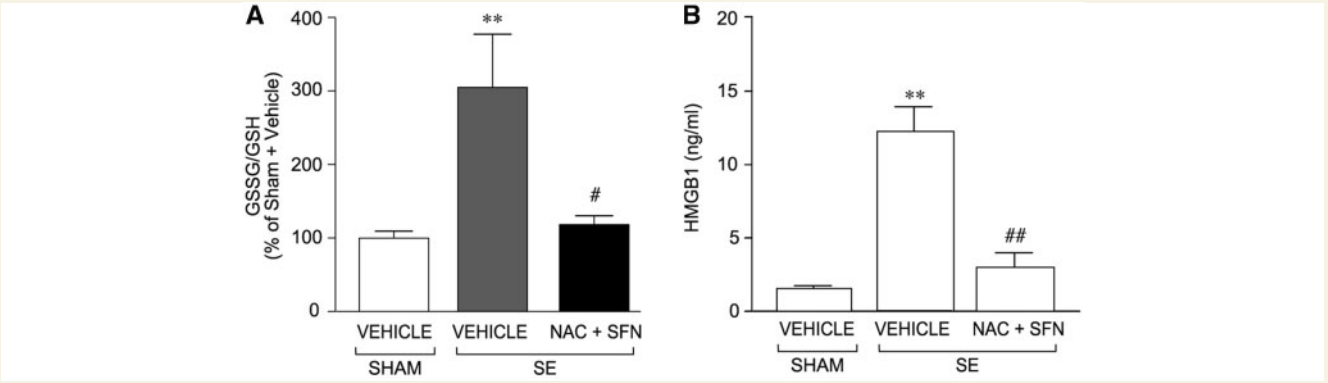

Figure 6 Oxidative stress markers and HMGB1 in blood. Histograms (mean ± SEM, $n = 9$ each group) report GSSG/GSH ratio (**A**) and total HMGB1 (**B**) in blood of status epilepticus-exposed rats injected with either vehicle or antioxidant drug combination (NAC + SFN) (same rats as in Fig. 3) compared 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-status epilepticus) then rats were followed up for monitoring SRS at 2 and 5 months post-status epilepticus. 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. $^{**}P < 0.01$ versus Sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus status epilepticus + vehicle by Kruskal-Wallis followed by Dunn's *post hoc* test. SE = status epilepticus.

(not shown). All rats were confirmed to be epileptic before the T-maze test (Fig. 3C) but they did not show behavioural seizures during the test.

Neurodegeneration in epileptic rats

At the end of behavioural testing, rats were killed for quantitative analysis of cell loss in Nissl-stained forebrain sections (Filibian *et al.*, 2012) of the ventral pole of the stimulated hippocampus (Fig. 4; rats are the same of Fig. 3). Vehicle-injected rats showed significant pyramidal cell loss in CA1 (Fig. 4A) and CA3/CA4 pyramidal cell layers (22 ± 8% decrease in cell number versus Sham, $P < 0.05$; not shown) and hilar interneurons (Fig. 4B). The antioxidant treatment reduced cell loss by half in CA1 ($P < 0.05$; Fig. 4A) and virtually prevented the neurodegeneration of hilar interneurons ($P < 0.01$; Fig. 4B). In particular, hilar calretinin- (but not somatostatin, not shown) positive cells were significantly protected by the drug combination (Fig. 4C). No significant neuroprotection was observed in CA3/CA4, the region of electrical stimulation, or in adjacent entorhinal cortex (not shown).

Effects of the antioxidant drug combination on HMGB1

We tested the novel hypothesis that reduction of oxidative stress during epileptogenesis prevents the nucleus-to-cytoplasm translocation of HMGB1 in the brain. We found that NAC + SFN decreased oxidative stress already 4 days post-status epilepticus (Fig. 5A, $n = 9$–11; protocol in Supplementary Fig. 1B); this antioxidant effect was similar when treatment was started 1 h (Fig. 5A) or 24 h (Supplementary Table 1) after status epilepticus onset. At 4 days post-status epilepticus, we found immunohistochemical evidence of nucleus-to-cytoplasm translocation of HMGB1 in activated astrocytes, microglia and brain endothelium of status epilepticus-exposed rats [$n = 5$; Fig. 5B(b versus a)], a phenomenon indicative of HMGB1 extracellular release. Clusters of CA1/CA3 pyramidal neurons with cytoplasmatic HMGB1 staining was observed in two of five status epilepticus-exposed rats (not shown). Such signal was absent in sham control rats ($n = 4$). The increase in the cytoplasmatic translocation of HMGB1 [Fig. 5B(c versus b)], was abolished by NAC + SFN. Notably, the changes in brain HMGB1, and the effects of treatment, were similarly detected in the blood of the same animals (Fig. 5C).

