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Reactive oxygen species mediate cognitive deficits in experimental temporal lobe epilepsy

Jennifer N. Pearson, BS^{1,†}, Shane Rowley, PhD^{1,†}, Li-Ping Liang, MD^{2,†}, Andrew M. White, MD³, Brian J. Day, PhD⁴, and Manisha Patel, PhD^{1,2,*}

¹Neuroscience Program, University of Colorado, Anschutz Medical Campus 80045

²Department of Pharmaceutical Sciences, University of Colorado, Anschutz Medical Campus 80045

³Department of Pediatrics, University of Colorado, Anschutz Medical Campus 80045

⁴National Jewish Health, Denver Colorado, 80206

Abstract

Cognitive dysfunction is an important comorbidity of temporal lobe epilepsy (TLE). However, no targeted therapies are available and the mechanisms underlying cognitive impairment, specifically deficits in learning and memory associated with TLE remain unknown. Oxidative stress is known to occur in the pathogenesis of TLE but its functional role remains to be determined. Here, we demonstrate that oxidative stress and resultant processes contribute to cognitive decline associated with epileptogenesis. Using a synthetic catalytic antioxidant, we show that pharmacological removal of reactive oxygen species (ROS) prevents 1) oxidative stress, 2) deficits in mitochondrial oxygen consumption rates, 3) hippocampal neuronal loss and 4) cognitive dysfunction without altering the intensity of the initial status epilepticus (SE) or epilepsy development in a rat model of SE-induced TLE. Moreover, the effects of the catalytic antioxidant on cognition persisted beyond the treatment period suggestive of disease-modification. The data implicate oxidative stress as a novel mechanism by which cognitive dysfunction can arise during epileptogenesis and suggest a potential disease-modifying therapeutic approach to target it.

Keywords

epilepsy; learning and memory; oxidative stress; reactive oxygen species; pilocarpine; mitochondrial dysfunction; neuronal loss

*To whom correspondence should be addressed: Manisha Patel, Ph.D., 12850 E. Montview Blvd V20-C238, Aurora, CO. 80045, Manisha.Patel@ucdenver.edu, 303-724-3606.

[†]Denotes equal contribution

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Introduction

Epilepsy is increasingly recognized as a spectrum disorder characterized by chronic, spontaneous seizures (SRS). Temporal Lobe Epilepsy (TLE) is the most prevalent form of acquired epilepsy and is defined by the appearance of SRS after a precipitating brain event such as trauma, ischemic stroke or status epilepticus (SE). In addition to experiencing unpredictable seizures, patients with TLE and indeed all epilepsies, tend to suffer from debilitating comorbidities. Up to half of the people with epilepsy experience one or more psychiatric or cognitive comorbidities (Wiebe & Hesdorffer, 2007). These comorbid conditions occur with epilepsy and impair quality of life. In fact, the burden of comorbidities can surpass those of the seizures themselves as quality of life correlates more with the comorbidities of epilepsy as opposed to the seizures alone (Gilliam et al, 2003). Prominent among these comorbidities of epilepsy is cognitive impairment (Institute of Medicine, 2012). Currently available anti-seizure drugs control seizures in roughly 60% of patients; however, they often exacerbate comorbidities such as cognitive impairment, particularly deficits in memory (Helmstaedter and Kurthen, 2001; Helmstaedter et al, 2003). Whereas the cognitive comorbidities of epilepsy are widely recognized for their frequent occurrence and debilitating nature, currently there are no targeted therapies to treat them or sufficient knowledge regarding their underlying mechanisms.

A growing body of research has implicated oxidative stress and mitochondrial dysfunction as contributing factors to numerous conditions with comorbid cognitive dysfunction including Alzheimer's disease (AD), Parkinson's disease (PD) and even normal aging (Butterfield, 2006; Lin and Beal, 2006). Excessive production of reactive oxygen species (ROS) leads to elevated levels of cellular macromolecule oxidation contributing to cell dysfunction and cell death (Hensley et al, 1995). ROS-induced neuronal death or processes underlying neuronal death could have a detrimental effect on areas of the brain controlling learning and memory processes such as the hippocampus, a brain region intimately associated with memory function. Samples obtained from patients with epilepsy show increased levels of oxidized proteins and lipids in serum, implicating oxidative stress as an ongoing process in these patients (Menon et al, 2012). Data from our laboratory have demonstrated increased steady-state levels of reactive species and impaired glutathione redox status in the hippocampus in two separate TLE models including the pilocarpine model and the kainic acid model (Liang et al, 2000, 2006, 2008; Patel et al, 2001, 2008; Ryan et al, 2013; Rowley et al, 2015). Collectively, this work shows that oxidative damage occurs during epileptogenesis and contributes to acute injury-induced neuronal damage (Liang et al, 2000; Fujikawa, 2005). Indeed, recent work from our laboratory in the kainic acid model of TLE has shown that attenuation of oxidative stress by a synthetic catalytic antioxidant porphyrin, $\text{Mn}^{\text{III}}\text{TDE-2-ImP}^{5+}$, is sufficient to protect mitochondrial function (Rowley et al, 2015). However, the functional role of oxidative stress in TLE or associated cognitive comorbidities is unknown. Furthermore, the role of neuronal damage *per se* on any functional deficits such as cognitive dysfunction associated with TLE is yet to be determined.

