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Review

Reactive oxygen species in status epilepticus

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ABSTRACT

There has been growing evidence for a critical role of oxidative stress in neurodegenerative disease, providing novel targets for disease modifying treatments. Although antioxidants have been suggested and tried in the treatment of epilepsy, it is only recently that the pivotal role of oxidative stress in the pathophysiology of status epilepticus has been recognized. Although conventionally thought to be generated by mitochondria, reactive oxygen species during status epilepticus and prolonged seizure are generated mainly by NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (stimulated by NMDA receptor activation). Excessive production of reactive oxygen species results in lipid peroxidation, DNA damage, enzyme inhibition, and mitochondrial damage, culminating in neuronal death. Antioxidant therapy has been hampered by poor CNS penetration and rapid consumption by oxidants. However, alternative approaches such as inhibiting NADPH oxidase or increasing endogenous antioxidant defenses through activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) could avoid these problems. Small molecules that increase Nrf2 activation have proven to be not only effective neuroprotectants following status epilepticus, but also potently antiepileptogenic. There are "Proceedings of the 7th London-Innsbruck Colloquium on Status Epilepticus and Acute Seizures".

1. Introduction

There has been a strong evolutionary drive for organisms and cells to use atmospheric dioxygen for respiration to meet energy demands. However, oxygen also naturally occurs in more reactive forms (such as singlet oxygen or H_2O_2) or as free radicals (atom or groups of atoms with an unpaired electron which act as electron acceptors) such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical (OH•); these are termed reactive oxygen species (ROS) [1]. Reactive oxygen species can be produced nonenzymatically by UV (ultraviolet) irradiation or enzymatically in the cells. Reactive oxygen species production varies greatly in different tissues depending on the availability of oxygen and expression of ROS-producing enzymes.

Compared to other organs, the brain is one of the greatest ROS producers because of its ~10-fold higher oxygen consumption and high metabolic rate compared to that of other tissues in the body [2]. The major ROS producers in the brain comprise NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX) (predominantly in two isoforms NOX2 and NOX4), mitochondria (electron transport chain, enzymes of TCA, and monoamine oxidases A and B [3]), xanthine oxidase, and lipoxygenase. Brain ROS generation occurs even under resting conditions and greatly increases during brain activity, so that an effective antioxidant system is necessary to protect cells against oxidative stress. The major endogenous antioxidants in the brain, glutathione and α-tocopherol (vitamin E) in lipids [4,5], play important protective roles along with antioxidant enzymes [2]. The dependence of the level of ROS production on cellular activity and the short lifetime of superoxide and its consequent limited diffusion make ROS ideal signaling molecules, necessary for physiological function [3]. In the brain, a plethora of processes have been identified that are regulated by moderate amounts of ROS. For example, NOX-derived ROS are instrumental in long-term potentiation (LTP), a process required for memory formation [6].

However, changes of the redox balance in the brain through an increase in ROS production or a decrease in the level of antioxidants can lead to oxidative damage, cellular dysfunction, and cell death. Excess ROS lead to lipid peroxidation and direct protein damage, which alters membrane and enzyme function, respectively, and subsequently destroys the cell [7]. The brain with its high content of polyunsaturated fatty acids is particularly prone to ROS-induced damage. Many neurological disorders are strongly associated with oxidative stress and excessive ROS production. Although energy deprivation plays a central role in

Abbreviations: KEAP1, Kelch-like ECH associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SE, status epilepticus.

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ischemia, significant reperfusion damage occurs through increased ROS production, particularly through NOX activation during reoxygenation [8]. There is increasing evidence for the involvement of oxidative stress in Alzheimer's disease observed both in postmortem tissue of patients with Alzheimer's disease [9] and also in cellular systems [10]. In Parkinson's disease, all the toxic proteins that generate the pathology have been shown to activate ROS production, and most of proteins, encoded by Parkinson's associated genes, are involved in mitochondrial or redox homeostasis [2]. Moreover, aggregated protein α-synuclein, which is involved in both sporadic and familial forms of Parkinson's disease, is able to produce superoxide in combination with heavy metal ions, so triggering neuronal cell loss [11]. Mitochondrial ROS overproduction is also evident in many neurodegenerative diseases including Huntington's disease, amyotrophic lateral sclerosis, progressive supranuclear gaze palsy, Friedreich's ataxia, and frontotemporal dementia [12–15]. Although antioxidant therapy has been proven to be effective for a number of neurodegenerative disorders in experiments on a cellular level, most of the clinical trials have failed to demonstrate neuroprotection or efficacy in patients. This failure to translate the positive effects of antioxidants in experimental systems is usually attributed to the difficulties in antioxidant delivery to cells in the brain, or to the chemical instability of the antioxidant.