Based on these findings, we measured the GSSG/GSH ratio as well as total HMGB1 in the blood of status epilepticus-exposed rats undergoing the therapeutic trial (same rats of Fig. 3). Blood was drawn by tail vein at the end of treatment (14 days post-status epilepticus). GSSG/GSH ratio (Fig. 6A; $P < 0.05$), total HMGB1 (Fig. 6B; $P < 0.01$) were increased in blood of status epilepticus-exposed rats compared to sham rats. All these effects were prevented by drug treatment (Fig. 6A and B). There was no correlation between the blood levels of total HMGB1 and total seizure number, their frequency and total and average seizure duration in rats (Spearman correlation test; not shown).

Comparison between early and delayed antioxidant treatment on hippocampal cell loss

Based on evidence that NAC + SFN reduced oxidative stress also after treatment was delayed to 24 h post-status epilepticus (Supplementary Table 1 versus 1 h post-status epilepticus in Fig. 5A), we determined whether this delayed treatment also afforded neuroprotection. Early treatment

for 4 days displayed neuroprotection in CA1 and hilus (Supplementary Table 2, $n = 4$), and this was similar to epileptic rats treated with NAC + SFN for 14 days (Fig. 4). The delayed treatment did not rescue CA1 pyramidal cell loss while it reduced hilar cell loss similarly to early treatment (Supplementary Table 2, $n = 5$). CA3/CA4 cell loss was not rescued by treatment either given at 1 h or 24 h post-status epilepticus, as in epileptic rats treated for 14 days.

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

We used immunohistochemistry to analyse the presence of oxidative stress markers in autoptic hippocampal specimens from patients experiencing status epilepticus and in surgically resected human hippocampal tissue from temporal lobe epilepsy. Figure 7 shows increased expression of iNOS (Fig. 7A and B), Xct (Fig. 7C and D) and Nrf2 (Fig. 7E and F) in NeuN-positive neuronal cells and GFAP-positive astrocytes in a patient who died 49 days after status epilepticus. 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 status epilepticus patient specimens evaluated between 1 and 49 days post-status epilepticus and in chronic epilepsy hippocampal tissue from patients with temporal lobe epilepsy (Supplementary Fig. 4). Notably, HMGB1 cytoplasmatic staining was increased in both neurons and astrocytes in adjacent slices (Supplementary Fig. 5), in accordance with previous findings in human temporal lobe epilepsy (Maroso *et al*, 2010).