Here, we tested the hypothesis that injury-mediated oxidative stress induces mitochondrial dysfunction and neuronal death leading to learning and memory deficits associated with

epileptogenesis. Using Mn^{III}TDE-2-ImP⁵⁺, we determined if pharmacological scavenging of reactive oxygen species (ROS) prevents 1) oxidative stress, 2) deficits in mitochondrial oxygen consumption rates (OCR), 3) hippocampal neuronal loss and 4) cognitive dysfunction in a rat model of TLE.

Materials and Methods

Reagents

All reagents were purchased from Sigma Aldrich or Fisher Scientific. Manganese (III) meso-tetrakis (di-N-ethylimidazole) porphyrin or Mn^{III}TDE-2-ImP⁵⁺ (also known in the literature as AEOL 10150) was pharmaceutical grade and obtained from Aeolus Pharmaceuticals.

Pilocarpine treatment

Animals were treated in accordance with NIH guidelines and all experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. Adult, male Sprague-Dawley rats (250–300g), purchased from Harlan Laboratories (Indianapolis, Indiana) were used in all experiments. Upon arrival, rats were individually housed on a 14/10 light/dark cycle (lights on at 6:00am and off at 8:00pm) with *ad libitum* access to both food (Harlan rat chow) and filtered water. After one week of acclimation, rats were randomly assigned to groups treated with saline or pilocarpine hydrochloride, to induce SE. Control rats were given scopolamine and saline instead of pilocarpine. Experimental rats were injected with scopolamine (1 mg/kg) 30 minutes prior to pilocarpine (340mg/kg) to limit peripheral cholinergic effects and diazepam (10 mg/kg) 90 minutes after pilocarpine to terminate SE. All rats were visually monitored during SE and behavioral seizures were scored using a modified Racine scale (Liang et al, 2000). Briefly, seizures were scored on the following scale: P1-freezing behavior, P2-head nodding, P3-unilateral forelimb clonus, P4-bilateral forelimb clonus with rearing, and P5-bilateral forelimb clonus with rearing, falling, and/or hind limb clonus. Only rats experiencing SE defined as: at least five stage P3 or higher seizures followed by a period of continuous seizure activity for at least two hours, were included in further analyses as SE is the best predictor for the development of chronic epilepsy. Rats given pilocarpine but not developing SE as described above were deemed “non-responders”. Non-responders provide an ideal control group to differentiate the effects of pilocarpine versus the effects of SE and as such, non-responders were tested for indices of oxidative stress, mitochondrial function and deficits in memory.

Catalytic antioxidant treatment

Rats were treated with the metalloporphyrin catalytic antioxidant, Mn^{III}TDE-2-ImP⁵⁺ (5mg/kg s.c.) or vehicle (saline for controls) starting 60 minutes after pilocarpine and continuing every 4 hours until sacrifice (for most endpoints-24 hours). For behavior testing, treatment with saline (controls) or Mn^{III}TDE-2-ImP⁵⁺ started 60 minutes after pilocarpine and continued every 4 hours for the first 48 hours followed by a gradual tapering of doses (q4 for 48 hours followed by q6, q12 and q24) ending two days before acclimation to behavioral testing parameters and five days before NOR learning and memory was tested.

Animals were divided into 4 groups: 1) Control (saline-saline) 2) Control+Mn^{III}TDE-2-ImP⁵⁺ 3) Pilo+saline and 4) Pilo+ Mn^{III}TDE-2-ImP⁵⁺. It was determined that for most end-points (indices of oxidative stress, oxygen consumption rates and cell death) the Mn^{III}TDE-2-ImP⁵⁺ alone group displayed no discernable difference compared to control animals and were therefore not included in further analyses.

Assessment of brain penetration of Mn^{III}TDE-2-ImP⁵⁺

We used the known plasma half-life and dosing paradigm of Mn^{III}TDE-2-ImP⁵⁺ (5mg/kg, s.c. q4h) as a guide to determine its brain penetration (O'Neill et al, 2010). Trunk blood was obtained and hippocampal brain regions were rapidly dissected 24h following dosing of Mn^{III}TDE-2-ImP⁵⁺ (5 mg/kg, s.c. q4h). Samples were randomized and blinded before detection of Mn^{III}TDE-2-ImP⁵⁺ was performed using HPLC (as described in O'Neill et al, 2010).