2. ROS as a pathogenic mechanism in status epilepticus and excitotoxicity

Pioneering studies in baboons by Meldrum and colleagues in the 1970's demonstrated that seizure activity, independent of any physiological compromise, can cause neuronal damage [16,17]. Further studies led to the idea that this damage was mediated through excitotoxicity from the excessive release of glutamate. As far back as 1957, glutamate was recognized as a potent neurotoxin [18]. Experiments on glutamate and its analogues demonstrated that there is a correlation between their excitatory and toxic actions;, that specific antagonists of their excitatory activity protect against their neurotoxicity, and that they are toxic to dendrites and cell bodies (dendrosomatotoxic) and axon-sparing. These observations led to the hypothesis that the toxic action was mediated through an action of glutamate at dendrosomatic receptors - the excitotoxic hypothesis [19]. Subsequent evidence indicated that ROS play a role in this excitotoxic damage [20], and later, ROS production was shown to be necessary for excitotoxic neuronal injury [21].

Oxidative stress during and after seizures was later shown to be instrumental in immediate and longer-term excitotoxic neuronal death [21,22], and the reduction of aconitase and glutathione shortly following status epilepticus (SE) strongly supports this hypothesis [23]. Excessive ROS production (especially in combination with nitric oxide to form peroxynitrite, a reactive nitrogen species) leads to lipid peroxidation, inactivation of enzymes, mitochondrial permeability transition pore opening, and DNA damage [24–26] (Fig. 1). Moreover, excessive ROS production can inhibit mitochondrial complex 1 activity, decrease mitochondrial membrane potential, and inhibit ATP production [27–29]. Ca^{2+}- and ROS-induced activation of the DNA-repair enzyme poly(ADP-ribose) (PAR) and poly(ADP-ribose) polymerases (PARP) depletes NADH and further reduces ATP production [30,31].

Human data have largely confirmed the animal studies. Oxidative stress markers are increased in the hippocampus of humans who died following SE or with chronic pharmacoresistant epilepsy [32]. A further recent study has indicated that patients with SE have reduced antioxidants and increased reactive oxygen and nitrogen species. Furthermore, the study also showed that catalase glutathione and total antioxidant capacity were lower, and malondialdehyde and nitric oxide levels were significantly higher in the patients with SE with comorbidity [33]. Moreover, in progressive myoclonic epilepsy, there is considerable evidence that ROS production is a key component of