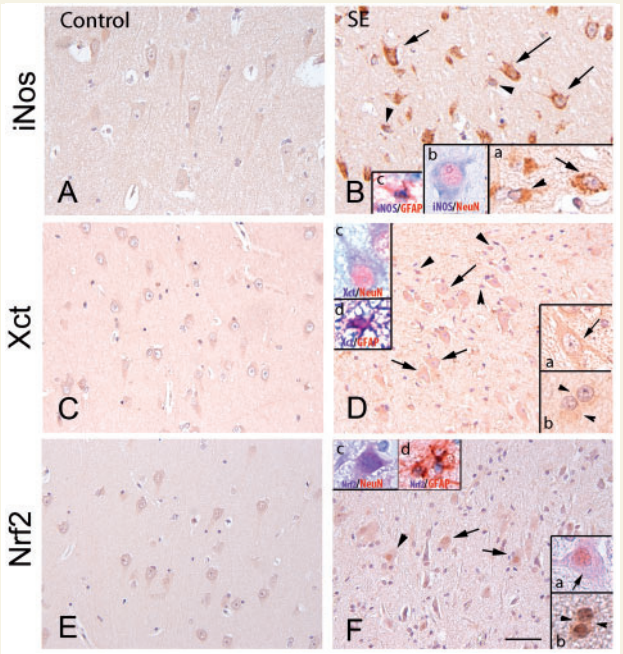

Figure 7 Oxidative stress in the hippocampus of patients with status epilepticus. Representative immunohistochemical micrographs of the CA1 region showing the expression of iNos (**A** and **B**), Xct (**C** and **D**) and Nrf2 (**E** and **F**) in control hippocampus (**A**, **C** and **E**) and in one representative patient who died after status epilepticus (49 days post-status epilepticus; **B**, **D** and **F**). Increased expression of these markers was observed in cells with neuronal (arrows in **B**, **D**, **F** and insets) and glial morphology (arrowheads in **B**, **D**, **F** and insets) compared to controls. Insets in **B** show iNos-positive neurons (arrow in inset **a**, and co-localization with NeuN in inset **b**) and astrocytes (arrowhead in inset **a**, and co-localization with GFAP in inset **c**). Insets in **D** show Xct-positive neurons (arrow in inset **a**, and co-localization with NeuN in inset **c**) and astrocytes (arrowhead in inset **b**, and co-localization with GFAP in inset **d**). Insets in **F** show Nrf2-positive neurons (arrow in inset **a**, and co-localization with NeuN in inset **c** and astrocytes; arrowhead in inset **b** and co-localization with GFAP in inset **d**). Insets in **F** show nuclear Nrf2 expression, denoting transcriptional activation of detoxifying enzymes. Scale bars in **A–F** = 80 μm; inset in **B** = 40 μm; insets in **B**, **D** and **F** = 25 μm. SE = status epilepticus.

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 (Rowley and Patel, 2013; Kovac *et al*., 2014). 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 (Waldbaum *et al*., 2010; Ryan *et al*., 2014; Bhuyan *et al*., 2015). It is likely that the mechanisms leading to oxidative tissue damage vary in the different phases of the epileptic process (Rowley and Patel, 2013). It remains to be determined if, and by which mechanisms, ROS generation contributes to the onset, progression and recurrence of spontaneous seizures. Importantly, whether targeting oxidative stress has any effect on seizure onset or progression is still unresolved.

Our novel findings show that an early and transient intervention with a specific combination of antioxidant drugs, namely NAC and SFN, mediates clinically relevant therapeutic effects in a status epilepticus rat model of acquired epilepsy. This combined treatment was more effective in rescuing mitochondrial dysfunction, and reducing oxidative stress during epileptogenesis than single drugs alone. The drug combination delayed epilepsy onset and blocked seizure progression. Accordingly, spontaneous seizure frequency was not modified at 2-month follow-up while it was drastically reduced at 5-month follow-up. Average SRS duration and incidence of rats developing epilepsy did not change. Notably, the drugs did not affect status

epilepticus severity and the associated acute cell loss, nor showed acute antiseizure effects. These data therefore provide evidence for a disease-modification effect mediated by this antioxidant treatment (Varvel *et al.*, 2015) and suggest that oxidative stress may play a role in the mechanisms of disease progression. In support, SFN was reported to suppress the progression of amygdala kindling in mice (Wang *et al.*, 2014) and chronic NAC treatment in neurotrauma-exposed animals normalized seizure threshold, which was reduced by brain injury (Silva *et al.*, 2011). Finally, chronic Nrf2 overexpression attained by gene therapy after epilepsy onset reduced SRS evoked by pilocarpine injection in mice (Mazzuferi *et al.*, 2013). Since Nrf2 coordinates the expression of numerous genes encoding detoxification, antioxidant and anti-inflammatory mediators, these pathways are likely to be relevant for the mechanisms of seizure recurrence (Mazzuferi *et al.*, 2013).

Our findings are apparently at variance with the lack of effect of a catalytic antioxidant porphyrin given post-status epilepticus on SRS, although both neuroprotection and rescue of behavioural deficit were attained in this study (Pearson *et al.*, 2015). 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 action of direct antioxidant used in a previous study (Pearson *et al.*, 2015) versus the Nrf2 inducer used in our study. The latter induces multiple genes, many of which encode endogenous antioxidants resulting in longer-lasting effects (Houghton *et al.*, 2013). 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 us to determine the effect of treatment on seizure progression. Similar effects on SRS progression were recently reported using an inducible nitric oxide inhibitor reducing reactive nitrogen species (Puttachary *et al*, 2016).