Measurement of redox biomarkers by HPLC

Glutathione (GSH), glutathione disulfide (GSSG), tyrosine and 3-nitrotyrosine (3-NT) assays were performed 24 hours after SE, blinded with an ESA (Chelmsford, MA) 5600 CoulArray HPLC equipped with eight electrochemical cells following the company instruction (ESA Application Note 70-3993) and previously described in the literature with small modifications (Lakritz et al, 1997; Liang et al, 2006). The potentials of the electrochemical cells were set at 400/450/500/570/630/690/830/860 mV vs. Pd. Analyte separation was performed using a TOSOHAAS (Montgomeryville, PA) reverse-phase ODS 80-TM C-18 analytical column (4.6 mm×250 cm; 5 µm particle size). A two-component gradient elution system was used with component A of the mobile phase composed of 50 mM NaH₂PO₄ pH 3.0, and component B composed of 50 mM NaH₂PO₄ and 50% methanol pH 3.0. An aliquot (20 µl) of the supernatant was injected into the HPLC. The level of 3-NT was expressed as a ratio of 3-NT to tyrosine. Samples were randomized and blinded before assessment.

Assessment of mitochondrial function

Synaptosomes were isolated from the hippocampus of control and treated animals and mitochondrial oxygen consumption ratios (OCR) 24 hours after SE and assessed using an Extracellular Flux Analyzer (Seahorse) according to methods previously described (Rowley et al, 2015). All samples were blinded and randomized before assessment.

Fluoro-Jade B analysis

Frozen sections (20 µm) were blinded, cut coronally through hippocampus and stained with Fluoro-Jade B (Histo-Chem) as previously described (Schmued and Hopkins, 2000). Briefly, the sections were hydrated and immersed in a solution of alcohol and 1% sodium hydroxide for 5 minutes. After a wash, the sections were placed into a solution of 0.06% potassium permanganate for 10 minutes and washed again for 2 minutes. The staining solution (0.004% Fluoro-Jade B in 0.1% acetic acid vehicle) was prepared fresh. After a 15 minute incubation in the staining solution, the sections were rinsed and dried. The sections were cleared by xylene for at least a minute before coverslipping with DPX. Imaging was

performed using a Nikon Eclipse TE2000-U microscope. Using Image-J, the Fluoro-Jade B positive signal of areas of interest (Hippocampal areas CA1, CA3 and hilus) was measured. The average of the fluorescent relative density was quantified for each animal and the group average was expressed as percentage of the control.

Video-EEG Implantation and Monitoring

Control rats were anesthetized with a combination of ketamine hydrochloride (100mg/kg), xylazine (10mg/kg) and isoflurane and placed into a stereotaxic apparatus. After preparation of the cranium, rats were implanted with bilateral subdural electrodes (4.0 mm posterior, 2.5 mm lateral relative to the bregma). Four stainless steel screws with attached teflon-coated stainless steel wire (PlasticsOne) were inserted into two craniotomies (cortical 1 and cortical 2) and two behind lambda (ground and reference). The screws and wire were secured to the skull using dental acrylic. Animals were allowed to recover for 2 weeks before recording began. Video (Virtual Dub) and EEG of SE (Pinnacle Technology Inc.) were obtained over a 24 hour period starting with a one hour baseline recording. Following the baseline recording, all animals were injected with scopolamine and 30 minutes later with pilocarpine followed by Mn^{III}TDE-2-IMP⁵⁺ or saline starting 1 hour after pilocarpine. Group treatment was randomly assigned using random.org, a random number generator. All EEG data were analyzed for average maximum electrographic power associated with SE (maximum amplitude-baseline) and power within various frequency bands using a Fast Fourier Transformation algorithm and custom-written software (White et al, 2006). Rats were continually monitored via 24/7 video-EEG for the following month to assess the development of epilepsy and to ensure that differences in seizure burden could not account for differences in learning and memory.

Behavioral Studies

Novel object recognition (NOR) testing was performed blinded and as previously described to assess non-hippocampal dependent memory (Pearson et al, 2014). Briefly, rats were acclimated to conditions approximating those of the NOR task over a series of days starting one week after SE. These acclimations included exposure to the dedicated testing room, behavioral arenas (matte black, expanded PVC) and stimulus objects ("Lil Buddies" brand dog toys). NOR testing consisted of two phases. During the first phase, rats were allowed to investigate two identical objects for 5 minutes, and returned to their home cage for a delay period. For this study, the NOR task was performed at two different delay lengths, 60 minutes to assess relatively short-term memory and at 24 hours to measure long-term memory (evidence suggests hippocampus is involved in long-term object recognition i.e. 24 hours see Hammond et al, 2004). After the delay, rats were returned to the same behavioral arena however one object was replaced with a novel object. Rats with intact recognition memory will notice this change and spend a greater proportion of time investigating the novel object. All tests were video recorded and behaviors of interest were quantified using specialized behavior recognition software (Topscore by Cleversys Inc., Reston, VA.) To determine the percent of total investigation time spent with the novel object the following equation was used: (duration sniffing novel object/[duration sniffing novel object + duration sniffing familiar object]).