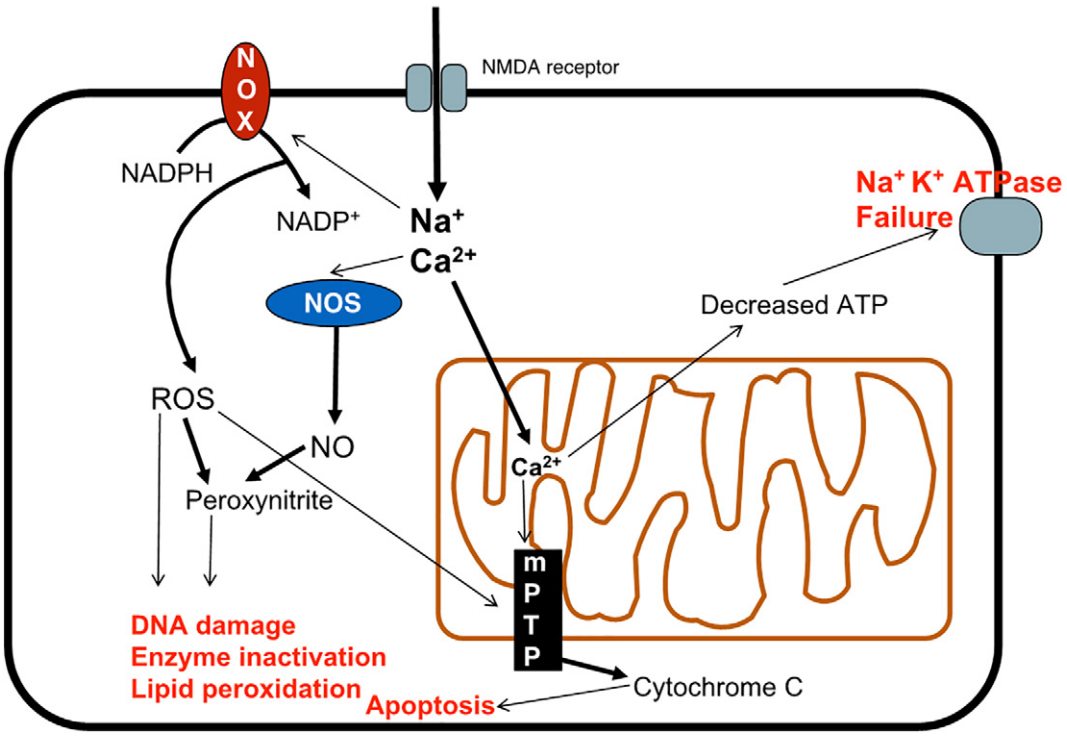

Fig. 1. Putative mechanisms leading to neuronal death following calcium (and sodium) entry through NMDA receptors. NMDA receptor activation and calcium entry activate several enzymes NADPH oxidase (NOX) and nitric oxide synthase (NOS). Reactive oxygen species (ROS) and nitric oxide form peroxynitrite, which is toxic to DNA, proteins, and lipids. Ca^{2+} from the cytosol is taken up by mitochondria; low Ca^{2+} load enhances ATP production, but excessive Ca^{2+} load along with ROS results in mitochondrial depolarization decreased ATP production, energy failure, and consequent failure to maintain cellular ionic gradients. Mitochondrial calcium accumulation and ROS contribute to the formation of the mitochondrial permeability transition pore (mPTP), which further disrupts mitochondrial function, but also permit cytochrome *c* into the cytosol where it can activate apoptotic pathways. From [44].

the pathophysiology [34]. Thus, animal and human data confirm that oxidative stress and excessive ROS production are notable features in SE.

3. Source of ROS in epilepsy

Mitochondria, the key organelles in ATP production and metabolism, have been assumed to be a major source of ROS production during seizures. Complex I and III are the primary sites of ROS production in mitochondria through "electron leak" from the electron transport chain [35]; indeed, it has been proposed that complex III is the main contributor to superoxide production during seizure activity [36]. Mitochondrial ROS production is critically dependent upon mitochondrial function and a hyperpolarized mitochondrial membrane potential [37]. Prolonged seizure activity, however, leads to depolarization of the mitochondrial membrane potential and mitochondrial failure [38]. Therefore, although mitochondria could serve as a significant source of ROS during brief seizures, it is difficult to see how they can contribute significantly during SE. Using robust protocols for real-time in vitro and ex vivo monitoring of ROS production during hyperexcitability, we were able to establish that mitochondria are not the main source of ROS during prolonged seizure activity [29,39]. Mitochondria are, however, likely a prominent target of ROS-induced damage during seizure activity, and permeability transition pore opening, which is strongly promoted by ROS, has been shown to lead to cell death during prolonged seizure-like activity [38].

In recent years, there has been growing evidence for a role of NOX in generating ROS in the brain. Nicotinamide adenine dinucleotide phosphate oxidases are enzymes specialized in ROS production, and their initial discovery was through the study of the respiratory burst in phagocytes and granulocytes [40]. We have identified NOX as an important source of hyperexcitability-induced ROS production and consequent neuronal damage [29] (Fig. 1). During electrically-induced SE, prominent ROS production and subsequent neuronal cell death in hippocampal subfields can be observed. This was suppressed by pharmacological NOX inhibition [39].

Nicotinamide adenine dinucleotide phosphate oxidases exist in different isoforms which have been shown to exert different functions, such as cellular signaling or regulation of cell growth [41]. Moreover, within the brain, these isoforms are differentially expressed in different cell types. In mammals, seven NADPH isoforms have been found, including NOX1–5 and two dual oxidases (DUOX1 and DUOX2) [41,42]. Nicotinamide adenine dinucleotide phosphate oxidases 1, 2, and 4 are expressed in the brain [42]. Several studies have looked into the role of NOX in epilepsy (for a summary, see table in [7]). It emerges that NOX2 plays a prominent role, which has been confirmed with more specific pharmacological antagonists [7]. Nicotinamide adenine dinucleotide phosphate oxidase 2 has been shown to be activated via NMDA receptor activation [43], a receptor which has a prominent role in seizure- and SE-induced neuronal death [44].