Oxidative stress has been implicated in cell loss and cognitive dysfunctions developing during epileptogenesis in different animal models (Rong *et al.*, 1999; Liang *et al.*, 2000; Barros *et al.*, 2007; Wang *et al.*, 2014; Pearson *et al.*, 2015). Both NAC and SFN were shown to provide neuroprotection in brain injury models (Knuckey *et al.*, 1995; Hong *et al.*, 2010; Mazzuferi *et al.*, 2013; Wang *et al.*, 2014), which is compatible with the role of ROS in glutamate excitotoxicity and in apoptotic cell death (Lafon-Cazal *et al.*, 1993; Reynolds and Hastings, 1995; Henshall and Murphy, 2008). Our antioxidant drug combination afforded neuroprotection and rescued cognitive deficits in a reference/working memory test by preventing the persistence of oxidative stress during epileptogenesis. We show that calretinin-positive cells in dentate hilus are rescued 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 (Gulyas *et al.*, 1999), 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 temporal lobe epilepsy, a phenomenon that may contribute to seizure generation and recurrence (Toth and Magloczky, 2014).

Early initiation of treatment (1 h post-status epilepticus) was more effective in reducing cell loss in CA1 than a delayed treatment schedule (24 h post-status epilepticus) while loss of hilar interneurons and oxidative stress were similarly prevented as assessed at 4 days post-status epilepticus. Thus, the data suggest that CA1 neurons are especially sensitive to oxidative damage occurring during the first 24 h post-injury. The resolution of oxidative stress at Day 4 post-status epilepticus by the delayed treatment warrents further investigations on its impact on disease onset and SRS progression.

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 (Dwivedi *et al.*, 2015) or using a metalloporphyrin catalytic antioxidant in a rat model of pilocarpine-induced epileptogenesis (Pearson *et al.*, 2015).

Disturbances in the normal redox state of the cells may contribute to the pathologic effects developing during epileptogenesis in various ways. 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 (Rowley and Patel, 2013). Moreover, neuronal excitability is controlled by glutamate and GABA, the biosynthesis of which depends on mitochondria (Kann *et al.*, 2005). 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 nucleus-to-cytoplasm translocation and subsequent cellular release of HMGB1, a pro-inflammatory molecules with ictogenic properties (Maroso *et al.*, 2010; Vezzani *et al.*, 2011*b*; Iori *et al.*, 2013; Balosso *et al.*, 2014). We previously showed that HMGB1 contributes to seizure generation and excitotoxic cell loss by activation of TLR4 and receptor for advanced glycation end-products (RAGE) (Maroso *et al.*, 2010; Iori *et al.*, 2013), and mice lacking either one of these receptors develop a milder form of epilepsy following status epilepticus (Iori *et al.*, 2013). Moreover, HMGB1 by activating TLR4 and RAGE, mediates cognitive dysfunctions in mice (Mazarati *et al.*, 2011). Overall, this evidence supports the novel concept that 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 HMGB1 translocation/extracellular release in brain tissue. Differently from astrocytes and neurons, we found that the HMGB1 translocation in microglia after status epilepticus was not associated with increased markers of oxidative stress in these cells. There are mechanisms underlying HMGB1 nucleus-to-cytoplasm translocation which may not depend on oxidative stress (Yu *et al.*, 2015). In particular, JAK/STAT1 activation is pivotal for HMGB1 hyperacetylation at nuclear localization sites and subsequent translocation in macrophages (Lu *et al.*, 2014). We cannot exclude, however, that the histological markers we have studied do not detect oxidative stress generated in microglia.

The brain changes measured during epileptogenesis in total HMGB1, 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 antioxidant drugs on their targets and possibly predicting their therapeutic effects.

The translation of our findings to the clinical setting is supported by our novel evidence that oxidative stress occurs in brain of patients experiencing status epilepticus, as well as in patients with drug-resistant temporal lobe epilepsy, 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 (Reagan-Shaw *et al.*, 2008), 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 (Holmay *et al.*, 2013). Moreover, NAC doses up to 3.6 g/day for several weeks have been used in neurological and psychiatric disorders (Deepmala *et al.*, 2015). 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 (Ben-Menachem *et al.*, 2000; Deepmala *et al.*, 2015). 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 (Cipolla *et al.*, 2015), and up to 27 mg were administered daily in autism spectrum disorders (Singh and Zimmerman, 2015) with signs of improvement and a safety profile.

In summary, our findings have high translational value since we report novel evidence that: (i) oxidative stress markers are induced in the hippocampus of humans who died following status epilepticus or with chronic pharmacoresistant epilepsy; and (ii) 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 (Pitkanen and Sutula, 2002; Schmidt and Sillanpaa, 2005).

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Supplementary material

Supplementary material is available at *Brain* online.

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