To assess hippocampal-dependent spatial memory, rats were tested in a standard Y-Maze (matte black, expanded PVC, 120° arm angles). Rats were placed into one arm of the Y-shaped maze with distal cues in sight and allowed to explore for eight minutes. Rats that have intact hippocampal-dependent spatial memory will remember the arm that was previously visited and will prefer to enter a new, unexplored arm. A successful alternation therefore consisted of consecutive arm entries (i.e. A-B-C) while an unsuccessful alternation was noted when the rat returned to the most recently explored arm (i.e. A-B-A). Total arm entries and sequence of arm entries was recorded and percent alternation was calculated as follows: [(the number of actual alternations ÷ the maximum possible alternations) × 100].

An observer blind to experimental condition and out of sight of animals observed all testing for signs of seizure activity (see modified Racine scale above). If a rat experienced a seizure before testing, they were tested at least one hour after the seizure. If a rat experienced a seizure during testing, the rat was excluded from analysis (one pilo-saline rat was excluded from the NOR60 task due to seizure during the test phase). While it is possible that rats may have experienced inter-ictal spiking during tests, multiple days of testing and well powered group numbers minimize the chances of spiking affecting overall test results.

Statistical analyses

Effects of group treatment and time on electrographical power during SE were compared using two-way analysis of variance (ANOVA). Effects of group treatment (control, pilo +saline, and pilo+Mn^{III}TDE-2-ImP⁵⁺) on indices of oxidative stress, oxygen consumption rates and neuronal death were analyzed using one-way ANOVA. The effects of seizures (control and pilo) and treatment group (saline and Mn^{III}TDE-2-ImP⁵⁺) on learning and memory were compared using two-way ANOVA. Significant main effects and interactions ($p < 0.05$) were probed using Bonferroni post-tests. All data is expressed as mean ± SEM unless otherwise noted. All analyses were performed using GraphPad Prism 5 software.

Results

Pharmacological removal of ROS does not alter severity of status epilepticus

Determination of therapeutic dosing and brain penetration of Mn^{III}TDE-2-ImP⁵⁺ was guided by its known plasma half-life and dosing frequency (5mg/kg s.c. q4h) to achieve therapeutic plasma levels in a rat model of acute lung injury (O'Neill et al, 2010). We determined if this dosing paradigm resulted in accumulation of the compound in the hippocampus of rats, an area of the brain susceptible to pilocarpine and important in learning and memory. After approximately 24 hours of this dosing paradigm and one hour after the last dose, the average concentration of Mn^{III}TDE-2-ImP⁵⁺ in the hippocampus was 91 ± 11.7 pmol/g tissue (mean ±S.E.M, n=4) and the average concentration in the plasma was 2.368 ± 0.068 μmol/g tissue (mean±S.E.M, n=4). This suggests that the drug passes the blood-brain barrier and reaches the brain. Based on the compound's antioxidant activity profile, these concentrations are in the range to exert antioxidant-mediated neuroprotection when administered every 4 hours (O'Neill et al, 2010). Therefore we established our dosing regimen for all studies to be one injection of Mn^{III}TDE-2-ImP⁵⁺ (5mg/kg, s.c.) every four hours until sacrifice.

Two measures were taken to ensure that the Mn^{III}TDE-2-ImP⁵⁺ dosing regimen did not affect the initiating insult, SE. First, we administered the compound one hour following pilocarpine, once SE was already initiated. Secondly, an analysis of maximal power during SE was performed. Specifically, maximum power was averaged every 15 minutes and plotted in Figure 1. No difference was detected between groups in maximal power at any point during SE ($p=0.62$; Figure 1A). Additionally, no differences were detected in any frequency band including delta 0–4 Hz ($p=0.72$, Figure 1B), theta 4–8 Hz ($p=0.93$, Figure 1C), alpha 8–13 Hz ($p=0.77$, Figure 1D), or betagamma 14 Hz ($p=0.84$, Figure 1E). To determine if the length of SE varied between groups, we analyzed time to 20% power, 10% power and 5% power (data not shown). No differences were detected between groups suggesting that length of SE was equivalent between groups. Analysis of behavioral seizures did not reveal any difference in latency to first behavioral seizure or in the frequency and severity of convulsive seizures during SE. Taken together the data suggest that treatment with Mn^{III}TDE-2-ImP⁵⁺ did not attenuate the overall severity of the initiating injury, SE.