Nicotinamide adenine dinucleotide phosphate oxidase may also contribute to the pathophysiology of SE in other ways Vasogenic edema formation during SE has also been linked to endothelin B (ETB) receptor-mediated astrocytic ROS production through NOX activation [45]. Nicotinamide adenine dinucleotide phosphate oxidase has also been identified as a pharmacological target for increased seizure susceptibility after systemic inflammation [46], and NOX inhibition with apocynin has been shown to lead to ameliorate seizure-induced reduction of adult hippocampal neurogenesis [47]. However, the precise roles of different NOX isoforms subtypes in hyperexcitability, seizures, and epilepsy are still unclear and await further studies using genetic ablation or isoform specific inhibitors.

Although NOX is an important mediator of ROS-induced cell damage in seizures and epilepsy, several other ROS sources exist in the cell such as xanthine oxidase, monoaminoxidase, and cyclooxygenase and lipoxygenase; these can also contribute to seizure-induced ROS [29,48,49]. During SE, the high metabolic demand results in excessive breakdown of ATP which enhances xanthine oxidase activity, and therefore contributes to a significant proportion of ROS production [29].

Establishing the sources of ROS at different time points and under different conditions in seizures, SE and epileptogenesis could reveal new important treatment targets. It is likely that different sources of ROS production are prominent at different phases of the disease – a phenomenon which has already been observed in ischemia reperfusion injury in the brain [8].

4. Neuroprotective and antiepileptogenic role of ROS inhibition following status epilepticus

The use of antioxidants for the treatment of epilepsy has a long history, with successful therapies being described as far back the 1970s [50]. The most extensively studied antioxidant therapy in epilepsy is α-tocopherol or vitamin E. Animal studies have confirmed its antioxidant efficacy and indicated antiepileptic effects. It was shown that pretreatment of rats with combined α-tocopherol and selenium prevented the development of iron-induced epileptiform activity in 72% of animals compared with 6% of untreated controls receiving an intracortical injection of aqueous iron [51]. However, an extensive study of α-tocopherol in four animal models of seizures and epileptogenesis confirmed its efficacy in the ferrous chloride model but not in the pentylenetetrazole threshold model, the maximal electroshock model, and the kindling model of epileptogenesis [52]. Furthermore, α-tocopherol demonstrated negligible effects in preventing seizures and oxidative injury in animal models of limbic epilepsy, such as amygdala-kindled seizures and electrically-induced SE [53].

Despite these findings, α-tocopherol administration following kainic acid-induced SE significantly reduced astrocytosis and microglia activation, emphasizing its antiinflammatory and neuroprotective potential [54]. This was confirmed in a recent study, which demonstrated that long-term treatment with 60 IU/kg/day of vitamin E prevented oxidative damage in the hippocampus and increased hilar parvalbumin expression in rats with epilepsy without a reduction in seizure frequency [55].

Numerous animal studies have demonstrated the use of other antioxidants such as vitamin C, N-acetyl-cysteine, coenzyme Q10, and various plant extracts or flavonoids for the treatment of SE, reduce lipid oxidation, and restore the activities of different antioxidant enzymes such as, superoxide dismutase, catalase, glutathione peroxidase, and the levels of glutathione in the rat hippocampus, striatum, or cortex [56–61]. Some antioxidants have shown particular promise including resveratrol, a naturally occurring antioxidant found in red grapes. Resveratrol given both before and after SE has been shown to decrease hippocampal neuronal cell death, mossy fiber sprouting, and abnormal neurogenesis [62,63]. Similarly, melatonin, a naturally occurring hormone that is a potent antioxidant and free radical scavenger attenuates seizure activity and neuronal damage in the hippocampus and piriform cortex following kainic acid-induced SE [64], and AEOL10150, a metalloporphyrin catalytic antioxidant, resulted in reduced mortality, neuronal death, and inflammation compared to control animals [65]. Antioxidant strategies, therefore, show considerable promise in neuroprotection in SE. However, administration of exogenous antioxidants provides specific challenges, and as a consequence, the results from many studies are mixed. The antioxidant has to cross the blood–brain barrier, and antioxidants get rapidly oxidized and so "consumed" through ROS scavenging, resulting in a short action. In addition, the translational potential of many of the studies is low as the antioxidant was given prior to the SE.