Treatment with Mn^{III}TDE-2-ImP⁵⁺ inhibits SE-induced oxidative stress

We sought to determine if Mn^{III}TDE-2-ImP⁵⁺ exerted antioxidant actions *in vivo* against SE-induced hippocampal oxidative stress. Based on its broad antioxidant actions against superoxide, hydrogen peroxide, lipid peroxides and peroxynitrite we tested its ability to prevent glutathione oxidation and preserve the ratio of reduced to oxidized glutathione (GSH:GSSG) (Kachadourian et al, 2004; Castello et al, 2008; O'Neill et al, 2011). Additionally, we tested its ability to prevent formation of 3-NT, an index of protein nitration. Assessment of oxidative stress parameters was performed 24 hours after SE, when previous data show these markers to be elevated (Liang et al, 2000; Ryan et al, 2013). A cohort of non-responders i.e. rats that received pilocarpine but did not develop SE, did not show deficits in oxidative stress parameters. Following pilocarpine-induced SE, Mn^{III}TDE-2-ImP⁵⁺ injections but not saline injections significantly prevented depletion of reduced GSH ($p<0.01$; Figure 2A) and accumulation of its disulfide, GSSG ($p<0.0001$; Figure 2B), thereby significantly improving the ratio of GSH:GSSG in the hippocampus ($p<0.001$; Figure 2C). Similarly, the ratio of 3-NT/tyrosine, which was increased in the hippocampus after pilocarpine ($p<0.0001$), was significantly decreased in rats treated with Mn^{III}TDE-2-ImP⁵⁺ compared to treatment with saline ($p<0.001$; Figure 2D). Therefore, scavenging ROS inhibited indices of oxidative stress allowing us to determine if oxidative stress confers any functional deficits in the pilocarpine model of TLE. Additionally, the lack of oxidative stress markers in rats receiving pilocarpine but not developing SE suggests that SE, and not merely pilocarpine treatment, is the causal factor in the development of oxidative stress.

Pharmacological removal of ROS ameliorates deficits in mitochondrial respiration

Mitochondria are the major site of ROS generation and therefore their major target. Using the catalytic antioxidant, Mn^{III}TDE-2-ImP⁵⁺, we asked if increased ROS were necessary to mediate deficits in mitochondrial oxygen consumption ratios (OCR), an index of mitochondrial dysfunction following SE. Pilocarpine-treated rats exhibit deficits in FCCP-stimulated mitochondrial OCR measured 48 hours after SE ($p<0.05$; Figure 3A). A time course of mitochondrial respiration revealed that these deficits appear 16 hours after SE and OCR stayed depleted relative to control levels at both 48 hours and 1 week after SE (Figure

3B). To determine the mechanistic role of ROS production in causing the mitochondrial dysfunction revealed by extracellular flux analysis, we assessed mitochondrial respiration in two groups of pilocarpine-treated rats injected with either saline or Mn^{III}TDE-2-ImP⁵⁺. Treatment with Mn^{III}TDE-2-ImP⁵⁺ resulted in mitochondrial OCR traces similar to that of a control trace (Figure 3C). Specifically, significant improvement in reserve capacity was observed in pilocarpine rats treated with Mn^{III}TDE-2-ImP⁵⁺ compared to that of saline treated pilocarpine rats ($p < 0.05$; Figure 3D). Deficits were not observed in rats treated with pilocarpine but not experiencing SE, suggesting that pilocarpine treatment alone does not induce deficits in mitochondrial OCR. Together with improvement of oxidative stress, these data indicate that deficits in mitochondrial OCR and other indices of dysfunction in TLE models are driven in part by ROS production resulting in impaired mitochondrial consumption of oxygen to form ATP.

Pharmacological removal of ROS attenuates SE-induced neuronal death

Mitochondria can regulate both apoptotic and necrotic cell death and have been implicated in the pathogenesis of various neurodegenerative diseases including AD and ALS. Neuronal death, particularly in the hippocampus, is a common feature of TLE and is thought to contribute to cognitive dysfunction (Pitkanen and Sutula, 2002). In epilepsy, key mediators of neuronal death are necrosis initiated by glutamate excitotoxicity and apoptosis (Ankarcrona et al, 1995; Reynolds and Hastings, 1995; Patel et al, 1996; Henshall and Murphy, 2008). Reactive species are contributors to glutamate excitotoxicity as well as apoptotic cell death (Lafon-Cazal et al, 1993; Reynolds and Hastings, 1995; Henshall and Murphy, 2008). Work from our laboratory has demonstrated the role of mitochondrial ROS in SE-induced neuronal death (Liang et al, 2000). Therefore, we evaluated the effects of scavenging ROS on SE-induced neuronal death using Fluoro-Jade B (FJB). FJB labeling of degenerating neurons in the hippocampus was quantified 24 hours after pilocarpine-induced SE. The pilocarpine group showed a significant increase in FJB labeling compared to controls in the hippocampal CA3 area ($p < 0.0001$; Figure 4A) and the hilus ($p < 0.0001$; Figure 4B), indicative of increased neuronal death. The pilocarpine group that received Mn^{III}TDE-2-ImP⁵⁺ had significantly reduced FJB staining compared to the saline-treated pilocarpine group in CA3 ($p < 0.05$; Figure 4A) and dentate hilus ($p < 0.05$; Figure 4B). Taken together, these data indicate that ROS contributes to SE-induced neuronal loss.

Pharmacological removal of ROS attenuates SE-induced memory deficits

To determine whether increased ROS production contributes to cognitive dysfunction in the pilocarpine model of TLE, rats were treated with Mn^{III}TDE-2-ImP⁵⁺ and tested in cognitive paradigms evaluating learning and memory. Testing was initiated one week after SE to allow the rats sufficient time to recover from SE. No differences in locomotion or indices of anxiety-like behavior were detected in the open field task and all rats were confirmed epileptic before being tested in the learning and memory tasks. Compared to saline, treatment with Mn^{III}TDE-2-ImP⁵⁺ significantly improved short-term recognition memory performance in pilocarpine-treated rats as measured by the Novel Object Recognition (NOR) task performed after a 60 minute delay ($p < 0.01$; Figure 5A). We next evaluated long-term recognition memory in the NOR task measured after a 24 hour delay (Figure 5B). Novelty preference ratios after the 24 hour delay were significantly improved in the pilo

+Mn^{III}TDE-2-ImP⁵⁺ group compared to pilo+saline group ($p < 0.05$) at a level similar to controls, suggesting that pharmacological removal of ROS attenuates deficits in long-term recognition memory in epileptic rats. To assess hippocampal-associated spatial memory, rats were tested in the Y-maze spontaneous alternation task (Figure 5C). Pilocarpine-induced SE regardless of treatment with Mn^{III}TDE-2-ImP⁵⁺ reduced the overall percent alternation indicative of deficits in spatial memory. Interestingly, a main effect of drug was detected indicating that regardless of seizure condition (control vs. pilocarpine) treatment with Mn^{III}TDE-2-ImP⁵⁺ improved spatial memory performance. Importantly, this effect represents a disease-modifying effect of scavenging of ROS on spatial memory as treatment with Mn^{III}TDE-2-ImP⁵⁺ was discontinued 3 weeks prior to the Y-Maze task. Non-responders did not display any deficits on learning and memory tasks, nor were any seizures observed, again supporting the notion that SE is the causal factor and not merely treatment with pilocarpine.

Pharmacological removal of ROS does not alter chronic epilepsy

Seizure burden during the time of behavioral studies (1 week and 3 weeks after SE) was not affected by treatment with Mn^{III}TDE-2-ImP⁵⁺ (Figure 6). Rats that were monitored for differences during SE remained under video-EEG observation for one month to monitor the development of epilepsy. There was no significant difference in the latency to the first spontaneous seizure (Figure 6A). Additionally, there were no differences detected in the frequency (Figure 6B), severity (Figure 6C) or duration (Figure 6D) of chronic seizures. This suggests that differences in learning and memory between groups are not the result of differences in the frequency or severity of spontaneous seizures. It is important to note that all animals treated with pilocarpine in the EEG cohort had at least one seizure and in most cases multiple seizures before learning and memory tasks began in the behavior cohort. This corroborates the observation that all rats were epileptic before testing.

Discussion

Using two relatively low stress learning and memory paradigms, the novel object task and the Y-maze, we have shown that rats, after pilocarpine-induced SE, display deficits in both recognition and spatial memory. Deficits were not observed in rats treated with pilocarpine but not experiencing SE, pointing to a causal role of SE in producing cognitive impairment. Catalytic removal of ROS improved short and long term recognition memory as well as spatial memory in epileptic rats even after discontinuation of Mn^{III}TDE-2-ImP⁵⁺ treatment, representing a disease modifying effect of antioxidant treatment against deficits in learning and memory. Additionally, we show that pharmacological removal of ROS and subsequent inhibition of oxidative stress is sufficient to attenuate mitochondrial dysfunction and hippocampal neuronal loss, providing a potential mechanism for cognitive impairment associated with epilepsy. Treatment with Mn^{III}TDE-2-ImP⁵⁺ had no effect on SE or spontaneous seizures occurring during periods of behavioral testing, suggesting that learning and memory improvement was not due to a reduction of overall seizure burden. It should be noted however that seizure burden was only evaluated during the first month after SE, and effects on chronic epilepsy cannot be ruled out. Taken together, our data suggest that injury-

induced ROS production may be a key driver in processes underlying cognitive dysfunction associated with epileptogenesis and therefore, a viable therapeutic target.