In view of this, we have investigated, other, more enduring, antioxidant strategies including decreasing ROS production by targeting NOX [39] and increasing endogenous antioxidant defenses through activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [32,66]. Nuclear factor erythroid 2-related factor 2 is a transcription factor that is negatively regulated by Kelch-like ECH associated protein 1 (KEAP1) [67]

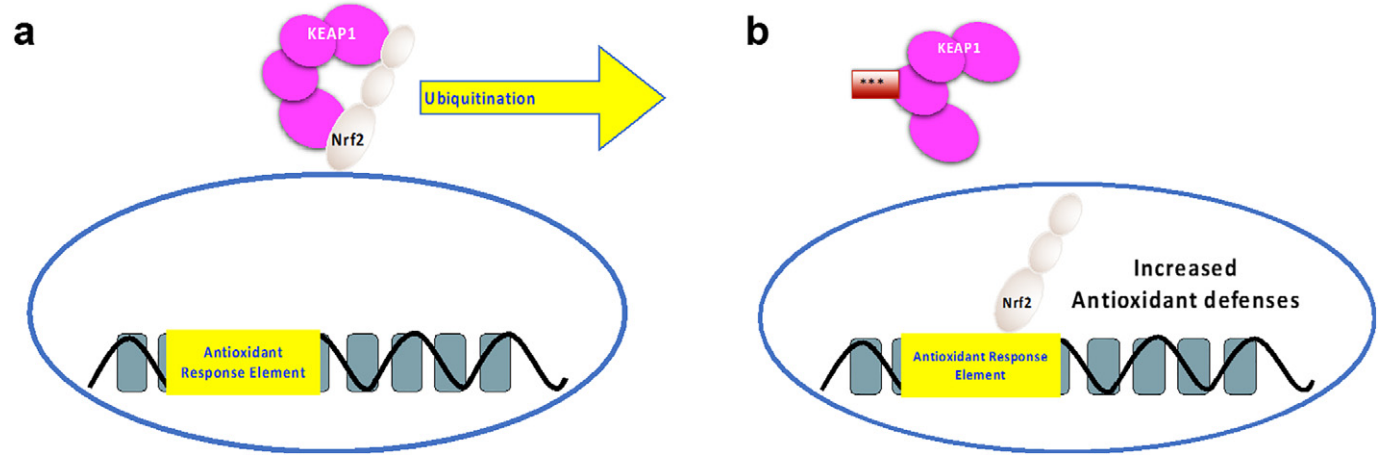

Fig. 2. Nrf2 and KEAP1. Nrf2 is bound by KEAP1 which prevents translocations into the nucleus and results in ubiquitination. *** represents endogenous reactive oxygen species or exogenous drug which bind to KEAP1 permitting Nrf2 translocation to the nucleus where it acts on the antioxidant response element to increase endogenous antioxidant defenses.

(Fig. 2). Nuclear factor erythroid 2-related factor 2 target proteins include not only antioxidant enzymes, but also enzymes involved in antiinflammation, detoxification, and metabolism [68]. Electrophiles and oxidants inactivate KEAP1 by chemically modifying critical cysteine sensors within the protein [69], leading to Nrf2 accumulation and increased transcription of Nrf2 target genes that encode antioxidants [70]. The long half-lives and the diverse nature of the upregulated proteins ensure long-lasting protection against multiple oxidants, offering protection against many pathologies [71]. In addition, Nrf2 activation results in an improvement of mitochondrial function, so increasing ATP production, which also can be neuroprotective [72]. Nuclear factor erythroid 2-related factor 2 increases after SE [73], probably as a response to increased ROS production. Genetically-induced overexpression of Nrf2 neuroprotects, reduces inflammation, and decreases subsequent seizure frequency following SE [73].