The compound used in these studies, $\text{Mn}^{\text{III}}\text{TDE-2-ImP}^{5+}$, is a small molecular weight, non-toxic catalytic antioxidant (Kachadourian et al, 2004; Castello et al, 2008). It possesses high superoxide dismutase activity as well catalase activity, enabling it to scavenge a broad range of reactive species including superoxide and hydrogen peroxide as well as peroxynitrite and lipid peroxides (Kachadourian et al, 2004; Castello et al, 2008). Like the endogenous antioxidant superoxide dismutase (SOD), this compound contains an active site metal that catalyzes the dismutation reaction of both superoxide and hydrogen peroxide catalytically, without requiring energy from the cell (Castello et al, 2008). $\text{Mn}^{\text{III}}\text{TDE-2-ImP}^{5+}$ has been proven efficacious at reducing oxidative stress in animal models of brain ischemic injury as well as radiation and vesicant-induced lung injury (Sheng et al, 2002; Rabbani et al, 2007; O'Neill et al, 2011; McGovern et al, 2011; Tewari-Singh et al, 2014). In the current study, we verified brain levels of the compound in plasma and hippocampus at concentrations known to exert antioxidant and neuroprotective effects (Sheng et al, 2002; O'Neill et al, 2011). Indeed, its ability to protect the glutathione redox status and prevent 3-NT adducts within the hippocampus following SE supports its antioxidant actions. Although the compound's relatively short half-life limited chronic dosing, a high potency to catalytically detoxify diverse reactive species together with the ability to penetrate the brain and inhibit oxidative damage in vivo, make $\text{Mn}^{\text{III}}\text{TDE-2-ImP}^{5+}$ a useful tool to investigate the role of oxidative stress in neurologic disorders.

A growing body of research suggests oxidative stress is both a cause and a consequence of epileptic seizures in humans and in animal models (Patel, 2004; Menon et al, 2012; Rumià et al, 2013). The functional role of oxidative stress in contributing to seizure-induced neuronal death largely comes from the demonstration that compounds with antioxidant-like properties exert neuroprotection (MacGregor et al, 1996; Tan et al, 1998; Rong et al, 1999; Gupta et al, 2000; Liang et al, 2008, 2012). The current study corroborates these findings and extends the literature to include mitochondrial dysfunction, specifically deficits in OCR, as a potential contributor to neuronal loss within the hippocampus (Rowley et al, 2015). We demonstrate alterations in oxidative phosphorylation assessed by OCR using real-time extracellular flux analysis. Specifically, our data suggest that synaptosomal mitochondria from epileptic animals have less metabolic reserve capacity and are unable to increase respiration to meet a large ATP demand. These findings compliment a recent study from our laboratory showing SE-induced mitochondrial dysfunction in the kainic acid model of TLE and attenuation by the same catalytic compound used in the current study (Rowley et al, 2015). Together with the findings that both SE and chronic seizures are sufficient to decrease activities of various mitochondrial enzymes and complexes of the electron transport chain, this supports the findings that there is persistent mitochondrial dysfunction in experimental TLE (Liang et al, 2000; Kudin et al, 2002; Chuang et al, 2004; Ryan et al, 2012; Rowley et al, 2015). Our data show that pharmacological removal of ROS inhibits deficits in mitochondrial respiration initiated by epileptogenic injury suggesting that ROS production may be an important driver of these deficits.

Attenuation of mitochondrial respiration deficits by $\text{Mn}^{\text{III}}\text{TDE-2-ImP}^{5+}$ was accompanied by inhibition of hippocampal neuronal loss. Mitochondria are key modulators of apoptotic and necrotic cell death, both of which have been implicated in seizure-induced neuronal loss (Shinoda et al, 2004a; Henshall 2007; Henshall and Murphy, 2008). Neuronal death is a key feature of refractory TLE, particularly in the temporal lobe which houses areas of the brain important for learning and memory. Removal of sclerotic tissue often improves seizure burden suggesting that neuronal death or the processes underlying it may play a role in epileptogenesis (Jobst and Cascino, 2015). Our data suggest that neuronal loss, while controversial in the propagation of epilepsy, may contribute to injury-induced memory impairment. However, given that scavenging of ROS produced only a moderate neuroprotective effect while completely attenuating deficits in memory, suggests that mechanisms underlying overt neuronal loss such as deficits in mitochondrial respiration or oxidative damage may be the underlying cause of memory impairment.

Although dozens of drugs have been developed to treat the seizures that characterize epilepsy, few if any drugs have been earmarked for the cognitive impairments that accompany the disease. The common belief is that if seizures are well controlled, memory deficits will subside. However, increasing evidence suggests that this is not the case. In some instances, memory impairment is evident at or before the first seizure, suggesting that memory deficits are not merely a side effect of frequent seizures (Pulliainen et al, 2000; Ogunrin et al, 2000). Indeed, an unexpected insight from our data was that protection of both neurons and learning and memory can be achieved without a concomitant decrease in seizure burden. This represents an important dissociation between seizures and cognitive impairment, and raises the possibility of pairing antioxidants with anti-seizure drugs as a combination therapy for achieving optimal control of seizures and cognitive comorbidities.

The results of this study have implications beyond the treatment of TLE and its comorbidities. Pilocarpine, a cholinergic agonist, produces long-term neurochemical and behavioral changes similar to changes induced by exposure to chemicals such as nerve agents (Jett, 2012). For these reasons, pilocarpine is often used as a surrogate of organophosphate nerve agents such as Soman and Sarin that irreversibly inhibit acetylcholinesterase. Seizure activity plays a critical role in mediating nerve agent neurotoxicity (Shih et al, 2003). The catalytic antioxidant used here, is currently undergoing evaluation as a medical countermeasure to chemical warfare agents. The demonstration that acute parenteral administration of $\text{Mn}^{\text{III}}\text{TDE-2-ImP}^{5+}$ confers neuroprotection against pilocarpine-induced oxidative stress, neuronal death and cognitive dysfunction suggest its translational utility to treat nerve agent toxicity. Studies are ongoing to develop an extended release drug product which would decrease the frequency of delivery used in these animal studies. However, it should be pointed out that orally active metalloporphyrins similar to $\text{Mn}^{\text{III}}\text{TDE-2-ImP}^{5+}$ with longer half-lives have been developed and have shown efficacy in attenuating epilepsy arising from mitochondrial disease and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism (Liang et al, 2008; Liang et al, 2012).

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Competing interests: Dr. Day is a consultant for and holds equity in Aeolus Pharmaceuticals that is developing metalloporphyrins as potential therapeutic agents.

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Highlights

- Catalytic removal of ROS improved memory in a rat model of temporal lobe epilepsy.
- Mitochondrial respiration deficits and neuronal death contributed to memory impairment.
- Catalytic removal of ROS offers disease-modifying effects for memory deficits in TLE.
- ROS mechanistically contributes to memory deficits comorbid with TLE.

Fig. 1. Electrophysiological power during SE is not affected by pharmacological removal of ROS. (A) Maximal power during SE ($p=0.6184$). (B) Power in Delta frequency range ($p=0.72$). (C) Power in Theta frequency range ($p=0.93$). (D) Power in Alpha frequency range ($p=0.77$) and (E) Power in BetaGamma frequency range ($p=0.84$). Analyzed via two-way repeated measures ANOVA $n=5$ /group.

Fig. 2.

Indices of oxidative stress are attenuated by treatment with Mn^{III}TDE-2-ImP⁵⁺. (**A**) Measures of reduced glutathione (GSH), and (**B**) oxidized glutathione (GSSG), the (**C**) ratio of GSH/GSSG, and (**D**) 3-nitrotyrosine (3-NT) formation. ***p* < 0.01, ****p* < 0.001 compared to control # < 0.01 ## < 0.001 compared to pilocarpine alone by one-way ANOVA, *n* = 5–8 in each group. NR= non-responder (rats treated with pilo but not developing SE).

Fig. 3.

Pharmacological removal of ROS attenuates SE-induced deficits in mitochondrial respiration. (A) Example traces of OCR from isolated hippocampal synaptosomes of control and treated rats. (B) Time course of deficits in mitochondrial reserve capacity in pilocarpine treated animals relative to control (C) Example bioenergetic profile traces from each group shown in (D) Mitochondrial reserve capacity. * $p < 0.05$ or ** $p < 0.01$ compared to control, # $p < 0.05$ compared to pilocarpine alone by one-way ANOVA, $n = 5$ in each group.

Fig. 4. Pharmacological removal of ROS attenuates SE-induced neuronal death 24 hours after SE. (A) quantification of relative fluorescence in CA3 ($p = 0.0002$) and (B) the hilus ($p < 0.0001$) *** $p < 0.001$ compared to control, # < 0.05 compared to pilocarpine alone by one-way ANOVA, $n = 6$ in each group.

Fig. 5. Pharmacological removal of ROS attenuates deficits in both recognition and spatial memory. (A) Performance on the novel object recognition task after a 60 minute delay and (B) after a 24 hour delay. (C) Performance on the Y-maze, a spatial memory task. * $p < 0.05$, ** $p < 0.01$ by two-way ANOVA Bonferroni post-test. M.E indicates a significant main effect of treatment, control vs. pilo ($p = 0.001$), m.e. indicates significant main effect of drug, saline vs. 10150 ($p = 0.03$). $n = 8-11$ /group.

Fig. 6. Pharmacological removal of ROS does not alter (A) the development of seizures or the latency to the first spontaneous seizure ($p = 0.25$) (B) the total number of seizures per week (treatment effect $p = 0.77$) (C) the average severity of the seizures (treatment effect $p = 0.30$) or (D) the average duration of seizures during the month of behavior testing ($p = 0.07$). $n = 5$ /group.