Nuclear factor erythroid 2-related factor 2 can be activated by sulforaphane, a naturally occurring substance obtained from cruciferous vegetables such as broccoli. Sulforaphane in combination with the antioxidant N-acetylcysteine given after SE decreased neuronal damage, subsequent seizures, and cognitive deficits [32]. Sulforaphane is hampered by poor penetration of the CNS, so we tested a CNS penetrant, potent, and specific inhibitor of KEAP1, RTA-408. RTA-408 given once daily over 3 days following SE gave almost complete neuroprotection in the hippocampus and reduced the occurrence of subsequent spontaneous seizures by ~94% [66] (Fig. 3).

5. Conclusion

It is now well-established that SE results in an increase in ROS, which reach toxic levels resulting in neuronal death. Targeting oxidative stress

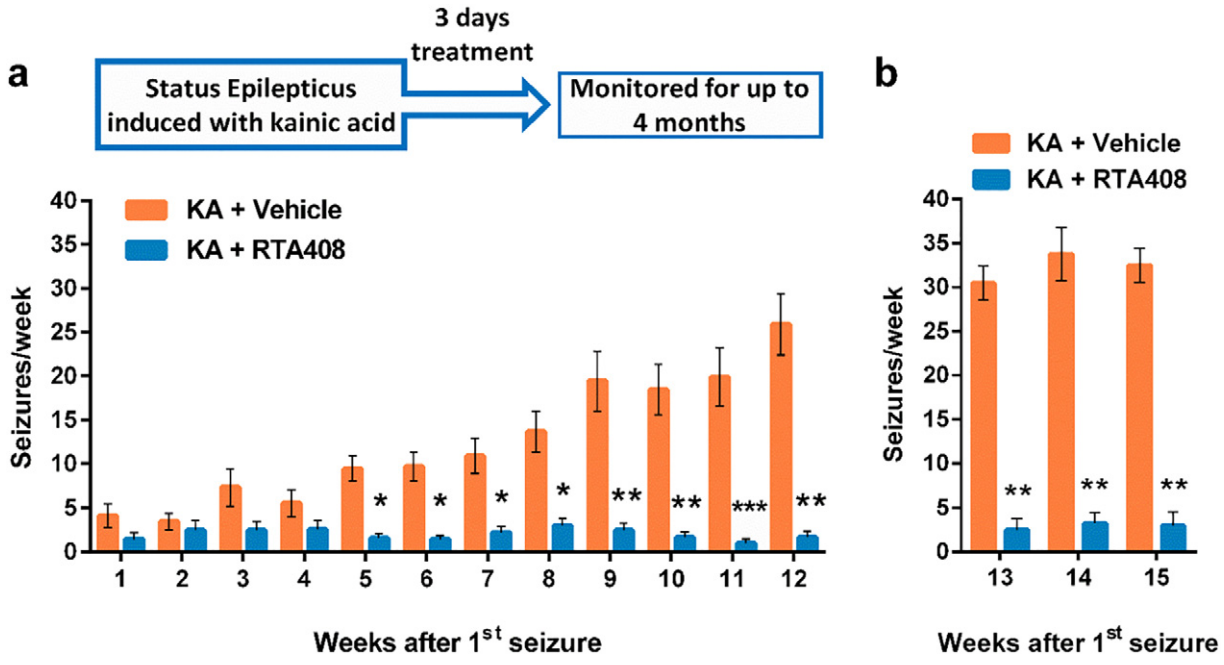

Fig. 3. KEAP1 inhibitor RTA-408 inhibits epileptogenesis and neuroprotects following status epilepticus. Status epilepticus was induced with kainic acid and terminated with diazepam after 2 h. The rats were then randomized to vehicle or RTA-408 once a day for three days and were then monitored for up to 4 months. Nine rats in each group were monitored for 12 weeks after first seizure (a) and, 4 of those rats in each group were monitored for 15 weeks following first seizure (b). *P < 0.05, **P < 0.01, and ***P < 0.001 by generalized log-linear mixed model followed by sequential Bonferroni post hoc test after.

following SE, using a variety of strategies, has been shown not only to neuroprotect, but also to prevent or ameliorate other consequences including cognitive decline and the occurrence of late, spontaneous seizures (epileptogenesis). There are a growing number of trials of potent and effective antioxidant strategies in other neurological disease and in other branches of medicine (in particular oncology) which could easily be repurposed for use in SE. Amongst the various strategies, activation of Nrf2, either alone or in combination with other antioxidant strategies, seems to hold the most promise.

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